A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Dietary effect of probiotics on innate immune response and disease resistance in aquaculture fish

Dong-Hwi Kim

Department of Marine Life Science

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

2018. 02

Dietary effect of probiotics on innate immune response and disease resistance in aquaculture fish

Dong-Hwi Kim

(Supervised by professor Moon-Soo Heo)

A thesis submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy

2018. 02.

This thesis has been examined and approved.

Thesis director, Gi-Young Kim, Prof. of Applied Marine Life Sciences

Joon-Bum Jeong, Prof. of Applied Marine Life Sciences

SeungHeon Lee, Prof. of Applied Marine Life Sciences

Chang-Nam Jin, Res. Prof. of Applied Marine Life Sciences

Moon-Soo Heo, Prof. of Applied Marine Life Sciences

2018.02.

Date

Department of Marine Life Science

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSI

CONTENT

국문초록

국내의 어류 양식 산업은 크게 성장하고 있지만, 양식 도중 발생하는 어류질병이 양식 산업 발전에 심각한 악 영향을 미치고 있다. 일반적으로 육상수조 양식의 경우 단위 면적당 생산량을 극대화 시키기 위해 고밀도 사육을 하고 있는데, 이러한 고밀도 사육은 증체율 저하 및 각종 질병 발생의 직접적인 원인을 제공하여 폐사에 따른 경제적 손실을 일으킨다. 양식 산업에서의 어류질병의 발생과 확산은 생산성을 현저하게 감소시킬 수 있으며, 이를 예방하기 위해 어류 양식장에서는 어류질병 관리 전략으로 항생제를 광범위하게 사용하고 있다. 하지만 일부 양식장에서는 어류질병의 치료 및 예방의 목적으로 항생제를 오남용 하고 있어 2차적인 문제를 야기시키며, 그 중 가장 대표적인 것이 항생제 내성균주와 항생제 잔존 어류이다.

지난 20년동안 어류 질병의 예방 및 관리에 있어서 probiotics의 적용은 많은 관심을 받고 있다. Probiotics로 인정받은 대부분의 미생물은 약물이나 식품으로 판매되고 있으며 여러 가지 측면에서 안전성이 확인되었다. 항생제보다 효과적이고 안전한 probiotics는 생물학적 제어로 그 쓰임이 증가하고 있다. 생균제로 가장 일반적으로 사용되는 유산균은 장내 미생물의 균형을 개선하는 probiotics로써 사용되고 있으며, 주로 *Bacillus* sp., *Pediococcus* sp., *Lactobacillus* sp., *Bifidobacterium* sp., *Lactococcus* sp. 등이 있으며 현재까지 정장작용, 면역증강작용, 영양학적 가치 증진, 간경화 개선 작용, 항암 작용 등에 대한 연구가 진행 중에 있다.

이에 따라 본 연구에서는 전통 발효 식품에서 분리한 *Bacillus amyloliquefaciens* JFP-02를 양식 어류에 급이 하였을 때 일반 사료와 비교하여

나타나는 성장도, 비특이적 면역반응, 병 저항성을 확인하여 새로운 사료첨가제로써의 응용 가능성을 확인하고자 하였다.

제주민속오일시장에서 전통 발효식품인 된장, 새우젓, 오징어젓, 전어젓, 꽃멸치젓, 갈치속젓, 자리젓에서 총 8 종의 probiotics 후보 균주를 분리하였다. NCBI 의 BLAST 를 이용하여 분리된 균주의 염기서열을 확인한 결과 *pediococcus acidilactici*, *Bacillus amyloliquefaciens*, *Lactobacillus parabuchneri*, *Enterococcus durans*, *Pediococcus pentosaceus*, *Lactobacillus sakei*, *Lactobacillus plantarum*, *Lactobacillus brevis* 로 동정 되었다.

분리된 probiotics 후보균주 중, pH, 온도, 배양조건, 인공 위액, 인공 담즙산, 내염성, 내열성에 대한 내성을 확인한 결과 *Bacillus amyloliquefaciens* JFP-02 을 공시균주로 선정하였다. *B. amyloliquefaciens* JFP-02 의 형태학적 특성은 집락의 모양은 고르지 못하고, 집락의 중앙이 볼록 솟아 있으며, 백금이로 취할 때 점성을 띈다. 장방출주사전자현미경을 이용하여 관찰한 결과 단간균으로 길이는 1.575 um, 폭은 0.727 um 으로 나타났다. 생육조건은 saccharose, yeast extract, $MgSO_4 \cdot 7H_2O$ 를 배지에 첨가하여 pH 9 로 조정한 후 30℃에서 생육 시킬 때 활성이 가장 높았다.

2015 년도와 2016 년도에 넙치 양식장 18 개소에서 총 13 개의 어류질병세균을 확보하였다. 분리된 균주의 염기서열을 확인한 결과 *Photobacterium damselae sub sp*. 2 종, *Edwardsiella tarda* 2 종, *Streptococcus parauberis* 2 종, *Streptococcus iniae*, *Tenacibaculum maritimum*, *Vibrio harveyi*, *Vibrio alginolyticus*, *Vibrio anguillarum*, *Staphylococcus caprae* 가 동정되었다.

넙치 양식장에서 분리한 어류질병세균을 대상으로 *B. amyloliquefaciens* JFP-02의 배양 조건에 따른 항균활성을 측정한 결과, pH4~pH9와 10℃~50℃에서 *T.*

maritimum, E. tarda 에서 가장 항균활성이 높았다. 또한 탄소원, 질소원, 무기염의 종류에 상관없이 *T. maritimum, E. tarda, V. campbellii S. caprae* 에 대하여 높은 항균활성을 보였다.

양식어류인 넙치, 돌돔, 강도다리를 대상으로 *B. amyloliquefaciens* JFP-02 를 급이하였을 때 나타나는 혈액학적 특성과 비특이적 면역반응, 어류질병에 대한 병 저항성 및 면역 유전자 발현량을 확인하였다.

넙치의 경우 8주동안 *B. amyloliquefaciens* JFP-02를 급이 시킨 후 혈액학적 분석 결과 hematocrit, total protein, glucose에서 컨트롤 그룹에 비해 유의적으로 증가하는 것을 확인한 반면, triglyceride, total cholesterol, AST, ALT는 컨트롤 그룹에 비해 감소 하는 것으로 확인되었다. 비특이적 면역반응인 라이소자임 활성과 대식세포 활성에서는 실험 후반부터 컨트롤그룹에 비해 유의적으로 증가하였으며, *S. iniae*를 이용한 병 저항성 실험 결과 컨트롤그룹에서 85%의 폐사율을 보인 반면, *B. amyloliquefaciens* JFP-02의 그룹에선 폐사율이 45%로 약 절반의 폐사율을 보였다.

돌돔의 경우 10주동안 *B. amyloliquefaciens* JFP-02를 급이 시킨 후 hematocrit, total protein, glucose의 결과 컨트롤그룹에 비해 *B. amyloliquefaciens* JFP-02의 그룹에서 유의적으로 활성이 증가하였다. triglyceride, total cholesterol의 결과 컨트롤 그룹에 비해 유의적으로 감소 하였으며, AST와 ALT는 실험 초기부터 꾸준히 감소 하였다. 라이소자임과 대식세포의 결과 컨트롤 그룹에 비해 유의적으로 증가 하였으며, *S. iniae*를 이용한 병 저항성 실험 결과 컨트롤 그룹이 20일째 폐사율이 90%인 반면, *B. amyloliquefaciens* JFP-02의 그룹에서 35%의 폐사율을 나타냈다.

강도다리를 이용한 실험 결과 성장률에서는 4주동안 *B. amyloliquefaciens* JFP-

02를 급이 하였을 때 혈액분석 결과 hematocrit, glucose, total protein, calcium은 컨트롤 그룹에 비해 유의적으로 증가하였으며, AST와 ALT는 컨트롤 그룹에 비해 감소하는 것을 확인하였다. 라이소자임 활성, 대식세포 활성은 컨트롤 그룹에 비해 유의적으로 증가하였으며, *S. parauberis*를 이용한 공격실험에선 컨트롤 그룹에선 20일째 75%의 폐사율을 보인 반면 *B. amyloliquefaciens* JFP-02그룹에선 45%의 폐사율을 보였다.

Real-time PCR을 이용하여 넙치의 TNFR-1과 IL-1b의 유전자 발현량을 확인한 결과 TNFR-1의 경우 신장을 제외한 장, 비장, 간에서 컨트롤 그룹보다 발현량이 적게 나왔으며, IL-1b의 경우 장과 간에선 컨트롤 그룹에 비해 적은 발현량을 보이고, 비장과 신장에서는 컨트롤 그룹에 비해 높은 발현량을 보였다. 돌돔의 경우 FAS의 경우 간에서 가장 많은 발현량을 보이는 반면, 신장에서 가장 적은 발현량을 보였다. Caspase 3의 경우 모든 장기에서 컨트롤에 비해 많은 발현량을 보였다. 강도다리의 경우 TNF와 IL-6의 유전자 발현량은 장에서 가장 많은 발현량을 보였으며, 신장에서 비교적 적은 발현량을 보였다.

본 연구에서는 제주도 전통발효 식품에서 분리한 *B.amyloliquefaciens* JFP-02가 probiotics가 가져야 할 특성을 지닌 것으로 사료되며, 배양조건에 따른 다양한 어류질병세균에 대한 항균활성을 확인할 수 있었다. 이러한 일련의 실험결과를 토대로 *B. amyloliquefaciens* JFP-02를 양식 어류에 적용하였을 비특이적 면역반응과 어류질병세균에 대하여 병 저항성을 가지는 것을 확인 하였으며, 향후 어류 양식산업에 도움이 될 것으로 사료된다.

LIST OF FIGURES

LIST OF TABLE

Part I. Characterization analysis of probiotics strains isolated from Jeju Island traditional foods

1.1 INTRODUCTION

The Fish aquaculture industry is an important industry responsible for future human protein supply. Future of the fish aquaculture industry is hopeful enough that a global futurist Alvin Toffler and the global economist Peter Drucker argue that “in the future of the 21st century, aquaculture is more promising then the internet”. According to the ‘FISH TO 2030: Prospects for fisheries and aquaculture’ jointly published by the World Bank, the Food and Agriculture Organization of the United Nations (FAO), International Food Policy Research Institute (IFPRI), the world fish supply rate in 2030 is 186 million tons, which is forecast to increase by 20.1% from 2011 (154 million tons). Although the middle class worldwide increased and fish consumption increased, but cannot cover demand for fish directly from the ocean. In 2030 half of the production will be aquaculture, and the production of cultured fish was expected to increase by 47.1% from 2011 (Msangi et al., 2013).

The incidence and spreads of fish diseases in the fish aquaculture industry can significantly reduce productivity, and to prevent antibiotics was widely used in fish disease management strategies in fish farms (Smith et al., 1994). However, some fish farms abuse antibiotics for the purpose of treatment and prevention of fish diseases, causing secondary problems, the most representative of which is antibiotic-resistant strains and antibiotic-remaining fish (Karunasagar et al., 1994; Witte et al., 1999). Since all antibiotics from 2012 on such problems, except for pharmaceutical use, other uses are prohibited, so the development of alternative substances for antibiotics for the prevention and treatment of diseases is urgent, for that purpose, research on various methods utilizing

probiotics and prebiotics has been attempted (Kim et al., 2017).

For the past decades, the application of probiotics in the prevention and management of fish disease has received more interest (Ganguly et al., 2010). Most probiotic microbes are marketed as drug or foodstuffs and safe application has been confirmed through long-term experience (Lim et al., 2011). Increased use of bacteria as biological controllers is effective and safer than antibiocis (Bansemir et al., 2006). Antibacterial activity in probiotic bacteria may occur by the production of bacteriocins (Avonts and Vust., 2010) which are antimictobial peptides or proteins that kill other related (narrow spectrum) or non-related (broad spectrum) microbiotas as one of the inherent defense mechanism of bacteria (Cotter et al., 2013). The mode of bacteriocin action includes destruction of cell walls, formation of pores in cell membrane, disruption of cell membrane followed by cell lysis and inhibition of nuclease activity (Lim et al., 2011).

Several studies have reported such antimicrobial protein or bacteriocins, derived mainly from lactic acid bacteria, as safe and effective natural food preservatives (Cleveland et al., 2001). They have been isolated primarily from meat and dairy products, are also nontoxic to humans, and leave no residues (Cotter et al., 2013). Bacteriocins are used as bio-preservatives of vegetable foods and beverages and their application appears to be a possible alternative to chemical compounds and antibiotics (Collins et al., 2010).

Korean traditional fermented seafood, Jeotgal is one of the most important foods in Korea and is fermented by naturally occurring microorganisms (Lee et al., 2014). It is prepared by adding 20-30% (w/w) salt to various types of seafood such as shrimp, oyster, shellfish, fish, fish eggs, and intestines, followed by preservation through fermentation (Guan et al., 2011). Several studies have reported the jeotgal fermentation process (Roh et al., 2010; Choi et al., 2013) which includes *Bacillus subtilis*, *Leuconostoc mesenteroides*, *Pediococcus halophilus* and other salt resistant aerobic and anaerobic bacteria (Lee., 1993). However, the molecular characterization of those bacterial species and their potential

application is scarce (Mohammadou et al., 2014). Thus, the present studies was carried out to isolated and characterize the bacterial strain *Bacillus amyloliquefaciens* from Jeju island traditional fermented food, and assess its probiotics.

1.2 MATERIALS AND METHODS

1.2.1 Isolated and storage of probiotics candidate strains

Fully ripened and ready to consume samples of Doenjang, Saeujeot, fermented Squid, salted Damselfish, salted guts of Hairtail, salted Gizzard shad, salted spratelloides gracilis were purchased from the local traditional markets on Jeju Island, South Korea (Fig. 1). Samples purchased were inoculated into MRSA (Man Rogosa Shape Agar, Difco., USA) diluted with 0.85% saline solution and incubated at 35℃ for 48 hours. The composition of the medium was shown in Table 1, 2. Single colony was isolated from the cultured strains and re-inoculated into MRSA and cultured at 35℃ for 48 hours. Inoculated into MRSB (Man Rogosa Shape Broth, Difco., USA) using the cultured strain, cultured at 35℃, 200 rpm for 48 hours, then stored at -80℃ in 20% (v/v) glycerol and used in the experiment (Kim et al., 2017).

Fig. 1. Isolated of probiotics microbes from Jeju Island traditional foods. (Doenjang, Saeujeot, fermented Squid, salted Damselfish, salted guts of Hairtail, salted Gizzard shad, salted Spratelloides gracilis)

Table 1. Composition of Man Rogosa Shape Agar (MRSA).

Ingredient	Amounts
Proteose Peptone No.3	10.0 g
Beef Extract	10.0 g
Yeast Extract	5.0 g
Dextrose	20.0 g
Polysorbate 80	1.0 g
Ammonium Citrate	2.0 g
Sodium Acetate	5.0 g
Magnesium Sulfate	0.1 g
Manganese Sulfate	0.05 g
Dipotassium Phosphate	2.0 g
Agar	15.0 g

Table 2. Composition of Man Rogosa Shape Broth (MRSB).

Ingredient	Amounts
Proteose Peptone No.3	10.0 g
Beef Extract	10.0 g
Yeast Extract	5.0 g
Dextrose	20.0 g
Polysorbate 80	1.0 g
Ammonium Citrate	2.0 g
Sodium Acetate	5.0 g
Magnesium Sulfate	0.1 g
Manganese Sulfate	0.05 g
Dipotassium Phosphate	2.0 g

1.2.2 Sequence analysis of probiotic candidate strains

For pure isolated probiotics candidate strains, DNA was extracted using 2.5% chelex (Chelex ® 100 Molecular Biology Grade Resin, Bio-RAD., USA), (Shen et al., 1999). The isolate strain was added in 2.5% chelex, reacted at 95℃, for 5 minutes, and stored at 4℃. 27 Forward (5′-AGAGTTTGATCCTGGCTCA-3′) primer and 1522 Reverse (5′-AAGGAGGTGATCCAGCCGCA-3′) primer were used for PCR. PCR was performed by adding 1 ul of DNA to AccuPower^{TM} PCR Pre-Mix (Bioneer., USA) and final volume of 25 ul. The PCR (Base for Gene Pro Thermal cycler, BIOER., USA) conditions were carried out Initial denaturation 5 min, denaturation at 94℃ for 1 min, annealing at 55℃ 1 min, extension ant 72℃ for 1 min at 30 cycles, and 72℃ final extension for 10 min. The amplified PCR product was confirmed by electrophoresis (MUpid® -ex, ADVANCE., USA) at 1% agarose gel.

1.2.3 Antibiotics susceptibility test of probiotics candidate strains

For antibiotics seven kind of amoxycillin (Table 3) were used, antibiotic resistant strains and susceptible strains were tested by using the Bauer-kirby test as an antibiotic commonly used to confirm the presence or absence of resistance of the stain type (Wayne., 1997). Inoculating the culture medium on MHA (Muller Hinton Agar, Difco., USA) and an antibiotic disc is placed on the medium a concentration gradient of the antibiotic occurs in the medium and shown the inhibition zone around the disc exhibits antibacterial activity. The inhibitory zone size varies according to solubility of the antibiotic and the degree of susceptibility of the bacterial. Probiotics candidate strains were pre-cultured in MRSB, then streaked on MHA and incubated for 48 hour, 35℃, secondly each of the antibiotic discs. The inhibitory zones for the antibiotic discs were measured to establish the existence and nonexistence of antibiotic resistance.

Table 3. Antibiotics list for selection of antibiotic resistant strains.

Antibiotics	**Concentrations (μg)**	**Diameter of inhibition zone (mm)**		
		Resistant	**Weakly sensitive**	**Sensitive**
Amoxycillin (AML)	10	≤ 13	14 - 17	≥ 18
Gentamicin (GN)	10	≤ 12	13 – 14	≥ 15
Erythromycin (EM)	15	≤ 13	14 – 22	≥ 23
Flofenicol (FFL)	30	≤ 12	13 – 17	≥ 18
Neomycin (NEO)	10	≤ 12	13 – 16	≥ 17
Oxytetracycline (OTC)	30	≤ 14	15 – 18	≥ 19
Penicillin (PE)	10 unit	≤ 19	20 – 27	≥ 28
Tetracycline (TC)	30	≤ 14	15 - 18	≥ 19

1.2.4 Optimal culture conditions of probiotics candidate strains

1.2.4.1 Optimal culture conditions according to pH

In order to confirm the degree of growth activity of the probiotics candidate strains according to the pH variation, pH was adjusted to 4 to 9 with 1 M HCl and 1 M NaOH in MRSB and then sterilized at 121℃ for 15 minutes. The prepared broth medium was inoculated with 1% of the pre-culture solution of the probiotics candidate strains and cultured at 35℃ and 200 rpm for 48 hours, then the turbidity of the culture solution was measured at 660 nm (Jang., 2012).

1.2.4.2 Optimal culture conditions according to temperature

In order to confirm the degree of growth activity of the probiotics candidate strains according to the temperature variation, 1% of the pre-culture solution was inoculated in the MRSB and incubated at 10℃, 20℃, 30℃, 40℃, 200 rpm after cultured for 48 hours, then the turbidity of the culture solution was measured at 660 nm (Jang., 2012).

1.2.4.3 Optimal culture conditions according to carbon source

In order to confirm the degree of growth activity of the probiotics candidate strains according to the culture medium composition, at least the growth medium GY (glucose 0.5%, yeast extract 0.1%, $MgSO_4 \cdot 7H_2O$ 0.02%) medium was treated with a representative carbon source dextrine, saccharose, sorbitol were added in an amount of 1% each and cultured at 35℃, 200 rpm for 48 hours, and the turbidity of the culture solution was measured at 660 nm, in the control group, a medium not containing glucose was used in the GY medium (Park et al., 1995; Sung et al., 2002).

1.2.4.4 Optimal culture conditions according to nitrogen source

In order to confirm the degree of growth activity of the probiotics candidate strains according to the culture medium composition, at least the growth medium GY medium was treated with a representative nitrogen source malt extract, peptone, yeast extract were added in an amount of 0.5% each and cultured at 35℃, 200 rpm for 48 hours, and the turbidity of the culture solution was measured at 660 nm, in the control group, a medium not containing yeast extract was used in the GY medium (Sung et al., 2002; OK and Choi., 2005).

1.2.4.5 Optimal culture conditions according to mineral source

In order to confirm the degree of growth activity of the probiotics candidate strains according to the culture medium composition, at least the growth medium GY medium was treated with a representative mineral source KH_2Po_4, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$ were added in an amount of 0.1% each and cultured at 35℃, 200 rpm for 48 hours, and the turbidity of the culture solution was measured at 660 nm, in the control group, a medium not containing $MgSO_4 \cdot 7H_2O$ was used in the GY medium (Ok and Choi., 2005; Kang et al., 1994).

1.2.5 Resistance analysis of probiotics candidate strains

1.2.5.1 Resistance to artificial gastric juice

To investigate resistance to artificial gastric juice each strain was diluted with 10^8 CFU/ml in a MRSB of pH 2.0 to 7.0 prepared by correcting 0.005 M sodium phosphate buffer with HCl utilizing the isolated probiotics candidate strains after inoculation it was allowed to stand at room temperature for 30 minutes, streaked on MRSA to measure number of bacteria was counted, and it was considered that the survival rate was not lower by 10 times or more as positive (Walker and Gilliland., 1993).

1.2.5.2 Resistance to artificial bile acids

To investigate the resistance to artificial bile acid each strain was diluted with 10^8 CFU/ml in a MRSB of 1 to 3% bile acid using the isolated probiotics candidate strains, it was inoculated at room temperature after standing for 30 minutes, it streaked on MRSA to measure the number of bacteria was counted, and it was considered that the survival rate was not lower by 10 times or more as positive (Kim et al., 2017).

1.2.5.3 Resistance to salt tolerance

To investigate the resistance to resistance to slat tolerance each strain was diluted with 10^8 CFU/ml in a MRSB of 0~50 ppt (Part Per Thousand) NaCl using a probiotics candidate strains it was inoculated at room temperature after standing for 30 minutes, it streaked on MRSA to measure the number of bacteria was counted, and it was considered that the survival rate was not lower by 10 times or more as positive (Kim et al., 2017).

1.2.5.4 Resistance to heart resistance

To investigate the resistance to resistance to heart tolerance each strain was diluted with 10^8 CFU/ml in a MRSB of probiotics candidate strains it was inoculated at room temperature after standing for water bath 60℃, 30 minutes, it streaked on MRSA to measure the number of bacteria was counted, and it was considered that the survival rate was not lower by 10 times or more as positive (Kim et al., 2017).

1.2.6 Probiotics strain selection

Confirmed the characteristics that probiotics strain should have through experiments on antibiotic susceptibility, artificial gastric juice, resistance to artificial bile acid, salt tolerance, heat resistance, optimal culture conditions, the greatest activity strain is selected.

1.2.7 Isolated of probiotics strain

1.2.7.1 Phylogenetic analysis

The nucleotide sequence analyzed confirmed the sequence shown in the nearest species as compared with the similar nucleotide of BLAST (Basic Local Alignment Search Tool, http://www.ncbi.nlm.nig.gov/blast) of NCBI (National Center Biotechnology Information). Multiple alignments of the nucleotide sequence of the isolated probiotics candidate strains and the base sequence indicated by BLAST, using the Clustal X program contained in Mega 7.0 software. Creation of tree topology was created by neighbor-joining method. For reliability evaluation of tree topology, performed bootstrap analysis applying 1,000 replications (Kim et al., 2017).

1.2.7.2 Morphological analysis

In order to investigate the morphological characteristics of the strain were observed using SEM (Field Emission Scanning Electron Microscope, JSM-6700F, JEOL LTd.). A membrane filter (Adventech., USA) in a agar medium, and a strain was streaked on the membrane filter using a loop. Pretreatment was carried out before the colonies of the strains were formed. In the pretreatment, filter was cut to 0.5 x 0.5 cm and then placed in 25% glutaraldehyde solution and fixed for 1 hour. Next it was washed twice with 1 M Phosphate buffer. After washed with water, the cut samples were dehydrated for 1 hour with 40, 50, 60, 70, 80, 90, 100% Ethyl Alcohol respectively. The dehydrated sample was immersed in a 1:1 mixed solution of isoamyl acetate and 100% ethyl alcohol for 1 hour and then left in a 100% isoamyl acetate solution for 1 hour. Finally it was dried using CO_2 gas, platinum was treated and observed using a sheet discharge SEM (Jang., 2012).

1.2.7.3 Biochemical analysis

In order to investigate biochemical characteristics, experiments were carried out using API 50 CHB kit (Bio Meriuex Co., France). MRSB was inoculated with the probiotics strain and cultured 30℃ for 48 hours and API 50 CHB kit was used according to the manufacture`s standard experimental method. Experimental results were obtained by incubating at 30℃ for 48 hours then readed the change in color (Jang., 2012).

1.2.8 Isolated and purification of bacteriocin from probiotics strain

Culture probiotics strain in MRSB was centrifuged at 12,000 rpm at 4℃ for 20 min then filtered with 0.2 um Nylon membrane filter (Advantech., USA) used a supernatant. Ammonium sulfate (Duksan., Korea) was added to the filtered supernatant at a concentration 50% (v/v) and allowed to stand at 4℃ for 12 hours followed by centrifuged at 12,000 rpm at 4℃ for 20 min, after recovering the precipitate, it was dissolved used 10 mM sodium phosphate buffer. Sephadex G-50 (Sigma., USA) was eluted from column with 10 mM sodium phosphate buffer at a rate 0.16 ml/min and fractionated 1 ml at a time (Sutyak et al., 2008).

1.2.9 Tricine SDS-PAGE

In order to measure the molecular weight of bacteriocin, tricine SDS-PAGE was performed. The Gel concentration was used at 12%, and the standard protein was pre-stanine Protein Marker (Elpis biotech., Korea). The purified sample was mixed with sample buffer at a ratio of 2:1 and heated at 100℃ for 3 minutes for electrophoresis. Electrophoresis running buffer (Tris base 15 g, Glycine 72 g, SDS 5 g, D.W 1 L) was diluted with 1X running buffer. The electrophoresed gel was stained with a staining solution (Coomassie brilliant blue R-250 (CBB R-250, Thermo scientific., USA) 2.0 g, Methanol (Sigma-Aldrich., USA) 400 ml, Acetic-acid (Sigma-Aldrich., USA) 100 ml, D.W 500 ml) for 30 minutes. Thereafter, it was decolorized with a Destaining solution (Methanol 400 ml, Acetic-acid 100 ml, Giycerine 50 ml, D.W 1,450 ml) and then washed with sterile distilled water for 6 hours (Kim et al., 2004).

1.2.10 Statistical analysis

All the tests were performed in triplicate. The data were subjected to statistical analysis using the SPSS (SPSS INC, version 18.0., USA). The statistical analysis was done by using one-way analysis of variance (ANOVA) followed by Tukey`s test ($P<0.05$) to compare the means between individual treatments. Result values were expressed as mean ± standard deviation (mean ± S.D) and percentage values were calculated by arcsine deformation value and statistically analyzed.

1.3 **RESULT**

1.3.1 Probiotics candidate strains

Eight species of probiotics candidate strains were isolated from Jeju Island traditional fermented foods: Doenjang, Saeujeot, fermented Squid, salted Damselfish, salted guts of Hairtail, salted Gizzard shad, salted Spratelloides gracilis. 1 species in fermented squid, 1 species in salted guts of Hairtail, 2 species in Doenjang, 2 species salted Damselfish, 2 species in salted Gizzard shad (Table 4). The isolated strains showed a stable growth MRSA at a culture temperature of 30 to 35℃, and were named JFP-01 to JFP-08.

Table 4. Culture conditions of isolated probiotics candidate strains from Jeju Island traditional foods.

Strain No.	Isolated source	Isolated temperature	Medium
JFP-01	Fermented squid	30℃	MRSA
JFP-02	Salted guts of Hairtail	35℃	MRSA
JFP-03	Doenjang	35℃	MRSA
JFP-04	Salted Damselfish	30℃	MRSA
JFP-05	Doenjang	35℃	MRSA
JFP-06	Salted Damselfish	35℃	MRSA
JFP-07	Salted Gizzard shad	30℃	MRSA
JFP-08	Salted Gizzard shad	35℃	MRSA

1.3.2 Sequence of probiotics candidate strains

The nucleotide sequence of the strains isolated by BLAST of NCBI was confirmed. JFP-01 isolated from fermented squid showed more than 99% homology with *Pediococcus acidilactici* of accession no. AB598949. JFP-02 isolated from Salted guts of Hairtail showed more than 99% homology with *Bacillus amyloliquefaciens* of accession no. KY773617. JFP-03 isolated from Doenjang showed more than 99% homology with *Lactobacillus parabuchneri* of accession no. AB368914. JFP-04 isolated from Salted Damselfish showed more than 100% homology with *Enterococcus durans* of accession no. KF768355. JFP-05 isolated from Doenjang showed more than 99% homology with *Pediococcus pentosaceus* of accession no. KF111710. JFP-06 isolated from Salted Damselfish showed more than 95% homology with *Lactobacillus sakei* of accession no. JX402124. JFP-07 isolated from Salted Gizzard shad showed more than 98% homology with *Lactobaccillus plantarum* of accession no. KF929420. JFP-08 isolated from Salted Gizzard shad showed more than 99% homology with *Lactobacillus brevis* of accession no. JQ063468 (Table 5).

Table 5. BLAST results of isolated probiotics candidate strains from Jeju Island traditional foods.

Strain No.	BLAST results		
	Accession No.	Species	Identities
JFP-01	AB598949	*Pediococcus acidilactici*	1516/1520 (99%)
JFP-02	KY773617	*Bacillus amyloliquefaciens*	1485/1487 (99%)
JFP-03	AB368914	*Lactobacillus parabuchneri*	1042/1050 (99%)
JFP-04	KF768355	*Enterococcus durans*	1007/1007 (100%)
JFP-05	KF111710	*Pediococcus pentosaceus*	951/952 (99%)
JFP-06	JX402124	*Lactobacillus sakei*	765/806 (95%)
JFP-07	KF929420	*Lactobacillus plantarum*	897/911 (98%)
JFP-08	JQ063468	*Lactobacillus brevis*	1019/1021 (99%)

1.3.3 Antibiotics susceptibility of probiotics candidate strains

Susceptibility tests of eight kinds of antibiotics were conducted using probiotics candidate strains. As a result, JFP-01, JFP-02 and JFP-04 showed sensitivity to 8 kinds of antibiotics, and JFP-05 were sensitive to seven kinds of antibiotics except tetracycline. JFP-07 was resistant to 6 antibiotics except gentamicin, flofenicol, JFP-03, JFP-06, JFP-08 showed resistance to 8 antibiotics (Table 6, Fig. 2). JFP-03, JFP-06, JFP- 07, JFP-08 which showed multiple drug resistance were isolated from Doenjang, Salted Damselfish, Salted Gizzard shad and its considered that the mutation of the strains was carried out during the culture. However for a more accurate cause, analysis of the gene plasmid of the strains isolated from the same sample as the separated isolate multiple drug resistance strains were considered to be necessary.

Table 6. Antibiotics susceptibility test of probiotics candidate strains from Jeju Island traditional foods.

(Inhibition zone (mm))

Probiotics / Antibiotics	JFP-01	JFP-02	JFP-03	JFP-04	JFP-05	JFP-06	JFP-07	JFP-08
AML	48	17	-	17	20	-	-	-
GN	34	41	-	14	15	-	26	-
EM	45	27	-	8	28	-	-	-
FFL	37	30	-	23	20	-	25	-
NEO	30	21	-	10	15	-	-	-
OTC	40	11	-	25	12	-	-	-
PE	55	17	-	50	32	-	-	-
TC	38	17	-	23	-	-	-	-

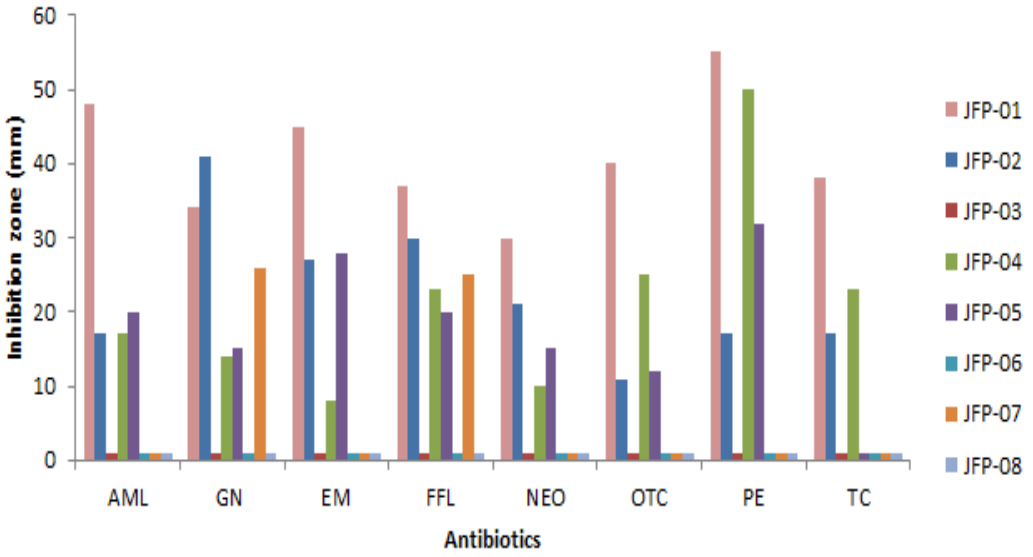


Fig. 2. Antibiotics susceptibility test of probiotics candidate strains from Jeju Island traditional foods.

1.3.4 Establish of optimal culture conditions for probiotics candidate strains

1.3.4.1 Establish of optimal culture conditions according to pH

As a result of investigating the growth activity showed at the time of cultured at 35℃ for 48 hours by modified the pH to range of 4 ~ 9 using the probiotics candidate strains, it was confirmed that in the order of JFP-07, JFP-05, JFP-06 its showed the highest growth activity (Fig. 3). In contrast, JFP-02, JFP-02 and JFP-04 showed low growth activity in this order. At pH 4, pH 5 all eight strains showed low growth activity, while JFP-05, JFP-08 showed the highest growth activity at pH 6. JFP-03, JFP-07 showed the highest growth activity at pH 7, JFP-01, JFP-02, JFP-04, JFP-06 showed the highest growth activity at pH 9. In these results, we confirmed the simple growth activity according to pH change, so we will mention in part. 2 of the production of antimicrobial activity substances according to pH change.

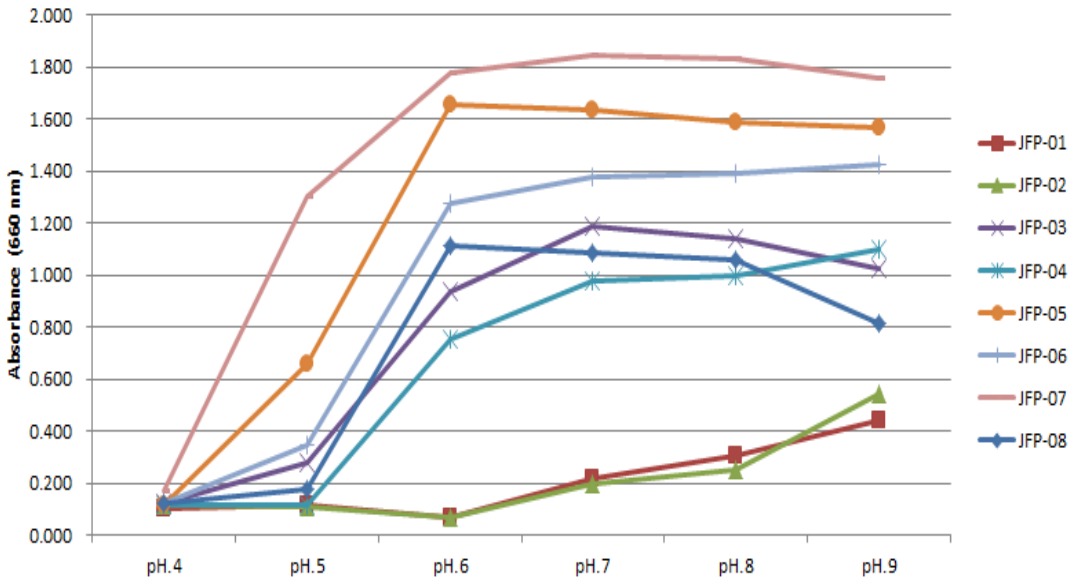


Fig. 3. Effect of pH on cell growth of probiotics candidate strains from Jeju Island traditional foods.

1.3.4.2 Establish of optimal culture conditions according to temperature

The probiotics candidate strains were used to modified the temperature at 10℃, 20℃, 30℃, 40℃ and the growth activity showed at the time of cultured for 48 hours was examined, as a result the growth activity of eight strains at 10℃ was incomplete (Fig. 4). JFP-07 had the highest growth activity at 20℃ and JFP-01 had the highest growth activity at 40℃. JFP-02, JFP-03, JFP-04, JFP-05, JFP-06 showed the highest growth activity at 30℃. In these results, we confirmed simple growth activity according to temperature change, so we will mention in part. 2 of the production of antimicrobial activity substances according to temperature change.

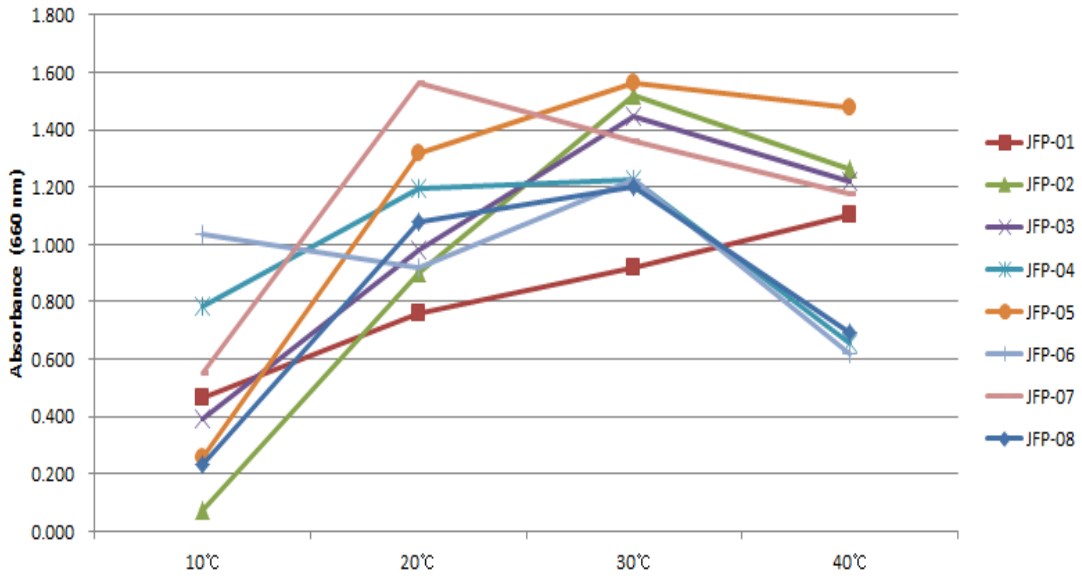


Fig. 4. Effect of temperature on cell growth of probiotics candidate strains from Jeju Island traditional foods.

1.3.4.3 Establish of optimal culture conditions according to carbon source

Growth activity of probiotics candidate strains were examined by adding 1% each of dextrine, saccharose and sorbitol which are representative carbon source, to GY medium to which glucose was not added. Seven strains except JFP-02 showed the highest growth activity (Fig. 5A) when dextrin was added and the growth activity of JFP-02 was highest when saccharose was added (Fig. 5B). In the case of sorbitol most strains showed highest growth activity in GY medium without added of sorbitol (Fig. 5C). Growth activity of eight probiotics candidate strains increased when dextrine, saccharose was added to GY medium and it was confirmed that when sorbitol was added, the growth activity of probiotics candidate strain was inhibited.

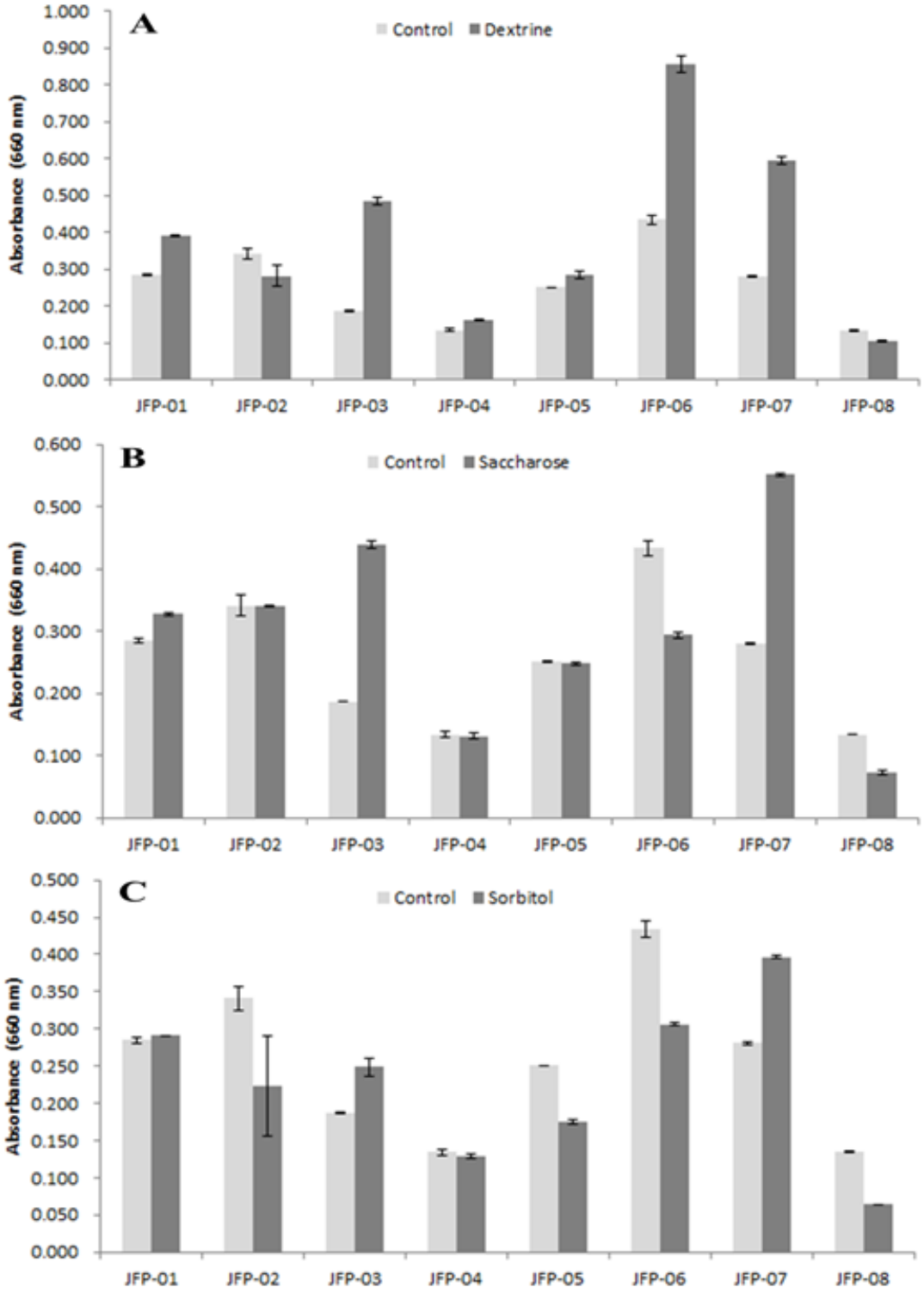


Fig. 5. Effect of carbon source in Dextrine (A), Saccharose (B), Sorbitol (C) on cell growth of probiotics candidate strains from Jeju Island traditional foods.

1.3.4.4 Establish of optimal culture conditions according to nitrogen source

Growth activity of probiotics candidate strains were examined by added 0.5% of representative nitrogen source malt extract, peptone, yeast extract to GY medium to which yeast extract was not added. JFP-03, JFP-04, JFP-05, JFP-06, JFP-07 showed the highest growth activity when peptone was added to GY medium (Fig. 6B), JFP-01, JFP-02, JFP-08 showed the highest growth activity when yeast extract was added (Fig. 6C). Conversely, in the group to which malt extract was added, it was confirmed that the growth activity remarkably decrease as compared to the GY medium (Fig. 6A). As a result, it was confirmed when peptone and yeast extract were added to the culture medium of the isolated probiotics candidate strains, the growth activity was increased although there was a difference between the strains.

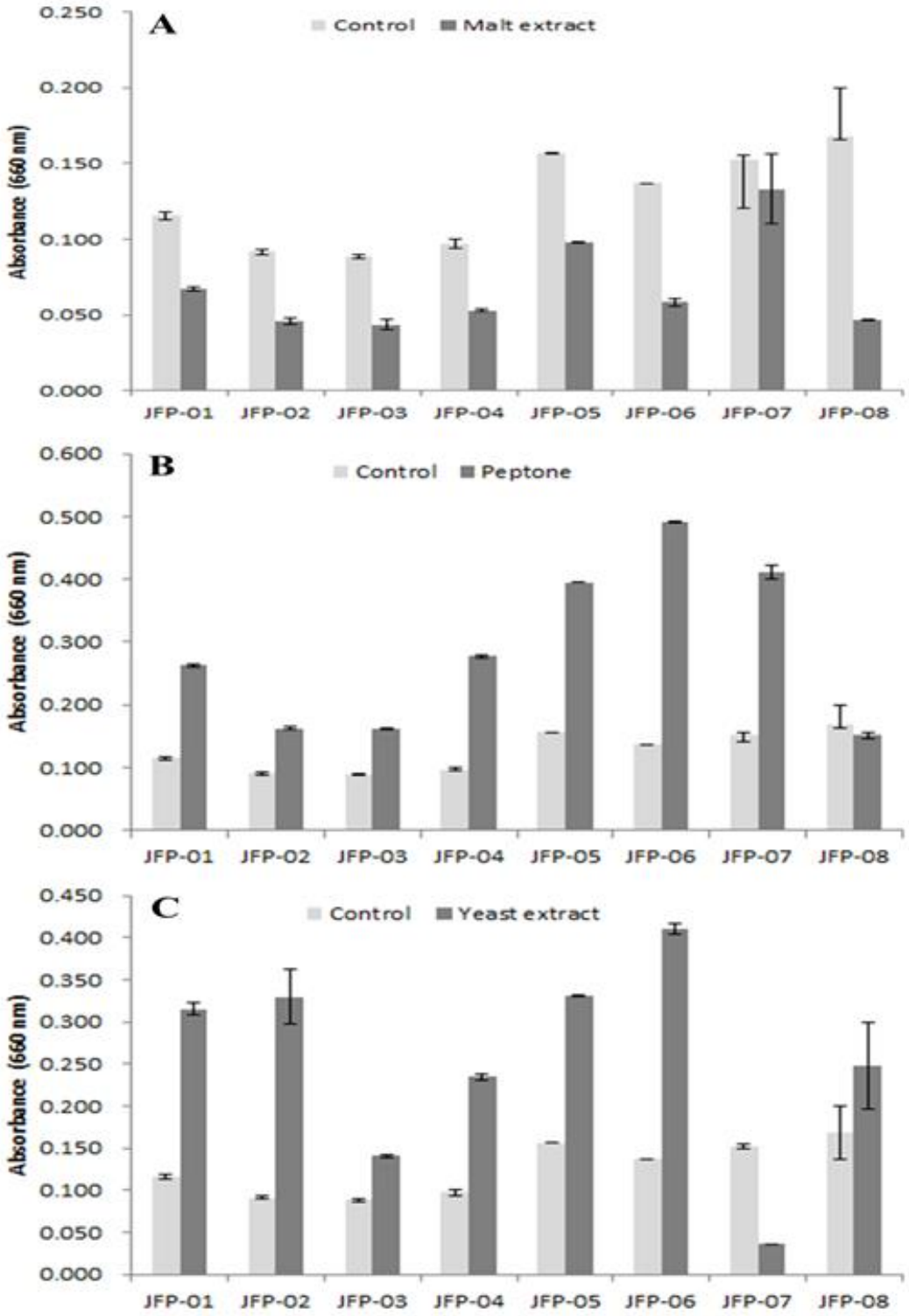


Fig. 6. Effect of nitrogen source in Malt extract (A), Peptone (B), Yeast extract (C) on cell growth of probiotics candidate strains from Jeju Island traditional foods.

1.3.4.5 Establish of optimal culture conditions according to mineral source

Growth activity of probiotics candidate strains were examined by added 0.1% each of representative mineral source KH_2Po_4, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$ to GY medium to which $MgSO_4 \cdot 7H_2O$ was not added. The growth activity of the probiotics candidate strains which can be seen in the medium supplemented with KH_2Po_4 was not significantly different from the control which is the control medium (Fig. 7A). JFP-01, JFP-02, JFP-03, JFP-04, JFP-06, JFP-07 showed the maximum growth activity when $MgSO_4 \cdot 7H_2O$ was added to GY medium (Fig. 7B), JFP-05 and JFP-08 showed the maximum growth activity when $FeSO_4 \cdot 7H_2O$ was added to GY medium (Fig 7C). As a result, it was confirmed when $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$ were added to the culture medium of the isolated probiotics candidate strains, there was a difference between the strains, but the growth activity increased.

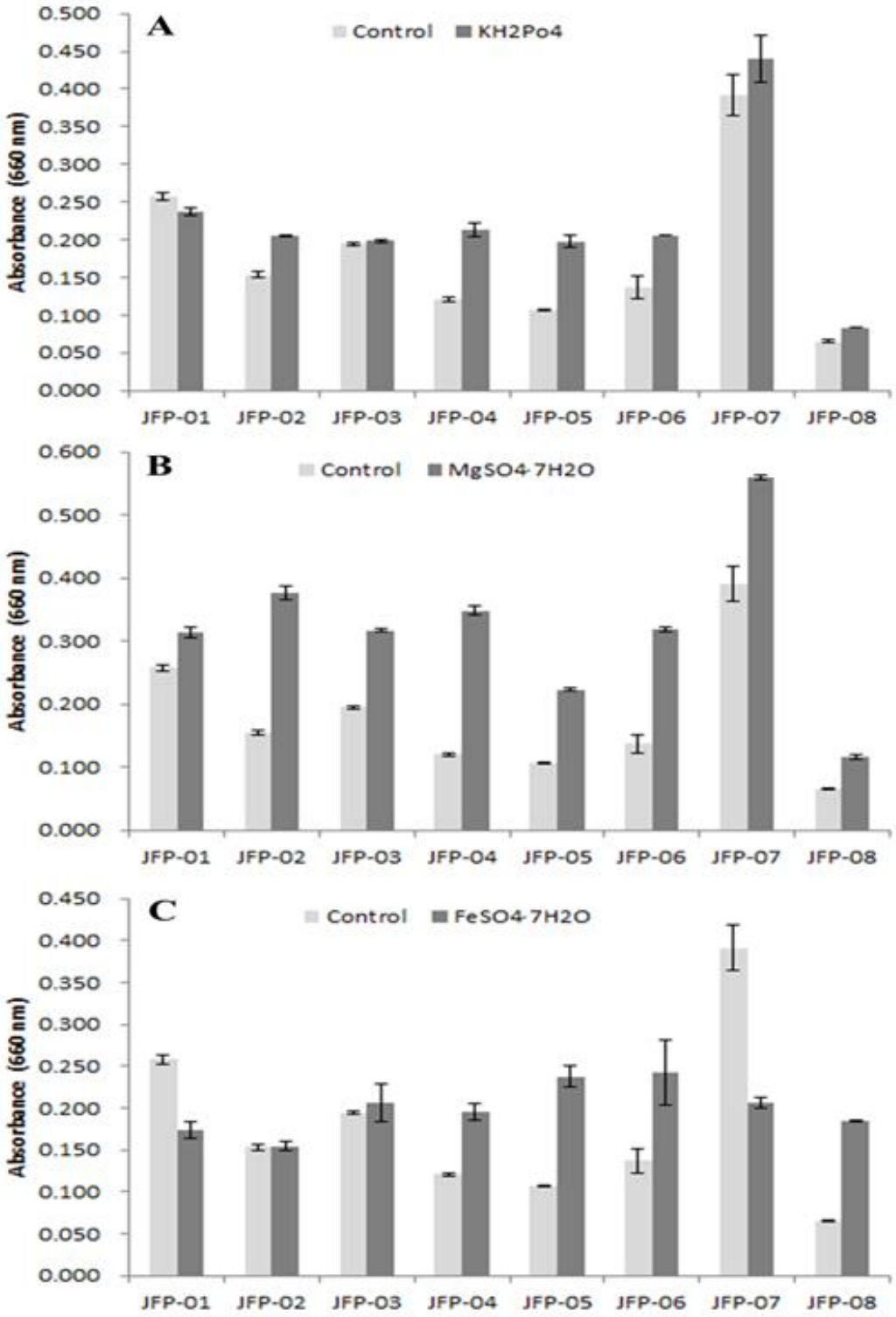


Fig. 7. Effect of mineral source in KH_2Po_4 (A), $MgSO_4 \cdot 7H_2O$ (B), $FeSO_4 \cdot 7H_2O$ (C) on cell growth of probiotics candidate strains from Jeju Island traditional foods.

1.3.5 Establish of resistance conditions of probiotics candidate strains

Resistance to artificial gastric juice, artificial bile acid, salt tolerance and heat resistance were confirmed using probiotics candidate strains (Table 7). In the case of resistance of artificial gastric juice at other concentrations except 2% positive eight strains showed whereas at 2% only JFP-02 and JFP-07 showed resistance. In the case of resistance to artificial bile acid except for 3% JFP-01, it showed both 1% and 2% resistance. In case of salt tolerance, 1%, 5% and 10% all showed resistance and heat resistance at 60℃ only JFP-02 and JFP-07 showed resistance.

Table 7. Resistance analysis of probiotics candidate strains from Jeju Island traditional foods.

Source / Probiotics	Artificial Gastric juice						Artificial bile acid			Salt tolerance			Heat resistance
	2%	3%	4%	5%	6%	7%	1%	2%	3%	1%	5%	10%	60℃
JFP-01	-	+	+	+	+	+	+	+	-	+	+	+	-
JFP-02	+	+	+	+	+	+	+	+	+	+	+	+	+
JFP-03	-	+	+	+	+	+	+	+	+	+	+	+	-
JFP-04	-	+	+	+	+	+	+	+	+	+	+	+	-
JFP-05	-	+	+	+	+	+	+	+	+	+	+	+	-
JFP-06	-	+	+	+	+	+	+	+	+	+	+	+	-
JFP-07	+	+	+	+	+	+	+	+	+	+	+	+	+
JFP-08	-	+	+	+	+	+	+	+	+	+	+	+	-

1.3.6 Probiotics strain selection

It has resistance to artificial gastric juice, artificial bile acid, salt tolerance, heat resistance, confirms the characteristics that probiotics should have and confirm the optimum culture conditions *Bacillus amyloliquefaciens* JFP-02, it was selected as a strain and using for future experiment. The optimal culture conditions were the highest growth activity by added saccharose as a carbon source, yeast extract as a nitrogen source, $MgSO_4 \cdot 7H_2O$ as a mineral source to MRS medium at pH 9 and 30℃.

1.3.7 Isolated of *Bacillus amyloliquefaciens* JFP-02

1.3.7.1 Phylogenetic characteristics

As a result of DNA PCR of *B. amyloliquefaciens* JFP-02 strain, a nucleotide sequence of 1,486 bp was obtained (Fig. 8). In addition, this isolated strain was genetically registered to NCBI and received and accession number of KM062020. Analysis of the nucleotide sequence of 1,486 bp used BLAST of NCBI showed homology of 99% or more with *B. amyloliquefaciens* and showed 99% homology with *Bacillus velezensis* and *Bacillus vallismortis*. Phylogenetic tree was created with the 16S rRNA base sequence of *Bacillus* spp. Most closely related strain and *B. amyloliquefaciens* JFP-02 (Fig. 9), and *Bacillus amyloliquefaciens* F10-1 over 99% showed homology (Table 8). However, *B. amyloliquefaciens* F10-1 was isolated from putrefied potatoes in China, and its considered that there is a problem that its guess that same strain, because it has different biochemical characteristics from *B. amyloliquefaciens* JFP-02.

001 GCTCAGGACG AACGCTGGCG GCGTGCCTAA TACATGCAAG TCGAGCGGAC
051 AGATGGGAGC TTGCTCCCTG ATGTTAGCGG CGGACGGGTG AGTAACACGT
101 GGGTAACCTG CCTGTAAGAC TGGGATAACT CCGGGAAACC GGGGCTAATA
151 CCGGATGGTT GTCTGAACCG CATGGTTCAG ACATAAAAGG TGGCTTCGGC
201 TACCACTTAC AGATGGACCC GCGGCGCATT AGCTAGTTGG TGAGGTAACG
251 GCTCACCAAG GCGACGATGC GTAGCCGACC TGAGAGGGTG ATCGGCCACA
301 CTGGGACTGA GACACGGCCC AGACTCCTAC GGGAGGCAGC AGTAGGGAAT
351 CTTCCGCAAT GGACGAAAGT CTGACGGAGC AACGCCGCGT GAGTGATGAA
401 GGTTTTCGGA TCGTAAAGCT CTGTTGTTAG GGAAGAACAA GTGCCGTTCA
451 AATAGGGCGG CACCTTGACG GTACCTAACC AGAAAGCCAC GGCTAACTAC
501 GTGCCAGCAG CCGCGGTAAT ACGTAGGTGG CAAGCGTTGT CCGGAATTAT
551 TGGGCGTAAA GGGCTCGCAG GCGGTTTCTT AAGTCTGATG TGAAAGCCCC
601 CGGCTCAACC GGGGAGGGTC ATTGGAAACT GGGGAACTTG AGTGCAGAAG
651 AGGAGAGTGG AATTCCACGT GTAGCGGTGA AATGCGTAGA GATGTGGAGG
701 AACACCAGTG GCGAAGGCGA CTCTCTGGTC TGTAACTGAC GCTGAGGAGC
751 GAAAGCGTGG GGAGCGAACA GGATTAGATA CCCTGGTAGT CCACGCCGTA
801 AACGATGAGT GCTAAGTGTT AGGGGGTTTC CGCCCCTTAG TGCTGCAGCT
851 AACGCATTAA GCACTCCGCC TGGGGAGTAC GGTCGCAAGA CTGAAACTCA
901 AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGCATGTG GTTTAATTCG
951 AAGCAACGCG AAGAACCTTA CCAGGTCTTG ACATCCTCTG ACAATCCTAG
1001 AGATAGGACG TCCCCTTCGG GGGCAGAGTG ACAGGTGGTG CATGGTTGTC
1051 GTCAGCTCGT GTCGTGAGAT GTTGGGTTAA GTCCCGCAAC GAGCGCAACC
1101 CTTGATCTTA GTTGCCAGCA TTCAGTTGGG CACTCTAAGG TGACTGCCGG
1151 TGACAAACCG GAGGAAGGTG GGGATGACGT CAAATCATCA TGCCCCTTAT
1201 GACCTGGGCT ACACACGTGC TACAATGGAC AGAACAAAGG GCAGCGAAAC
1251 CGCGAGGTTA AGCCAATCCC ACAAATCTGT TCTCAGTTCG GATCGCAGTC
1301 TGCAACTCGA CTGCGTGAAG CTGGAATCGC TAGTAATCGC GGATCAGCAT
1351 GCCGCGGTGA ATACGTTCCC GGGCCTTGTA CACACCGCCC GTCACACCAC
1401 GAGAGTTTGT AACACCCGAA GTCGGTGAGG TAACCTTTAT GGAGCCAGCC
1451 GCCGAAAGTG GGACAGATGA TTGGGGTGAA GTCTAA

Fig. 8. 16S rRNA sequence of the *B. amyloliquefaciens* JFP-02.

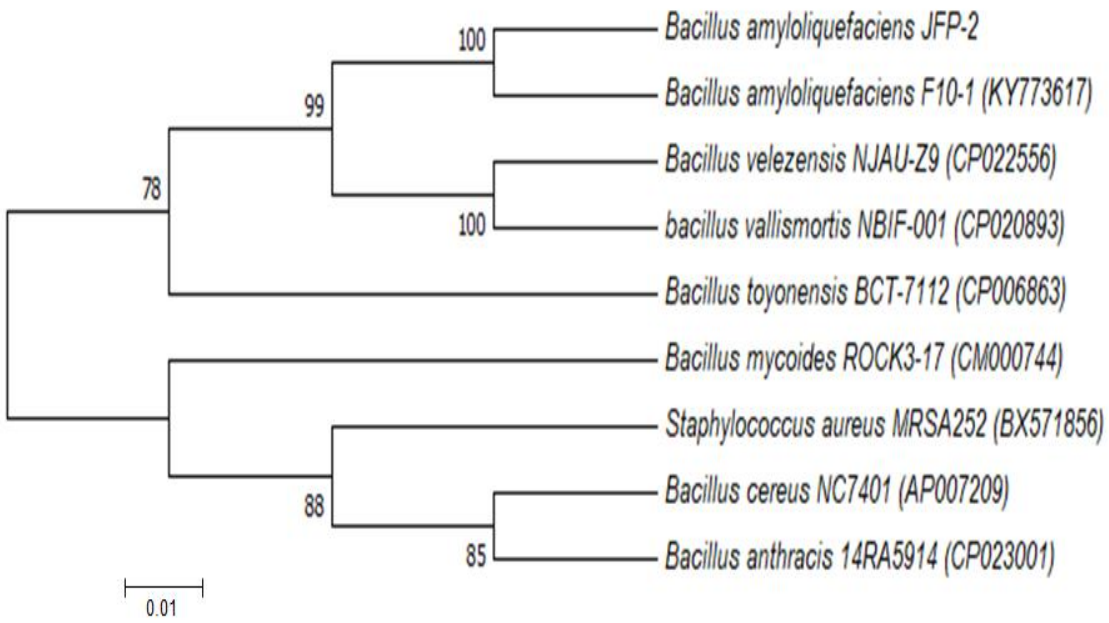


Fig. 9. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain JFP-02 within the radiation of the genus *Bacillus* spp. Bootstrep percentage (From 1,000 replications)>50% are shown at branch points. Bar, 0.01 substitutions per nucleotide position.

Table 8. Sequence similarity between *B. amyloliquefaciens* JFP-02 and *B. amyloliquefaciens* F10-1. (*) - same nucleotide; (-) - sequence not available, different nucleotide.

```
JFP-02  GCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATG
F10-1   GCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATG
        ************************************************************

JFP-02  GGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTG
F10-1   GGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTG
        ************************************************************

JFP-02  CCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTCTG
F10-1   CCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTCTG
        ************************************************************

JFP-02  AACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCC
F10-1   AACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCC
        ************************************************************

JFP-02  GCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGC
F10-1   GCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGC
        ************************************************************

JFP-02  CGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC
F10-1   CGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC
        ************************************************************

JFP-02  GGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGC
F10-1   GGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGC
        ************************************************************

JFP-02  CGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAA
F10-1   CGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAA
        ************************************************************

JFP-02  GTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTA
F10-1   GTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTA
        ************************************************************

JFP-02  ACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTAT
F10-1   ACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATT
        ************************************************************

JFP-02  GGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTC
F10-1   GGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTC
        ************************************************************
```

Table 8. Sequence similarity between *B. amyloliquefaciens* JFP-02 and *B. amyloliquefaciens* F10-1. (*) same nucleotide, (-) : sequence not available, different nucleotide.

```
JFP-02  AACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGA
F10-1   AACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGA
        ************************************************************

JFP-02  ATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAG
F10-1   ATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAG
        ************************************************************

JFP-02  GCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAG
F10-1   GCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAG
        ************************************************************

JFP-02  GATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGG
F10-1   GATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGG
        ************************************************************

JFP-02  TTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACG
F10-1   TTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACG
        ************************************************************

JFP-02  GTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCA
F10-1   GTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCA
        ************************************************************

JFP-02  TGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGA
F10-1   TGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGA
        ************************************************************

JFP-02  CAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGT
F10-1   CAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGT
        ************************************************************

JFP-02  TGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
F10-1   TGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
        ************************************************************

JFP-02  TTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAA
F10-1   TTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAA
        ************************************************************

JFP-02  ACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTA
F10-1   ACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTA
        ************************************************************
```

Table 8. Sequence similarity between *B. amyloliquefaciens* JFP-02 and *B. amyloliquefaciens* F10-1. (*) same nucleotide, (-) : sequence not available, different nucleotide.

```
JFP-02  CACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAA
F10-1   CACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAA
        **************************************************

JFP-02  TCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGC
F10-1   TCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGC
        **************************************************

JFP-02  TGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCT
F10-1   TGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCT
        **************************************************

JFP-02  TGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGT
F10-1   TGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGT
        **************************************************

JFP-02  AACCTTTATGGAGCCAGCCGCCGAAAGTGGGACAGATGATTGGGGTGAAGTC-TA
F10-1   AACCTTTATGGAGCCAGCCGCCGAAGGTGGGACAGATGATTGGGGTGAAGTCGTA
        ************************* ************************* **

JFP-02  A
F10-1   A
        *
```

1.3.7.2 Morphological characteristics

B. amyloliquefaciens JFP-02 was cultured in MRSA medium to confirm the morphological characteristics (Fig. 10). The shape of colony was uneven, the center of colony was convex, and loop draws a considerable viscosity when taken. As a result of observation used SEM, it was in the form of single bacillus, the length was 1.575 um, and the width was 0.727 um (Fig. 11).

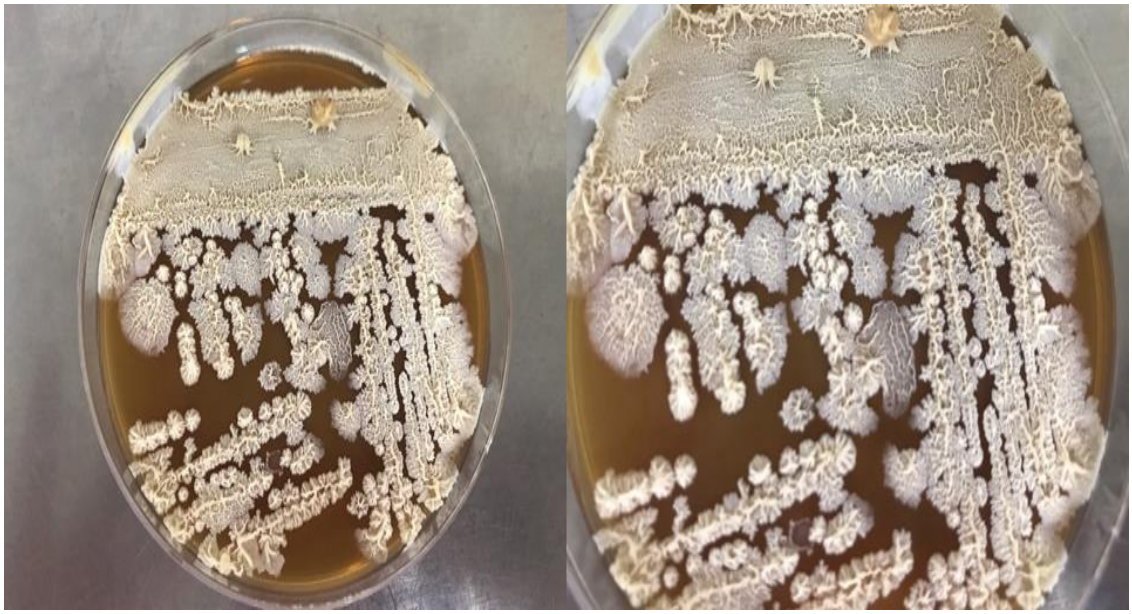


Fig. 10. Morphological characterization of *B. amyloliquefaciens* JFP-02. Cells were grown at 30℃ for 24hours.

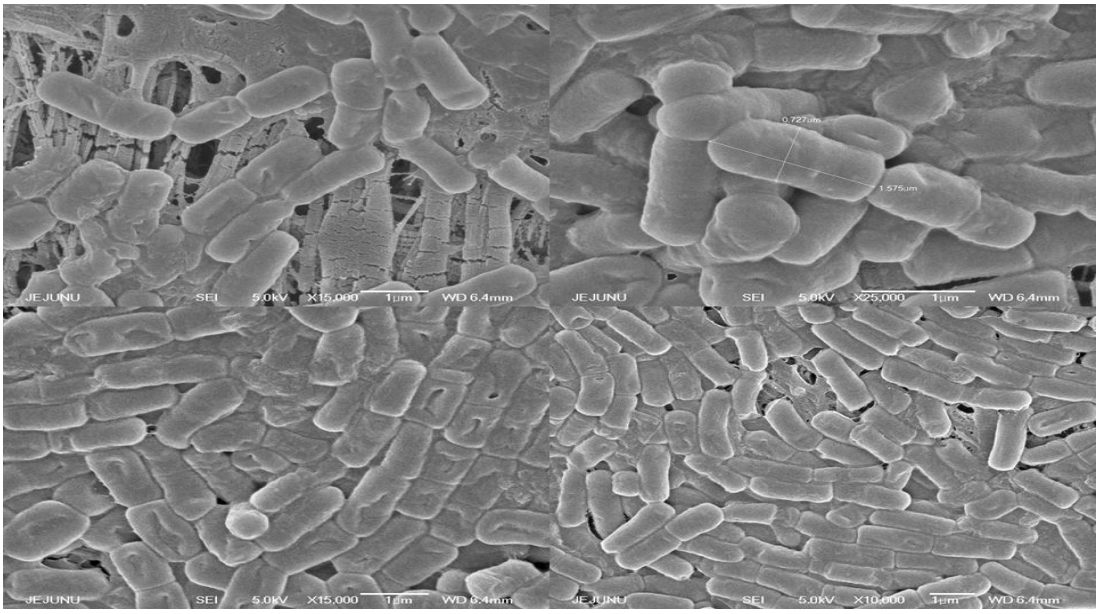


Fig. 11. Scanning electron micrograph of *B. amyloliqufaciens* JFP-02. Cells were grown at 30℃ for 24hours.

1.3.7.3 Biochemical characteristics

Carbohydrate sources an important role in antimicrobial substance production, and experiments were conducted used API 50 CHB kit in order to acidic production ability from glucose. As a result of investigating acidic production ability from around all 49 species, 26 kinds of glycerol and others showed a positive reaction, and 21 kinds such as erythritol and others showed a negative reaction (Fig. 12). Used API 50 CHB kit showed 98% similarity when compared with standard *B. amyloliquefaciens* (Table 9).

Fig. 12. Biochemical characteristics of different carbohydrate source in *B. amyloliquefaciens* JFP-02 isolated from Jeju Island traditional food by API 50 CHB.

Table 9. Biochemical characteristics of different carbohydrate source in *B. amyloliquefaciens* JFP-02 isolated from Jeju Island traditional food by API 50 CHB.

No.	Carbohydrate source	Utilization		No.	Carbohydrate source	Utilization	
		Standard	JFP-02			Standard	JFP-02
0	Control	-	-	25	Esculin	+	+
1	Glycerol	+	+	26	Salicin	+	+
2	Erythritol	-	-	27	Celibiose	+	+
3	D-arabinose	-	-	28	Maltose	+	+
4	L- arabinose	+	+	29	Lactose	+	+
5	Ribose	+	+	30	Melibiose	+	+
6	D-xylose	+	+	31	Sucrose	+	+
7	L- xylose	-	-	32	Trehalose	+	+
8	Adonitol	-	-	33	Inulin	+	-
9	B-Methyl-D-Xylose	-	-	34	Melezitose	-	-
10	Galactose	+	+	35	Raffinose	+	+
11	Glucose	+	+	36	Starch	+	+
12	Fructose	+	+	37	Glycogen	+	+
13	Mannose	+	+	38	Xylitol	-	-
14	Sorbose	-	-	39	Gentiobiose	+	+
15	Rhamnose	-	-	40	D-turRanose	+	-
16	Dulcitol	-	-	41	D-lyxose	-	-
17	Inositol	+	+	42	D-tagatose	-	-
18	Mannitol	+	+	43	D-fucose	-	-
19	Sorbitol	+	+	44	L-fucose	-	-
20	α-Methyl-D-Mannoside	-	-	45	D-arabitol	-	-
21	α-Methyl-D-Glucoside	+	+	46	L-arabitol	-	-
22	N-Acetyl-Glucosamine	+	+	47	Gluconate	-	-
23	Amygdalin	+	+	48	2-Keto-Gluconate	-	-
24	Arbutin	+	+	49	5-Keto-Gluconate	-	-

1.3.8 Confirmation of bacteriocin by tricine SDS-PAGE

In order to measure the molecular weight of purified bacteriocin, it was confirmed used 12% tricine SDS-PAGE (Fig. 13). As a result of compared the molecular weight with the standard protein, it was confirmed that a band appeared around 37 kDa. Previous studies, the results of the research that the bacteriocin of *B. amyloliquefaciens* was around 37 kDa have been reported (Wang et al., 2016). It's conceivable that *B. amyloliquefaciens* JFP-02 also produces bacteriocin.

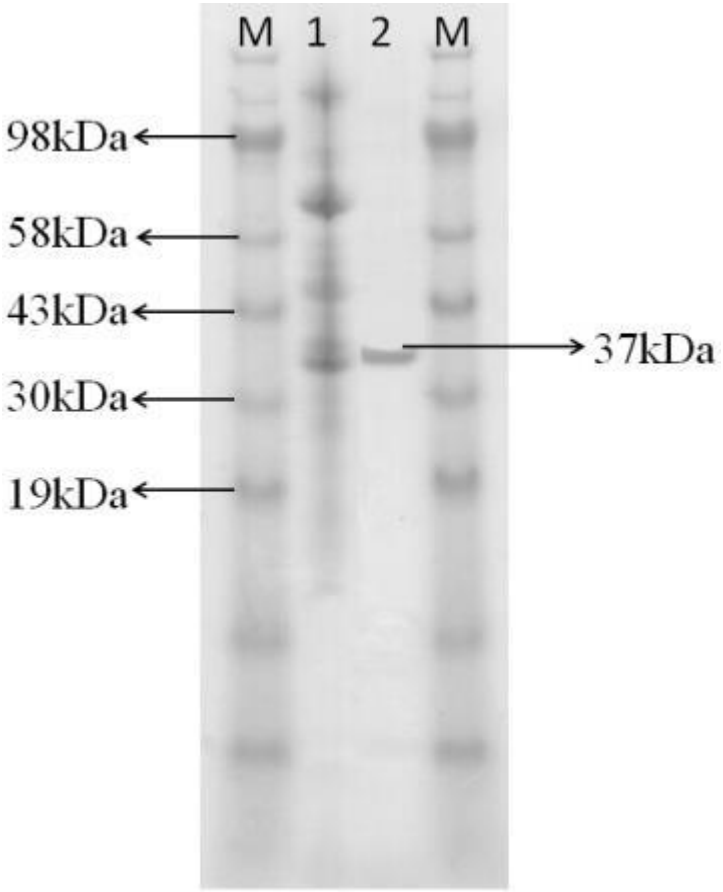


Fig. 13. SDS-PAGE of partially purified bacteriocin *B. amyloliquefaciens* JFP-02. Line (M) – protein size marker; Line (1) – Cell free culture supernatant; Line (2) – Purified bacteriocin JFP-02.

1.4 **DISCUSSION**

In chapter 1, we tried to confirm the characteristics by isolated probiotics strain from Jeju Island traditional fermented foods and to confirm the possibility of future using as probiotics. Eight species of probiotics candidate strains were isolated from Jeju Island traditional fermented foods: Doenjang, Saeujeot, fermented Squid, salted Damselfish, salted guts of Hairtail, salted Gizzard shad, salted Spratelloides gracilis. As a result of confirmed the nucleotide sequence of the isolated strains used BLAST of NCBI, it was identified by *pediococcus acidilactici*, *Bacillus amyloliquefaciens*, *Lactobacillus parabuchneri*, *Enterococcus durans*, *Pediococcus pentosaceus*, *Lactobacillus sakei*, *Lactobacillus plantarum*, *Lactobacillus brevis* (Table 5).

Susceptibility tests of eight kinds of antibiotics were conducted using probiotics candidate strains. If probiotics strain was resistant to antibiotics and used as a probiotic in the future, there is a possibility of harmful effect on the human body and fish body. All eight antibiotics used in this experiment were sensitive to both antibiotics of JFP-01, JFP-02, JFP-04 (Table 6, Fig. 2). The remaining probiotics candidate strains were resistant to at least one antibiotic at most and all eight. This is considered to have been mutated in the process of cultured the strains. However for a more accurate cause, analysis of the gene plasmid of the strains isolated from the same sample as the separated isolate multiple drug resistance strains were considered to be necessary.

The results of confirmed the optimum growth of pH of the probiotics candidate strains (Fig. 3), JFP-05, JFP-08 at pH 6, JFP-03, JFP-07 at pH 7, JFP-01, JFP-02, JFP-04, JFP-06 at pH 9 then showed the highest growth activity. In these results, we confirmed the simple growth activity according to pH change, so we will mention in part. 2 of the production of antimicrobial activity substances according to pH change. The results of confirmed the optimum growth of temperature of the probiotics candidate strains (Fig. 4), JFP-07 at 20℃,

JFP-01 at 40℃, JFP-02, JFP-03, JFP-04, JFP-05, JFP-06 at 30℃ then showed the highest growth activity. In these results, we confirmed simple growth activity according to temperature change, so we will mention in part. 2 of the production of antimicrobial activity substances according to temperature change.

As a result of confirmed the showed growth activity when carbon source, nitrogen, mineral source were added to the growth medium, for carbon source was confirmed that JFP-02 was saccharose (Fig. 5B), 7 kinds except for JFP-02 were dextrin, the growth activity was the highest when added (Fig. 5A). In case of nitrogen source, JFP-03, JFP-04, JFP-05, JFP-06, JFP-07 showed that when peptone was added (Fig. 6B), JFP-01, JFP-02, JFP-08 when yeast extract was added, its showed the highest growth activity (Fig. 6C). In case of mineral source, JFP-01, JFP-02, JFP-03, JFP-04, JFP-06, JFP-07 showed the highest growth activity when $MgSO_4 \cdot 7H_2O$ was added (Fig. 7B), JFP-05, JFP-08 showed the highest growth activity when $FeSO_4 \cdot 7H_2O$ was added (Fig. 7C). Bases on these results, probiotics candidate strains can expect highest growth activity when saccharose, dextrine, peptone, yeast extract, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$ were added to the medium. Resistance to artificial gastric juice, artificial bile acid, salt tolerance and heat resistance were confirmed as a result JFP-02, JFP-07 showed resistance to artificial gastric juice of 2% and heat resistance of 60℃ (Table 7). Accordingly the probiotics strain was selected as *Bacillus amyloliquefaciens* JFP-02.

As a result of DNA PCR of *B. amyloliquefaciens* JFP-02 strain, a nucleotide sequence of 1,486 bp was obtained (Fig. 8) and NCBI and received and accession number of KM062020. Phylogenetic tree was created with the 16S rRNA base sequence of *Bacillus* spp. Most closely related strain and *B. amyloliquefaciens* JFP-02 (Fig. 9), and *Bacillus amyloliquefaciens* F10-1 over 99% showed homology (Table 8). However, *B. amyloliquefaciens* F10-1 was isolated from putrefied potatoes in China, and its considered that there is a problem that its guess that same strain, because it has different biochemical

characteristics from *B. amyloliquefaciens* JFP-02.

B. amyloliquefaciens JFP-02 was the shape of colony was uneven, the center of colony was convex (Fig. 10) and loop draws a considerable viscosity when taken. As a result of observation used SEM, it was in the form of single bacillus, the length was 1.575 um, and the width was 0.727 um (Fig. 11). Biochemical characteristics API 50 CHB kit of investigating acidic production ability from around all 49 species, 26 kinds of glycerol and others showed a positive reaction, and 21 kinds such as erythritol and others showed a negative reaction (Fig. 12). Used API 50 CHB kit showed 98% similarity when compared with standard *B. amyloliquefaciens* (Table 9).

As a result of compared the molecular weight with the standard protein, it was confirmed that a band appeared around 37 kDa (Fig. 13). Previous studies, the results of the research that the bacteriocin of *B. amyloliquefaciens* was around 37 kDa have been reported (Wang et al., 2016). It's conceivable that *B. amyloliquefaciens* JFP-02 also produces bacteriocin.

Through these series of experiment results, it`s seems that *B. amyloliquefaciens* JFP-02 isolated from Jeju Island traditional food in possesses characteristics of probiotics and when compared with previous studies, its seems produce bacteriocin, an antimicrobial substance. Thus suggest *B. amyloliquefaciens* JFP-02 can be using as future probiotic.

Part II. Antimicrobial activity of *Bacillus amyloliquefaciens* JFP-02 on fish disease pathogens isolated from Jeju Island fish farm

2.1 INTRODUCTION

The domestic fish aquaculture industry is growing greatly, but fish diseases that occur during the aquaculture have serious adverse effect on the development of the aquaculture industry. Generally high density breeding is carried out in order to maximize the production amount per unit area, but these high density breeding is accompanied by a decrease in the gain increase rate, occurrence of various disease to cause a direct economic loss (Statistical years book., 2016).

In the case of Jeju Island olive flounder farm, mortality situation to be 4,519 ton (production amount 21,139 ton) in 2010, 2,427 ton (production amount 22,823 ton) in 2011, 5,601 ton (production amount 24,575 ton) in 2012, 5,760 ton (production amount 23,002 ton) in 2013, 6,710 ton (production amount 26,283 ton) in 2014, 6,928 ton (production amount 27,142 ton) in 2015 then increased years. In addition the amount of damage by mortality was investigated to be 29.4 billion won in 2010, 37.6 billion won in 2011, 51.3 billion won in 2012, 40.3 billion won in 2013, 48.5 billion won in 2014, 52.9 billion won in 2015 (Kim et al., 2017; Statistics Korea., 2016).

There are bacterial diseases, viral diseases, parasitic diseases caused by diseases that developin the process of olive flounder farm. Bacterial diseases include *Edwardsiell* spp., *Streptococcus* spp., *Vibrio* spp., *Aeromonas* spp. Viral diseases include Koi Herpesvirus (KHV), Infections Pancreatic Necrosis (IPN) and Viral Hemorrhagic Septicemia (VHS). Parasitic diseases include *Scuticociliatosis, Ichthyophthirius multifilis, Trichodipdoma* spp.,

Microcotyle sebastis (Lee et al., 2007; Nguyen and Kanai., 1999).

As a result the mortality rate due to fish diseases was estimated, in 1996 the mortality rate due to bacterial diseases reached about 70.7%, the parasitic diseases 21.9% and the viral diseases 4.6% were collected. This is considered to be the time when the development of antibiotics for bacterial diseases is showed and the mortality rate due to bacterial diseases appeared high. However in 2014 when the development of antibiotics was done the mortality rate due to bacterial diseases will be reduced to 41.6%, but parasitic diseases is 19.6%, viral diseases is 10.8%, bacterial disease combined infection was compiled to 11.7% (National Institute of Fisheries Science). It is noteworthy that bacterial diseases decreased with the use of antibiotics, while complex infections increased. Combined infection of bacterial diseases reduced the effect of using a single antibiotic and increased the number of aquaculture farm abusively using some antibiotics.

Recently due to the worldwide problem of antibiotic abuse, FDA and WHO recommend reduction of food corps, livestock, fish antibiotic usage. However in some aquaculture farms, consumer disbelief is still spreading socially due to problems such as antibiotic residual toxicity in antibiotic abuse and emergence of resistant bacteria against antibiotics. Therefore, in order to solve these problems, by administering substances that have demonstrated medium antibacterial activity and immune effect, such as natural substance, to cultured fish rather than chemical substances, the disease resistance and fecundity efficiency and promotion of growth, immunity effect on research.

In this study, we confirmed the antimicrobial activity against fish disease pathogens isolated from Jeju Island olive flounder farm, used *Bacillus amyloliquefaciens* JFP-02, to confirm the possibility of preventive and therapeutic methods for fish disease pathogens.

2.2 MATERIALS AND METHODS

2.2.1 Isolated of fish disease pathogens in 2015

In order to retain fish disease pathogens, 10 sites out of the olive flounder farm of Jeju Island can be randomly selected and breeding water, drainage water, influent water, were collected in a sterilized sample bottle and the olive flounder was collected used sterilized zipper bag. The liver and kidney separated from olive flounder and breeding water, drainage water, influent water were diluted with 0.8% saline solution in 1 ml and then smeared on each medium and incubated at 25℃ for 48 hours (Jung et al., 2009; Woo et al., 2010). The medium used were Marine Agra (MA, Difco., USA), TCBS (Difco., USA), Mannitol Salt Agar (MAN, BD., USA), Brain Heart Infusion Agar (BHIA, Difco., USA), MacConkey Sorbitol Agar (MACs, Difco., USA), Oxford Medium Base (Oxford, Difco., USA), Coliform Agar (Coli, Merck., USA), Nutrient Agar (NA, Difco., USA), Salmonella Shigella Agar (SS, Difco., USA), Mannitol Egg Yolk Polymyxin Agar (MYP, OXOID., USA), MRSA. The compositions are the same as Table 10.

Table 10. Composition of mediums

Media	Ingredient	Amounts
Marine Agar	Peptone	5.0 g
	Yeast Extract	1.0 g
	Ferric Citrate	0.1 g
	Sodium Chloride	19.45 g
	Magnesium Chloride	8.8 g
	Sodium Sulfate	3.24 g
	Calcium Chloride	1.8 g
	Potassium Chloride	0.55 g
	Sodium Bicarbonate	0.16 g
	Potassium Bromide	0.08 g
	Strontium Chloride	34.0 mg
	Boric Acid	22.0 mg
	Sodium Silicate	4.0 mg
	Sodium Fluoride	2.4 mg
	Ammonium Nitrate	1.6 mg
	Disodium Phosphate	8.0 mg
	Agar	15.0 g
TCBS Agar	Yeast Extract	5.0 g
	Proteose Peptone No.3	10.0 g
	Sodium Citrate	10.0 g
	Sodium Thiosulfate	10.0 g
	Oxgall	8.0 g
	Saccharose	20.0 g
	Sodium Chloride	10.0 g
	Ferric Ammonium Citrate	1.0 g
	Bromthymol Blue	0.04 g
	Thymol Blue	0.04 g
	Agar	15.0 g
Mannitol Egg Yolk Polymyxin Agar	Meat Extract	1.0 g
	Peptone	10.0 g
	Mannitol	10.0 g
	Sodium Chloride	10.0 g
	Phenol Red	0.025 g

	Agar	12.0 g
Brain Heart Infusion Agar	Calf Brains, Infusion from 200 g	7.7 g
	Beef Heart, Infusion from 250 g	9.8 g
	Proteose Peptone	10.0 g
	Dextrose	2.0 g
	Sodium Chloride	5.0 g
	Disodium Phosphate	2.5 g
	Agar	15.0 g
Mannitol Salt Agar	Beef Extract	1.0 g
	Pancreatic Digest of Casein	5.0 g
	Peptic Digest of Animal Tissue	5.0 g
	Sodium Chloride	75.0 g
	D-Mannitol	10.0 g
	Phenol Red	0.025 g
	Agar	15.0 g
MacConkey Agar	Pancreatic Digest of Gelatin	17.0 g
	Peptones (meat and casein)	3.0 g
	Lactose	10.0 g
	Bile Salts No. 3	1.5 g
	Sodium Chloride	5.0 g
	Agar	13.5 g
	Neutral Red	0.03 g
	Crystal vioret	1.0 mg
Oxford Medium Base	Pancreatic Digest of Casein	8.9 g
	Proteose Peptone No. 3	4.4 g
	Yeast Extract	4.4 g
	Tryptic Digest of Beef Heart	2.7 g
	Starch	0.9 g
	Sodium Chloride	4.4 g
	Esculin	1.0 g
	Ferric Ammonium Citrate	0.5 g
	Lithium Chloride	15.0 g
	Agar	15.3 g
Coliform Agar	Chromogenic mix	20.3 g
	Yeast Extract	3.0 g
	Peptone	5.0 g
	Lactose	2.5 g

	Sodium Chloride	5.0 g
	Di-Sodium hydrogen phosphate	3.5 g
	Potassium di-hydrogen phosphate	1.5 g
	Neutral red	0.03 g
	Agar	15.0 g
Nutrient Agar	Beef Extract	3.0 g
	Peptone	5.0 g
	Agar	15.0 g
Salmonella Shigella Agar	Beef Extract	5.0 g
	Pancreatic Digest of Casein	2.5 g
	Peptic Digest of Animal Tissue	2.5 g
	Lactose	10.0 g
	Bile Salts	8.5 g
	Sodium Citrate	8.5 g
	Sodium Thiosulfate	8.5 g
	Ferric Citrate	1.0 g
	Neutral Red	0.025 g
	Agar	13.5 g
	Brilliant Green	0.330 mg

2.2.2 Isolated of fish disease pathogens in 2016

In order to retain fish disease pathogens, 10 sites out of the olive flounder farm of Jeju Island can be randomly selected and breeding water, drainage water, influent water, were collected in a sterilized sample bottle and the olive flounder was collected used sterilized zipper bag. The liver and kidney separated from olive flounder and breeding water, drainage water, influent water were diluted with 0.8% saline solution in 1 ml and then smeared on each medium and incubated at 25℃ for 48 hours (Jung et al., 2009; Woo et al., 2010). The used mediums were the same as in 2015.

2.2.3 Sequence analysis of fish disease pathogens

For pure isolated probiotics candidate strains, DNA was extracted using 2.5% (Shen et al., 1999). The isolate strain was added in 2.5% chelex, reacted at 95℃, for 5 minutes, and stored at 4℃. 27 Forward (5′-AGAGTTTGATCCTGGCTCA-3′) primer and 1522 Reverse (5′-AAGGAGGTGATCCAGCCGCA-3′) primer were used for PCR. PCR was performed by adding 1 ul of DNA to AccuPower^{TM} PCR Pre-Mix and final volume of 25 ul. The PCR conditions were carried out Initial denaturation 5 min, denaturation at 94℃ for 1 min, annealing at 55℃ 1 min, extension ant 72℃ for 1 min at 30 cycles, and 72℃ final extension for 10 min. The amplified PCR product was confirmed by electrophoresis at 1% agarose gel.

2.2.4 Antibiotics susceptibility test of fish disease pathogens

For antibiotics seven kind of amoxycillin (Table 3) were used, antibiotic resistant strains and susceptible strains were tested by using the Bauer-kirby test as an antibiotic commonly used to confirm the presence or absence of resistance of the stain type (Wayne., 1997). The experimental principle is the same as 1.2.3. Fish disease pathogens were pre-cultured in BHIB (Brain Heart Infusion Broth, Difco., USA), then streaked on MHA and incubated for 48 hour, 35℃, secondly each of the antibiotic discs. The inhibitory zones for the antibiotic discs were measured to establish the existence and nonexistence of antibiotic resistance.

2.2.5 Antimicrobial activity of probiotics candidate strains against fish disease pathogens

The antimicrobial activity performance of fish disease pathogens of the isolated probiotics candidate strains were measured using the paper disc method (Lee and Park., 2010). 100 ul of probiotics candidate strains pre-culture in 8 mm paper disc were dispensed and dried at 35℃ for 24 hours. The fish disease pathogens were pre-culture in BHIB and streaked on MHA, and then the paper disc of disc at regular intervals was raised and cultured 25℃ for 48 hours. After cultured the presence or absence of inhibition zone formation was confirmed around the paper disc, and antibacterial activity performance was confirmed.

2.2.6 Antimicrobial activity of *Bacillus amyloliquefaciens* JFP-02 against fish disease pathogens according to optimal culture conditions

2.2.6.1 Antimicrobial activity according to pH variation

In order to confirm the antimicrobial activity against fish disease pathogens according to pH variation using the *B. amyloliquefaciens* JFP-02, pH was adjusted to 4 to 9 with 1 M HCl and 1 M NaOH in MRSB and then sterilized at 121℃ for 15 minutes. The prepared broth medium was inoculated with 1% of the pre-culture solution of the *B. amyloliquefaciens* JFP-02 and cultured at 30℃ and 200 rpm for 48 hours. 100 ul of *B. amyloliquefaciens* JFP-02 pre-culture in 8 mm paper disc were dispensed and dried at 30℃ for 24 hours. The fish disease pathogens were pre-culture and streaked on MHA, and then the paper disc of disc at regular intervals was raised and cultured 30℃ for 48 hours. After cultured the presence or absence of inhibition zone formation was confirmed around the paper disc, and antibacterial activity performance was confirmed (Yoon et al., 2010; Lee et al., 2011).

2.2.6.2 Antimicrobial activity according to temperature variation

In order to confirm the antimicrobial activity against fish disease pathogens according to temperature variation using the *B. amyloliquefaciens* JFP-02, 1% of the pre-culture solution was inoculated in the MRSB and incubated at 10℃, 20℃, 30℃, 40℃, 200 rpm after cultured for 48 hours. 100 ul of *B. amyloliquefaciens* JFP-02 pre-culture in 8 mm paper disc were dispensed and dried at 30℃ for 24 hours. The fish disease pathogens were pre-culture and streaked on MHA, and then the paper disc of disc at regular intervals was raised and cultured 30℃ for 48 hours. After cultured the presence or absence of inhibition zone formation was confirmed around the paper disc, and antibacterial activity performance was confirmed (Yoon et al., 2010; Lee et al., 2006).

2.2.6.3 Antimicrobial activity according to carbon source variation

In order to confirm the antimicrobial activity against fish disease pathogens according to carbon source variation using the *B. amyloliquefaciens* JFP-02, GY (yeast extract 0.1%, $MgSO_4 \cdot 7H_2O$ 0.02%) medium was treated with a representative carbon source dextrine, saccharose, sorbitol were added in an amount of 1% each and cultured at 30℃, 200 rpm for 48 hours. 100 ul of *B. amyloliquefaciens* JFP-02 pre-culture in 8 mm paper disc were dispensed and dried at 30℃ for 24 hours. The fish disease pathogens were pre-culture and streaked on MHA, and then the paper disc of disc at regular intervals was raised and cultured 30℃ for 48 hours. After cultured the presence or absence of inhibition zone formation was confirmed around the paper disc, and antibacterial activity performance was confirmed (Kang et al., 2011; Park et al., 2013).

2.2.6.4 Antimicrobial activity according to nitrogen source variation

In order to confirm the antimicrobial activity against fish disease pathogens according to nitrogen source variation using the *B. amyloliquefaciens* JFP-02, GY (glucose 0.5%, $MgSO_4 \cdot 7H_2O$ 0.02%) medium was treated with a representative nitrogen source malt extract, peptone, yeast extract were added in an amount of 0.5% each and cultured at 30℃, 200 rpm for 48 hours. 100 ul of *B. amyloliquefaciens* JFP-02 pre-culture in 8 mm paper disc were dispensed and dried at 30℃ for 24 hours. The fish disease pathogens were pre-culture and streaked on MHA, and then the paper disc of disc at regular intervals was raised and cultured 30℃ for 48 hours. After cultured the presence or absence of inhibition zone formation was confirmed around the paper disc, and antibacterial activity performance was confirmed (Kang et al., 2011; Park et al., 2013).

2.2.6.5 Antimicrobial activity according to mineral source variation

In order to confirm the antimicrobial activity against fish disease pathogens according to mineral source variation using the *B. amyloliquefaciens* JFP-02, GY (glucose 0.5%, yeast extract 0.1%) medium was treated with a representative mineral source KH_2Po_4, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$ were added in an amount of 0.1% each and cultured at 30℃, 200 rpm for 48 hours. 100 ul of *B. amyloliquefaciens* JFP-02 pre-culture in 8 mm paper disc were dispensed and dried at 30℃ for 24 hours. The fish disease pathogens were pre-culture and streaked on MHA, and then the paper disc of disc at regular intervals was raised and cultured 30℃ for 48 hours. After cultured the presence or absence of inhibition zone formation was confirmed around the paper disc, and antibacterial activity performance was confirmed (Kang et al., 2011; Park et al., 2013).

2.2.7 Statistical analysis

All the tests were performed in triplicate. The data were subjected to statistical analysis using the SPSS (SPSS INC, version 18.0., USA). The statistical analysis was done by using one-way analysis of variance (ANOVA) followed by Tukey`s test ($P<0.05$) to compare the means between individual treatments. Result values were expressed as mean ± standard deviation (mean ± S.D) and percentage values were calculated by arcsine deformation value and statistically analyzed.

2.3 **RESULTS**

2.3.1 Isolated of fish disease pathogens from olive flounder farm in 2015

The number of viable cell counted by streaked each medium used of breeding water, drainage water, influent water, olive flounder collected at 10 olive flounder farms in Jeju Island (Fig. 14). As a result, in the MAN medium in which *Staphylococcus aureus*, can growth, the viable cell count was measured at two places, Coli medium can growth *E. coli* and *Listeria monocytogenes* can growth in Oxford medium, the number of viable bacterial was measured. The number of viable bacteria was measured in nine places in the BHIA medium where *Streptococcus* spp. and *Edwardsiella* spp. can growth, among which the largest number was observed in the breeding water and kidney.

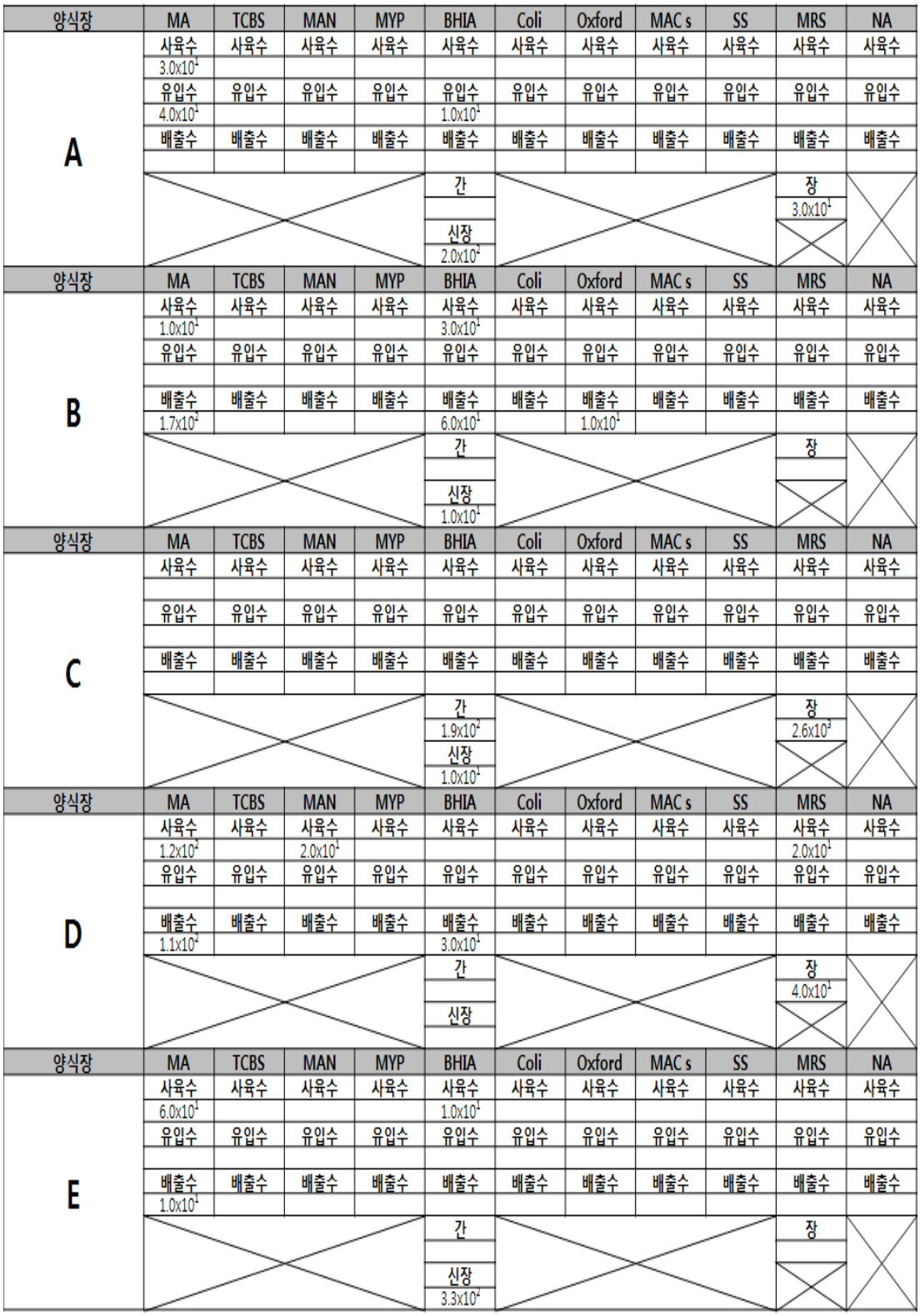


Fig. 14. Viable cell count of isolated fish disease pathogens from olive flounder aquaculture farms in 2015. (CFU/ml)

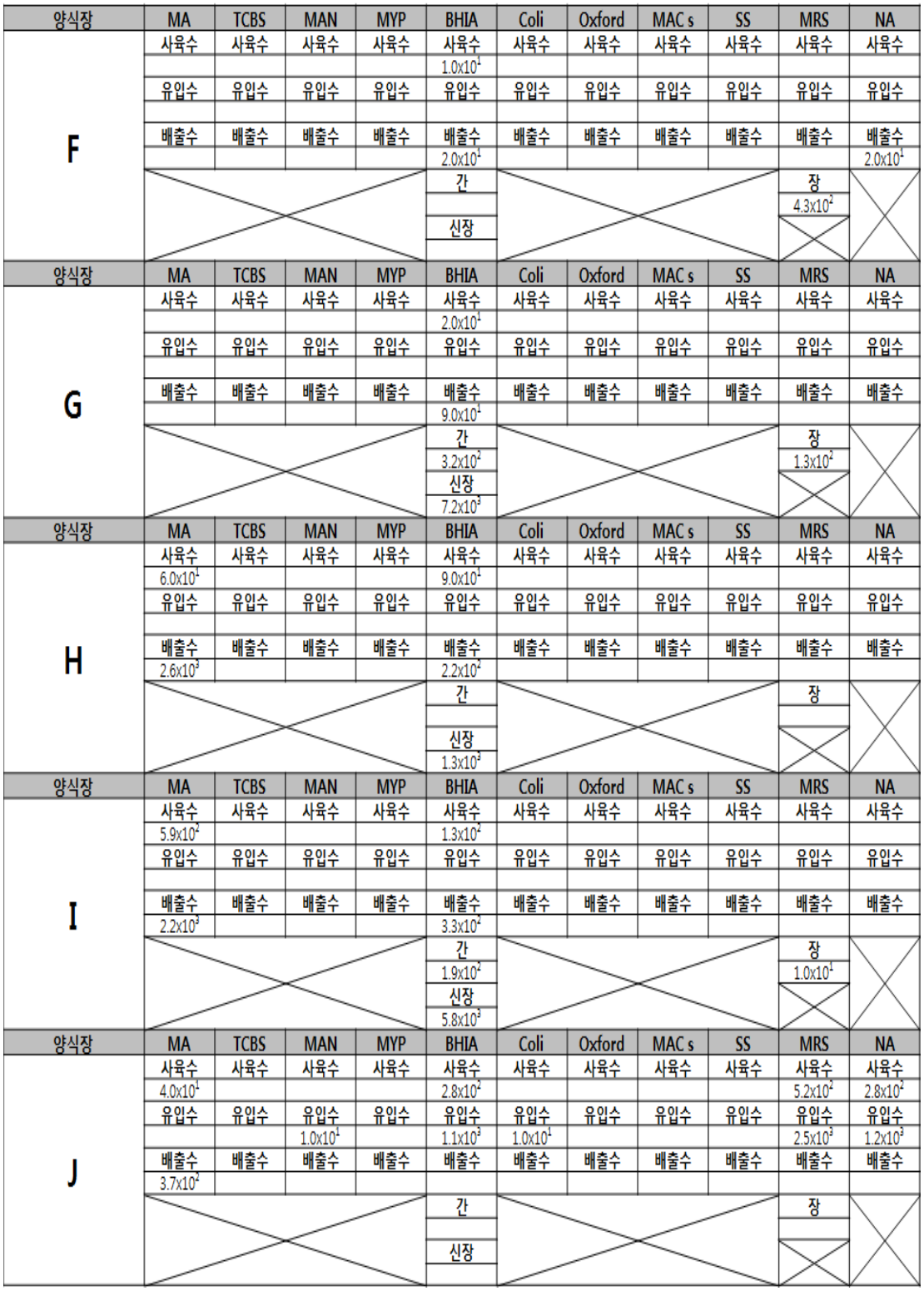


Fig. 14. Viable cell count of isolated fish disease pathogens from olive flounder aquaculture farms in 2015. (CFU/ml)

2.3.2 Isolated of fish disease pathogens from olive flounder farm in 2015

Used BLAST of NCBI, four species of fish disease pathogens in bacterial isolated from olive flounder farm was obtained. Isolated of *Photobacterium damselae* sub spp. showing symptoms such as nodules were showed in the kidney, necrosis is accompanied, appetite deterioration, body color blackening, fin, mouth, gill cover in erythema appears, and fin and abdomen redness and bleeding, abdominal distention, intestinal ulcer, hernia a symptomatic of *Edwardsiella tarda*. Also, isolated of *Streptococcus parauberis* showing symptoms such as eyes bulging, overhang, body color blackening, gill redness, abdominal distension, kidney bedbug, and *Streptococcus iniae* showing symptoms such as petechial, congestion, brain injurt, body color blackening, mouth redness, exophthalmos, abdominal distension, hernia were sympathetic (Table 11). Fish disease pathogens of the isolated were named JFM-001 to JFM004.

Table 11. BLAST results of isolated fish disease pathogens strains from olive flounder aquaculture farms.

Strain No.	BLAST results		
	Accession No.	Species	Identities
JFM-001	KF956381	*Photobacterium damselae* sub sp.	1022/1039 (98%)
JFM-002	KF646671	*Edwardsiella tarde*	1042/1054 (99%)
JFM-003	AY942570	*Streptococcus pararuberis*	1004/1009 (99%)
JFM-004	KC748467	*Streptococcus iniae*	967/1001 (97%)

2.3.3 Antibiotics susceptibility test of fish disease pathogens in 2015

Antibiotics susceptibility test of fish disease pathogens were performed on eight antibiotics commonly used in fish farm (Table 12, Fig. 15). The JFM-001 strain was only resistance to amoxycillin, penicillin, and the other antibiotics showed sensitivity. The JFP-002 strain was sensitive only to gentamicin, flofenicol, neomycin and the other antibiotics showed resistance. The JFM-003 strain was susceptible to all antibiotics except oxytetracycline, and the JFM-004 strain was susceptible to all antibiotics except oxtetracycline, penicillin. Comprehensively 4 strains were sensitive only to gentamicin, flofenicol, and neomycin.

Table 12. Antibiotics susceptibility test of fish disease pathogens from olive flounder aquaculture farms. (Inhibition zone (mm))

pathogens / Antibiotics	JFM-001	JFM-002	JFM-003	JFM-004
Amoxycillin	-	-	22	14
Gentamicin	45	28	24	32
Erythromycin	48	9	27	36
Flofenicol	43	35	32	37
Neomycin	32	27	16	18
Oxytetracycline	15	-	8	10
Penicillin	-	-	24	9
Tetracycline	25	-	17	24

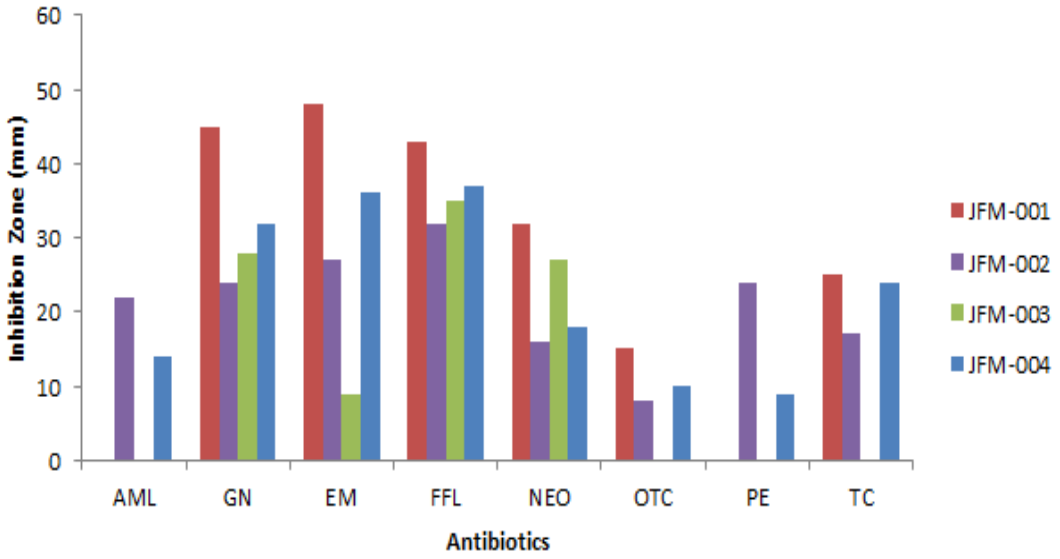


Fig. 15. Antibiotics susceptibility test of fish disease pathogens from olive flounder aquaculture farms.

2.3.4 Antimicrobial activity of probiotics candidate strains against fish disease pathogens

As a result of confirmed the antimicrobial activity against fish disease pathogens using probiotics candidate strains isolated from Jeju Island traditional fermented foods JFP-01, JFP-02 showed antimicrobial activity against four types of fish disease pathogens, JFP-02 showed the highest antimicrobial activity (Table 13). JFP-03, JFP-04, JFP-05 showed antibacterial activity only with JFM-002, JFM-003, JFM-004, and JFP-06, JFP-08 showed antimicrobial activity only with JFM-004, JFP-07 showed no antimicrobial activity against four kinds fish disease pathogens (Fig. 16).

Table 13. Antimicrobial activity of probiotics candidate strains for fish disease pathogens from olive flounder aquaculture farms. (Inhibition zone (mm))

Fish disease	Probiotics candidate strains							
	JFP-01	JFP-02	JFP-03	JFP-04	JFP-05	JFP-06	JFP-07	JFP-08
JFM-001	12	30	-	-	-	-	-	-
JFM-002	12	25	12	11	16	-	-	-
JFM-003	16	27	17	19	21	-	-	-
JFM-004	14	27	13	16	16	14	-	11

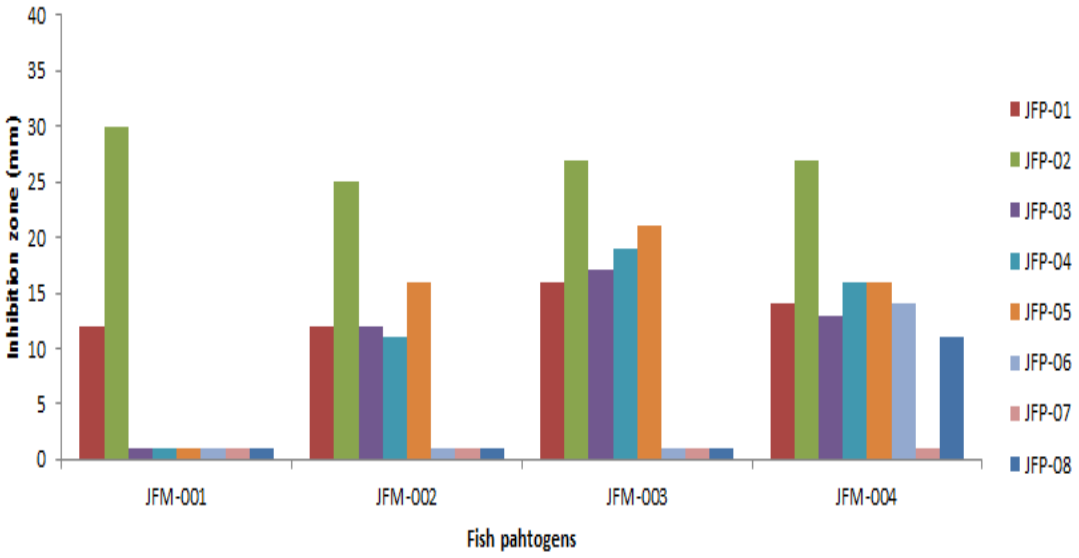


Fig. 16. Antimicrobial activity of probiotics candidate strains for fish disease pathogens from olive flounder aquaculture farms.

2.3.5 Isolated of fish disease pathogens from olive flounder farm in 2016

The number of viable cell counted by streaked each medium used of breeding water, drainage water, influent water, olive flounder collected at 10 olive flounder farms in Jeju Island (Fig. 17). As a result, in the Coli medium can growth *E. coli* and SS medium ca growth *Edwaedsiella tarda* the viable cell count was measured at one place. Oxford medium can growth *L. monocytogenes* and the viable cell count was measured at two place for breeding water, two place for drainage water, three place for influent water. The number of viable bacteria was measured in fifteen places in the BHIA medium where *Streptococcus* spp. and *Edwardsiella* spp. can growth, among which the largest number was observed in the liver and kidney of olive flounder.

양식장	MA	TCBS	MAN	MYP	BHIA	Coli	Oxford	MACs	SS	MRS	NA
K	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수
	$5.1x10^4$										
	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수
	$2.3x10^4$										
	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수
	$1.5x10^3$										
					간					장	
					$2.0x10^2$					$1.1x10^2$	
					신장						
					$6.0x10^2$						

양식장	MA	TCBS	MAN	MYP	BHIA	Coli	Oxford	MACs	SS	MRS	NA
L	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수
	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수
	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수
						$2.3x10^3$					
					간					장	
					$1.1x10^3$					$1.3x10^2$	
					신장						
					$1.3x10^3$						

양식장	MA	TCBS	MAN	MYP	BHIA	Coli	Oxford	MACs	SS	MRS	NA
M	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수
	$1.3x10^3$										
	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수
	$3.9x10^5$										
	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수
	$3.0x10^2$										
					간					장	
										$2.0x10^2$	
					신장						

양식장	MA	TCBS	MAN	MYP	BHIA	Coli	Oxford	MACs	SS	MRS	NA
N	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수
	$1.0x10^2$										
	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수
	$3.0x10^2$										
	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수
					간					장	
					$2.4x10^3$					$2.0x10^2$	
					신장						

Fig. 17. Viable cell count of isolated fish disease pathogens from olive flounder aquaculture farms in 2016. (CFU/ml)

양식장	MA	TCBS	MAN	MYP	BHIA	Coli	Oxford	MACs	SS	MRS	NA
O	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수
							$1.9x10^2$				
	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수
	$1.1x10^2$						$1.3x10^2$				
	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수
					$3.0x10^1$		$2.1x10^2$				
					간					장	
					$4.1x10^2$					$2.1x10^2$	
					신장						
					$2.3x10^2$						

양식장	MA	TCBS	MAN	MYP	BHIA	Coli	Oxford	MACs	SS	MRS	NA
P	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수
	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수
	$1.0x10^5$						$1.8x10^2$				
	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수
	$4.0x10^2$										
					간					장	
					$3.2x10^3$					$2.9x10^2$	
					신장						
					$1.0x10^3$						

양식장	MA	TCBS	MAN	MYP	BHIA	Coli	Oxford	MACs	SS	MRS	NA
Q	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수
	$1.0x10^2$						$4.0x10^2$			$1.9x10^2$	
	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수
										$4.6x10^2$	
	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수
					$3.0x10^1$						
					간					장	
					$2.4x10^2$					$9.0x10^2$	
					신장						
					$1.1x10^2$						

양식장	MA	TCBS	MAN	MYP	BHIA	Coli	Oxford	MACs	SS	MRS	NA
R	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수	사육수
	$4.0x10^3$				$9.0x10^1$				$2.0x10^2$	$3.0x10^2$	
	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수	유입수
	$3.0x10^2$						$1.1x10^2$			$2.1x10^3$	
	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수	배출수
					$2.2x10^2$		$1.0x10^2$				
					간					장	
										$1.0x10^2$	
					신장						

Fig. 17. Viable cell count of isolated fish disease pathogens from olive flounder aquaculture farms in 2016. (CFU/ml)

2.3.6 Isolated of fish disease pathogens from olive flounder farm in 2016

Used BLAST of NCBI, nine species of fish disease pathogens in bacterial isolated from olive flounder farm was obtained. Isolated of *Tenacibaculum maritimum*, *Staphylococcus caprae*, *Edwardsiella tarda*, *Streptococcu parauberis*, *Photobacterium damselae* showing symptoms such as body color blackening, fin and mouth redness, gill erosion were sympathetic. Isolated of *Vibrio harveyi*, *Vibrio campbellii* showing gram negative bacteria symptoms such as abdominal distension, hernia is external symptoms and hemorrhagic revenge, liver hemorrhage, spleen atrophy is internal symptoms were sympathetic. Isolated of *Vibrio alginolyticus* showing symptoms such as intestine and gallbladder putrefaction hemorrhage and ulcer, body color blackening, body bleeding were sympathetic. Isolated of *Vibrio anguillarum* showing symptoms such as body color blackening, body bleeding, exophthalmos and redness, visceral spot bleeding were sympathetic (Table 14). Fish disease pathogens of the isolated were named JM-01 to JM-09.

Table 14. BLAST results of isolated fish disease pathogen strains from olive flounder aquaculture farms.

Strain No.	BLAST results		
	Accession No.	Species	Identities
JM-01	NBRC1009111	*Tenacibaculum maritimum*	1132/1168 (97%)
JM-02	ATCC15947	*Edwardsiella tarda*	1082/1117 (97%)
JM-03	DSM6631	*Streptococcus parauberis*	1164/1222 (95%)
JM-04	NBRC15634	*Vibrio harveyi*	1146/1181 (97%)
JM-05	ATCC33539	*Photobacterium damselae*	1170/1196 (98%)
JM-06	ATCC25920	*Vbrio campbellii*	1123/1176 (95%)
JM-07	NBRC15630	*Vibrio alginolyticus*	1175/1219 (96%)
JM-08	NBRC13266	*Vibrio anguillarum*	1145/1177 (97%)
JM-09	DSM20608	*Staphylococcus caprae*	1133/1177 (96%)

2.3.7 Antibiotics susceptibility test of fish disease pathogens in 2016

Antibiotics susceptibility test of fish disease pathogens were conducted on eight kind antibiotics same as in 2015 (Table 15, Fig. 18). Only antibiotics flofenicol showed sensitivity of all nine species of fish disease pathogens. Amoxycillin showed sensitivity only to JM-01, JM-02, JM-03, JM-09, and gentamicin showed sensitivity to JM-01, JM-02, JM-03, JM-05, JM-07, JM-08, JM-09. Erythromycin showed sensitivity to JM-01, JM-03, JM-05, JM-08 and JM-06, JM-07 showed resistance to neomycin. Oxtetracycline showed resistance to JM-01, JM-02, JM-05, and penicillin showed sensitivity only to JM-01, JM-03, and tetracycline showed resistance to JM-02, JM-05, JM-06. It was confirmed that fish disease pathogens from aquaculture farm comprehensively showed sensitivity with gentamicin, flofenicol, and neomycin.

Table 15. Antibiotics susceptibility test of fish disease pathogens from olive flounder aquaculture farms. (Inhibition zone (mm))

pathogens / Antibiotics	JM-01	JM-02	JM-03	JM-04	JM-05	JM-06	JM-07	JM-08	JM-09
AML	30	19	34	-	-	11	-	10	19
GN	35	21	14	12	14	11	13	23	23
EM	41	0	27	12	16	11	13	23	-
FFL	39	28	27	26	24	20	40	34	28
NEO	26	25	13	19	14	11	12	17	23
OTC	10	9	29	25	10	18	21	22	32
PE	31	9	40	-	-	19	-	-	17
TC	17	11	25	25	9	-	27	32	34

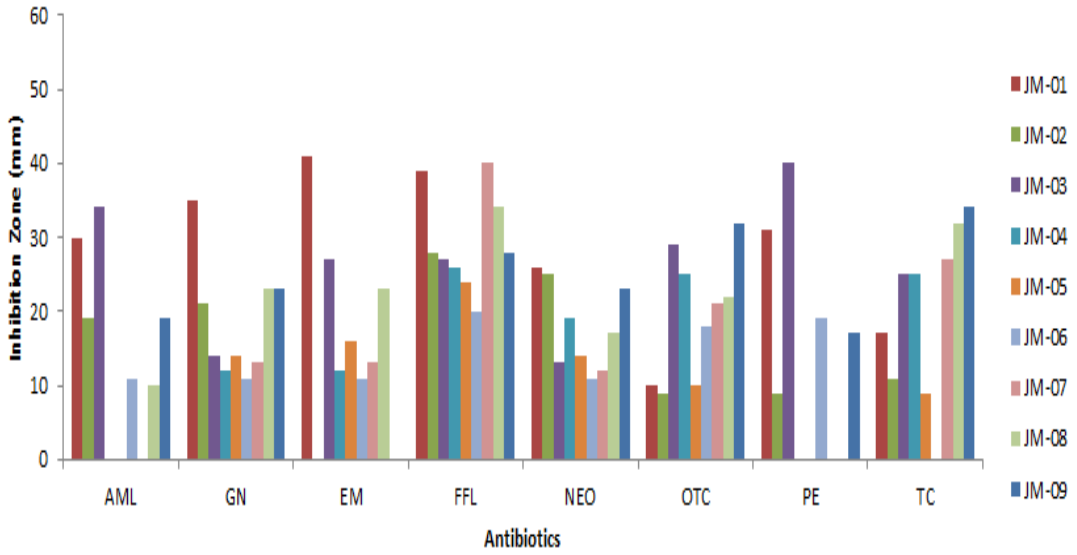


Fig. 18. Antibiotics susceptibility test of fish disease pathogens from olive flounder aquaculture farms.

2.3.8 Antimicrobial activity of *Bacillus amyloliquefaciens* JFP-02 according to optimal culture conditions

2.3.8.1 Effect of antimicrobial activity according to pH variation

Used *B. amyloliquefaciens* JFP-02, pH was adjusted to 4~9, cultured at 30℃ for 48 hours, and was placed in an 8 mm paper disc to confirm antimicrobial activity against fish disease pathogens, the antimicrobial activity performance of all fish disease pathogens except JM-05 was showed (Table 16, Fig. 19, 20). JM-01, JM-02, JM-07 showed inhibition zones all pH conditions. Confirmed according to pH variation, pH 6 showed antimicrobial activity against 8 kind of fish disease pathogens, shoed the most antimicrobial activity, then showed antimicrobial activity against 5 kind fish disease pathogens at pH 4 and pH 5, four species in pH 7, seven species in pH 8, six species in pH 9 showed antimicrobial activity against fish disease pathogens. The growth activity by according to pH described in the chapter 1 showed the highest growth activity at pH 9, but it was confirmed that the antimicrobial activity effect was highest at pH 6 apart from the growth activity.

Table 16. Antimicrobial activity for fish disease pathogens according to pH variation of *B. amyloliquefaciens* JFP-02. (Inhibition zone (mm))

pathogens / pH	JM-01	JM-02	JM-03	JM-04	JM-05	JM-06	JM-07	JM-08	JM-09
pH 4	35	19	0	13	0	20	15	0	0
pH 5	30	25	0	12	0	21	13	0	0
pH 6	45	40	19	12	0	20	11	16	34
pH 7	35	21	0	0	0	0	12	0	27
pH. 8	47	26	19	11	0	20	12	0	37
pH. 9	43	21	19	13	0	0	12	0	38

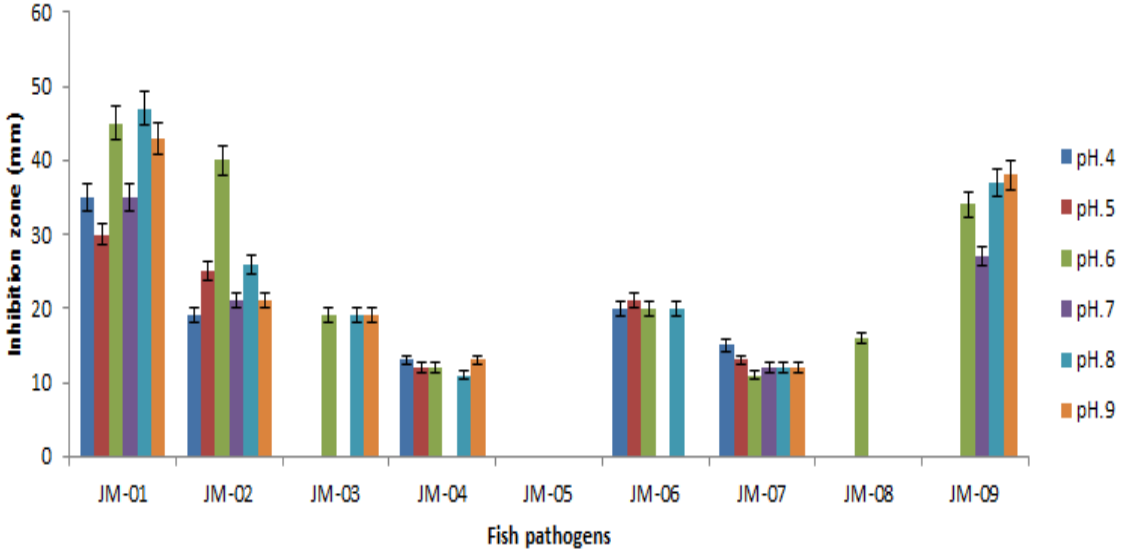


Fig. 19. Antimicrobial activity for fish disease pathogens according to pH variation of *B. amyloliquefaciens* JFP-02.

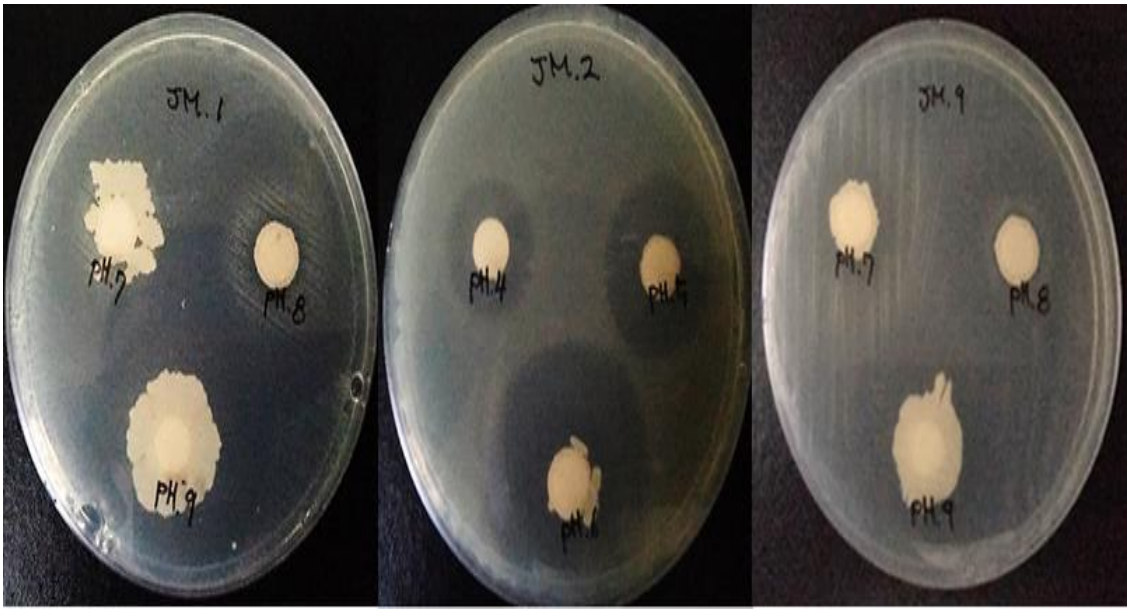


Fig. 20. Antimicrobial activity for fish disease pathogens according to pH variation of *B. amyloliquefaciens* JFP-02.

2.3.8.2 Effect of antimicrobial activity according to temperature variation

Used *B. amyloliquefaciens* JFP-02, temperature was adjusted to 10℃ ~ 50℃, cultured for 48 hours, and was placed in an 8 mm paper disc to confirm antimicrobial activity against fish disease pathogens, only 20℃ showed antimicrobial activity performance of all fish disease pathogens (Table 17, Fig. 21, 22). Confirmed according to temperature variation, it shoed antimicrobial activity against eight kind of fish disease pathogens at 10℃ and 30℃, then showed antimicrobial activity against seven kind of fish disease pathogens at 50℃, and five kind of fish disease pathogens at 40. The highest growth activity was observed at 30℃ in the growth according to the temperature described in chapter 1, but the highest antimicrobial activity was observed at 20℃.

Table 17. Antimicrobial activity for fish disease pathogens according to temperature variation of *B. amyloliquefaciens* JFP-02. (Inhibition zone (mm))

pathogens / temperature	JM-01	JM-02	JM-03	JM-04	JM-05	JM-06	JM-07	JM-08	JM-09
10℃	50	49	20	15	0	19	16	17	34
20℃	49	45	20	11	18	20	17	16	35
30℃	47	50	20	11	0	19	18	14	35
40℃	42	45	21	13	0	21	0	0	0
50℃	49	45	23	14	17	0	20	0	30

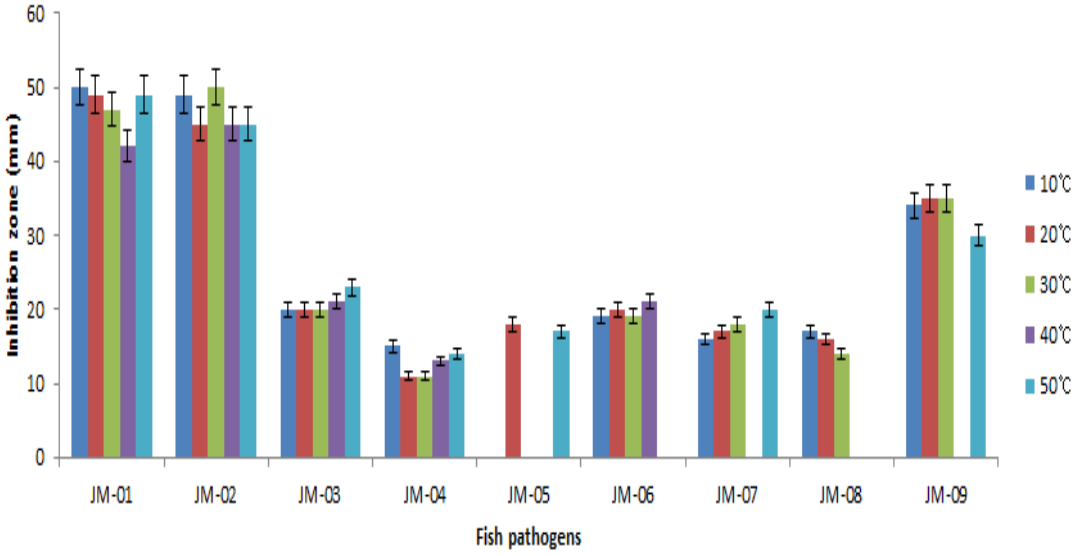


Fig. 21. Antimicrobial activity for fish disease pathogens according to temperature variation of *B. amyloliquefaciens* JFP-02.

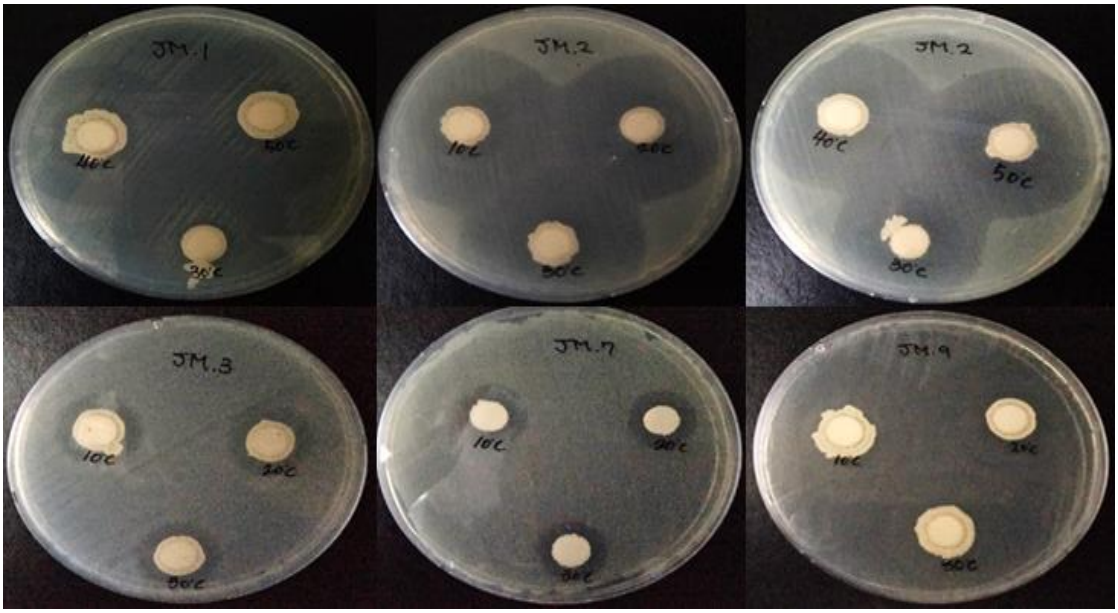


Fig. 22. Antimicrobial activity for fish disease pathogens according to temperature variation of *B. amyloliquefaciens* JFP-02.

2.3.8.3 Effect of antimicrobial activity according to carbon source variation

As a result of confirmed the antimicrobial activity of *B. amyloliquefaciens* JFP-02 against fish disease pathogens by added 1% each of dextrine, saccharose, sorbitol which were representative carbon sources to GY medium without glucose to except for JM-04, JM-07, JM-08 antimicrobial activity were showed in other fish disease pathogens (Fig. 23, 24). When dextrin was added, it showed the highest antimicrobial activity performance, and then showed antimicrobial activity performance in order of sorbitol and saccharose. As with the establishment of optimal growth activity according to the carbon source described in chapter 1, highest antimicrobial activity performance was confirmed when dexrine was added. It`s conceivable that when dextrin among carbon source was added to the medium component of *B. amyloliquefaciens* JFP-02, it`s showed highest activity.

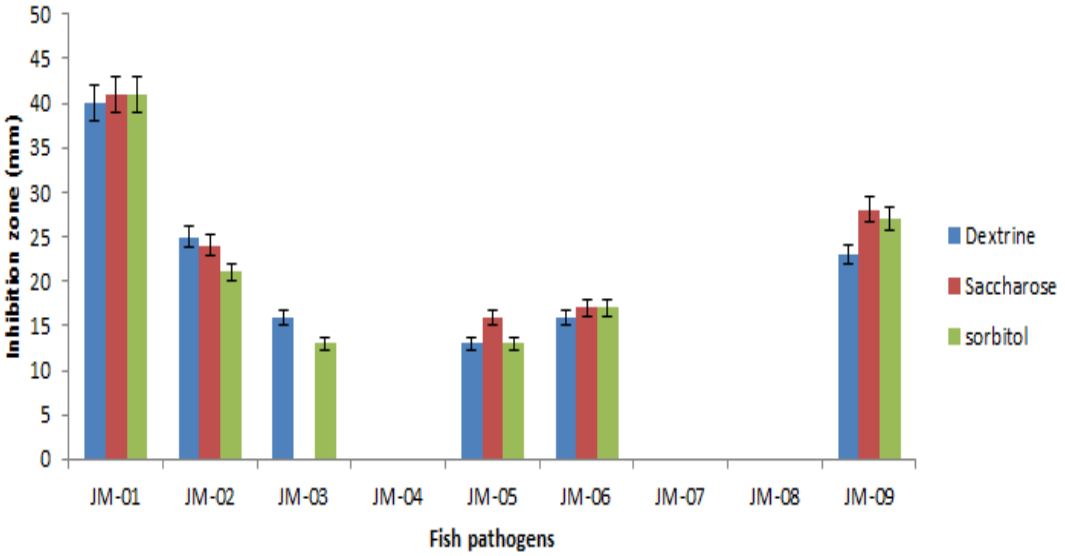


Fig. 23. Antimicrobial activity for fish disease pathogens according to carbon source variation of *B. amyloliquefaciens* JFP-02.

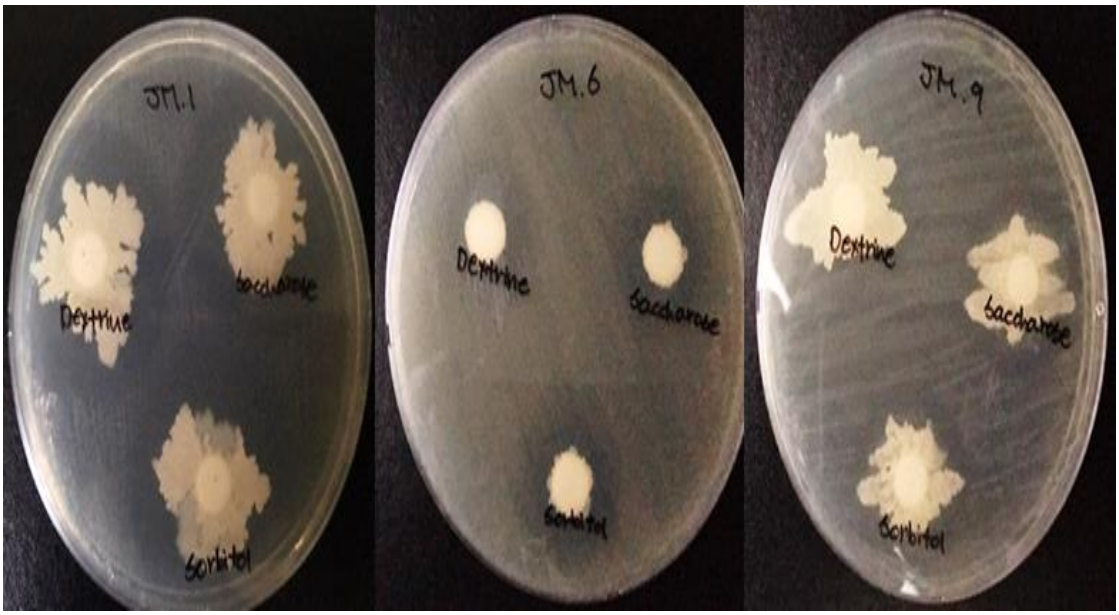


Fig. 24. Antimicrobial activity for fish disease pathogens according to carbon source variation of *B. amyloliquefaciens* JFP-02.

2.3.8.4 Effect of antimicrobial activity according to nitrogen source variation

As a result of confirmed the antimicrobial activity of *B. amyloliquefaciens* JFP-02 against fish disease pathogens by added 0.5% each of malt extract, peptone, yeast extract which were representative nitrogen sources to GY medium without yeast extract to except for JM-03, JM-05 antimicrobial activity were showed in other fish disease pathogens (Fig. 25, 26). When yeast extract was added, it showed the highest antimicrobial activity performance, and then showed antimicrobial activity performance in order of peptone and malt extract. As well as establishing optimal growth according to the nitrogen source described in chapter 1, highest antimicrobial activity performance was confirmed when yeast extract was added. Conversely, it was confirmed that not only the growth activity and antimicrobial activity at the time of added of malt extract were lowered. . It`s conceivable that when yeast extract among nitrogen source was added to the medium component of *B. amyloliquefaciens* JFP-02, it`s showed highest activity.

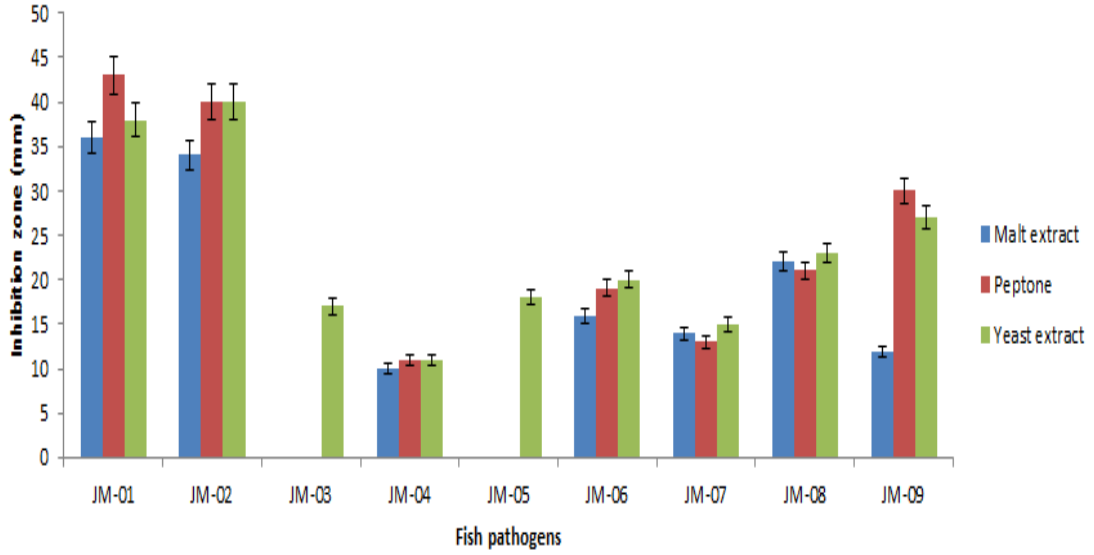


Fig. 25. Antimicrobial activity for fish disease pathogens according to nitrogen source variation of *B. amyloliquefaciens* JFP-02.

Fig. 26. Antimicrobial activity for fish disease pathogens according to nitrogen source variation of *B. amyloliquefaciens* JFP-02.

2.3.8.5 Effect of antimicrobial activity according to mineral source variation

As a result of confirmed the antimicrobial activity of *B. amyloliquefaciens* JFP-02 against fish disease pathogens by added 0.1% each of KH_2Po_4, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$ which were representative mineral sources to GY medium without $MgSO_4 \cdot 7H_2$ to only KH_2Po_4 were showed antimicrobial activity in fish disease pathogens (Fig. 27, 28). When KH_2Po_4 was added, it showed the highest antimicrobial activity performance in fish disease pathogens except JM-02 and JM-04, and then showed antimicrobial activity performance in order of $MgSO_4 \cdot 7H_2O$ and $FeSO_4 \cdot 7H_2O$. In contrast to the establishment of optimum growth activity according to the mineral source described in chapter 1, highest antimicrobial activity performance was confirmed when KH_2Po_4, and $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$ which showed highest growth activity was added it was confirmed that the antimicrobial activity was. It`s conceivable that when KH2Po4 among mineral source was added to the medium component of *B. amyloliquefaciens* JFP-02, it`s showed highest activity.

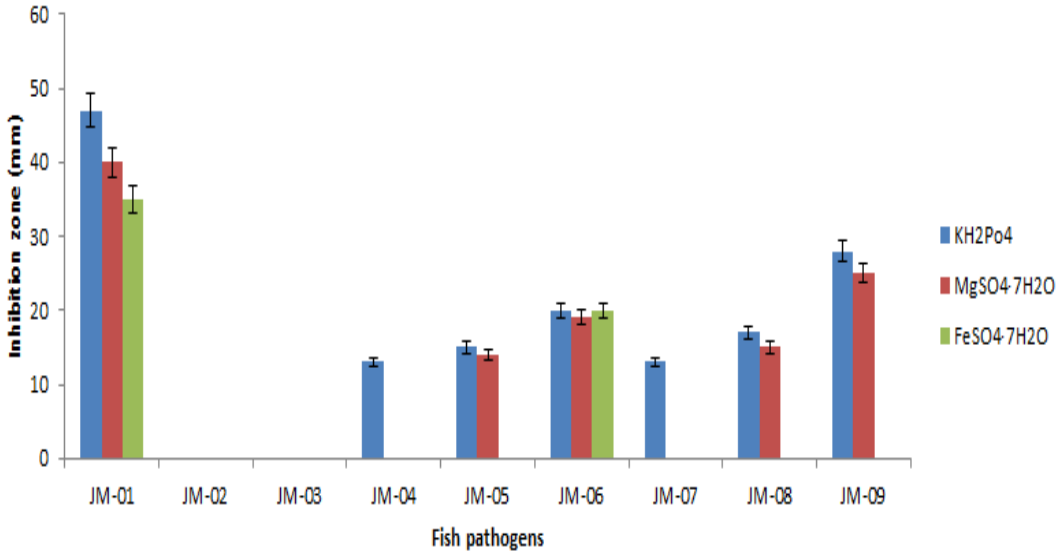


Fig. 27. Antimicrobial activity for fish disease pathogens according to mineral source variation of *B. amyloliquefaciens* JFP-02.

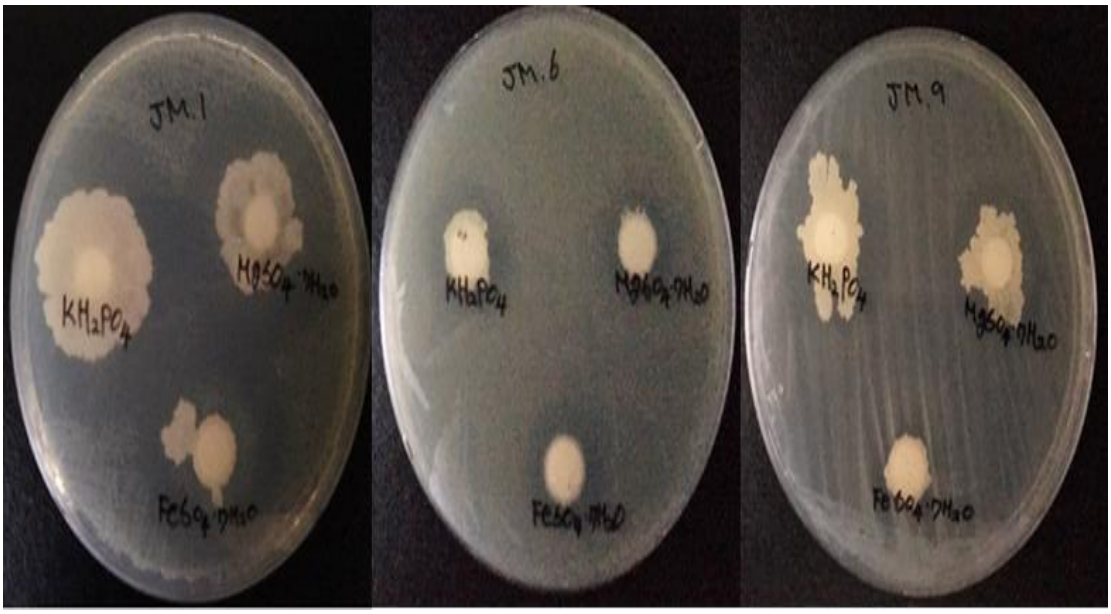


Fig. 28. Antimicrobial activity for fish disease pathogens according to mineral source variation of *B. amyloliquefaciens* JFP-02.

2.4 **DISCCUSION**

In chapter 2, we tried to confirmed the antimicrobial activity against fish disease pathogens isolated from Jeju Island olive flounder aquaculture farm, used *Bacillus amyloliquefaciens* JFP-02. In 2015 and 2016 a total 13 species fish disease pathogens were isolated at 18 olive flounder aquaculture farms site. Confirmed the sequence of the isolated strains, *Photobacterium damselae sub sp*. 2 species, *Edwardsiella tarda* 2 species, *Streptococcus parauberis* 2 species, *Streptococcus iniae*, *Tenacibaculum maritimum*, *Vibrio harveyi*, *Vibrio alginolyticus*, *Vibrio anguillarum*, *Staphylococcus caprae* (Table 11, 14).

Antibiotics susceptibility test were conducted to confirm the presence or absence of antibiotic resistance of fish diseases pathogens. Both 13 species fish disease pathogens showed sensitivity only to gentamicin, flofenicol, and neomycin. In addition, although there were deviations for each fish disease pathogens, it showed much resistance in amoxycillin, erythromycin, oxytetracycline, penicillin (Table 12, 15).

As a result of measuring antimicrobial activity used probiotics candidate strains for fish disease pathogens isolated from Jeju Island olive flounder aquaculture farm in 2015, it was selected as the probiotics *B. amyloliquefaciens* JFP-02 for four species of dish disease pathogens, it was confirmed that the antimicrobial activity was much highest than the other probiotics candidate strains (Table 13).

Antimicrobial activity was according to the culture conditions of *B. amyloliquefaciens* JFP-02 was measured for fish disease pathogens isolated from olive flounder aquaculture farm in 2016. Measurement results of antimicrobial activity according to pH variable, JM-01 and JM-02 showed highest antimicrobial activity from pH 4 to pH 9, whereas JM-05 had not antimicrobial activity (Table 16). In addition, although the growth activity according to pH described in chapter 1 showed the highest growth activity at pH 9, it was

confirmed that the antimicrobial activity effect was highest at pH 6, apart from the growth activity. Antimicrobial activity according to temperature variable, JM-01 and JM-02 showed highest antimicrobial activity to 10℃ to 50℃, JM-05 showed antimicrobial activity only at 20℃ and 50℃ (Table 17). As with pH, the growth activity showed the highest growth activity at 30℃, but apart from this, it was confirmed that the antimicrobial activity effect was highest at 20℃.

As a result of confirmed the antimicrobial activity effect according to carbon source, nitrogen source, mineral source which were the main components of the medium, it showed the highest antimicrobial activity in JM-01 and JM-09, regardless of the kinds of carbon source, JM-04, JM-07, JM-08 did not measure antimicrobial activity (Fig. 23). As with the establishment of optimal growth activity according to the carbon source described in chapter 1, highest antimicrobial activity performance was confirmed when dexrine was added. The effect of antimicrobial activity according to nitrogen source showed the highest antimicrobial activity in JM-01 and JM-02, regardless of the kinds nitrogen source, conversely when added malt extract and peptone JM-03 and JM-05 did not measure antimicrobial activity (Fig. 25). As with the establishment of optimal growth activity according to the carbon source described in chapter 1, highest antimicrobial activity performance was confirmed when yeast extract was added. The antimicrobial activity effect according to mineral source showed the highest antimicrobial activity in JM-01 and JM-06, regardless of the kind nitrogen source and JM-02, JM-03 did not measure antimicrobial activity (Fig. 27). Growth activity according to mineral source showed low growth activity at the time od added KH_2Po_4, but antimicrobial activity showed the highest effect.

This study confirmed the antimicrobial activity of *B. amyloliquefaciens* JFP-02 against various fish disease pathogens according to culture conditions, the results of can be good basis for inhibition of fish disease pathogens in the future.

Part III. Dietary effect of *B. amyloliquefaciens* JFP-02 on innate immune response and disease resistance in aquaculture fish

3.1 INTRODUCTION

According to the season, Jeju Island shore region can be coasted on the Chinese continental coastal waters, southern sea water, yellow sea bottom cold water, with different properties such as tsushima current and yellow sea current, because it`s influenced of warm current fish of the good fishing ground where abundant fishery resources are distributed is formed. Jeju Island aquaculture fishery, when started production of olive flounder seedlings in 1980, on the basis of 2016 yearly 557 points in 457 ha, the aquaculture fishery in the olive flounder is the main (Marine Fishery Status., 2017).

Production amount and production value of foam olive flounder steadily increase compared to the initial stage. Looking at the production situation, it will be 21,139 ton (production amount 262,900 million won) in 2010, 22,823 ton (production amount 251,205 million won) in 2011, 24,575 ton (production amount 267,914 million won) in 2011, 23,002 ton (production amount 266,267 million won) in 2013, 26,283 ton (production amount 239,528 million won) in 2014, 27,142 ton (production amount 291,684 million won) in 2015, 26,098 ton (production amount 331,334 million won) in 2016 (Marine Fishery Status., 2017). Looking at the exportation and amount of export of olive flounder, it will be 3,872 ton (amount of export 44,560 thousand dollars) in 2010, 3,071 ton (amount of export 41,169 thousand dollars) in 2011, 2,923 ton (amount of export 38,729 thousand dollars) in 2012, 2,473 ton (amount of export 36,590 thousand dollars) in 2013, 2,182 ton (amount of export 26,168) in 2014, 1,856 ton (amount of export 24,833 thousand dollars) in 2015, 1,733 ton (amount of export 25,743 thousand dollars) in 2016 (Marine Fishery Status.,

2017).

Based on these statistics, the production of olive flounder at Jeju Island accounts for more than 50% of the nation`s production, and it`s positioned as an important industry in Jeju Island. However, various diseases occurring in the olive flounder aquaculture farm are recognized as a major problem in the aquaculture industry. To control fish disease, at the same time various studies such as probiotics were tried in order to increase immune activity (Kim et al., 2017). Development of excellent probiotics not only prevents indiscriminate antibiotic marketing in fish farms, but also contributes to consumer`s perception of the safety of aquaculture fish.

The supplementation of fish diets with probiotics modulate specific functions of the gut and immune system and enhance disease protection (Gullian et al., 2004; Nayak., 2010). The most commonly used probiotics in aquaculture are *Bacillus* sp., *Pediococcus* sp., *Lactobacillus* sp., *Bifidobacterium* sp., *Lactococcus* sp (Abdel-Tawwab et al., 2008; Aly et al., 2008; Pal et al., 2007). *Bacillus* species are nonpathogenic aerobic gram-positive bacteria that are administrated orally to enhance gastro-intestinal microbial populations (Zhou et al., 2010; Ridha and Azad., 2012; Ling-Hong et al., 2016). Most probiotics microbes are marketed as drug or foodstuffs and their safe application has been confirmed (Mayra-Makien and Bigert., 1993).

In this study, confirmed to applicability of *B. amyloliquefaciens* JFP-02 when fed to aquaculture fish compared with normal diet confirmed growth and innate immune response and disease resistance.

3.2 MATERIALS AND METHODS

3.2.1.1 Fish (olive flounder (*Paralichthys olivaceus*))

Olive flounder (105±0.26 g) was purchased at an aquaculture farm in Jeju Island Jocheon-eup and used it for experiments. The purchased olive flounder was moved to the laboratory of Jeju National University and fed the basic feed (Table 18) for 2 weeks and domestication to the environment. We used a rearing aquarium of 500 mm x 160 mm, and in three groups, 30 olive flounder were cultures in each tank and ventilation twice a day. The measured water quality parameters were: temperature 19.0±0.5℃, pH 8.54±0.21, salinity 32.61±0.09‰, dissolved oxygen 7.47±1.27 mg/L, and photoperiod 10 h light: 10h dark cycle. Fish were fed with a basal diet ad libitum twice a day at 10:00, and 18:00 h at a rate of 4% of their body weight. Experimental culture was carried out for 8 weeks. We performed random sampling at the time of blood collection and dissection (Kim et al., 2017).

3.2.1.2 Experimental diet

B. amyloliquefaciens JFP-02, a probiotics strain was added to the basic feed for produced the experimental feed and the concentrations were adjusted to 1.4 x 10^4 CFU/100 g, 1.4 x 10^6 CFU/100 g. The basic feed not added anything was used in the control group (Kim et al., 2017).

Table 18. Formulation and proximate composition of the basal diets for olive flounder.

Ingredients (g/100 g diet)	Composition (%)
Fish meal	50.0
Soybean meal	8.0
Defatted rice bran	10.0
Wheat flour	13.0
Fish oil [a]	3.0
Soy lecithin	1.0
a-Potato	4.0
a-cellulose [b]	1.0
*Lj*LP	0.0
Blood meal	2.0
Dextrin	2.0
Casein [b]	2.0
EPA + DHA [a]	0.5
Vitamin and minerals premix	2.0
Proximate analysis (% dry matter basis)	
Moisture	10.0
Crude protein	49.9
Crude lipid	10.7
Crude ash	11.2
Crude carbohydrate	18.2

[a] E-Wha oil, Pusan, Korea

[b] United States Biochemical (Cleveland, OH) 4412

[c] Premix (g/100 g) contains DL-calcium pantothenate, 0.5; choline bitartrate, 10; inositol, 0.5; menadione, 0.02; niacin, 0.5; pyridoxine-HCl, 0.1; riboflavin, 0.1; thiamine mononitrate, 0.1; DL-a-tocopheryl acetate, 0.2; retinyl acetate, 0.02; biotin, 0.01; folic acid, 0.02; B12, 0.0002; Cholecalciferol, 0.008:a-cellulose, 85.0

[d] Premix (g/100 g) contains Al, 0.12; Ca, 500; Cl, 10; Cu, 0.5; Co, 0.9; Na, 0.13; Mg, 50; P, 5000; K, 425; Zn, 0.3; Fe, 4; I, 0.5; Se, 0.02; Mn, 0.90

3.2.2.1 Fish (rock bream (*Oplegnathus fasciatus*))

Rock bream (25.4±0.13 g) was purchased at an aquaculture farm in Jeju Island Chuja-myeon and used it for experiments. The purchased rock bream was moved to the laboratory of Jeju National University and fed the basic feed (Table 19) for 2 weeks and domestication to the environment. We used a rearing aquarium of 1,000 L and in two groups, 35 rock bream were cultures in each tank and ventilation twice a day. The measured water quality parameters were: temperature 20.1±0.5℃, pH 7.5±0.6, salinity 33.0±0.7‰, dissolved oxygen 8.4±0.6 mg/L, and photoperiod 10 h light: 10h dark cycle. Fish were fed with a basal diet ad libitum twice a day at 10:00, and 18:00 h at a rate of 5% of their body weight. Experimental culture was carried out for 10 weeks. We performed random sampling at the time of blood collection and dissection (Kim et al., 2017).

3.2.2.2 Experimental diet

B. amyloliquefaciens JFP-02, a probiotics strain was added to the basic feed for produced the experimental feed and the concentrations were adjusted to 1.4 x 10^6 CFU/100 g. The basic feed not added anything was used in the control group (Kim et al., 2017).

Table 19. Dietary formulation and proximate composition of basal diet for rock bream.

Ingredients (g/100g diet)	Composition (%)
Fish meal	57.0
Wheat flour	20.0
Soy bean oil	5.7
Fish oil [a]	5.3
Mineral premix [c]	2.5
Vitamin premix [d]	3.0
Cellulose [b]	2.5
Binder	2.0
Antifungal	0.3
Antioxidant	0.35
Proximate analysis (% dry matter basis)	
Crude protein	39.48
Crude Lipid	19.7
Ash	9.7
Fiber	2.7
Moisture	8.2
NFE [e]	17.9
Gross energy (MJ/kg) [f]	22.06

[a] E-Wha oil, Pusan, Korea

[b] United States Biochemical (Cleveland, OH) 44122

[c] Premix (g/100 g) contains DL-calcium pantothenate, 0.5; choline bitartrate, 10; inositol, 0.5; menadione, 0.02; niacin, 0.5; pyridoxine-HCl, 0.1; riboflavin, 0.1; thiamine mononitrate, 0.1; DL-a-tocopheryl acetate, 0.2; retinyl acetate, 0.02; biotin, 0.01; folic acid, 0.02; B12, 0.0002; Cholecalciferol, 0.008; a-cellulose, 85.0

[d] Premix (g/100 g) contains Al, 0.12; Ca, 500; Cl, 10; Cu, 0.5; Co, 0.9; Na, 0.13; Mg, 50; P, 5000; K, 425; Zn, 0.3; Fe, 4; I, 0.5; Se, 0.02; Mn, 0.90

[e] Nitrogen-free extract (NFE) – dry matter – (crude protein + crude lipid + ash + fibre)

[f] Gross energy (MJ/kg) calculated according to 23.6 KJ/g for protein, 39.5 KJ/g for lipid and 17.0 kJ/g for NFE

3.2.3.1 Fish (starry flounder (*Platichthys stellatus*))

Starry flounder (17.8±2.97 g) was purchased at an aquaculture farm in Jeju Island Namwon-eup and used it for experiments. The purchased starry flounder was moved to the laboratory of Jeju National University and fed the basic feed (Table 20) for 2 weeks and domestication to the environment. We used a rearing aquarium of 500 mm x 160 mm and in two groups, 39 starry flounder were cultures in each tank and ventilation twice a day. The measured water quality parameters were: temperature 21.2±0.3℃, pH 7.9±0.3, salinity 32.1±0.3‰, dissolved oxygen 7.54±0.3 mg/L, and photoperiod 10 h light: 10h dark cycle. Fish were fed with a basal diet ad libitum twice a day at 10:00, and 18:00 h at a rate of 2% of their body weight. Experimental culture was carried out for 4 weeks. We performed random sampling at the time of blood collection and dissection (Park et al., 2016).

3.2.3.2 Experimental diet

B. amyloliquefaciens JFP-02, a probiotics strain was added to the basic feed for produced the experimental feed and the concentrations were adjusted to 1.4 x 10^6 CFU/100 g. The basic feed not added anything was used in the control group (Kim et al., 2017).

Table 20. Ingredient and proximate composition of the basal diet for starry flounder.

Ingredients	Composition (%)
Casein, vitamin-free [a]	22.0
fish meal [b]	48.0
Dextrin	21.0
Vitamin premix [c]	2.5
Mineral premix [d]	3.0
Carboxymethyl cellulose	3.0
Choline salt	0.5
Proximate analysis (% dry matter basis)	
Crude protein	48.8
Crude lipid	9.3
Crude fiber	1.4
Ash	14.0
N-free extract [e]	26.5

[a] Serva, Feinbiochemica, Heidelberg, Germany

[b] E-Wha oil, Pusan, Korea;

[c] Vitamin premix contained the following amount which were diluted in cellulose (g/kg premix): L-ascorbic acid, 121.2; DL-a-tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-D-pantothenate, 12.7; *myo*-inositol, 181.8; D-biotin, 0.27; folic acid, 0.68; *P*-aminobenzoic acid, 18.2; menadione, 1.8; rethinyl acetate, 0.73; cholecalciferol, 0.003; cyanocobalamin, 0.003.

[d] Mineral premix contained the following ingredients (g/kg premix): $MgSO_4 \cdot 7H_2O$, 80.0; $NaH_2PO_4 \cdot 2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4 \cdot 7H_2O$, 20.0; Ca-lactate, 356.5; CuCl, 0.2; $AlCl_3 \cdot 6H_2O$, 0.15; KI, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4 \cdot H_2O$, 2.0; $CoCl_2 \cdot 6H_2O$, 1.0

[e] Calculated by difference (100 – crude protein – crude lipid – crude fiber – ash)

3.2.4 Growth survey

At the finish of the experiment, final weight was measured and the weight gain, feed conversion ratio, specific growth rate into their respective calculation formulas. The calculation formulas are as follows (Kim et al., 2017).

WG (%) = [(final weight – initial weight) / initial weight] x 100

FCR (%) = feed consumed / weight gain

SGR (%) = [(final body weight – initial body weight) / time (days)] x 100

3.2.5 Hematological analysis

In the hematological analysis was once every two weeks (once a week for starry flounder), random sampling of experimental fish that blood collection and dissection. Blood collection was used 1 ml syringe from the tail of the experimental fish, and the whole blood was used for hematocrit analysis which is a measure of anemia. In the analysis method of hematocrit, after filling the blood with micro-hematocrit capillary tubes (Fisherbrand., USA), the entrance to clay plate (Cha-seal, KIMBLE., USA) was prevented, then the value was measured micro-hematocrit centrifuge (HHC-24, Hanshin-medical.,. Korea) at 12,000 rpm for 5 minutes (Kim., 2016). Blood sampled was allowed to store at room temperature for 1 hour, then the serum was separated using a centrifuge at 12,000 rpm for 10min and stored at -80℃. Used the separated serum, TP (total protein) showing the amount of protein in the serum, an important energy source in the body glucose, TC (total cholesterol) which is a measure of hyperlipidemia, triglyceride which is regarded as a cause of arteriosclerosis, hepatotoxicity it was used for ALT (alanine aminotransferase), AST (aspartate aminotransferase) analysis (Jang., 2012). Serum was injected into VetScan vs2 (Calxis, Uiwang., Korea) used a comprehensive kit (Calxis, Uiwang., Korea) and analyzed.

3.2.6 Innate immune response of aquaculture fish

3.2.6.1 Lysozyme activity

Lysozyme is a glycoside hydrolase that catalyzes the hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan, which is the major component of gram-positive bacterial cell wall. Lysozyme, also known as muramidase or N-acetylmuramide glycanhydolase is an antimicrobial enzyme produced by animals that forms part of the innate immune system. In this turbidimetric assay, 0.03% lyophilized *Micrococcus lysodeikticus* (0.05 M sodium phosphate buffer, pH 6.2) was used as a substrate. 10 ul of fish serum were added to 250 ul of a bacterial suspension in a 96-well plate. The reduction was measured with a microplate reader (Biorad, Ramsey, MN., USA) at 22℃ and 490 nm after 0.5 and 4.5 min of incubation. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001/min (Kim et al., 2017).

3.2.6.2 Macrophage activity

Macrophage is distributed in all tissues in the object and is responsible for immunity, and is an innate immune system that catches bacteria and the like entering from the outside and phagocytosis. When a macrophage ingests a pathogen, the pathogen becomes trapped in a phagosome, which then fuses with a lysosome. Within the phagolysosome, enzymes and toxic peroxides digest the pathogen. The intracellular macrophage activity was fish serum with 25 ul of NBT (nitro blue tetrazolium, Sigma-aldrich., USA) in 175 ul culture medium for 2 hours at 28℃. The supernatants were removed and the cells were fixed with 100% (v/v) methanol for 5 min. Each well was washed twice with 125 ul of 70% (v/v) methanol. The fixed cells were allowed to air dry. The reduced NBT was dissolved used 125 ul of 2N potassium hydroxide and 150 ul of DMSO. Optical density was measured by spectrophotometry at 650 nm (Secombes and Fletcher., 1992).

3.2.7 Total RNA

The organs (liver, kidney, and spleen, intestine) of the experimental fish were extirpation and place in a 1.5 ml tube and stored at -80℃ until RNA was separated. Separation of total RNA was performed according to the protocol of Hybrid-R^{TM} kit (Gene All., Korea). Organs were homogenized to 1 x 10^7 cells used Ribo Ex^{TM} (Gene All., Korea), stored at room temperature for 5 minutes, and then centrifuged at 12,000 rpm, 4℃ for 10 minutes. After taken only the supernatant, added 200 ul of chloroform, and stored at room temperature for 2 minutes, centrifuge at 12,000 rpm for 15 minutes and taken only the supernatant. RB1 buffer in the supernatant to a total volume of 1 ml, and then 700 ul added to mini spin column and centrifuge at 10,000 rpm for 30 seconds. After added 500 ul of SW1, centrifuge at 10,000 rpm for 30 seconds, and 500 ul of RNW were added, centrifuge at 10,000 rpm for 30 seconds. Centrifuge again at 10,000 rpm for 1 minute, and added to 100 ul of Nuclease-free water, which transfer to a new mini spin column was added, stored for 1 minute, then centrifuge at 10,000 rpm for 1 minute to obtain total RNA.

3.2.8 cDNA

In order to synthesize cDNA used total RNA, it was carried out according to the protocol of cDNA Synthesis kit (PhileKorea., Korea). To synthesize cDNA, 5x cDNA synthesis mix and RTase (with RNase inhibitor) were respectively quantitated with RNA of experimental fish and reacted at 42℃ for 30minutes and at 70℃ for 10 minutes according to concentration to synthesize cDNA.

3.2.9 Real-time PCR

For the immune gene expression analysis when fed with *B. amyloliquefaciens* JFP-02 in feed used Real-time PCR. Table 21, Table 22, Table 23 showed the immune gene primer of olive flounder, rock bream, and starry flounder. To prepare the PCR reaction solution, 2.5 ul of each primer (10 uM), 1 ul of the template solution (1 ug cDNA), and DEPC-treated water were added to 12.5ul of Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent technologies, CA., USA) a final volume was made 25 ul. In the real-time PCR conditions, after reaction at 95℃ for 10 minutes, and reactions at 95℃ for 30 seconds, 60℃ for 60 seconds, 72℃ for 60 seconds were performed in one cycle for a total 40 cycles. After amplification, fluorescence data acquired from 0.1℃/seconds at 65℃ to 95℃ and the melting curve was analyzed (Kim et al., 2016). The result was analyzed using LightCycler ® Nano SW 1.0 (Roche., USA) and the relative expression ration (R) of mRNA was calculated according to the formula $2^{-\Delta\Delta Ct} = 2^{-(\Delta Ct\,[test]-\Delta Ct\,[\beta\text{-actin}])}$ (Jang ., 2014). Real-time PCR efficiencies were acquired by the amplification of dilution series of cDNA according to the equation $10^{(-1/slope)}$ and were consistent between target genes and β-actin. The results are presented as means with standard deviations.

Table 21. Quantitative Real-time PCR primer sequence of olive flounder immune genes.

Gene	Primer sequences 5` -3`	Product size (bp)	Accession Number	Tm	Reference
TNFR-1 – F	AGATCCATGACCTGCTGA	145	AB080946	50	Jang., 2014
TNFR-1 - R	GGACCTCTCATAGGCACA				
IL-1b – F	TGCACCCTTCACCCACCA	117	AB070835	60	Jang., 2014
IL-1b – R	CGACACGCTCCAGATGCA				
β-actin - F	AGGCGCAGAGCCTTGATG	191	HQ386788	57	Jang., 2014
β-actin - R	GTCAAGCGCCAAAAATAACTG				

Table 22. Quantitative Real-time PCR primer sequence of *Oplegnathus fasciatus* immune genes.

Gene	Primer sequences 5` -3`	Accession Number	Reference
β-actin – F	CAGGGAGAAGATGACCCAGA	FJ945145	Zenke *et al*., 2010
β-actin – R	CATAGATGGGCACTGTGTGG		
FAS – F	GTTTCGTGCGTCGTTTATCA	AB619804	Jeong *et al*., 2011
FAS – R	CAAACCTGCAGCACACAGACA		
Caspase 3 – F	TGAGGGTGTGTTCTTTGGTACGGA	JQ315116	Elvitigala *et al*., 2012
Caspase 3 – R	TTCCCACTAGTGACTTGCAGCGAT		

Table 23. Quantitative Real-time PCR primer sequence of starry flounder immune genes.

Gene	Primer sequences 5` -3`	Product size (bp)	Reference
TNF – F	TGAGGGATGACCGAACCAC	148	Tong *et al*., 2015
TNF – R	GGACTGGCAGCAGAAAGAAGA		
IL-6 – F	ACAGACACAGCAGATTGCCATAGA	197	Tong *et al*., 2015
IL-6 – R	GCTCCCATCCATCCCTCTTAC		
β-actin – F	GATGCTGTTGTAGGTGGT	106	Tong *et al*., 2015
β-actin – R	AAAGCCAACAGGGAGAAG		

3.2.10 Challenge test

The fish disease pathogens used for the challenge test were pre-cultured for 48 hours at 25℃ in 1.5% BHIB using *Streptococcus iniae* (JFM-004) and *Streptococcus parauberis* (JM-03). After feeding (8 weeks of the olive flounder; 10 weeks of the rock bream; 4 weeks starry flounder) the group of control group and *B. amyloliquefaciens* JFP-02, 200 ul of *S. iniae* and *S. parauberis* at a concentration of 1.2 x 10^6 CFU/ml were injected intraperitoneally into the fish body and then the cumulative mortality (%) was calculated (Kim et al., 2017).

3.2.11 Statistical analysis

All the tests were performed in triplicate. The data were subjected to statistical analysis using the SPSS (SPSS INC, version 18.0., USA). The statistical analysis was done by using one-way analysis of variance (ANOVA) followed by Tukey`s test ($P<0.05$) to compare the means between individual treatments. Result values were expressed as mean ± standard deviation (mean ± S.D) and percentage values were calculated by arcsine deformation value and statistically analyzed.

3.3 **RESULTS**

3.3.1.1 Growth of olive flounder

Dietary influence of *B. amyloliquefaciens* JFP-02 in olive flounder for 8 weeks (Table 24). The highest weight gain, FCR and SGR occurred with the *B. amyloliquefaciens* JFP-02 fed fish group compared with the control. There were no significant differences among the *B. amyloliquefaciens* JFP-02 diet group. These results suggest that there is a difference in the growth of olive flounder according to the concentration of *B. amyloliquefaciens* JFP-02.

Table 24. Growth performance of olive flounder fed with diets enriched *B. amyloliquefaciens* JFP-02.

Parameters	Experimental diets		
	Control	P1	P2
Initial weight (g)	105±0.26^{a}	104±0.11^{a}	106±0.02^{a}
Final weight (g)	112±0.26^{a}	116±0.23^{ab}	121±0.08^{b}
WG (%)	6.67±0.19^{a}	11.54±0.13^{ab}	14.15±0.21^{b}
FCR (%)	1.14±0.14^{a}	1.27±0.07^{ab}	1.29±0.18^{b}
SGR (%)	0.32±0.12^{a}	0.87±.031^{b}	0.94±0.16^{b}

Probiotic enriched feed P1=1.4 x 10^{4} CFU/100g feed; P2= 1.4 x 10^{6} CFU/100g feed. *Values (Mean ± SD) in the same row different superscripts are significantly different from each other ($P<0.05$); WG (%) = [(final weight – initial weigh) / initial weight] x 100; FCR (%) = feed consumed / weight gain; SGR (%) = [(final body weight – initial body weight) / time (days)] x 100.

3.3.1.2 Hematological analysis of olive flounder

Results of hematocrit as compared with the control group of 8 weeks, *B. amyloliquefaciens* JFP-02 group increased significantly, and at the finish of the experiment, compared was *B. amyloliquefaciens* JFP-02 (42.6 ±0.79) with the control group (34.27±2.33), which was higher than that in the control group (Fig. 29). Results of TP as compared with the control group of 4 weeks and 8 weeks, *B. amyloliquefaciens* JFP-02 group increased significantly, and at the finish of the experiment, compared was *B. amyloliquefaciens* JFP-02 (1.19±0.07) with the control group (0.78±0.04), which was higher than that in the control group (Fig. 29). Results of glucose as compared with the control group of 8 weeks, *B. amyloliquefaciens* JFP-02 group increased significantly, and at the finish of the experiment, compared was *B. amyloliquefaciens* JFP-02 (102.33±1.76) with the control group (57.33±1.45), which was higher than that in the control group (Fig. 29). Results of triglyceride as compared with the control group of 6 weeks, *B. amyloliquefaciens* JFP-02 group decreased significantly, and at the finish of the experiment, compared was *B. amyloliquefaciens* JFP-02 (2.07±0.03) with the control group (2.47±0.03), which was lower than that in the control group (Fig. 30). Results of TC as compared with the control group of 8 weeks, *B. amyloliquefaciens* JFP-02 group decreased significantly, and at the finish of the experiment, compared was *B. amyloliquefaciens* JFP-02 (100.0±1.45) with the control group (115.0±2.89), which was lower than that in the control group (Fig. 30). Results of AST and ALT steadily decreased from the start of the experiment as compared with the control group, and at the finish of the experiment, compared was *B. amyloliquefaciens* JFP-02 (AST-8.0±3.51; ALT-1.33±0.33) with the control group (AST-28.33±2.03; ALT-2.67±0.33), which was lower than that in the control group (Fig. 30).

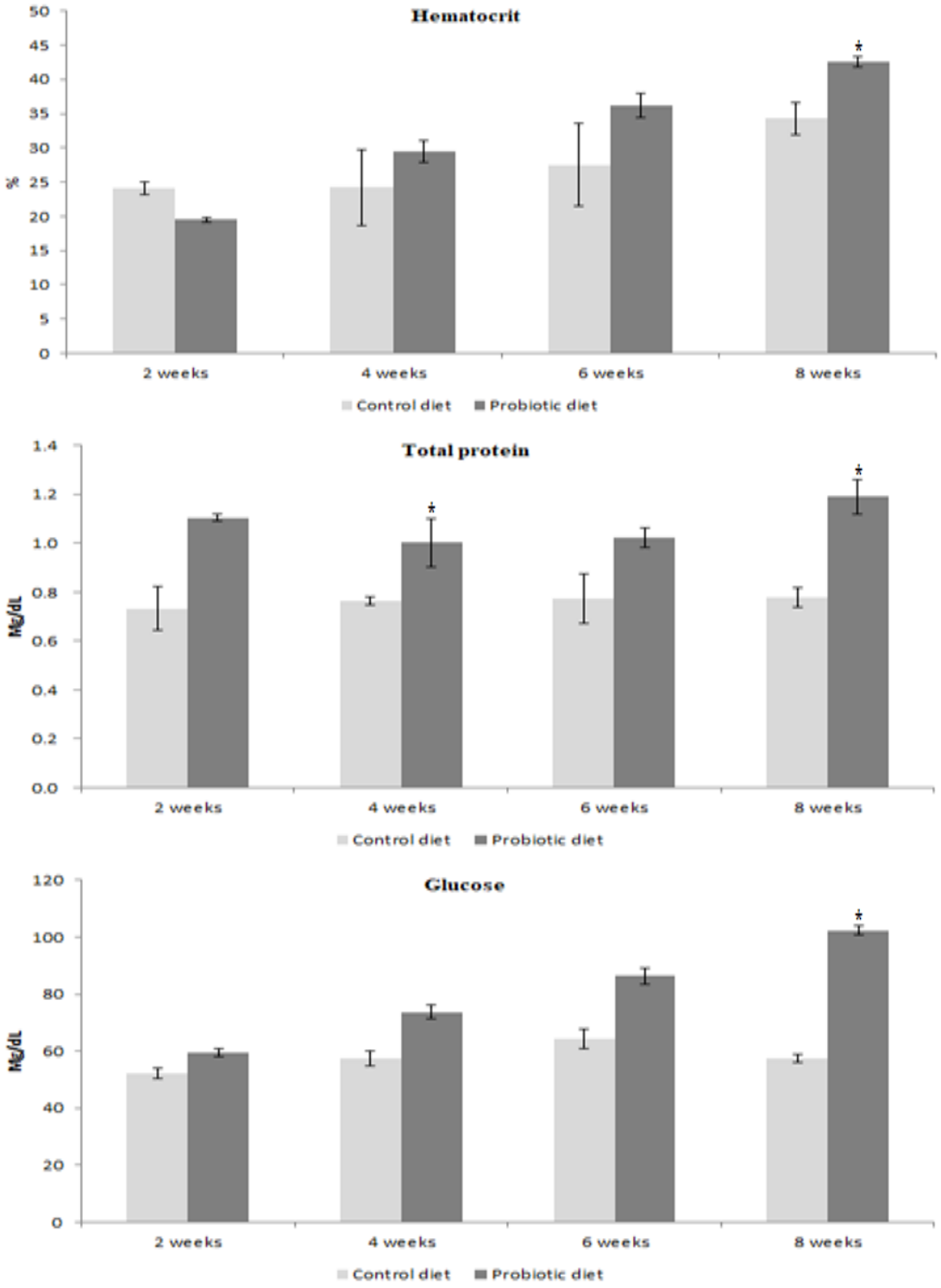


Fig. 29. Serum biochemical analysis (Hematocrit, Total protein, Glucose) of olive flounder fed with control and *B. amyloliquefaciens* JFP-02 enriched probiotic diet. Values are mean ± SE (n=5).

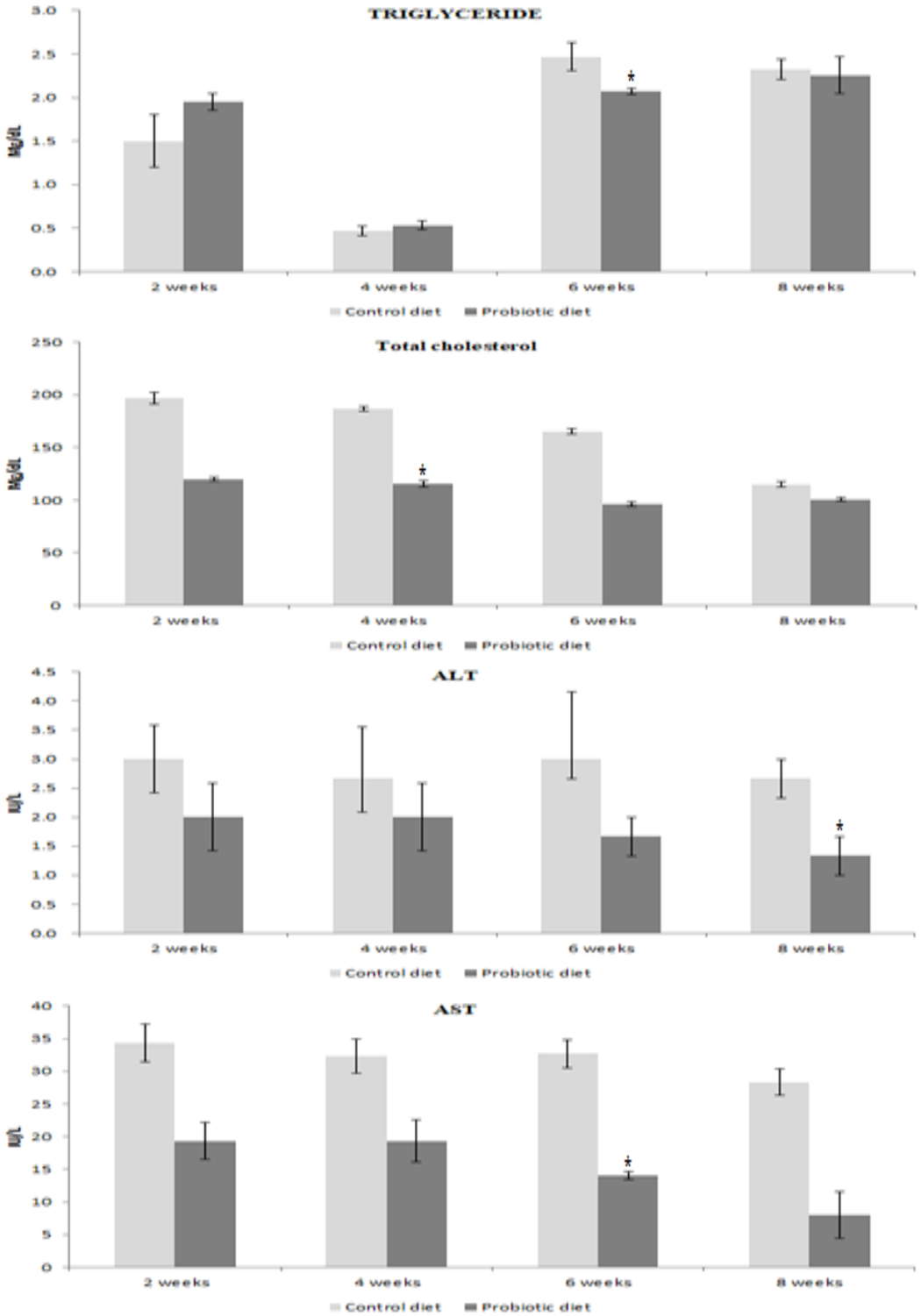


Fig. 30. Serum biochemical analysis (TRIGLYCERIDE, Total cholesterol, ALT, AST) of olive flounder fed with control and *B. amyloliquefaciens* JFP-02 enriched probiotic diet. Values are mean ± SE (n=5).

3.3.1.3 Innate immune response of olive flounder

Results of lysozyme activity as compared with the control group of 6 weeks and 8 weeks, *B. amyloliquefaciens* JFP-02 group increased significantly, and at the finish of the experiment, the lysozyme activity was higher in the group fed with *B. amyloliquefaciens* JFP-02 (38.37±0.59) than in the control group (34.38±1.06) (Fig. 31). Results of macrophage activity as compared with the control group of 8 weeks, *B. amyloliquefaciens* JFP-02 group increased significantly, and at the finish of the experiment, the lysozyme activity was higher in the group fed with *B. amyloliquefaciens* JFP-02 (0.42±0.009) than in the control group (0.35±0.00) (Fig. 31).

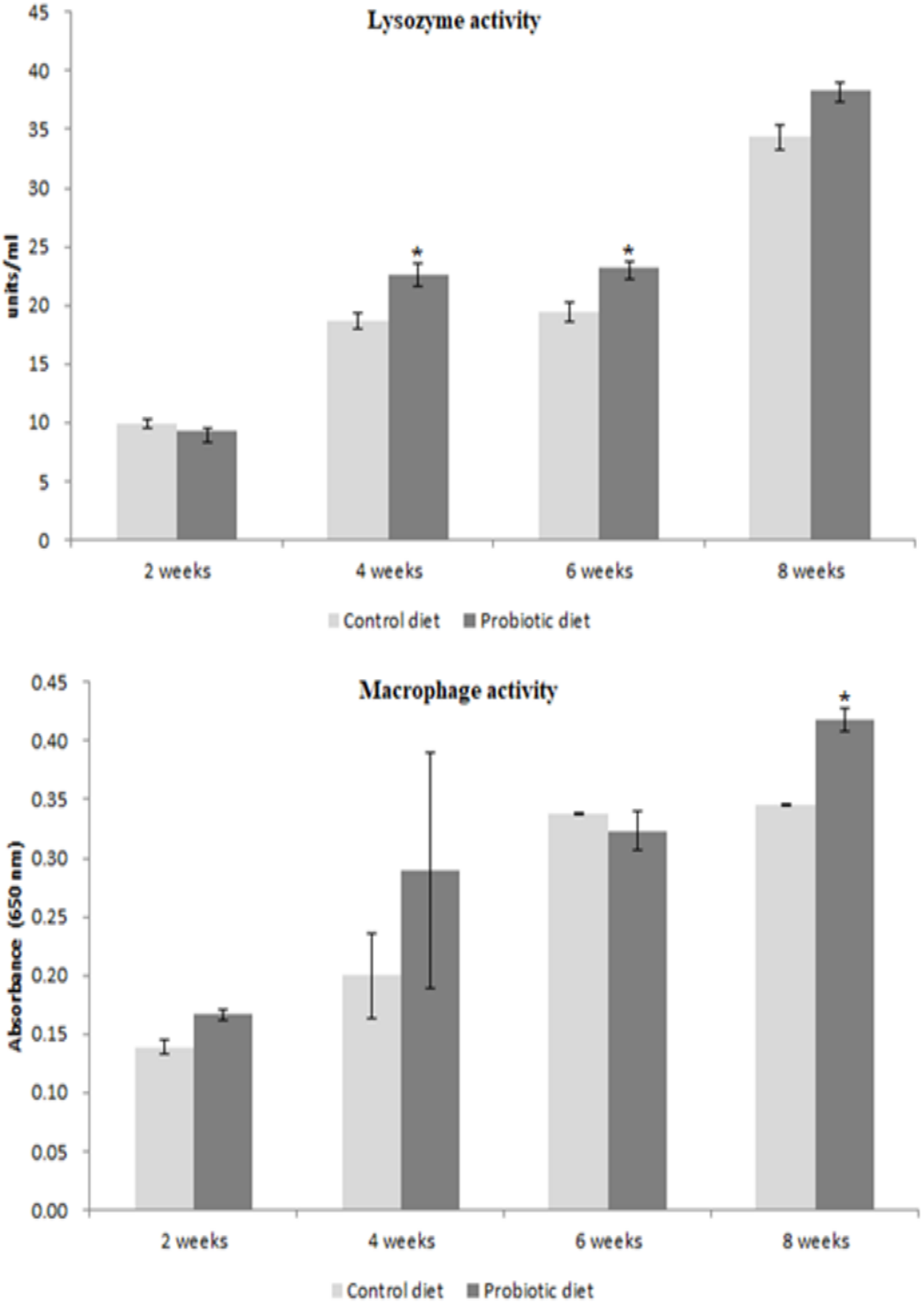
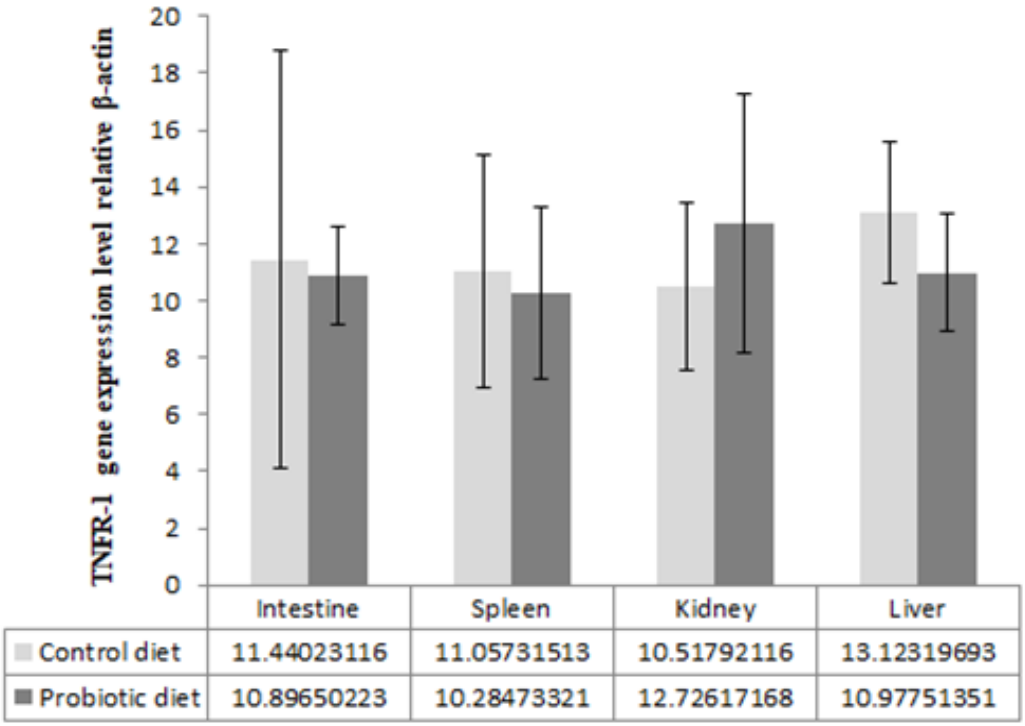


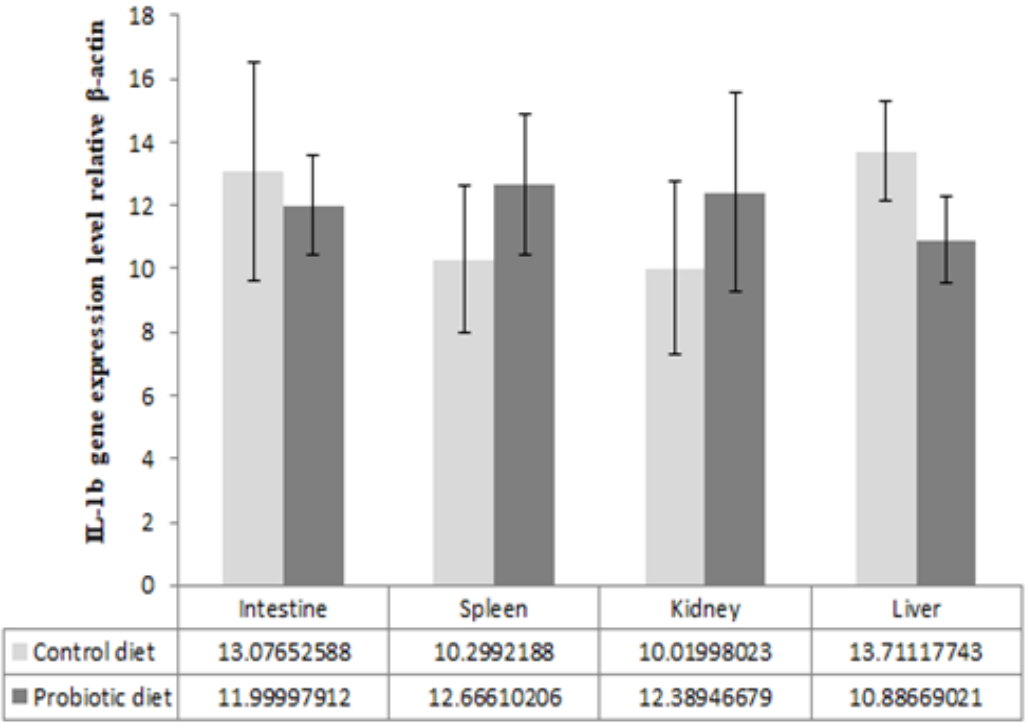
Fig. 31. Serum lysozyme and macrophage activity of olive flounder fed with control and *B. amyloliquefaciens* JFP-02 enriched probiotic diet. Values are mean ± SE (n=5).

3.3.1.4 Real-time PCR expression of immune gene in olive flounder

In the last week of the experiment, fish were collected from the control group and *B. amyloliquefaciens* JFP-02 group, and spleen, kidney, liver, intestine total RNA was separated, and real-time PCR was performed after cDNA synthesis using the primer TNFR-1 and IL-1b, the gene expression level was confirmed. In the case of TNFR-1, the expression level was smaller than that in the intestine, kidney, and liver excluding the kidney, the expression level was lower in the case of IL-1b than in the control group, and in the spleen, kidney, the expression level was higher spleen and kidney than in the control group (Fig. 32). This is thought to inhibited formation of inflammatory cytokines.



	Intestine	Spleen	Kidney	Liver
Control diet	11.44023116	11.05731513	10.51792116	13.12319693
Probiotic diet	10.89650223	10.28473321	12.72617168	10.97751351



	Intestine	Spleen	Kidney	Liver
Control diet	13.07652588	10.2992188	10.01998023	13.71117743
Probiotic diet	11.99997912	12.66610206	12.38946679	10.88669021

Fig. 32. Relative cDNA in various tissues of olive flounder. Intestine, spleen, kidney, liver were examined. The all samples were normalized using β-actin expression as an internal control. Values are mean ± SE (n=5).

3.3.1.5 Disease resistance of olive flounder

As a result of the challenge experiment with *S. iniae* inoculated (1.2 x 10^6 CFU/ml) with *B. amyloliquefaciens* JFP-02 for 8 weeks, the control group showed mortality rate from 4th day and *B. amyloliquefaciens* JFP-02 group the mortality rate was showed from the 6th day (Fig. 33). On the 20th day at the finish of the experiment, the control group showed a mortality rate of 85%, the *B. amyloliquefaciens* JFP-02 group showed mortality rate of 45% showed, high survival rate and high *S. iniae* disease resistance showed.

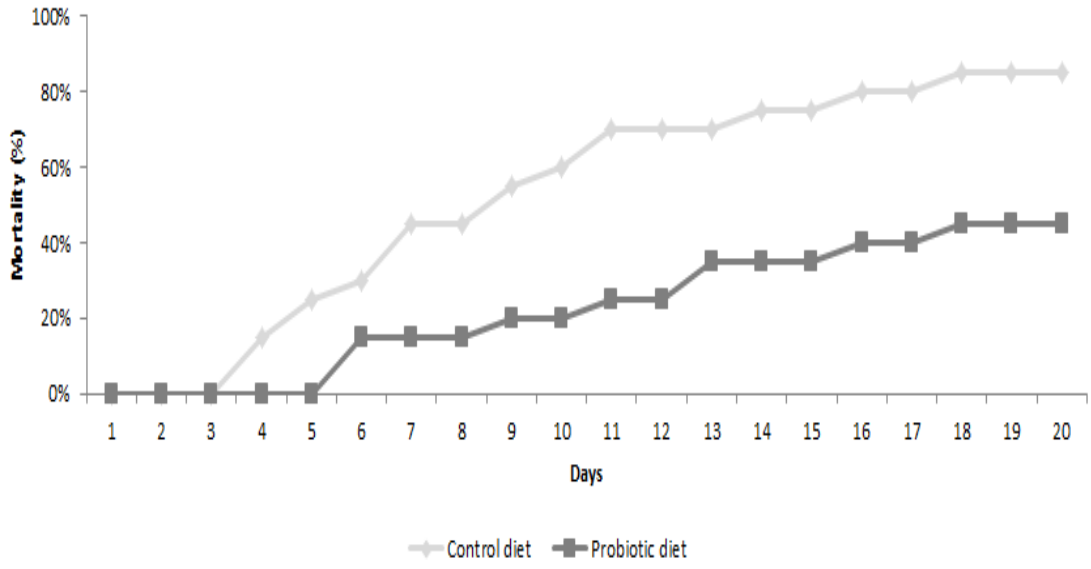


Fig. 33. Cumulative mortality of olive flounder fed with control and *B. amyloliquefacien* JFP-02 enriched probiotic diet. Values are mean ± SE (n=5).

3.3.2.1 Growth of rock bream

Growth performance of both treatment groups of fish (control and *B. amyloliquefaciens* JFP-02 feed) are shown in Table 25. In the *B. amyloliquefaciens* JFP-02 fed fish group FCR decreased 1.86±0.20, WG and SGR increased 32.76±1.32 and 2.56±0.14 compared to the control fish group. These results were thought to be effective when B. *amyloliquefaciens* JFP-02 was added at the concentration of 1.4 x 10^6 CFU/100 g for the growth performance of the rock bream.

Table 25. Growth performance of rock bream fed with diets enriched *B. amyloliquefaciens* JFP-02.

Parameters	Experimental diets	
	Control	Probiotic
Initial weight (g)	25.4±0.13[a]	24.9±0.21 [a]
Final weight (g)	31.28±2.25[a]	33.06±1.87[b]
WG (%)	23.16±1.14[a]	32.76±1.32[b]
FCR (%)	2.22±0.09[a]	2.56±0.14[b]
SGR (%)	2.18±0.22[c]	1.86±0.20[a]

*Data are presented as mean ± SD (n = 10). Values in each row with different superscripts show significant difference ($P < 0.05$); WG (%) = [(final weight – initial weigh) / initial weight] x 100; FCR (%) = feed consumed / weight gain; SGR (%) = [(final body weight – initial body weight) / time (days)] x 100.

3.3.2.2 Hematological analysis of rock bream

Results of hematocrit as compared with the control group of 8 weeks and 10 weeks, *B. amyloliquefaciens* JFP-02 group increased significantly, and at the finish of the experiment, compared was *B. amyloliquefaciens* JFP-02 (59.17±0.95)with the control group (37.7±0.33), which was higher than that in the control group (Fig. 34).). Results of TP as compared with the control group of 8 weeks and 10 weeks, *B. amyloliquefaciens* JFP-02 group increased significantly, and at the finish of the experiment, compared was *B. amyloliquefaciens* JFP-02 (1.4±0.1) with the control group (0.7±0.12), which was higher than that in the control group (Fig. 34). Results of glucose as compared with the control group of 8 weeks and 10 weeks, *B. amyloliquefaciens* JFP-02 group increased significantly, and at the finish of the experiment, compared was *B. amyloliquefaciens* JFP-02 (83.6±2.40) with the control group (34.6±0.88), which was higher than that in the control group (Fig. 34). Results of triglyceride as compared with the control group of 8 weeks, *B. amyloliquefaciens* JFP-02 group decreased significantly, and at the finish of the experiment, compared was *B. amyloliquefaciens* JFP-02 (2.3±0.33) with the control group (3.3±0.3), which was lower than that in the control group (Fig. 35). Results of TC as compared with the control group of 8 weeks and 10 weeks, *B. amyloliquefaciens* JFP-02 group decreased significantly, and at the finish of the experiment, compared was *B. amyloliquefaciens* JFP-02 (55.0±0.57) with the control group (179.3±2.3), which was lower than that in the control group (Fig. 35). Results of AST and ALT steadily decreased from the start of the experiment as compared with the control group, and at the finish of the experiment, compared was *B. amyloliquefaciens* JFP-02 (AST-10.3±0.88; ALT-1.67±0.66) with the control group (AST-48.0±1.52; ALT-3.0±1.15), which was lower than that in the control group (Fig. 35).

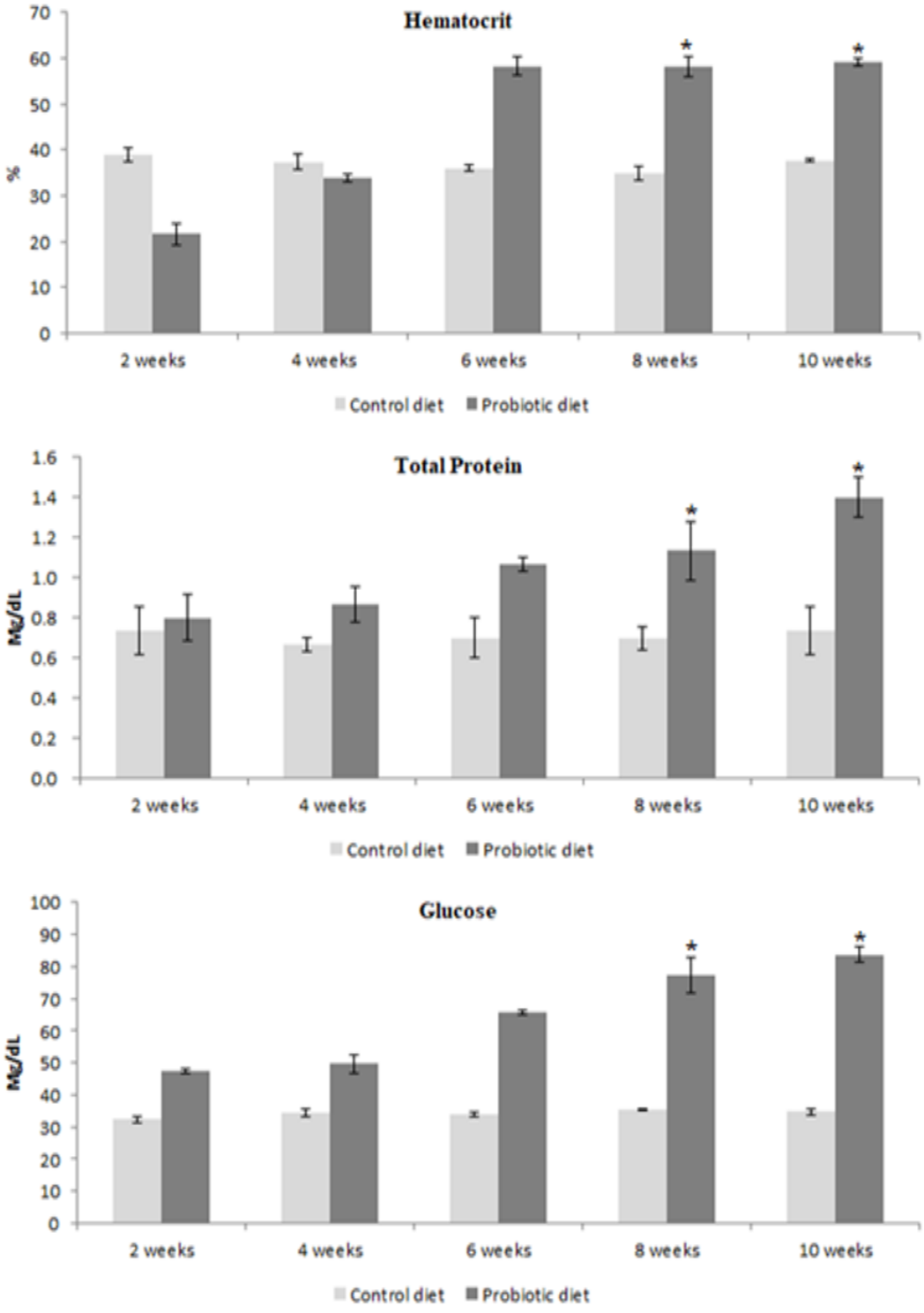


Fig. 34. Serum biochemical analysis (Hematocrit, Total protein, Glucose) of rock bream fed with control and *B. amyloliquefaciens* JFP-02 enriched probiotic diet. Values are mean ± SE (n=5).

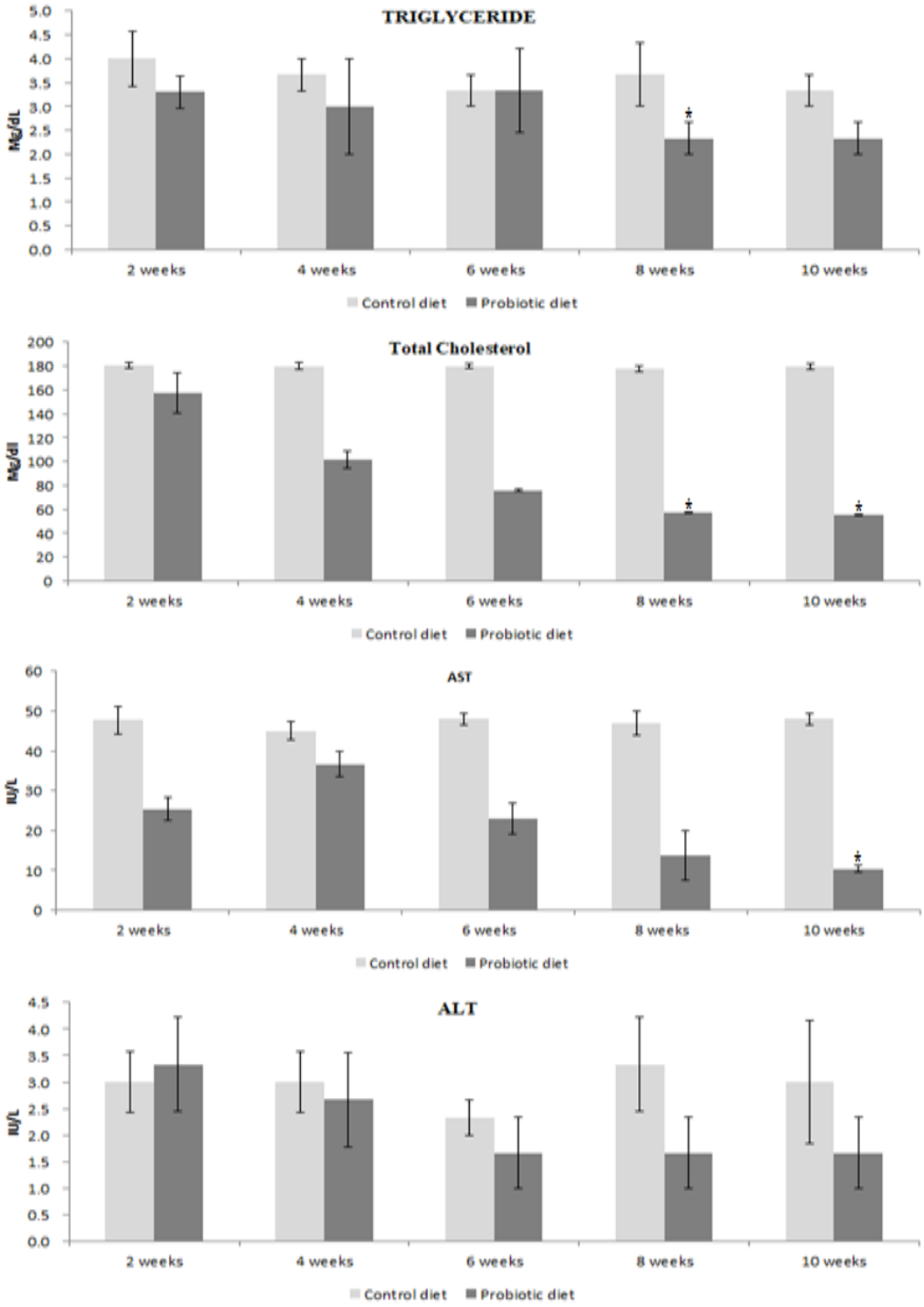


Fig. 35. Serum biochemical analysis (TRIGLYCERIDE, Total cholesterol, ALT, AST) of rock bream fed with control and *B. amyloliquefaciens* JFP-02 enriched probiotic diet. Values are mean ± SE (n=5).

3.3.2.3 Innate immune response of olive flounder

Results of lysozyme activity as compared with the control group of 10 weeks, *B. amyloliquefaciens* JFP-02 group increased significantly, and at the finish of the experiment, the lysozyme activity was higher in the group fed with *B. amyloliquefaciens* JFP-02 (32.65±0.27) than in the control group (12.92±0.31) (Fig. 36). Results of macrophage activity as compared with the control group of 8 weeks, *B. amyloliquefaciens* JFP-02 group increased significantly, and at the finish of the experiment, the lysozyme activity was higher in the group fed with *B. amyloliquefaciens* JFP-02 (1.36±0.01) than in the control group (1.16±0.03) (Fig. 36).

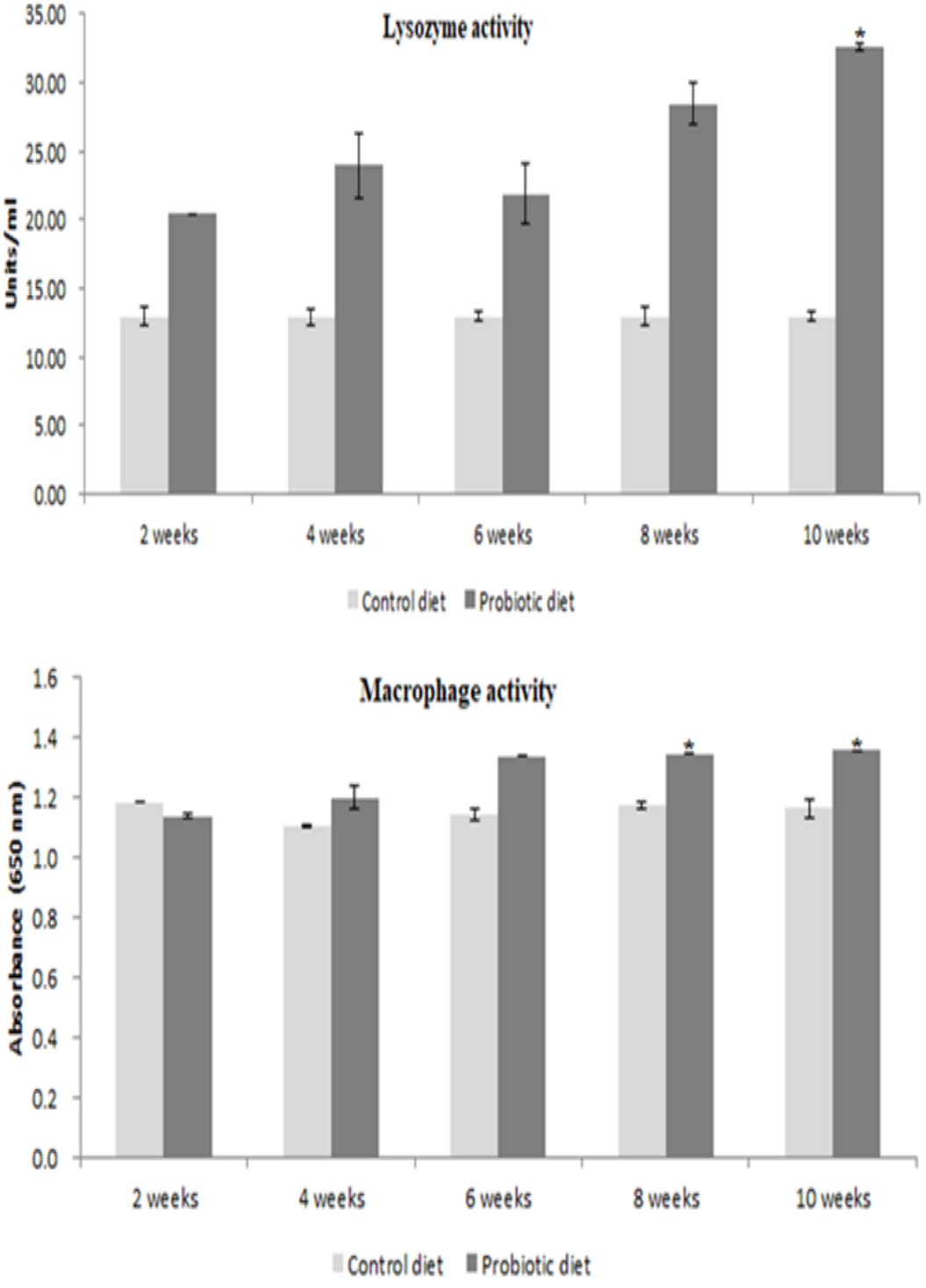
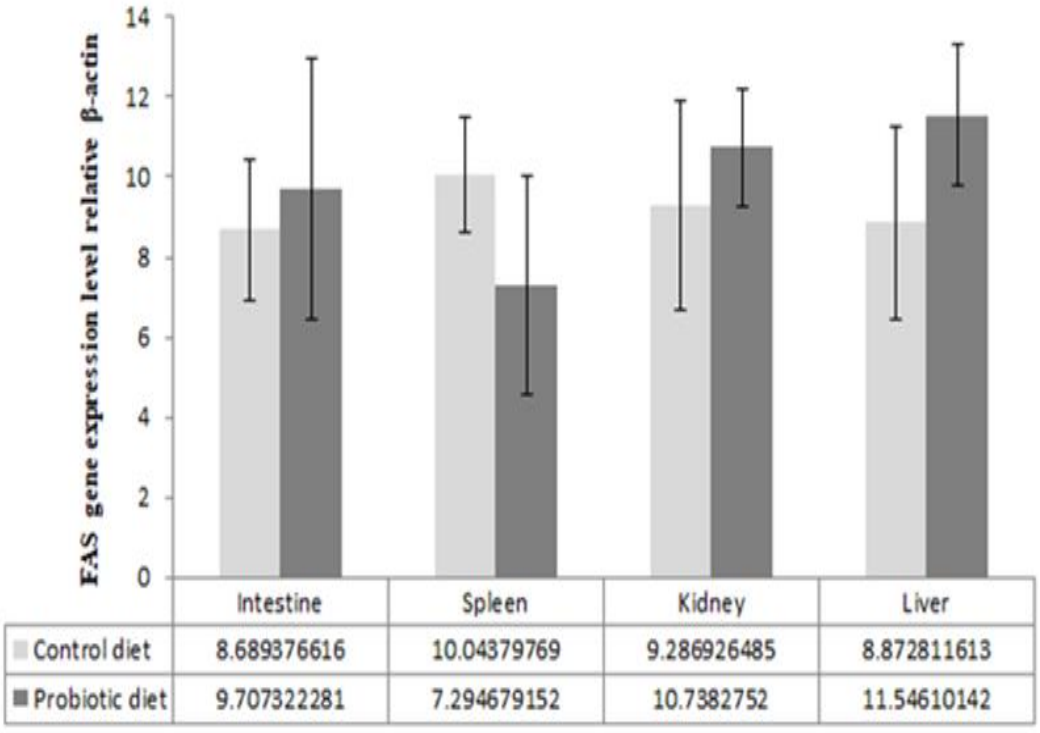


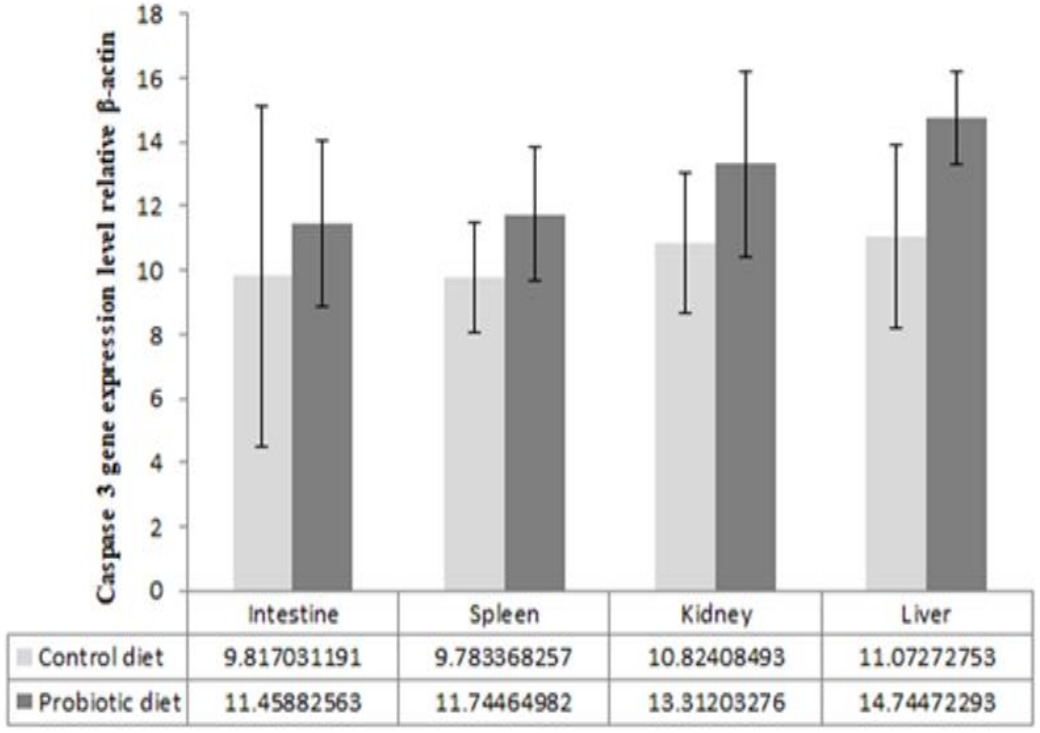
Fig. 36. Serum lysozyme and macrophage activity of rock bream fed with control and *B. amyloliquefaciens* JFP-02 enriched probiotic diet. Values are mean ± SE (n=5).

3.3.2.4 Real-time PCR expression of immune gene in rock bream

In the last week of the experiment, fish were collected from the control group and *B. amyloliquefaciens* JFP-02 group, and spleen, kidney, liver, intestine total RNA was separated, and real-time PCR was performed after cDNA synthesis using the primer FAS and Caspase 3 the gene expression level was confirmed. In the case of FAS, the expression level was highest than that in the liver, whereas the lowest expression level was showed in the kidney. In the case of Caspase 3, it showed high expression than the control in all organs (Fig. 37). It is thought that this promotes the expression of immune genes in the fish body.



	Intestine	Spleen	Kidney	Liver
Control diet	8.689376616	10.04379769	9.286926485	8.872811613
Probiotic diet	9.707322281	7.294679152	10.7382752	11.54610142



	Intestine	Spleen	Kidney	Liver
Control diet	9.817031191	9.783368257	10.82408493	11.07272753
Probiotic diet	11.45882563	11.74464982	13.31203276	14.74472293

Fig. 37. Relative cDNA in various tissues of rock bream. Intestine, spleen, kidney, liver were examined. The all samples were normalized using β-actin expression as an internal control. Values are mean ± SE (n=5).

3.3.2.5 Disease resistance of rock bream

As a result of the challenge experiment with *S. iniae* inoculated (1.2 x 10^6 CFU/ml) with *B. amyloliquefaciens* JFP-02 for 10 weeks, the control group showed mortality rate from 5^{th} day and *B. amyloliquefaciens* JFP-02 group the mortality rate was showed from the 6^{th} day (Fig. 38). On the 20^{th} day at the finish of the experiment, the control group showed a mortality rate of 90%, the *B. amyloliquefaciens* JFP-02 group showed mortality rate of 35% showed, high survival rate and high *S. iniae* disease resistance showed.

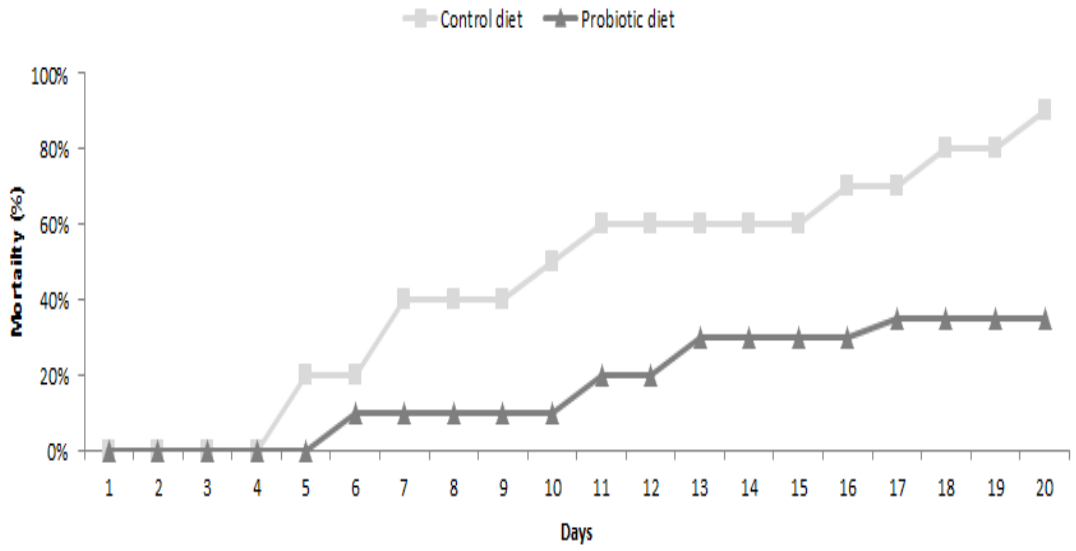


Fig. 38. Cumulative mortality of rock bream fed with control and *B. amyloliquefacien* JFP-02 enriched probiotic diet. Values are mean ± SE (n=5).

3.3.3.1 Growth of starry flounder

Growth performance of both treatment groups of fish (control and *B. amyloliquefaciens* JFP-02 feed) are shown in Table 26. In the *B. amyloliquefaciens* JFP-02 fed fish group FCR decreased 1.75±0.09, WG and SGR increased 53.1±2.76 and 16.79±0.14 compared to the control fish group. These results were thought to be effective when B. *amyloliquefaciens* JFP-02 was added at the concentration of 1.4 x 10^6 CFU/100 g for the growth performance of the starry flounder.

Table 26. Growth performance of starry flounder fed with diets enriched *B. amyloliquefaciens* JFP-02.

Parameters	Experimental diets	
	Control	Probiotic
Initial weight (g)	17.8±2.97[a]	17.7±1.87[a]
Final weight (g)	25.9±3.82[a]	27.1±2.85[ab]
WG (%)	45.5±2.14[a]	53.1±2.76[b]
FCR (%)	2.17±0.12[a]	1.75±0.09[b]
SGR (%)	14.45±0.28[c]	16.79±0.14[c]

*Data are presented as mean ± SD (n = 10). Values in each row with different superscripts show significant difference ($P < 0.05$); WG (%) = [(final weight – initial weigh) / initial weight] x 100; FCR (%) = feed consumed / weight gain; SGR (%) = [(final body weight – initial body weight) / time (days)] x 100.

3.3.3.2 Hematological of starry flounder

Results of hematocrit as compared with the control group of 4 weeks, *B. amyloliquefaciens* JFP-02 group increased significantly, and at the finish of the experiment, compared was *B. amyloliquefaciens* JFP-02 (86.06±1.06) with the control group (75.16±4.57), which was higher than that in the control group (Fig. 39). Results of TP as compared with the control group of 4 weeks and 8 weeks, *B. amyloliquefaciens* JFP-02 group increased significantly, and at the finish of the experiment, compared was *B. amyloliquefaciens* JFP-02 (3.3±0.05) with the control group (3.1±0.06), which was higher than that in the control group (Fig. 39). Results of glucose as compared with the control group of 8 weeks, *B. amyloliquefaciens* JFP-02 group increased significantly, and at the finish of the experiment, compared was *B. amyloliquefaciens* JFP-02 (39±1.15) with the control group (29±0.58), which was higher than that in the control group (Fig. 39). Results of AST and ALT steadily decreased from the start of the experiment as compared with the control group, and at the finish of the experiment, compared was *B. amyloliquefaciens* JFP-02 (AST-16±0.58;ALT-22±0.57) with the control group (AST-44±2.31; ALT-48±2.30), which was lower than that in the control group (Fig. 40). Results of calcium as compared with the control group of 4 weeks, *B. amyloliquefaciens* JFP-02 group increased significantly, and at the finish of the experiment, compared was *B. amyloliquefaciens* JFP-02 (11.6±0.35) with the control group (9.6±0.29), which was higher than that in the control group (Fig. 40).

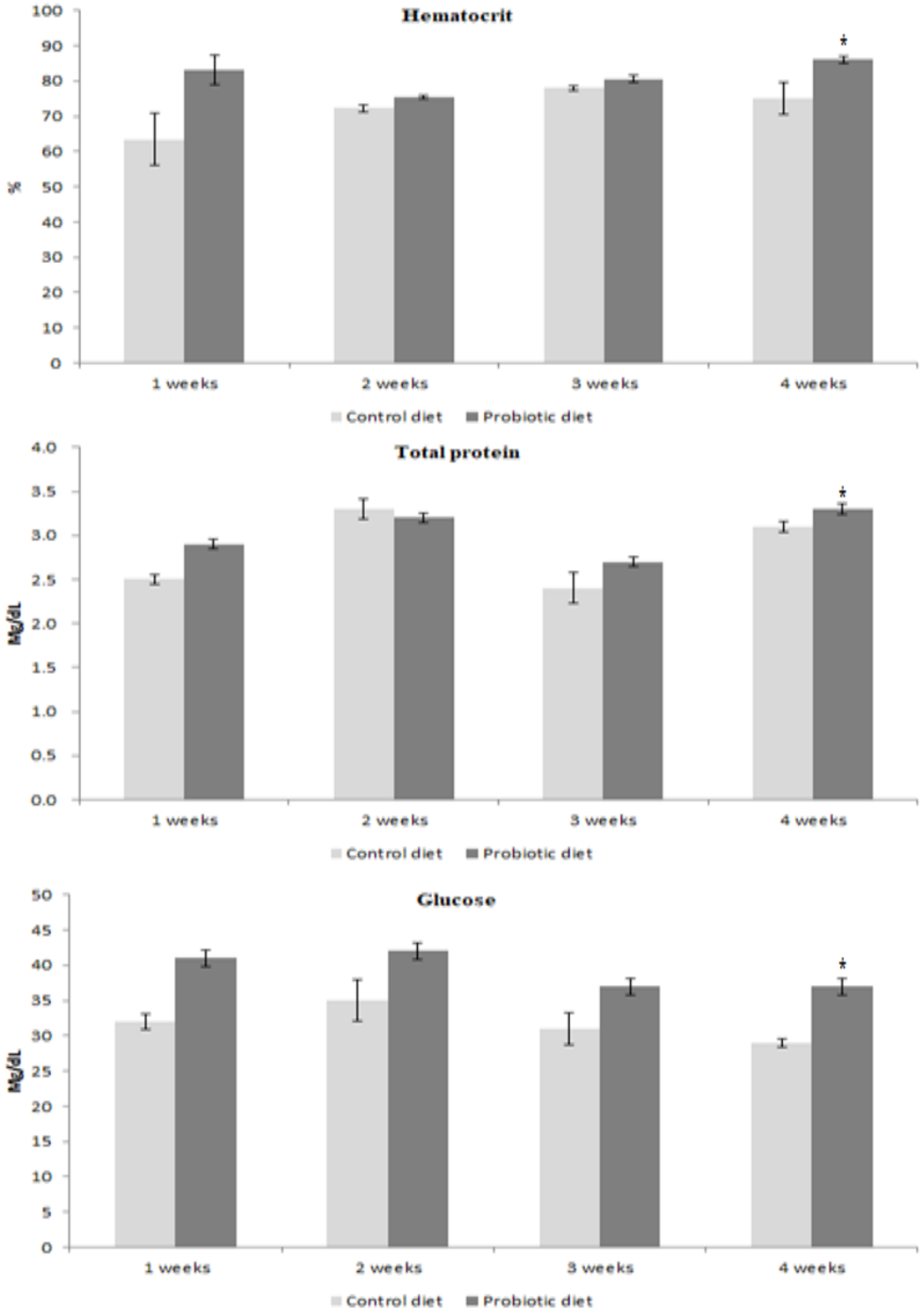


Fig. 39. Serum biochemical analysis (Hematocrit, Total protein, Glucose) of starry flounder fed with control and *B. amyloliquefaciens* JFP-02 enriched probiotic diet. Values are mean ± SE (n=5).

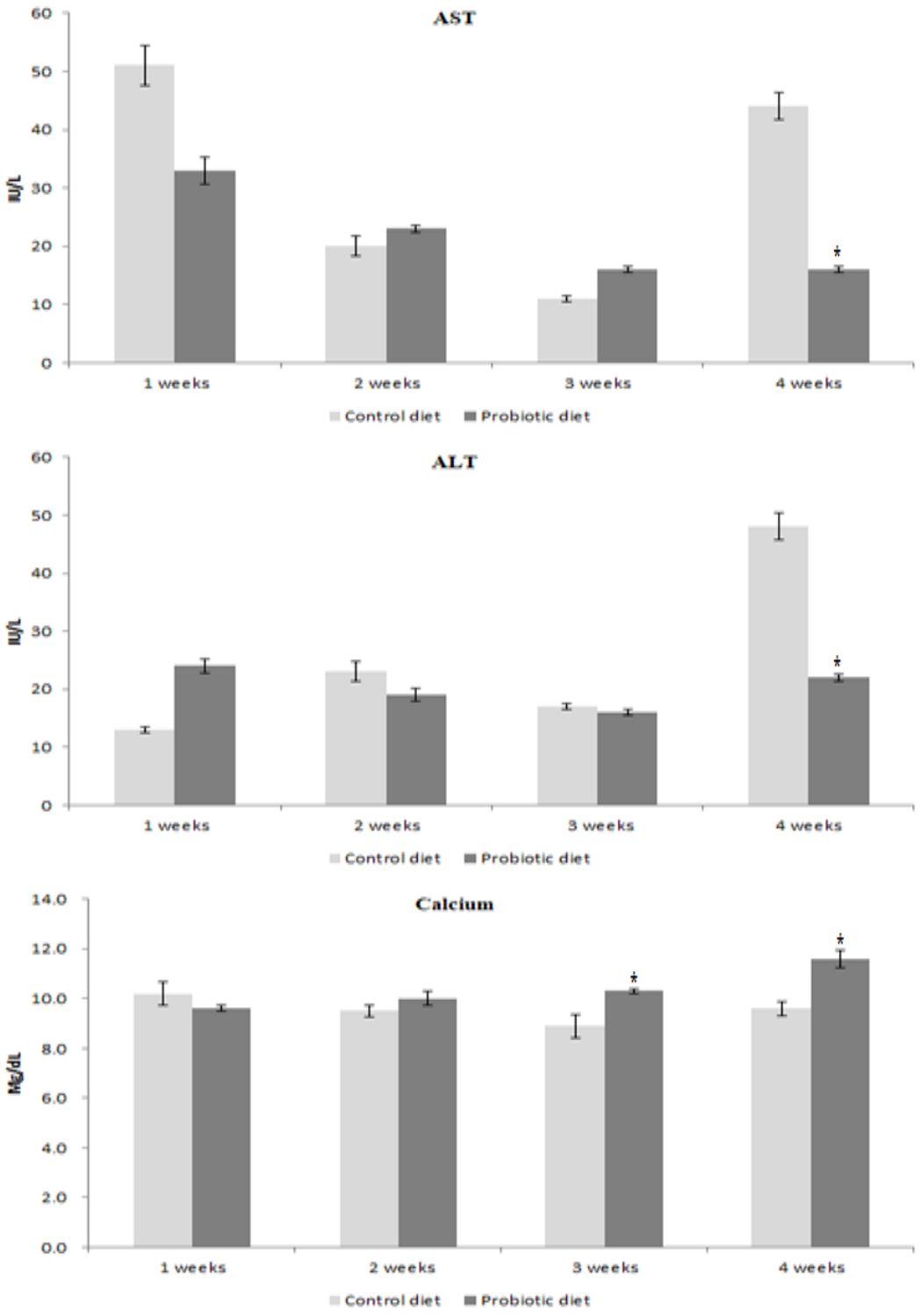


Fig. 40. Serum biochemical analysis (ALT, AST, Calcium) of starry flounder fed with control and *B. amyloliquefaciens* JFP-02 enriched probiotic diet. Values are mean ± SE (n=5).

3.3.3.3 Innate immune response of starry flounder

Results of lysozyme activity as compared with the control group of 4 weeks, *B. amyloliquefaciens* JFP-02 group increased, and at the finish of the experiment, the lysozyme activity was higher in the group fed with *B. amyloliquefaciens* JFP-02 (20.20±0.79) than in the control group (18.71±0.13) (Fig. 41). Results of macrophage activity as compared with the control group of 2 weeks, *B. amyloliquefaciens* JFP-02 group increased, and at the finish of the experiment, the lysozyme activity was higher in the group fed with *B. amyloliquefaciens* JFP-02 (0.20±0.01) than in the control group (0.19±0.01) (Fig. 41).

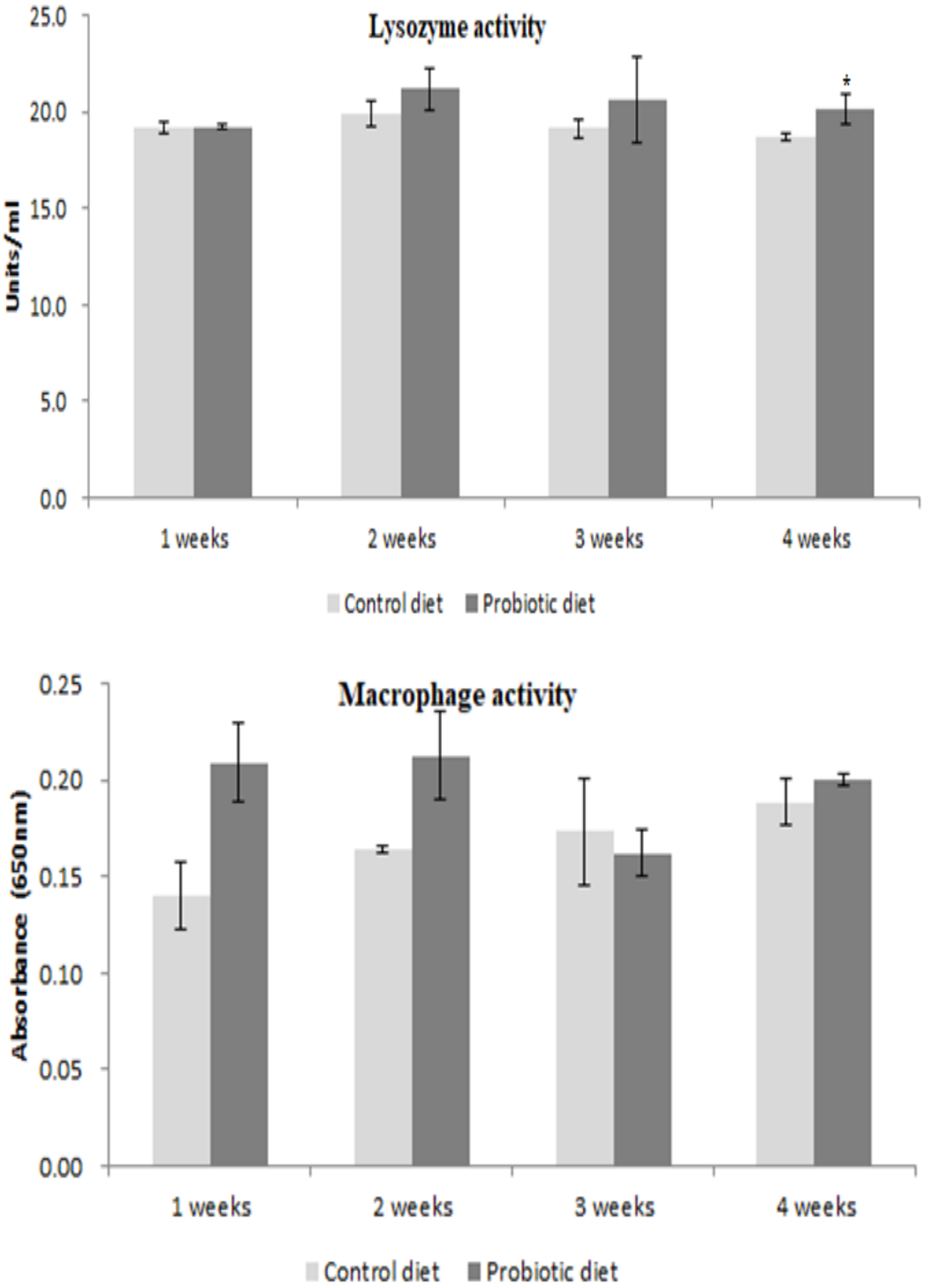
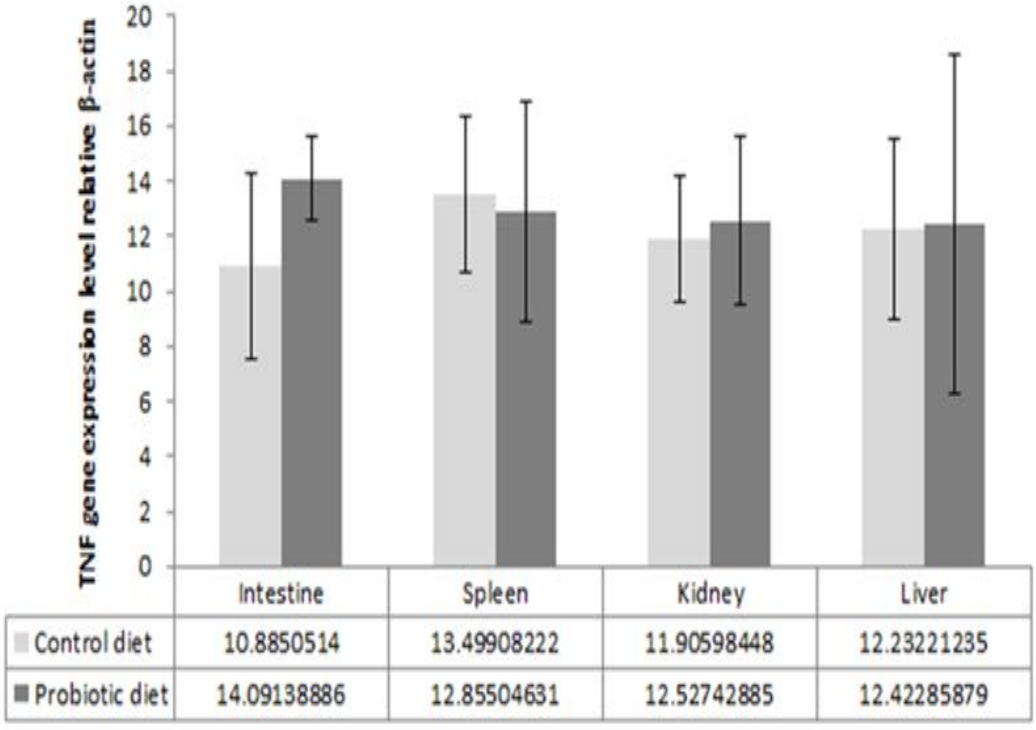


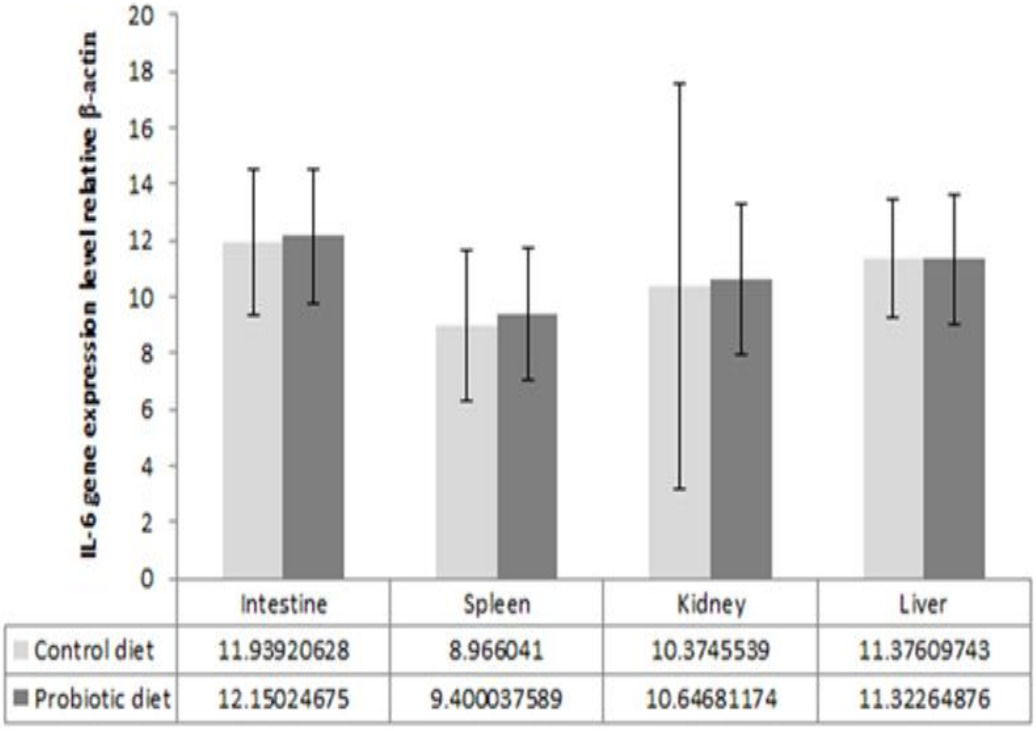
Fig. 41. Serum lysozyme and macrophage activity of starry flounder fed with control and *B. amyloliquefaciens* JFP-02 enriched probiotic diet. Values are mean ± SE (n=5).

3.3.3.4 Real-time PCR expression of immune gene in starry flounder

In the last week of the experiment, fish were collected from the control group and *B. amyloliquefaciens* JFP-02 group, and spleen, kidney, liver, intestine total RNA was separated, and real-time PCR was performed after cDNA synthesis using the primer TNF and IL-6, the gene expression level was confirmed. In the case of TNF and IL-6, the expression amount was showed highest in the intestine, and the expression amount in the kidney was comparatively lower (Fig. 42). These results are considered to promote expression of immune genes in fish body especially intestine.



	Intestine	Spleen	Kidney	Liver
Control diet	10.8850514	13.49908222	11.90598448	12.23221235
Probiotic diet	14.09138886	12.85504631	12.52742885	12.42285879



	Intestine	Spleen	Kidney	Liver
Control diet	11.93920628	8.966041	10.3745539	11.37609743
Probiotic diet	12.15024675	9.400037589	10.64681174	11.32264876

Fig. 42. Relative cDNA in various tissues of starry flounder. Intestine, spleen, kidney, liver were examined. The all samples were normalized using β-actin expression as an internal control. Values are mean ± SE (n=5).

3.3.3.5 Disease resistance of starry flounder

As a result of the challenge experiment with *S. parauberis* inoculated (1.2 x 10^6 CFU/ml) with *B. amyloliquefaciens* JFP-02 for 4 weeks, the control group showed mortality rate from 6th day and *B. amyloliquefaciens* JFP-02 group the mortality rate was showed from the 7th day (Fig. 43). On the 20th day at the finish of the experiment, the control group showed mortality rate of 75%, the *B. amyloliquefaciens* JFP-02 group showed mortality rate of 45% showed, high survival rate and high *S. parauberis* disease resistance showed.

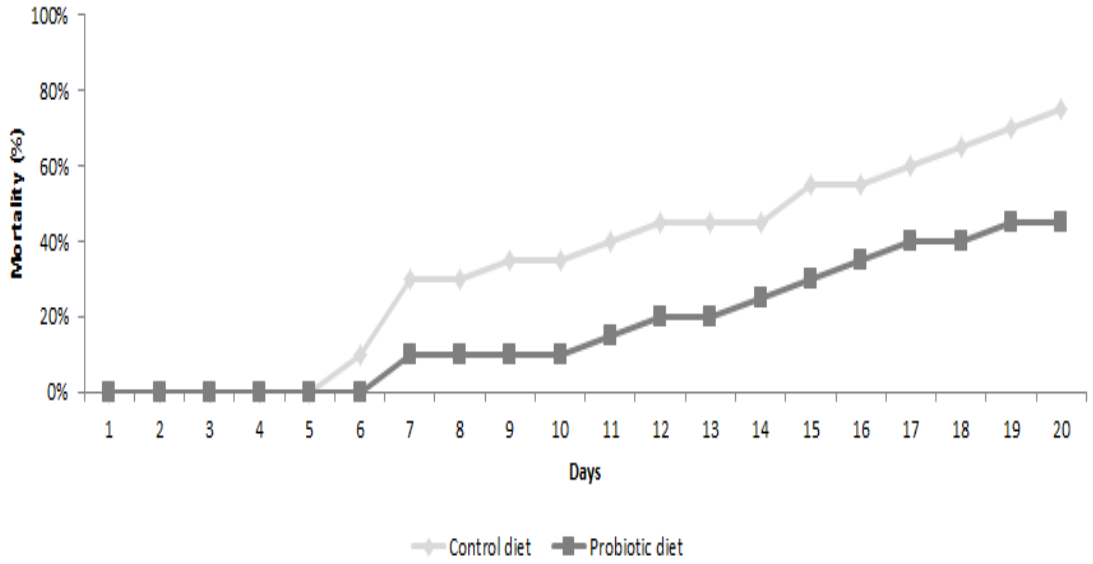


Fig. 43. Cumulative mortality of starry flounder fed with control and *B. amyloliquefacien* JFP-02 enriched probiotic diet. Values are mean ± SE (n=5).

3.4 **DISCUSSION**

Dietary effect of our lab isolated probiotic bacterial strain *Bacillus amyloliquefacens*-JFP2 from Jeju Island's traditional fermented seafood, jeotgal was assessed for growth and innate immune parameters of olive flounder, rock bream, starry flounder.

Fish fed probiotic enriched feed showed significant increase in growth, FCR and survival rate compared to control diet fed fish. Similarly, significant effects of probiotic enriched diet on growth performance have also been reported in red sea bream (Dawood et al., 2016), rohu (Ghosh et al., 2003), Nile tilapia (Aly et al., 2008), Siberian sturgeon (Pourgholam et al., 2016) and Pla Pho (*Pangasius bocourti*) (Meng-Umphan, 2009).

B. amyloliquefaciens enriched probiotic diet fed fish show a significant decrease in cholesterol and triglyceride, levels compared to the control diet fed fish (Fig. 30, 35). Similar declining pattern of triglycerides and total serum cholesterol levels were reported in tilapia fed with feed containing *Bacillus cereus* (Wang et al., 2015). However, levels of total protein and glucose rose significantly ($P<0.05$) in probiotic fed fish compared to the control diet fed fish (Fig. 29, 34). Likewise increased glucose levels were reported in Rogu, *Labeo rohita* and Nile tilapia, *Oreochromis niloticus* when fed diets enriched with probiotic bacteria (Mohapatra et al., 2014; Reda and Selim, 2015). In general, an increase in serum protein level is an indicator of innate immunity, which is considered important in invertebrates and a fundamental defense mechanism of fish (Ellis, 1990). Such mechanisms form a series of essential functions that keep host cells alive, healthy, and protected from pathogens. The use of *Bacillus* species has been shown to elicit inhibitory responses to numerous fish diseases, such as red mouth disease, edwardsiellosis, furunculosis, lactococcusis, streptococcusis, vibriosis and aeromoniasis (Harikrishnan et al., 2010; Brunt et al., 2007; Ridha et al., 2012; Kumar et al., 2006; Taoka et al., 2006; Kamgar et al., 2012). ALT and AST, two liver enzymes, decreased in the probiotic diet

fish group compared to the control (Fig. 40). Results indicate that probiotic diet could decrease the level of liver enzymes. This is in accordance with the results of Lin and Luo, (2011).

Among the innate immune parameters, respiratory burst and lysozyme activity were found to rise significantly ($P<0.05$) in the fish fed with probiotic feed compared to the control (Fig. 31, 36, 41). Similar results were observed after feeding diets formulated with probiotic bacteria in rogu and mrigala (Mohapatra et al., 2014; Bandyopadhyay, et al., 2015). Respiratory burst activity due to an increase in the oxidation level in phagocytes stimulated by foreign agents is considered to be an important indicator of innate defense mechanism in fish (Miyazaki, 1998), where $O2^-$ is the first product to be released (Lee et al., 2000). In fish the $O2^-$production is generally affected after activation of phagocytes since it triggers production of superoxide anion ($O2^-$) and its reactive derivatives (i.e. hydrogen peroxide and hydroxyl radicals) associated with intense oxygen consumption, called the respiratory burst (Secombes and Fletcher, 1992). The reactive species are capable of destroying the invading pathogens (Hassett and Cohen, 1989). In the present study, administration of probiotic enhanced diet improved the production of ROS and RNS by peripheral blood leucocytes and oxygen radicals during the process of oxidative burst from week 1-6. Production of ROS and RNS or NO is a crucial mechanism limiting the growth of fish pathogens (Olivier et al., 1985).

In the case of TNFR-1, the expression level was smaller than that in the intestine, kidney, and liver excluding the kidney, the expression level was lower in the case of IL-1b than in the control group, and in the spleen, kidney, the expression level was higher spleen and kidney than in the control group (Fig. 32).In the case of FAS, the expression level was highest than that in the liver, whereas the lowest expression level was showed in the kidney. In the case of Caspase 3, it showed high expression than the control in all organs (Fig. 37). In the case of TNF and IL-6, the expression amount was showed highest in the

intestine, and the expression amount in the kidney was comparatively lower (Fig. 42).

The enhancement of the innate immune response with a *B. amyloliquefaciens* enriched probiotic diet, decreased mortality rate (Fig. 33, 38, 43) thereby protecting the fish against *S. iniae S. parauberis*. In addition, feed utilization increases thereby decreasing the amount of feed necessary for fish growth, and thus reducing production costs. Based on these series of experimental results, we have confirmed that *Bacillus amyloliquefaciens* JFP-02 has an innate immune reaction applied to aquaculture fish and disease resistance against fish disease pathogens, and it seems to be useful for the fish aquaculture industry.

CONCLUSION

The domestic fish aquaculture industry is growing greatly, but fish diseases that occur during the aquaculture have serious adverse effect on the development of the aquaculture industry. Generally high density breeding is carried out in order to maximize the production amount per unit area, but these high density breeding is accompanied by a decrease in the gain increase rate, occurrence of various disease to cause a direct economic loss The incidence and spreads of fish diseases in the fish aquaculture industry can significantly reduce productivity, and to prevent antibiotics was widely used in fish disease management strategies in fish farms (Smith et al., 1994). However, some fish farms abuse antibiotics for the purpose of treatment and prevention of fish diseases, causing secondary problems, the most representative of which is antibiotic-resistant strains and antibiotic-remaining fish (Karunasagar et al., 1994; Witte et al., 1999).

For the past decades, the application of probiotics in the prevention and management of fish disease has received more interest (Ganguly et al., 2010). Most probiotic microbes are marketed as drug or foodstuffs and safe application has been confirmed through long-term experience (Lim et al., 2011). Increased use of bacteria as biological controllers is effective and safer than antibiocis (Bansemir et al., 2006). The supplementation of fish diets with probiotics modulate specific functions of the gut and immune system and enhance disease protection (Gullian et al., 2004; Nayak., 2010). The most commonly used probiotics in aquaculture are *Bacillus* sp., *Pediococcus* sp., *Lactobacillus* sp., *Bifidobacterium* sp., *Lactococcus* sp (Abdel-Tawwab et al., 2008; Aly et al., 2008; Pal et al., 2007).

In this study, the compared to control feed when fed aquaculture fish with *Bacillus amyloliquefaciens* JFP-02 isolated traditional fermented foods, also growth performance, innate immune response, disease resistance confirmed the possibility of application as a

new feed additive.

Eight species of probiotics candidate strains were isolated from Jeju Island traditional fermented foods: Doenjang, Saeujeot, fermented Squid, salted Damselfish, salted guts of Hairtail, salted Gizzard shad, salted Spratelloides gracilis. As a result of confirmed the nucleotide sequence of the isolated strains used BLAST of NCBI, it was identified by *pediococcus acidilactici*, *Bacillus amyloliquefaciens*, *Lactobacillus parabuchneri*, *Enterococcus durans*, *Pediococcus pentosaceus*, *Lactobacillus sakei, Lactobacillus plantarum*, *Lactobacillus brevis* (Table 5).

It`s resistance to artificial gastric juice, artificial bile acid, salt tolerance, heat resistance, confirms the characteristics that probiotics should have and confirm the optimum culture conditions *Bacillus amyloliquefaciens* JFP-02. *B. amyloliquefaciens* JFP-02 was the shape of colony was uneven, the center of colony was convex (Fig. 10) and loop draws a considerable viscosity when taken. As a result of observation used SEM, it was in the form of single bacillus, the length was 1.575 um, and the width was 0.727 um. The optimal culture conditions were the highest growth activity by added saccharose as a carbon source, yeast extract as a nitrogen source, $MgSO_4 \cdot 7H_2O$ as a mineral source to MRS medium at pH 9 and 30℃.

In 2015 and 2016 a total 13 species fish disease pathogens were isolated at 18 olive flounder aquaculture farms site. Confirmed the sequence of the isolated strains, *Photobacterium damselae sub sp*. 2 species, *Edwardsiella tarda* 2 species, *Streptococcus parauberis* 2 species, *Streptococcus iniae*, *Tenacibaculum maritimum*, *Vibrio harveyi*, *Vibrio alginolyticus*, *Vibrio anguillarum*, *Staphylococcus caprae* (Table 11, 14).

As a result of measured the antimicrobial activity according to the culture conditions of *B. amyloliquefaciens* JFP-02 for fish disease pathogens isolated from the olive flounder farm, *T. maritimum, E. tarda* at pH 4 to pH 9and 10℃ to 50℃ the antimicrobial activity

was the highest. In addition, it showed highest antimicrobial activity against *T. maritimum*, *E. tarda*, *V. campbellii S. caprae* of carbon source, nitrogen source and mineral source.

Confirmed of the hematological characteristics, innate immune response, disease resistance, and immune genes expression level showed when *B. amyloliquefaciens* JFP-02 was fed for olive flounder, rock bream, and starry flounder of aquaculture fish.

In the case of olive flounder, confirmed that hematocrit, total protein, glucose increased significantly compared to the control group by hematological analysis after fed *B. amyloliquefaciens* JFP-02 for 8 weeks, it was confirmed that triglyceride, total cholesterol, AST, ALT decreased compared to the control group. Lysozyme activity and macrophage activity which is an innate immune response, increase significantly compared with the control group from the latter part of the experiment and showed mortality rate of 85% in the control group of the disease resistance experiment using *S. iniae*, than *B. amyloliquefaciens* JFP-02 group showed mortality rate of 45%.

In the case of rock bream, confirmed that hematocrit, total protein, glucose increased significantly compared to the control group by hematological analysis after fed *B. amyloliquefaciens* JFP-02 for 10 weeks, it was confirmed that triglyceride, total cholesterol in deceased significantly compared to the control group and AST, ALT decreased. Lysozyme activity and macrophage activity which is an innate immune response, increase significantly compared with the control group and showed mortality rate of 90% in the control group of the disease resistance experiment using *S. iniae*, than *B. amyloliquefaciens* JFP-02 group showed mortality rate of 35%.

In the case of starry flounder, confirmed that hematocrit, total protein, glucose, calcium increased significantly compared to the control group by hematological analysis after fed *B. amyloliquefaciens* JFP-02 for 4 weeks, it was confirmed that AST, ALT in deceased compared to the control group. Lysozyme activity and macrophage activity which is an innate immune response, increase significantly compared with the control group and

showed mortality rate of 75% in the control group of the disease resistance experiment using *S. parauberis*, than *B. amyloliquefaciens* JFP-02 group showed mortality rate of 45%.

In the case of olive flounder, TNFR-1 the expression level was smaller than that in the intestine, kidney, and liver excluding the kidney, the expression level was lower in the case of IL-1b than in the control group, and in the spleen, kidney, the expression level was higher spleen and kidney than in the control group (Fig. 32). In the case of rock bream, FAS the expression level was highest than that in the liver, whereas the lowest expression level was showed in the kidney. In the case of Caspase 3, it showed high expression than the control in all organs (Fig. 37). In the case of starry flounder TNF and IL-6, the expression amount was showed highest in the intestine, and the expression amount in the kidney was comparatively lower (Fig. 42).

In this study, *B.amyloliquefaciens* JFP-02 isolated from Jeju Island traditional foods had characteristic of probiotic, and it's possible to confirm the antimicrobial activity against various fish disease pathogens according to culture conditions. Series of experimental results, it was confirmed that *B. amyloliquefaciens* JFP-02 has an innate immune response and disease resistance against fish disease pathogens to aquaculture fish. It seems to be useful for the fish aquaculture industry in the future.

REFERENCE

Abdel-Tawwab, M., Abdel-Rahman, A. M., Ismael, N. E., 2008. Evaluation of commercial live bakers' yeast, *Saccharomyces cerevisiae* as a growth and immunity promoter for Fry Nile tilapia, *Oreochromis niloticus* (L.) challenged in situ with *Aeromonas hydrophila*. Aquaculture, 280(1)**:** 185-189.

Ajani, E. K., Bamidele, S. F., 2014. Use of lactic acid bacteria from Nile tilapia *Oreochromis niloticus* as probiotics for sustainable production and improvement in fish welfare. The Israeli Journal of Aquaculture-Bamidgeh, 66**:** 977-989.

Aly, S. M., Ahmed, Y. A. G., Ghareeb, A. A. A., Mohamed, M. F., 2008. Studies on *Bacillus subtilis* and *Lactobacillus acidophilus*, as potential probiotics, on the immune response and resistance of Tilapia nilotica (*Oreochromis niloticus*) to challenge infections. Fish & shellfish immunology, 25(1)**:** 128-136.

Aly, S. M., Mohamed, M. F., John, G., 2008. Effect of probiotics on the survival, growth and challenge infection in Tilapia nilotica (*Oreochromis niloticus*). Aquaculture research, 39(6)**:** 647-656.

Avonts, L., De Vuyst, L., 2001. Antimicrobial potential of probiotic lactic acid bacteria. Mededelingen (Rijksuniversiteit te Gent. Fakulteit van de Landbouwkundige en Toegepaste Biologische Wetenschappen), 66(3b)**:** 543-550.

Bandyopadhyay, P., Sarkar, B., Mahanty, A., Rathore, R. M., Patra, B. C., 2015. Dietary Administered *Bacillus* sp. PP9 Enhances Growth, Nutrition and Immunity in *Cirrhinus mrigala* (Hamilton). Proceedings of the National Academy of Sciences, India Section B: Biological Sciences, 85(3)**:** 759-766.

Bansemir, A., Blume, M., Schröder, S., Lindequist, U., 2006. Screening of cultivated seaweeds for antibacterial activity against fish pathogenic bacteria. Aquaculture, 252(1)**:** 79-84.

Brunt, J., Newaj-Fyzul, A., Austin, B., 2007. The development of probiotics for the control of multiple bacterial diseases of rainbow trout, *Oncorhynchus mykiss* (Walbaum). Journal of Fish Diseases, 30(10)**:** 573-579.

Choi, E. J., Lee, S. H., Jung, J. Y., & Jeon, C. O., 2013. *Brevibacteriumjeotgali* sp. nov., isolated from jeotgal, a traditional Korean fermented seafood. International journal of systematic and evolutionary microbiology, 63(9)**:** 3430-3436.

Cleveland, J., Montville, T. J., Nes, I. F., Chikindas, M. L., 2001. Bacteriocins: safe, natural antimicrobials for food preservation. International journal of food microbiology, 71(1)**:** 1-20.

Collins, B., Cotter, P. D., Hill, C., Ross, R. P., 2010. Applications of lactic acid bacteria-produced bacteriocins. Biotechnology of lactic acid bacteria: Novel applications, 89-109.

Cotter, P. D., Ross, R. P., Hill, C., 2013. Bacteriocins—a viable alternative to antibiotics?. Nature Reviews Microbiology, 11(2)**:** 95-105.

Dawood, M. A., Koshio, S., 2016. Recent advances in the role of probiotics and prebiotics in carp aquaculture: a review. Aquaculture, 454**:** 243-251.

Ellis, A, E., 2990. In: Stolen, J, S., Fletcher, T, C., Anderson, D, P., Robertson, B, S., VanMuisvinkel, W, R., editors. Lysozyme assay in techniques in fish immunology. Fair Haven, NJ, USA: SOS publications. 101-103.

Elvitigala, D. A. S., Whang, I., Premachandra, H. K. A., Umasuthan, N., Oh, M. J., Jung, S. J., Lee, J., 2012. Caspase 3 from rock bream (*Oplegnathus fasciatus*): genomic characterization and transcriptional profiling upon bacterial and viral inductions. Fish & shellfish immunology, 33(1)**:** 99-110.

FAO, 2016. The State of World Fisheries and Aquaculture 2016. Fisheries and Aquaculture Department, FAO, Rome.

Ganguly, S., Paul, I., Mukhopadhayay, S. K., 2010. Application and effectiveness of immunostimulants, probiotics, and prebiotics in aquaculture: a review. Israeli Journal of Aquaculture-Bamidgeh, 62(3)**:** 130-138.

Ghosh, K., Sen, S. K., Ray, A. K., 2003. Supplementation of an isolated fish gut bacterium, *Bacillus circulans*, in formulated diets for rohu, *Labeo rohita*, fingerlings. Israeli Journal of Aquaculture-Bamidgeh, 55**:**13-21.

Guan, L., Cho, K. H., Lee, J. H., 2011. Analysis of the cultivable bacterial community in jeotgal, a Korean salted and fermented seafood, and identification of its dominant bacteria. Food microbiology, 28(1)**:** 101-113.

Gullian, M., Thompon, F., Rodriguez, J. 2004. Selection of probiotic bacteria and study of their immunostimulatory effect in *Penaeus vannamei*. Aquaculture, 233(1)**:** 1-14.

Harikrishnan, R., Balasundaram, C., Heo, M. S., 2010. *Lactobacillus sakei* BK19 enriched diet enhances the immunity status and disease resistance to *streptococcosis* infection in kelp grouper, *Epinephelus bruneus*. Fish & shellfish immunology, 29(6)**:** 1037-1043.

Hassett, D, J., Cohen, M, S., 1989. Bacterial adoption to oxidative stress: implications of pathogensesis and interaction with phagocytic cell. Federation of American Societies for Experimental Biology, 3**:** 1574-1581.

Hwang, U. G., Lee, J. S., Kang, J. C., 2013. Reponses of the Hepatic Microsomal Cytochrome P450 Monooxygenase System in Rock Bream *Oplegnathus fasciatus* Exposed to Tributyltin (TBT). Fisheries and aquatic sciences, 16(4)**:** 261-265.

Jang, I. S., 2012. Isolation and Cultural Characterization of Antibacterial Substance Produced Bacterium, *Bacillus* sp. IS-2 and Supplementation Infeed on Immune Response, Disease resistance in olive flounder, *Paralichthys olivaceus*. Jeju National University.

Jang, Y. H., 2014. Identification, characterization, rapid detection technique development and vaccine production of major fish pathogen, *Pseudomonas anguilliseptica* isolated from farmed olive flounder, *Paralichthys olivaceus*, Jeju National University.

Jeong, J. M., Kim, J. W., Park, H. J., Song, J. H., Kim, D. H., Park, C. I., 2011. Molecular cloning and characterisation of the rock bream, *Oplegnathus fasciatus*, Fas (CD95/APO-1), and its expression analysis in response to bacterial or viral infection. Results in immunology, 1(1)**:** 11-17.

Jung, M. H., Nikapitiya, C., Song, J. Y., Lee, J. H., Lee, J., Oh, M. J., Jung, S. J., 2014. Gene expression of pro-and anti-apoptotic proteins in rock bream (*Oplegnathus fasciatus*) infected with megalocytivirus (family Iridoviridae). Fish & shellfish immunology, 37(1)**:** 122-130.

Jung, S. H., Choi, D.L., Kim, J. W., Jo, M. R., Jee, B. Y., Seo, J. S., 2009. Pharmacokinetics of oxolinic acid in cultured olive flounder *Paralichthys olivaceus* by oral administration, injection and dipping. Journal of Fish Pathology. 22(2): 125-135.

Kamgar, M., Ghane, M., 2012. Evaluation of *Bacillus subtilis* effect as probiotic on hematological parameters of rainbow trout, *Oncorhynchus mykiss* (Walbaum) following

experimental infection with *Streptococcus iniae*. Journal of Fisheries and Aquatic Science, 7(6)**:** 422-430.

Kang, D. H., Bae, H. K., Kim, H. S., 2011. Isolation and antibacterial activity of *Actinomycetes* producing growth inhibition compounds against multi-antibiotic resistant *Pseudomonas aeruginosa*. KSBB Journal, 26(1)**:** 19-26.

Karunasagar, I., Pai, R., Malathi, G. R., Karunasagar, I., 1994. Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection. Aquaculture, 128(3)**:** 203-209.

Kim, D. H., Park, S. H., Kim, J. H., Lee, H. R., Heo, M. S., 2017. Screening of Antimicrobial Activity of Marine-Derived Biomaterials against Fish Pathogens. Microbiology and Biotechnology Letter, 44(4)**:** 535-539.

Kim, D. H., Subramanian, D., Heo, M. S., 2017. Dietary Effect of Probiotic Bacteria, *Bacillus amyloliquefaciens*-JFP2 on Growth and Innate Immune Response in Rock Bream *Oplegnathus fasciatus*, Challenged with *Streptococcus iniae*. Israeli Journal of Aquaculture-Bamidgeh, 69**:** 1354-1365.

Kim, D. H., Subramanian, D., Park, S. H., Jang, Y. H., Heo, M. S., 2017. Assessment and Potential Application of the Probiotic Strain, *Bacillus amyloliquefaciens* JFP2, Isolated from Fermented Seafood-Jeotgal in Flounder *Paralichthys olivaceus* Juveniles. Israeli Journal of Aquaculture-Bamidgeh, 69**:** 1352-1364.

Kim, K. M., Lee, J. U., Kim, J. W., Han, S. J., Kim, K. D., Jo, J. Y., 2008. Daily feeding rates of parrot fish *Oplegnathus fasciatus* fed extruded pellet at the different water temperatures. Journal of Aquaculture, 21(4)**:** 294-298.

Kim, J. H., Jeong, D. S., Kang, J. C., 2013. Toxic effects on the nonspecific immune

system of the rock bream *Oplegnathus fasciatus* upon exposure to di-2-ethylhexyl phthalate. Fisheries and aquatic sciences, 16(3)**:** 171-176.

Kim, P. I ., Chung, K. C., 2004. Production of an antifungal protein for control of *Colletotrichum lagenarium* by *Bacillus amyloliquefaciens* MET0908. FEMS Microbiology Letters, 234(1)**:** 177-183.

Kim, S. M., 2016. Analysis of the cause of emaciation disease in cultured olive flounder, *Paralichthys olivaceus* in Korea, Jeju National University.

Ko, G. Y., Lim, S. J., Kim, S. S., Oh, D. H., Lee, K. J., 2008. Effects of dietary supplementation of Scoria on growth and protein digestibility in juvenile parrot fish *Oplegnathus fasciatus* and olive flounder *Paralichthys olivaceus*. Journal of Aquaculture, 21(3)**:** 133-138.

Kumar, R., Mukherjee, S. C., Prasad, K. P., Pal, A. K., 2006. Evaluation of *Bacillus subtilis* as a probiotic to Indian major carp *Labeo rohita* (Ham.). Aquaculture Research, 37(12)**:** 1215-1221.

Kumar, N. R., Raman, R. P., Jadhao, S. B., Brahmchari, R. K., Kumar, K., Dash, G., 2013. Effect of dietary supplementation of *Bacillus licheniformis* on gut microbiota, growth and immune response in giant freshwater prawn, *Macrobrachium rosenbergii* (de Man, 1879). Aquaculture international, 21(2)**:** 387-403.

Lee, C. H., 1993. Fish fermentation technology, pp. 189-279. In Lee, C. H., Steinkraus, K. H, Reilly, P. J. A (eds), Fish fermentation technology in Korea. United Nations University Press, Tokyo, Japan.

Lee, C. H., Kim, P. Y., Ko, C. S., Oh, D. C., Kang, B. J., 2007. Biological characteristics of *Streptococcus iniae* and *Streptococcus parauberis* isolated from cultured flounder,

Paralichthys olivaceus, in Jeju. Journal of Fish Pathology. 20**:** 33-40.

Lee, D. C., Lee, J. I., Kim, D. H., Cho, M. Y., Kim, J. W., 2011. Efficacy of a vaccine against *Streptococcus parauberis* infection in starry flounder *Platichthys stellatus* Pallas. Journal of fish pathology, 24(3)**:** 189-195.

Lee, D, C., Rieman, B, E., Thompson, W., 2000. Bayesian viability assessment module (BayVAM): A tool for investigation population dynamics and relative viability of resident and anadromous salmonids. USDA Forest Service, Boise, ID.

Lee, E. H., Jang, K. I., Bae, I. Y., Lee, H. G., 2011. Antibacterial effects of leek and garlic juice and powder in a mixed strains system. Korean Journal of Food Science and Technology, 43(4)**:** 518-523.

Lee, K. S., Oh, C. S., Lee, K. Y., 2006. Antimicrobial effect of the fractions extracted from a lotus (*Nelumbo nucifera*) leaf. Journal of the Korean Society of Food Science and Nutrition, 35(2)**:** 219-223.

Lee, K. W., Park, K. S., 2010. Antibiotic-resistance profiles and the identification of the ampicillin-resistance gene of *Vibrio parahaemolyticus* isolated from seawater. Korean Journal of Fisheries and Aquatic Sciences, 43**:** 637-641.

Lee, S. H., Jung, J. Y., Jeon, C. O., 2014. Effects of temperature on microbial succession and metabolite change during saeu-jeot fermentation. Food microbiology, 38**:** 16-25.

Li, P. Y., Wang, J. Y., Song, Z. D., Zhang, L. M., Zhang, H., Li, X. X., Pan, Q., 2015. Evaluation of soy protein concentrate as a substitute for fishmeal in diets for juvenile starry flounder (*Platichthys stellatus*). Aquaculture, 448**:** 578-585.

Lim, S. J., Oh, D. H., Khosravi, S., Cha, J. H., Park, S. H., Kim, K. W., Lee, K. J., 2013.

Taurine is an essential nutrient for juvenile parrot fish *Oplegnathus fasciatus*. Aquaculture, 414**:** 274-279.

Lim, J. H., Jeong, H. Y., Kim, S. D., 2011. Characterization of the bacteriocin J4 produced by *Bacillus amyloliquefaciens* J4 isolated from Korean traditional fermented soybean paste. Journal of the Korean Society for Applied Biological Chemistry, 54(3)**:** 468-474.

Lin, S., Luo, L., 2011. Effects of different levels of soybean meal inclusion in replacement for fish meal on growth, digestive enzymes and transaminase activities in practical diets for juvenile tilapia, *Oreochromis niloticus*× *O. aureus*. Animal Feed Science and Technology, 168(1)**:** 80-87.

Ling-Hong, M., Ming-Chun, R., Xian-Ping, G., Jun, X., Jian, Z., Liu, B., Liang-Kun, P., 2016. Utilization and Metabolism of α-Starch by Black Carp (*Mylopharyngodon piceus*) Juveniles. The Israeli Journal of Aquaculture-Bamidgeh, 68**:** 1259-1269.

Mayra-Makinen, A., Bigret, M. A. R. C., 2004. Industrial use and production of lactic acid bacteria. Food science and technology-New York-marcel dekker, 139**:** 175-198

Msangi, S., Kobayashi, M., Batka, M., Vannuccini, S., Dey, M. M., Anderson, J. L., 2013. Fish to 2030: prospects for fisheries and aquaculture. World Bank Report, 83177-GLB**:** 102.

Meng-Umphan, K., 2009. Growth performance, sex hormone levels and maturation ability of Pla Pho (*Pangasius bocourti*) fed with *Spirulina* supplementary pellet and hormone application. International journal of Agriculture and Biology, 11**:** 458-462.

Miyazaki, Tougo., 1998. A simple method to evaluate respiratory burst activity of blood phagocytes from Japanese flounder. Fish Pathology, 33**:** 141-142.

Mohammadou, B. A., Le Blay, G., Mbofung, C. M., Barbier, G., 2014. Antimicrobial activities, toxinogenic potential and sensitivity to antibiotics of Bacillus strains isolated from *Mbuja*, an Hibiscus sabdariffa fermented seeds from Cameroon. African Journal of Biotechnology, 13(35)**:** 3617-3627.

Mohapatra, S., Chakraborty, T., Prusty, A. K., PaniPrasad, K., Mohanta, K. N., 2014. Beneficial effects of dietary probiotics mixture on hemato-immunology and cell apoptosis of *Labeo rohita* fingerlings reared at higher water temperatures. PloS one, 9(6)**:** e100929.

Ministry for Food Agriculture Forestry and Fisheries. 2016. Statistical year book of maritime affairs and fisheries.

Nayak, S. K., 2010. Probiotics and immunity: a fish perspective. Fish & Shellfish immunology, 29(1)**:** 2-14.

Nguyen, H. T, Kanai, K., 1999. Selective agars for the isolation of *Streptococcus iniae* from Japanese flounder, *Paralichthys olivaceus*, and its cultural environment. Journal of Applied Microbiology, 86**:** 769-776.

Olivier, G., Evelyn, T. P. T., Lallier, R., 1985. Immunity to aeromonas salmonicida in coho salmon (*oncorhynchus kisutch*) inducfd by modified freund's complete adjuvant: Its non-specific nature and the probable role of macrophages in the phenomenon. Developmental & Comparative Immunology, 9(3)**:** 419-432.

Ortuno, J., Cuesta, A., Rodriguez, A., Esteban, M, A., Meseguer, J. 2002. Oral administration of yeast, *Saccharomyces cerevisiae*, enhances the cellular innate immune response of gilthead seabream (*Sparus aurata* L.). Veterinary immunology and Immunopathology, 85**:** 41-50.

Pal, D., Joardar, S. N., Roy, B., 2007. Immunostimulatory effect of a yeast (*Sacchromyces cereciasiae*) cell wall feef supplement on rohu (*Labeo rohita*), an India major carp. Israeli Journal of Aquaculture-Bamidgeh, 59(3)**:** 175-181.

Park, S. Y., Yoo, J. C., Seong, C. N., Cho, S. S., 2013. Isolation and characterization of *Bacillus subtilis* MP56 with antimicrobial activity against MDR (multi drug resistant) strains. The Korean Journal of Microbiology, 49(1)**:** 90-94.

Park, Y., Moniruzzaman, M., Lee, S., Hong, J., Won, S., Lee, J. M., Bai, S. C., 2016. Comparison of the effects of dietary single and multi-probiotics on growth, non-specific immune responses and disease resistance in starry flounder, *Platichthys stellatus*. *F*ish & shellfish immunology, 59**:** 351-357.

Pourgholam, M. A., Khara, H., Safari, R., Sadati, M. A. Y., Aramli, M. S., 2016. Dietary Administration of *Lactobacillus plantarum* Enhanced Growth Performance and Innate Immune Response of Siberian Sturgeon, *Acipenser baerii*. Probiotics and antimicrobial proteins, 8(1)**:** 1-7.

Raida, M. K., Larsen, J. L., Nielsen, M. E., Buchmann, K., 2003. Enhanced resistance of rainbow trout, *Oncorhynchus mykiss* (Walbaum), against *Yersinia ruckeri* challenge following oral administration of *Bacillus subtilis* and *B. licheniformis* (BioPlus2B). Journal of Fish Diseases, 26(8)**:** 495-498.

Reda, R. M., Selim, K. M., 2015. Evaluation of *Bacillus amyloliquefaciens* on the growth performance, intestinal morphology, hematology and body composition of Nile tilapia, *Oreochromis niloticus*. Aquaculture international, 23(1)**:** 203-217.

Ridha, M. T., Azad, I. S., 2012. Preliminary evaluation of growth performance and immune response of Nile tilapia *Oreochromis niloticus* supplemented with two putative

probiotic bacteria. Aquaculture Research, 43(6)**:** 843-852.

Roh, S. W., Kim, K. H., Nam, Y. D., Chang, H. W., Park, E. J., Bae, J. W., 2010. Investigation of archaeal and bacterial diversity in fermented seafood using barcoded pyrosequencing. The ISME journal, 4(1)**:** 1-16.

Seconbes, C, J., Flecher, T, C., 1992. The role of phagocytes in the protective mechanism in fish. Annual. Review of Fish Diseases, 2**:** 53-71.

Shen, P., Fan, X. R., Li, G. W., 1999. Beijing: Higher Education Press. Experiment of Microbiology.

Smith, P., Hiney, M. P., Samuelsen, O. B., 1994. Bacterial resistance to antimicrobial agents used in fish farming: a critical evaluation of method and meaning. Annual review of fish diseases, 4**:** 273-313.

Soltanzadeh, S., Fereidouni, A. E., Ouraji, H., Khalili, K. J., 2016. Growth performance, body composition, hematological, and serum biochemical responses of beluga (*Huso huso*) juveniles to different dietary inclusion levels of faba bean (*Vicia faba*) meal. Aquaculture international, 24(1)**:** 395-413.

Sutyak, K. E., Wirawan, R. E., Aroutcheva, A. A., Chikindas, M. L., 2008. Isolation of the *Bacillus subtilis* antimicrobial peptide subtilosin from the dairy product-derived *Bacillus amyloliquefaciens*. Journal of applied microbiology, 104(4)**:** 1067-1074.

Taoka, Y., Maeda, H., JO, J, Y., Jeon, M, J., Bai, S, C., Lee, W, J., Koshio, S., 2006. Growth, stress tolerance and nonspecific immune response of Japanese flounder *Paralichthys olivaceus* to probiotics in a closed recirculation system. Fisheries Science, 72**:** 310-321.

Tong, Y., Sun, X., Wang, B., Wang, L., Li, Y., Tian, J., Zheng, M., 2015. Transcriptome of intraperitoneal organs of starry flounder *Platichthys stellatus* challenged by *Edwardsiella ictaluri* JCM1680. Chinese journal of oceanology and limnology, 33(1)**:** 20-27.

Walker, D. K., Gilliland, S. E., 1993. Relationships among bile tolerance, bile salt deconjugation, and assimilation of cholesterol by *Lactobacillus acidophilus*1. Journal of dairy science, 76(4)**:** 956-961.

Wang, H., Yang, L., Ping, Y., Bai, Y., Luo, H., Huang, H., Yao, B., 2016. Engineering of a *Bacillus amyloliquefaciens* strain with high neutral protease producing capacity and optimization of its fermentation conditions. PloS one, 11(1)**:** e0146373.

Wang, M., Liu, G., Lu, M., Ke, X., Liu, Z., Gao, F., Yu, D., 2017. Effect of *Bacillus cereus* as a water or feed additive on the gut microbiota and immunological parameters of Nile tilapia. Aquaculture Research, 48(6)**:** 3163-3173.

Wayne., 1997. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disk susceptibility tests. Approved standard M2-A6. Wayne, Pa: National Committee for Clinical Laboratory Standards.

Wang, X., Kim, K. W., Bai, S. C., Huh, M. D., Cho, B. Y., 2003. Effects of the different levels of dietary vitamin C on growth and tissue ascorbic acid changes in parrot fish (*Oplegnathus fasciatus*). Aquaculture, 215(1)**:** 203-211.

Witte, W., Klare, I., Werner, G., 1999. Selective pressure by antibiotics as feed additives. Infection, 27**:** S35-S38.

Woo, H. W., Lee, J. H., Kim, Y. K., Cho, M. Y., Jung, S. H., Kim, J. W., Park, S. I., 2010. Effects of garlic *Allium sativum* extract immersion on the immune responses of olive

flounder *Paralichthys olivaceus* prechallenged with pathogenic bacteria. Journal of Fish Pathology. 23(2)**:** 199-209.

Yoon, S. Y., Lee, S. Y., Kim, K. B. W. R., Song, E. J., Lee, S. J., Lee, C. J., Ahn, D. H., 2010. Antimicrobial activity of the *Sargassum fulvellum* ethanol extract and the effect of temperature and pH on their activity. Korean Journal of Food Science and Technology, 42(2)**:** 155-159.

Zenke, K., Nam, Y. K., Kim, K. H., 2010. Development of siRNA expression vector utilizing rock bream β-actin promoter: a potential therapeutic tool against viral infection in fish. Applied microbiology and biotechnology, 85(3)**:** 679-690.

Zhou, X., Tian, Z., Wang, Y., Li, W., 2010. Effect of treatment with probiotics as water additives on tilapia (*Oreochromis niloticus*) growth performance and immune response. Fish physiology and biochemistry, 36(3)**:** 501-509.

ACKOWLEDGEMENT

박사학위 논문이 나오기까지 많은 분들의 도움이 있었습니다. 그 중에서 이 논문이 완성될 수 있도록 학부생부터 지금까지 많은 관심과 지도편달을 아낌없이 해주시고, 항상 믿어주시고 한 길만 가게끔 기회를 주신 허문수 교수님께 머리 숙여 깊은 감사의 인사를 올립니다. 그리고 대학원 기간 동안 학술적인 조언을 많이 해주시고 바쁘신 가운데 학위논문을 검토해주신 김기영 교수님, 정준범 교수님, 이승헌 교수님, 진창남 연구교수님, 대학원 기간 동안 여러 분야에서 학문적으로 많은 가르침을 주신 송춘복 교수님, 최광식 교수님, 이영돈 교수님, 이제희 교수님, 여인규 교수님, 전유진 교수님, 이경준 교수님, 정석근 교수님, 박상률 교수님께도 진심으로 감사의 인사를 드립니다. 지금까지 실험실 생활을 하면서 많은 조언과 힘을 준 영환이형, 영건이형, 만철이형, 창식이형, 주상이형, 윤범이형, 익수형, 용재형, 창영이형 너무나 감사 드립니다. 실험 할 때 여러모로 서포트 해준 승현이, 소현이, 민선, 경미, 지운, 하리, 향실, 다예, 지현이, 해리, Dr. Dharaneedharan에게 진심으로 감사의 인사를 드립니다.

그리고 대학생활 동안 항상 저를 친동생처럼 챙겨주고, 힘들 때마다 위로해주고, 많은 도움 준 송헌이형, 승민이형, 박사학위 논문 나올 때까지 많은 도움을 준 해양생물이용공학실험실, 바이러스실험실, 어류육종실험실, 양어사료실험실 분자유전실험실, 어류분자생리실험실 원들에게 감사의 인사를 드립니다.

마지막으로 지금의 제가 있기까지 항상 믿어주고 다독여주고, 응원해준 세상에서 가장 소중한 우리 가족 어머니, 아버지, 그리고 하나뿐인 자랑스러운 동생 동성이, 도영이누나, 연희누나, 미연이에게 항상 고마웠다고 말씀 드립니다. 또한 누구보다 기뻐해주시고 항상 보고 싶은 법륜당 태륜스님께 이 논문을 바칩니다.