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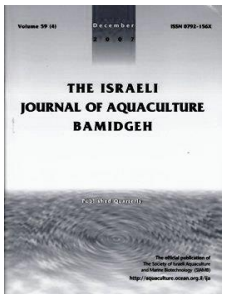

Assessment and Potential Application of the Probiotic Strain, *Bacillus amyloliquefaciens* JFP2, Isolated from Fermented Seafood-Jeotgal in Flounder *Paralichthys olivaceus* Juveniles

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Abstract

The strain *Bacillus amyloliquefaciens* JFP2 was isolated from Jeotgal, traditional fermented seafood from Jeju Island, South Korea to assess its probiotic and antibacterial effect against fish pathogens. The isolate JFP2 was identified using scanning electron microscopic analysis and 16S rRNA gene sequence analysis. The isolated strain, *B. amyloliquefaciens* JFP2 showed a broad spectrum of antibacterial activity against both Gram-positive and Gram-negative fish pathogenic bacteria namely *Aeromonas hydrophila*, *Edwardsiella tarda*, *Photobacterium damselase sup-sps*, *Photobacterium phosphoreum*, *Streptococcous parauberis*, *S. iniae* and *Vibrio anguillaram*. The bacteriocin JFP2 with a molecular mass of 37 kDa that was purified by ammonium sulphate precipitation followed by carboxymethyl-Sephadex column chromatography, showed antibacterial activity at a range of temperatures, from 4º-45ºC and at a range of pH values from 2-12. However, loss of antibacterial activity was observed after treating the bacteriocin with proteolytic enzymes such as pepsin, proteinase K, and trypsin. The mode of antibacterial action shown by the bacteriocin JFP2 was bacteriolytic in nature, resulting in cell wall degradation of *A. hydrophila.* Dietary inclusion of the isolated JFP2 strain showed improved growth performance in juvenile flounder along with increased disease resistance against *Streptococcus iniae.* These results indicate that the bacteriocin JFP2 belongs to class IIIa bacteriocin that could be used as a potential alternative antibacterial agent to control pathogenic diseases in aquaculture and seafood industries.

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Introduction

The occurrence and spread of diseases in the aquaculture industry could significantly decrease productivity and encourage fish farmers to use antibiotics extensively as a strategy in disease management. However, for the past two decades, the application of probiotics in the prevention and management of fish diseases 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., 2005). Increased use of probiotic bacteria as biological controllers is effective and safer than antibiotics (Bansemir et al., 2006). Antibacterial activity in probiotic bacteria may occur by the production of bacteriocins (Avonts and Vuyst, 2001) which are antimicrobial 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 proteins 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). The present study was carried out to isolate and characterize the bacterial strain *Bacillus amyloliliquefaciens* JFP2 from Jeju Island's traditional fermented seafood jeotgal, and assess its probiotic antimicrobial properties along with its dietary influence on *Paralichthys olivaceus* growth and disease resistance.

Materials and Methods

Jeju Island's traditional fermented food. Fully ripened and ready to consume samples of salted, fermented seafood product *Jeotgal,* were purchased from the local traditional markets on Jeju Island, S. Korea and used for the isolation of bacteria. The pH of the samples was measured using an Orion 3-star Benchtop pH meter (Thermo Fisher Scientific, Rockford, Ill., U.S.A.), and the percentage of salt was measured using a Pocket PAL-03S portable refractometer (ATAGO, Japan). Sampling for bacterial isolation was carried out in three replications immediately after collection of the samples.

Isolation and characterization of bacteria. About 10mg of *Jeotgal* sample was homogenized and 10^3-fold diluted with a sterile saline solution (0.85%, w/v). The diluted suspension was plated on marine agar (MA; Difco., U.S.A.) supplemented with 1%, 2%, 5%, 10%, and 15% of NaCl, and incubated aerobically at 30 °C for 3-5 d. Clearly isolated colonies were subcultured on MRS agar media (de Man Rogosa and Sharp) (MRSB; Difco., USA), and incubated at 37°C for 48 hrs. After incubation, the cultures were repropagated twice to insure purity and then stored at -80°C with 20% sterile glycerol for further use. Strains with good antimicrobial ability were selected for further investigation.

Strain identification was carried out using conventional morphological and biochemical tests as described by Shen et al. (1999). Molecular confirmation of the identified strain was done with 16S Rrna gene sequences analysis which includes; 1) the genomic DNA of the identified strain JFP2 was isolated; 2) 16S rRNA gene was amplified from chromosomal DNA by PCR using universal oligo-nucleotide primers (16s forward

primer: 5′- AGAGTTTGATCCTGGCTCAG -3′ and 16s Reverse Primer: 5′- GGTTACCTTGTTACGACTT -3′); 3) The amplicon was sequenced and compared with the 16S rRNA sequences in the GenBank database by BLASTN; 4) Multiple sequence alignment was done using CLUSTAL X software and a phylogenetic tree was constructed by the neighbor-joining method using MEGA (Version 4.1) software. The confidence level of each branch (1000 repeats) was tested by bootstrap analysis.

Antibiotic sensitivity and resistance pattern of strain JFP2. Antibiotic resistance and susceptibility of the isolated strain was analyzed by disc diffusion method as recommended by the national committee for clinical laboratory standards (Wayne, 1997). Cells from 48 h old cultures were diluted in 1:20 LB broth and 100 µl (10^5 CFU/ml bacteria) was swabbed on top of the nutrient agar plates and allowed to dry for 10 mins. Various antibiotic impregnated discs (Life Technology, S. Korea) were placed on the surface of the swabbed plates and left for 30 mins at room temperature for the diffusion of the antibiotics. The plates were then incubated for 20 h at 37°C, after which the strain JFP2 was classified as sensitive or resistant to an antibiotic based on the zone of growth inhibition (ZI) given in standard antibiotic chart.

Probiotic properties of the strain JFP2

Resistance to acidic gastric conditions and high temperature. To determine the resistance to acidic conditions of stomach, viable cells were counted during incubation for 120 min at 37℃ in MRS broth adjusted to pH 2.0 using 0.1 N HCl. The number of viable cells was determined by plating samples on MRS solid medium and incubating at 37℃ for 48 h. The resistance of selected strain to bile salts secreted into duodenum was tested by the growth of the strain JFP2 in the presence of bile salt oxgall (Sigma, S. Korea) added to the concentration of 3%(w/v) by the method described by Walker and Gilliland (1993). The growth of strain JFP2 was monitored during incubation at 37℃ statically up to 7h by reading at 600nm optical density. The isolate was cultured in MRS broth for 24h at 37℃ statically, and then heated to 50℃ and 60℃ for 30 min. After heat treatment, viable cells were counted by plating the heated culture on MRS solid medium.

Proteolytic activity. The proteolytic activity was measured by growing JFP2 strain in 10% skim milk at 37°C for 42 h. The absorbance was read at 650 nm with a spectrophotometer (Libra S22 UV/Visible, Biochrom, England) (Citi et al., 1963). The results were expressed as mg/mL tyrosine by means of reference to a calibration curve.

Evaluation of cell surface hydrophobicity. The cell surface hydrophobicity assay was conducted according to the method described by Lee et al. (2011) with slight modifications. Freshly prepared cells were centrifuged at 8000 rpm for 10 min. The cells of the isolated JFP2 strain cultured at 37°C in MRS broth were harvested by centrifugation (10000 ×g, 10 min at 4°C) at the early growth phase (12 h culture), washed twice with PBS (pH 7.0), resuspended in PBS and the absorbance was measured at 580 nm (reading 1). One ml of hydrocarbon (Toluene, hexadecane or Xylene) was added to 3ml of washed cells, and mixed thoroughly for 2 min using a vortex. The phases were allowed to separate at room temperature for 30 min, after which 1 ml of the upper phase was removed and the absorbance was determined at OD 580nm (reading 2). Percentage hydrophobicity was calculated as: (OD 580 nm of cells (reading 1) - OD 580 nm of aqueous phase (reading 2)/OD580nm of cells (reading 1)×100= % hydrophobicity.

Anitoxidant ability of the strain JFP2. The DPPH radical-scavenging capacity of the strain JFP2 was determined according to the method described by Li et al. with some modifications (2012) where 1.0, 1.5, 2.0 and 2.5 ml of freshly prepared JFP2 cells (10^7 CFU/ml), was added to 1.0 ml methanolic DPPH radical solution (0.05 mM). The mixture was mixed vigorously and incubated at room temperature in the dark for 30 min. The controls included only deionized water and DPPH solution. The blanks contained only methanol and the cells. The absorbance of the resulting solution was measured in triplicate at 517nm, after centrifugation at 12000 rpm for 10 min.

Antimicrobial properties of the strain JFP2. The isolated JFP2 bacterial strain was tested for the production of antimicrobial substance activity of the cell free culture supernatant (CFCS) using disc diffusion method along with broth micro-dilution assay to determine the minimum inhibitory concentration (MIC) of the JFP2 strain. CFCS was prepared by centrifugation (8000 ×g, 5 mins, 4°C) of JFP2 culture grown on MRS broth

at 37°C for 48 h, adjustment to pH 7.0 with 1N NaOH and filter sterilized through 0.45 µm pore Millipore membrane filter.

Antimicrobial assay against fish pathogens. Antibacterial activity of the isolated JFP2 strain was determined against seven major fish pathogens namely *Aeromonas hydrophila, Edwardsiella tarda, Photobacterium damselase* sup-sps, *Photobacterium phosphoreum* (Gram negative bacteria) and *Streptococcous parauberis, S. iniae, Vibrio anguillarum* (Gram positive bacteria) which were isolated from infected flounder collected from fish farms located in and around Jeju island. Fish pathogens were cultured in Brain Heart Infusion (BHI) broth at 37ºC on a shaking incubator (at 190 rpm) overnight. For the disc diffusion assay, a lawn of bacterial culture was prepared by spreading 100 µL culture broth (10^7 CFU/mL) of each test organism on solid BHI agar plates. Paper discs impregnated with JFP2 CFCS were air dried and laid on BHI agar plates previously inoculated with pathogens and allowed to stand for 15 mins at 4ºC aseptically. Later the plates were incubated at 37ºC for 24 h. For micro-dilution assay, pathogenic bacteria were cultured overnight at 37°C in BHI broth and adjusted to a final density of 10^7 CFU/mL by 0.5 McFarland standards. Then, in a 96-well plate, 90 µL of BHI broth was added, and 10 µL of CFCS was diluted eight times serially. Thus, 10 µL of bacterial culture was inoculated and then the 96-well plate was incubated at 37°C for 12 h in a shaking incubator rotating at 150 rpm. The concentration at which there was no, or minimally observable bacterial growth (as noted by least visible bacterial growth), was taken as the MIC. Tetracycline was used as positive control and test results were scored when a zone of inhibition was observed after the incubation period. Both assays were performed in triplicate and the mean ± standard error (SE) was calculated.

Column purification and SDS-PAGE separation of antibacterial substance – bacteriocin. For bacteriocin production, the isolated strain JFP2 was grown in MRS broth for 24 h at 37°C and CFCS prepared as stated before. The CFCS containing crude bacteriocin was filtered through 0.45-µm filter membranes, and proteins were precipitated with ammonium sulphate at 50% saturation by constant stirring overnight for 16 h at 4°C. Initial purification was achieved by 0-50% ammonium sulfate salting out to the culture supernatant. The precipitated crude extract was harvested by centrifugation at 10,000 g for 20 min and dissolved in the 10 mM Tris-HCl buffer (pH 8.0). The concentrated solution was loaded on to a column (1.6×10 cm) of anion-exchange diethylaminoethyl (DEAE)-Sepharose Fast Flow (Life Technology, Korea), previously equilibrated with 10 mM Tris-HCl buffer (pH 8.0), and the column was eluted with a linear gradient of 0-0.5 M NaCl in 0.1M Tris·HCl buffer (pH. 8.0) at a flow rate of 1 mL/min. The active fractions were pooled and applied to a column (2.6×60 cm) of Sephacryl S-100 HR (Life Technology, Korea), and eluted with 10 mM Tris · HCl buffer (pH 8.0) at 1 mL/min. The fractions showing antibacterial activity were pooled and lyophilized to characterize their biochemical properties. Protein concentration was determined by using BCA method with bovine serum albumin as a standard (Sigma, USA) and the molecular weight was determined by SDS-PAGE separation. The purified bacteriocin was detected through 12% separating and 5% stacking gel with acrylamide and bis-acrylaminde ratio 30:1 under denaturing condition. 15 µl of bacteriocin (5 mg/ml) mixed in 4:1 ratio with denaturing sample buffer was loaded per well along with CFCS and blank medium as negative controls in separate wells. Electrophoresis was done at 20mA for first 30 mins followed by 30mA for 2 h. Then the gel was removed and stained with Coomassie brilliant blue G-250 and de-stained to visualize the separated protein bands.

Antibacterial mechanism of bacteriocin against Aeromonas hydrophila. Late-log phase of *Aeromonas hydrophila* culture in BHI broth maintained at 37ºC for 12h was used to explore the antibacterial mechanism of partially purified bacteriocin of *B. amyloliquefaciens* JFP2 by cell wall destruction procedure (Borrero et al., 2011). Bacterial cell with viable density of 10^6 CFU/mL was pelletized and suspended in 5 mL of 10 mM sodium phosphate buffer (pH 7.0). To this 100 µL of bacteriocin was added and allowed to stand for 15 mins at 37ºC and then centrifuged at 4ºC for 2000 rpm, supernatant was collected and rinsed in 10 mL of potassium buffer (*p*H 7.0). SDS (4%, w/v) was added and mixed for 90 min at room temperature. The mixture was treated for 15 min at 100ºC, and the cell wall was collected by centrifugation at 14,000×g for 15 min at 4º C.

The mixture was resuspended in 10 mL of 0.1% Triton X-100 (Sigma-Aldrich) and was stirred for 30 min at room temperature. After centrifugation, the mixture was resuspended in 1 mL of 5 mM sodium phosphate buffer (pH 7.0), and the optical density was measured at 600 nm.

*Sensitivity of bacteriocin to different p*H*, temperatures, and enzymes.* To study temperature sensitivity of JFP2 bacteriocin, the aliquots of partially purified bacteriocin (4,000 AU/mL) were exposed to different temperatures: 4, 30, 45, 60 and 75ºC for 30 min and residual activity was measured using spot-on-lawn method (Paik and Glatz, 1995) through dilution of bacteriocin against the indicator lawn of bacteria namely *Aeromonas hydrophila*. The titer was the least dilution showing inhibition zone expressed in antimicrobial activity unit per milliliter (AU/mL). For stability at different *p*H, the aliquots were adjusted to range between 2.0-12.0 *p*H using 2N HCL and 2N NaOH, and incubated for 2 hours. The residual activity was measured after neutralizing the aliquots to *p*H 6.0. To investigate enzyme sensitivity, partially purified and lyophilized bacteriocin JFP2 (4,000 AU/mL) was treated with degrading enzymes (Sigma, Korea) such as lysozyme (10,000 AU/mL), α-amylase (100 AU/mL) and proteinase K (200 AU/mL) and incubated at 37ºC for 2 hours. The untreated purified bacteriocin and saline were used as positive and negative controls respectively. The residual activity was measured as the diameter of inhibition zone (mm).

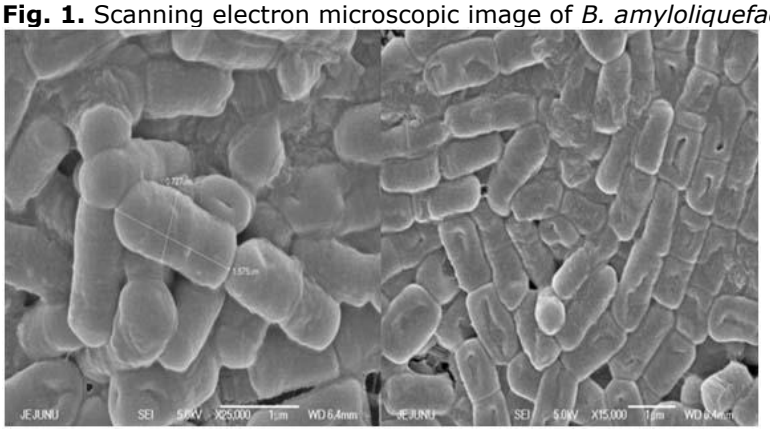
Dietary effect on olive flounder disease resistance. Isolated *B. amyloliquefaciens*-JFP2 culture was suspended in sterile saline water and sprayed over the flounder fish feed (Suhyup, S.Korea) in concentrations of 1.4×10^4 (P1), and 1.4×10^8 (P2) CFU/100 g, excluding control feed (C); stored at 4°C until use. About 90 healthy olive flounder (105±0.26 g) purchased from a flounder fish farm located in Jeju Island were randomly grouped as Control (C), Probiotic1 (P1), and Probiotic2 (P2), in triplicate (n=10). Fish were fed standard culture practice at 4% body weight for 30 days. Dietary performance was evaluated through estimation of nutritional indices such as live weight gain (LWG), feed conversion ratio (FCR), specific growth rate (SGR), and disease resistance was assessed by challenge with *Streptococcus iniae;* survival rate was recorded for 20 days.

Statistical analysis. Data were statistically analyzed using SPSS version 11.0. One way analysis of variance (ANOVA) and Duncan's Multiple Range Test were applied to compare means between treatments.

Results

Among the hundred and eight different bacterial species isolated from Jeju Island's traditional fermented seafood product, isolate JFP2 was selected for further strain characterization based on its abundant growth in MRS selective medium and its wide spectrum of antimicrobial ability (data not shown). The staining test and scanning electron microscope analysis revealed it to be a Gram-positive bacterium with rod-shaped (0.7-0.9 µm thick, 1.5-3.0 µm long) cells. (Fig. 1). Biochemical tests confirmed that the investigated bacterial species was aerobic, testing positive for catalase, nitrate reduction, indole, capable of starch and gelatin hydrolysis, and negative for methyl red and Voges Proskauer assays.

Fig. 1. Scanning electron microscopic image of *B. amyloliquefaciens* JFP2



The 16S rRNA gene sequence of isolate JFP2 showed high similarities (>99%) to *Bacillus amyloliquefaciens* and the phylogenetic tree constructed based on the 16S rRNA gene sequences confirmed this. (Fig. 2). The isolate was designated as JFP2. Except for chloramphenicol, the isolated strain JFP2 was sensitive to all the other tested antibiotics namely erythromycin, vancomycin, tetracycline, streptomycin, kanamycin, ampicillin, penicillin, neomycin, oxytetracyclin (Table 1).

Fig. 2. Phylogenetic tree constructed using the 16S rRNA gene sequences of *B. amyloliquefaciens* JFP2.

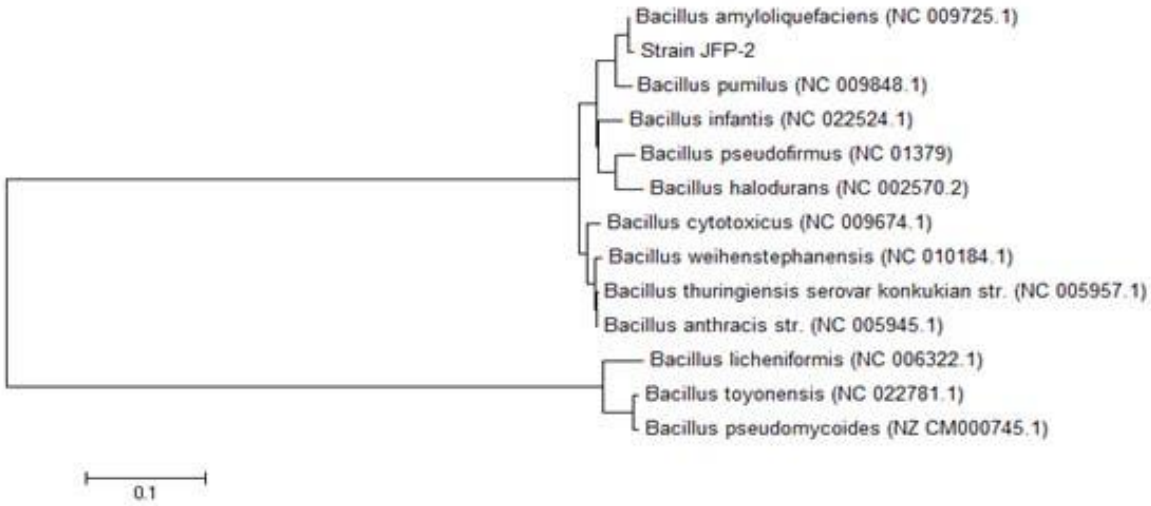


Table 1. Antibiotic resistance of *B. amyloliquefaciens* JFP2

Antibiotics (50µg/mL)	*ZI (mm)*
Streptomycin	16±0.12
Kanamycin	12±0.32
Erythromycin	17±0.21
Vancomycin	14±0.4
Ampicillin `	18±0.11
Tetracycline	15±0.13
Penicillin	16±0.23
Oxytetracyclin	14±0.13
Neomycin	15±0.11
Chloramphenicol	3±0.1

Probiotic properties of B. amyloliquefaciens JFP2. Viability of the isolated JFP2 strain at low pH and high temperature conditions are shown in Figure 3. The result indicated that cell viability increased slightly at pH 2.0 while at pH 3.0 and 4.0 the strain JFP2 survived and grew. Survival ability of strain JFP2 in the presence of bile salt (Oxgall, 0.3%) is also presented in Figure 3. Results revealed that the strain was able to grow in the presence of oxgall.

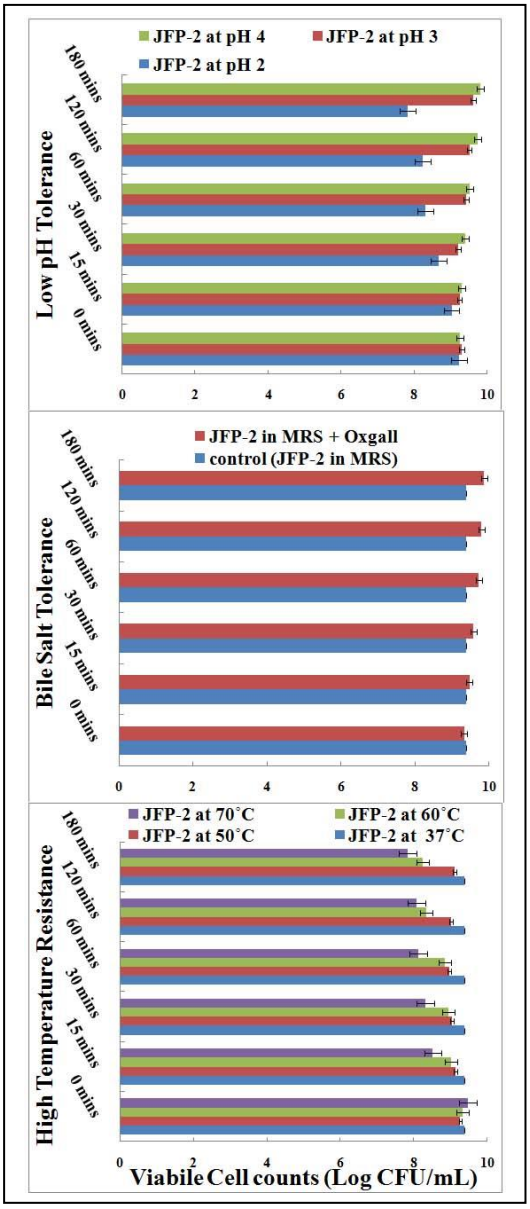


Fig. 3. Effect of low pH, high temperature and bile salts on viability of *B. amyloliquefaciens* JFP2.

Positive proteolytic activity of strain JFP2 was determined with 0.057 mg/ml tyrosine liberation. Strain JFP2 also exhibited high hydrophobicity expressed as 97.43%, 92.54%, and 79.56%, of microbial adhesion to hexadecane, toluene and xylene respectively (Fig. 4). A significant dose-dependent inhibition of DPPH activity was shown by the strain JFP2, with the highest free radical-scavenging activity (73.5%) occurring at 6 ml of cells (10^6 CFU/ml) (Fig. 5).

Fig. 4. Hydrophobicity of *B. amyloliquefaciens JFP2 against various solvents*

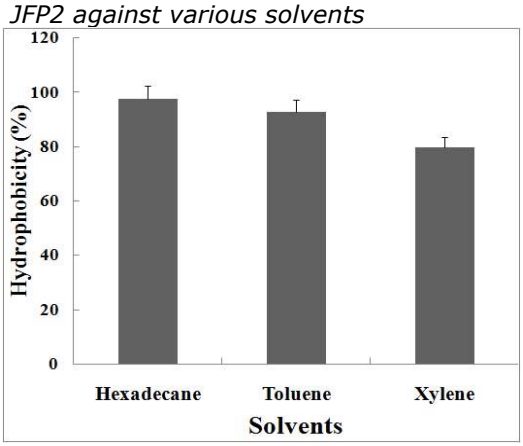
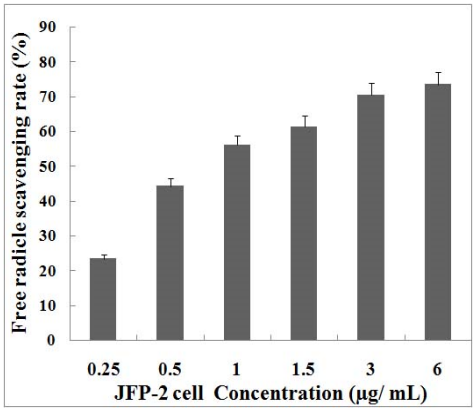


Fig.5. Antioxidant activities of *B amyloliquefaciens JFP2*



Antibacterial properties of B. amyloliquefaciens JFP2. Antibacterial effect of the isolated strain JFP2 against the five pathogens appears in Table 2.

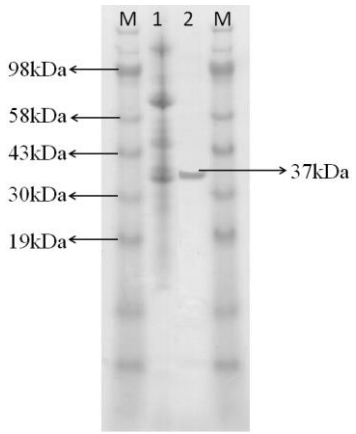
Table 2. Antimicrobial activity of bacteriocin JFP2 from *B. amyloliquefaciens* against seven major fish pathogens

Indicator fish pathogens	*Zone of inhibition (mm)*[a]	*Minimum inhibitoryconcentration (µg/mL)*
Aeromonas hydrophila	14.4±013	12.5
Edwardsiella tarda	17.2±0.35	7.5
Photobacterium damselase	13.3±0.14	25.0
Photobacterium phosphoreum	13.21±0.2	25.0
Streptococcous parauberis	14.24±0.23	12.5
S. iniae	12.92±0.11	25.0
Vibrio anguillarum	12.43±0.21	25.0

[a] Antibacterial activities were monitored by growing the bacteria in BHI and incubated at 37°C for 17 h.

Among the seven pathogenic microbes, significant growth inhibition occurred against *E. tarda* with the maximum zone inhibition of 17.2±0.35 mm along with the MIC value of 7.5 µg/mL. This was followed by *Aeromonas hydrophila* (14.4±013mm)*, Streptococcus parauberis* (14.24±0.23mm), *Photobacterium damselase* (13.32±0.14mm), *Photobacterium phosphorem* (13.21±0.2mm), *S. iniae* (12.92±0.11) and *Vibrio anguillaram* (12.43±0.21). The presence of an antibacterial substance was assessed by ammonium sulfate precipitation and CM-Sephadex column purification, which was then separated by SDS-PAGE to reveal a single protein band of 37kDa (Fig. 6).

Fig. 6. SDS-PAGE of partially purified bacteriocin JFP2. Lane M - protein size marker; Lane 1 – Cell free culture supernatant; Lane 2 - purified bacteriocin JFP2.



The residual antibacterial activity of *B. amyloliquefaciens* JFP2 bacteriocin after treatment with various temperature, pH and enzymes can be seen in Table 3.

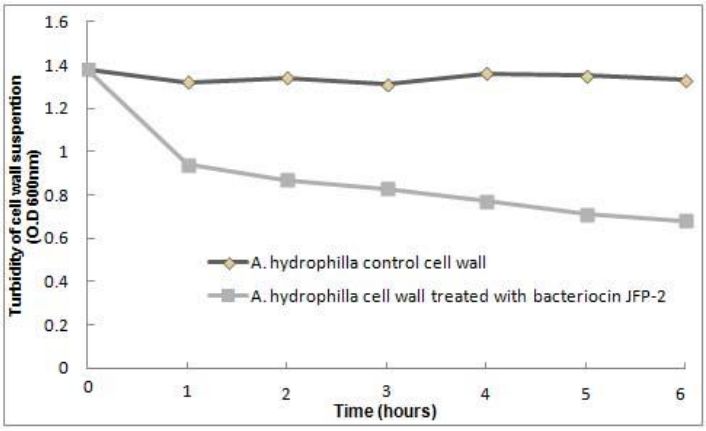
Table 3. Effect of pH, temperature & enzymes on antibacterial activity of bacteriocin JFP2 against *A. hydrophila*

Treatment [a]	*Residual antimicrobial activity (%)*[b]
pH	
2	70
4	85
6	100
7	100
8	100
9	100
10	98
11	95
12	95
Temperature (ºC)	
4	90
30	94
	82
45	40
60	5
75	95
	95
Enzymes	99
α-Amylase	15
α-Glucosidase	12
Lipase	10
Pepsin	
Proteinase K	
Trypsin	

[a]The final enzyme concentration was 15 mg/mL. [b]The relative residual activity was measured by the modified spot-on-lawn method, and % activity is indicated by comparing with untreated bacteriocin JFP2

The residual bacteriocidal activity of bacteriocin JFP2 between 4ºC-45ºC exposure ranged from 94-40%. Activity was only 5% at 60ºC. In the range of pH 2-12, bactericidal activity was between 70-100%. However, enzymatic degradation of bacterioncin JFP2 with proteniase K, pepsin and trypsin showed a great loss of antimicrobial activity. This indicates the proteinaceous nature of the bacteriocin (Table 2). Cell wall degradation assay showed decreased optical density with increased degradation of *A. hydrophila* cells by bacteriocin JFP2 when incubated for 5 hours at 37ºC (Fig. 7).

Fig. 7. Cell wall degradation of *Aeromonas hydrophila* by bacteriocin JFP2.



Dietary influence of B. amyloliquefaciens JFP2 in olive flounder. The highest live weight gain, FCR and SGR occurred with the probiotic fed fish group compared with the control (Table 4). There were no significant differences among the probiotic diet group. Fish fed *B. amyloliquefaciens* JFP2 enriched probiotic diet had higher survival rate (P1:93.86% and P2:95.31%) than the control diet fed fish group (64.32%).

Table 4. Dietary influence of *B. amyloliquefaciens* JFP2 on growth and survival of flounder fish challenged with *S. iniae.*

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}
LWG (%)	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±0.31^{b}	0.94±0.16^{b}
Survival rate (%)	64.32±2.13^{a}	93.86±2.16^{b}	95.31±2.09^{b}

Probiotic enriched feed P1=1.4 × 10^{4} CFU/100 g feed; P2=1.4 × 10^{8} CFU/100 g of control feed. *Values (Mean ± SD) in the same row with different superscripts are significantly different from each other (P<0.05); LWG%: [final weight (g) - initial weight (g)/ initial weight (g)] ×100; SGR = [In final body weight - In initial body weight/time (days)] ×100; FCR= Feed consumed / Weight gain; SR= [end number of the alive fish/the beginning number of the fish]× 100.

Discussion

The morphological, phylogenetic, and biochemical results suggest that JFP2 isolate is a member of the *Bacillus* species. Previous studies have also reported the presence of *Bacillus* spp. from similar fermented food products (Nam and Ahn 2015; Namgung et al., 2010). Resistance to antibiotics was strain-dependent. Previous studies have reported that *B. subtilis,* a major fermenting bacterial species of Mbuja, exhibited 18%-64% resistance to tetracycline and chloramphenicol while *B. thuringiensis* showed resistance to ampicillin and chloramphenicol (Mohammadou et al., 2014).

The ability of microorganisms to survive in the gastrointestinal tract is one of the main important characteristics required for probiotic bacteria. The acidic conditions (pH 2) in the stomach and the bile salt concentration in the gastrointestinal track are adverse conditions that probiotic bacteria must overcome when taken orally (Prasad et al., 1998) to allow their colonization in the intestinal surface. Growth at pH 2 and in 0.3% oxgall suggests that *Bacillus amyloliquefaciens* JFP2 is able to withstand these harsh gastric

environments. The strain *B. amyloliquefaciens* JFP2 also grew at high temperatures which could be a positive trait for commercial use. The isolate JFP2 showed a positive proteolytic activity with highly expressed hydrophobicity and significant antioxidant capacity. All these suggest that the isolated strain *B. amyloliquefaciens* JFP2 can be used as a potential probiotic bacteria (Arusu et al., 2014).

Bacillus amyloliquefaciens JFP2 showed a wide spectrum of antibacterial activity against the seven major fish pathogens (Table 3). The antibacterial activity of the strain JFP2 was not only evident against Gram-positive bacteria but also against Gram-negative ones, with maximum inhibition against *E. tarda* (17.2±0.35 mm). A similar antagonistic effect of bacteriocin was produced by *Bacillus* subtilis against common fish pathogens such as *Aeromonas hydrophila, Edwardsiella tarda*, *Pseudomonas aeruginosa, Pseudomonas fluorescens, Flavobacterium branchiophilum* (Nayak and Mukherjee, 2011). The isolated strain JFP2 showed a significant antibacterial effect and the JFP2 CFCS was subjected to Sephadex G-100 gel filtration and SDS-PAGE separation of 37 kDa bacteriocin. These were consistent with previous reports on similar molecular weight of bacteriocin produced by *B. amyloliquefaciens J4,* 39 kDa (Lim et al., 2011) and *Bacillus* sp. SW1, 38 kDa (Kim et al., 2014), and higher than the other bacteriocin produced by *B. subtilis* YS-1005, 27 kDa (Kim et al., 1999), and *B. subtilis* YU-1432, 29 kDa (Lee and Lee, 2011). Additionally, bacteriocin JFP2 residual activity was similar to other bacteriocins produced by *L. lactis* ssp*. Diacetilactis* (Kojic et al., 1991) but in contrast, Lactacin F, after exposure at 50ºC for 30 min, no activity was identified (Kim et al., 2005). After exposure to proteniase K there was loss of activity and pepsin and trypsin confirmed their proteinaceous nature which was concurrent with previous studies (Nilsen et al., 2003; Nam and Ahn, 2015).

Antibacterial mechanisms of bacteriocins are broadly divided into three types: bacteriostatic, bactericidal, and bacteriolytic (Cotter et al., 2013). In the present study, the mechanism of bacteriocin JFP2 was determined the cell wall degradation method. The bacteriocin JFP2 was treated with cell walls of *A. hydrophila* for 5 hours at 37ºC. The optical density was decreased with increased cell wall degradation by the bacteriocin JFP2 (Fig. 7). Our results showed a bacteriolytic mode of the antibacterial mechanism of bacteriocin JFP2 isolated from the Jeju Island's traditional fermented food against *A. hydrophila*. The bacteriocin protein that kills other bacterial cells through cell wall degradation are class III bacteriocins (Bastos et al., 2010). The isolated strain *B. amyloliquefaceins* JFP2 also produces a large 37 kDa, heat stable antibacterial protein and because of its cell wall degradation mode it can be classified as class IIIa bacteriocin molecule. However, bacteriocin JFP2 need further studies to confirm its classifications.

The results of the present study showed that Jeju Island's traditional fermented sea food Jeotgal harbors probiotic bacteria such as *B. amyloliquefaciens* which produce bacteriocin JFP2. This can be used as a potential antibacterial agent against fish pathogenic bacteria. Recently, *in-vitro* antibacterial, antifungal and antioxidant properties of *B. amyloliquefaciens* isolated from soil sludge have proved that the *B. amyloliquefaciens* could be potent as probiotic bacteria (Kadaikunnan et al., 2105). Moreover, flounder juveniles fed with *B. amyloliquefaciens*-JFP2 enriched feed can overcome *S. iniae* infection. A detailed biochemical and immunological study on the dietary effect of this potential bacterium is currently being carried out (data not shown). Based on our results we believe that *B. amyloliquefaciens* JFP2 and/or its bacteriocin can be used as a bio-control agent of pathogenic bacteria if supplemented by feed industries in aquaculture feeds.

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