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Soybeans Fermented with *Bacillus polyfermenticus* KJS-2 Protects *Oplegnathus fasciatus* from Iridovirus and Pathogenic Bacterial Infection

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Lyophilized powder (BP2FS) of soybeans fermented with *Bacillus polyfermenticus* KJS-2 (*B. polyfermenticus* KJS-2) exhibited *in vitro* antibacterial activities against eight pathogenic bacteria. BP2FS was used as a fodder additive for *Oplegnathus fasciatus* (*O. fasciatus*) culture. One group (UFD) of *O. fasciatus* was fed a commercial fodder, while another group (FD) was fed the same fodder, but including BP2FS (6×10^4 cfu g^{-1} fodder), two times daily for 120 days. The mean body weight of the FD group (67.29±12.62 g) was higher than that of the UFD group (56.56±8.21 g) after 120 days. The survival rate of FD was 80% compared to 40% for the UFD group. Cumulative mortalities in the FD and UFD groups were 18.95% and 60.98% respectively. *B. polyfermenticus* KJS-2 was isolated from the intestines of the FD group and the number of viable colonies was estimated to be 1.04×10^4 cfu g^{-1}. Iridovirus and *Vibrio vulnificus* was detected in the organs of the UFD group but not in the FD group. All of the infected fish showed typical clinical symptoms of hemorrhage in their tail fins. Dissection of the infected internal organs revealed liver congestion and spleen enlargement - typical symptoms caused by iridovirus infection. These results clearly show that BP2FS is highly beneficial in preventing *O. fasciatus* from iridovirus infection.

Key words : *Bacillus polyfermenticus* KJS-2, fermented soybeans, *Oplegnathus fasciatus*, antimicrobial activity, iridovirus

Introduction

Striped beakperch, *Oplegnathus fasciatus* (*O. fasciatus*), is one of the most economically important fish in Korea. This species is widely distributed in the Pacific Ocean, including southern parts of the Korean peninsula, Japan, Taiwan, and Hawaii [23]. The culture of *O. fasciatus* is hampered by infectious diseases caused by bacteria, parasites and viruses. Viruses are the most causative infectious agents in aquaculture, leading to significant economic losses. For example, an iridovirus-like pathogen was first isolated from the redfin perch [18]. Iridovirus and nodavirus have also been identified as the major pathogens in grouper culture at the fry and fingerling stages. In fish, iridovirus cause necrosis, sleepy grouper disease, and other systemic diseases [19]. Large-scale mortality was first reported in cultured *O. fasciatus* from the southern coastal areas of the Korean peninsula during August to September 1998 [15]. The percentage of total mortality was estimated to be 60%. According to [13], the initial signs of iridovirus infection are reduced feed intake, lethargy and a darkened body, with a typical swimming pattern at the edge of an aquatic plant in the terminal stages of the disease. [25] cloned a small subunit of the ribonucleotide reductase (RNRS) gene from the red sea bream iridovirus (RSIV) and successfully amplified virus-specific DNAs with a primer set based on the RNRS gene. Characteristics of the iridovirus-infected fish included the appearance of Feulgen-positive enlarged cells in the kidney, spleen, heart, liver and gills. The spleen showed extensive necrosis, haemorrhage, and enlarged cells, mainly around the splenic pulp [13]. *O. fasciatus* infected by a virus is also highly susceptible to pathogenic bacteria, several of which, *Streptococcus iniae* has been previously reported to cause mortality in fish [27].

Appropriate probiotics applications have been shown to improve intestinal microbial balance, thus leading to improved nutrition absorption [8,21,26] and reduced pathogenic problems in the gastrointestinal tract [4,9]. Probiotics have been used across of range of aquatic organisms, includ-

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ing salmon and shrimp infected with pathogenic bacteria [1,6,31]. In 1933, Dr. Terakado isolated several endospore-forming rods from the air, at least four of which were used to make the commercial mixed strain product 'Bispan', which has since been used for the treatment of long-term intestinal disorders for human and animals. 'Bispan' strains are described in the Japanese Pharmacopoeia as amylolytic bacilli [20]. *B. polyfermenticus* KJS-2 was identified from the 'Bispan' strains and used in this study.

In this paper, we report on the antibacterial activity and preventive efficacy of soybeans fermented with *B. polyfermenticus* KJS-2 against iridovirus and pathogenic bacteria. DNA amplification method using polymerase chain reaction (PCR) was performed to determine the infection of iridovirus in *O. fasciatus*. In addition, various tissues obtained from the viral-infected moribund fish were subjected to cellular analysis through hemacolor staining.

Materials and Methods

Microorganisms

B. polyfermenticus KJS-2 was isolated from commercial 'Bispan' composed of several species of *Bacillus* strains. The basal medium used for growth of bacteria was tryptic soy broth (TSB; Becton and Dickinson, Franklin Lakes, NJ, USA). The microorganisms used for antimicrobial activity determination were *Streptococcus iniae* ATCC 29178, *Streptococcus parauberis* DSM 6631, *Vibrio harveyi* ATCC 14126, *Vibrio ordalii* KCCM 41669, *Vibrio vulnificus* ATCC 27562, *Flexibacter tractuosus* KCTC 2670, *Edwardsiella tarda* ATCC 15947, and *Lactococcus garviae* KCCM 40698. All microorganisms used in this exercise were obtained from the American Type Culture Collection (ATCC), the Korea Culture Center of Microorganisms (KCCM) or the Korean Collection for Type Cultures (KCTC).

Fermentation

Three ml of TSB medium was used for the growth of the bacterium. One ml from the 3 ml culture was inoculated into 100 ml of TSB medium in a 500 ml flask. All flasks were incubated in a rotary shaker (Seyoung scientific, Korea) under 200 rpm at 37°C for 16 hrs. Bacterial concentrations were determined by measuring optical density (OD) at 600 nm using a UV/vis spectrometer (Simadzu, Japan), by second the culture with OD=1.2 was used as a seed for solid-state fermentation. One-hundred grams of soybean (Hamyang, Korea) was placed in a 1 l Erlenmeyer flask and autoclaved twice at 121°C for 20 min at an interval of 5 hrs to kill all spore-forming microorganisms. The autoclaved soybeans were cooled to room temperature, after which, 30 ml of the seed culture (described above) was added to soybeans and mixed. Flasks were incubated statically at 40°C for 72 hrs. The culture product was then lyophilized and ground to powder. The product was stored at 4°C until used. The powder was labeled as "BP2FS". It was then used for antimicrobial activity test and as an additive to the commercial fodder Futra Larvae (Alleraqua, Denmark). *B. polyfermenticus* KJS-2 in the product was included to 1×10^{10} cfu g^{-1}. The 1 g of BP2FS was admixed with 1 kg of the fodder and used.

Antimicrobial activity of BP2FS

Antimicrobial activity of BP2FS was assayed by the spot-on-lawn method [20]. 10^7 cells of each of the indicated strains (*Streptococcus iniae* ATCC 29178, *Streptococcus parauberis* DSM 6631, *Vibrio harveyi* ATCC 14126, *Vibrio ordalii* KCCM 41669, *Vibrio vulnificus* ATCC 27562, *Flexibacter tractuosus* KCTC 2670, *Edwardsiella tarda* ATCC 15947, and *Lactococcus garviae* KCCM 40698) were overlaid on tryptic soy agar (TSA; Becton and Dickinson, Franklin Lakes, NJ, USA) with 2.5% NaCl (w/v). Seven strains, except for *Streptococcus parauberis* DSM 6631 which was incubated at 37°C, were incubated at 25°C. BP2FS and fermented soybeans without *Bacillus polyfermenticus* KJS-2 (FS) were suspended in 50% acetone to the concentration of 1 or 0.5 w/v. When the agar hardened, 20 µl of BP2FS was spotted on the plate. The activity was determined by clear zone.

Survival and growth rates of *Oplegnathus fasciatus*

The body weight of fingerlings was approximately 5 grams each when purchased from Sebo Susan (Tongyoung, Korea). We grew the fish in two separate groups (10,000 fish each) for 120 days, one (labeled as UFD) fed with the commercial fodder Futra Larvae (Alleraqua, Denmark) and the other (termed as FD) fed with the Futra Larvae containing BP2FS. Prior to the feeding exercise, each group of the fish was stocked in an enclosed net-cage in the sea and fed commercial fodder for a week to allow them to acclimatize. Feed mass was maintained at 6% of total body weight. The two groups were fed twice daily for 16 weeks (From July 16, 2007 to October 20, 2007). The FD group was fed with BP2FS corresponding to approximately 0.6% of the fodder by

weight, which is equivalent to 6×10^4 cfu g^{-1} fodder. Soybeans fermented with *B. polyfermenticus* KJS-2 was increased fodder efficacy of fish than *B. polyfermenticus* KJS-2 in the former experiment (data not shown). Survival and growth rate were determined every 10 days for 120 days. Mortalities were monitored twice daily for 16 weeks. The water temperature ranged from 18°C at the beginning to 27°C at the end of the experiment because of the natural fluctuation of sea water temperature.

Detection of virus and pathogenic bacteria from the spleen and kidney

To detect virus infection, total DNA and total RNA were isolated from tissues and used as template for PCR. In order to isolate total RNA, the spleens and kidneys (50 mg) of iridovirus infected five fish were homogenized with a teflon homogenizer in 1ml of 0.1% DEPC (diethylpyrocarbonate) solution and centrifuged at 12,000 rpm for 10 min at 4°C to remove insoluble materials. Subsequently, 0.2 ml of the supernatant was transferred to a microcentrifuge tube, where 0.2 ml of chloroform and 1 ml of Trizol reagent (Invitrogen, USA) were added. The mixture was manually shaken vigorously for 15 sec, and centrifuged at 12,000 rpm for 15 min at 4°C. After adding 0.5ml of isopropanol and 1 ml of Trizol reagent to 0.5ml of the supernatant solution, the precipitate was centrifuged again at 12,000 rpm for 15 min at 4°C, and the supernatant was removed. The RNA pellet was washed twice with 75% ethanol, dried and redissolved in 40 μl of 0.1% DEPC and stored at -80°C until used. cDNA was synthesized in a final volume of 20 μl of reaction mixture containing 4 μl of first strand buffer (5x), 1 μl of 0.1 M DTT, 10.8 μl of 0.1% DEPC, 1 μl of 10 mM dNTP, 1 μl of random primer, 0.2 μl of Superscript II R (Invitrogen, USA), and 2 μl of total RNA.

In order to isolate total DNA, 50 mg of spleen and kidney were homogenized with a teflon homogenizer in a 1ml of PBS. After adding 0.2 ml of tissue lysis buffer and 40 μl of proteinase to the disrupted tissues, the sample was manually shaken vigorously for 15 sec. The tube was incubated for 60 min at 55°C and further incubated for 10 min at 72°C after addition of 200 μl of binding buffer. The mixture was centrifuged and the supernatant was transferred to a fresh tube. Subsequently, 100 μl of isopropanol was added to the supernatant and the resulting DNA collected by centrifugation at 8,000 rpm for 1 min at 4°C. The DNA pellet was washed with 75% ethanol, redissolved in 40 μl in TE buffer and stored at -80°C until used. PCR reaction were performed in a final volume of 20 μl of reaction mixture containing Accupower™ PCR premix (Bioneer, Korea), 16 μl of distilled water, 1 μl of each 10 pM primer, and 2 μl of template DNA. The virus-specific primers for PCR are designed based on known results (Table 1) [3,7,16,22,30]. The amplification was performed with a Thermocycler T Gradient (Biometra, Germany) (Table 1). Amplification conditions for each primer were optimized to give the expected size of DNA according to results in Table 2. PCR products were analyzed in 1.5% agarose gel containing ethidium bromide (0.5 μg/ml) and visualized with a UV transilluminator. DNAs from iridovirus, Hirame Rhabdovirus, Birnavirus, viral nervous necrosis virus, and viral hemorrhagic septicemia virus were used as templates for positive control. In order to isolate strains, the spleens and kidneys (50 mg) of iridovirus infected five fish were homogenized with a teflon homogenizer in 1ml of 10 mM phosphate buffer solution (pH 6.8) and centrifuged at 12,000 rpm for 10 min at 4°C to get the supernatant. Subsequently, the supernatant was transferred to a microcentrifuge tube and diluted to 10 mM phosphate buffer solution (pH 6.8). Infection of tissues by patho-

Table 1. PCR profiles for virus gene amplification

	Step					
	Temp (°C), Time (sec)					
Genes	Pre-Denaturing	Denaturing	Annealing	Polymerization	Termination	Cycles
DPTF-Irido	94, 300	94, 30	55, 60	72, 60	72, 300	30
GP-HRV		94, 30	55, 30	72, 45		30
MCP-Birna		94, 30	55, 60	72, 60		30
CP-VNN		95, 40	50, 40	72, 40		25
NP-VHS		94, 30	52, 30	68, 60		35

DPTF-Irido: DNA polymerase type-B family of iridovirus; GP-HRV: glycoprotein of Hirame Rhabdovirus; MCP-Birna: major capsid protein of Birnavirus; CP-VNN: coat protein of viral nervous necrosis virus; NP-VHS: nucleoprotein of viral hemorrhagic septicemia virus.

Table 2. PCR primers used for gene amplification

Genes	Nucleotide sequences of primers	Sizes of PCR products (bp)	Genbank accession numbers
DPTF-Irido	Forward: GTGACTGCACACCAATGGAC Reverse: GGCTTTCTCAATCAGCTTGC	698	AY779031
GP-HRV	Forward: ACCCTGGGATTCCTTGATTC Reverse: TCTGGTGGGCACGATAAGTT	533	AF104985
MCP-Birna	Forward: GCACCACGAAGGTACGAAAT Reverse: GTACGTTGCCGTTTCCTGAT	597	AF342727
CP-VNN	Forward: CGGATACGTTGTTGTTGACG Reverse: CAACAGGCAGCAGAATTTGA	758	AY140798
NP-VHS	Forward: GAGAGAACTGGCCCTGACTG Reverse: ATGATCCGTCTGGCTGACTC	444	Y18263

genic bacteria was determined using Easy 24E Plus kit (Komed, Korea) according to manufacturer's recommendations. The viable cell count was also evaluated using TSA to estimate the total *B. polyfermenticus* KJS-2 population in intestine. *B. polyfermenticus* KJS-2 was selected easily on plate because colony shape of *B. polyfermenticus* KJS-2 grown on TSA was visibly distinguished from other *Bacillus* strains. The *B. polyfermenticus* KJS-2 was producing opaque, dark-yellow colonies with round and flat shape, while other *Bacillus* strains were forming milky-white colonies with uneven and rough edges.

Hemacolor staining

Chemicals for hemacolor staining were purchased from Merck Diagnostica (Darmstadt, Germany). Cells on slide glass were dried for at least 5 min, fixed with hemacolor fixative solution for 5 sec and stained for 10 sec in hemacolor red reagent and 10 sec in hemacolor blue reagent. The cells were rinsed to remove excess dye out of slide glasses and dried. The enlarged cells were observed by light microscopy (×400).

Results

Antimicrobial activity of BP2FS

The spot-on-lawn method was used to assess antimicrobial activities of BP2FS in three Gram-positive and five Gram-negative bacteria, all of which are known as pathogenic fish bacteria. The formation of a clear zone of more than 2 mm was considered as significant antibacterial activity. Antibacterial activities in seven harmful microorganisms including Gram-positive and Gram-negative were clear (Table 3). However, the inhibition zone on *Edwardsiella tarda* ATCC 15947 was not significantly clear. FS showed no effect of antimicrobial activities against all pathogenic bacteria. These results indicate that BP2FS has a broad spectrum of antibacterial activities in Gram-positive and the Gram-negative bacteria.

Table 3. Antibacterial activities of BP2FS and FS by spot-on-lawn method

Microorganisms	Culture Medium	Incubation temperature (°C)	Inhibition	
			BP2FS	FS
Gram-positivebacteria				
Streptococcusparauberis DSM 6631	TSA+2.5% NaCl	37	+	-
Streptococcusiniae ATCC 29178	TSA+2.5% NaCl	25	+	-
Lactococcusgarviae KCCM 40698	TSA+2.5% NaCl	25	+	-
Gram-negativebacteria				
Flexibactertractuosus ATCC 23168	TSA+2.5% NaCl	25	+	-
Vibrioharveyi KCCM 40866	TSA+2.5% NaCl	25	+	-
Vibriovulnificus ATCC 27562	TSA+2.5% NaCl	25	+	-
Vbrioordalii KCCM 41669	TSA+2.5% NaCl	25	+	-
Edwardsiellatarda ATCC 15947	TSA+2.5% NaCl	25	+/-#	-

#, not significantly inhibited.

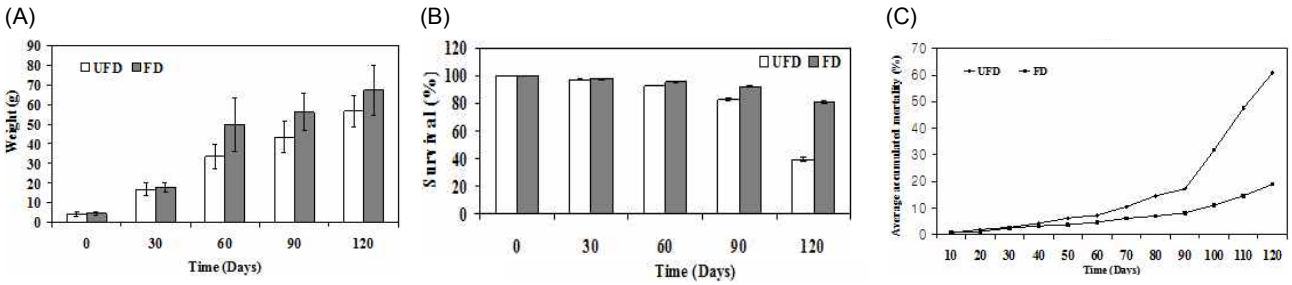

Fig. 1. (A) Average weights, (B) survivals and (C) cumulative mortality of *Oplegnathus fasciatus* during 120 days of culture.

Effects of BP2FS on *Oplegnathus fasciatus*

The effects of BP2FS as a fodder additive on body weight, survival rate and mortality were examined. Mean weights in the FD group (67.29±12.62 g) were slightly higher than the equivalent in the UFD group (56.56±8.21 g; Fig. 1). However, there was a dramatic difference between the survival rate of FD and UFD group. After 120 days, the survival rate of FD was 80% compared with 40% in the UFD group. Based on this result, cumulative mortalities of *O. fasciatus* in the FD and UFD group were 18.95% and 60.98% respectively. The colony numbers of *B. polyfermenticus* KJS-2 in intestine of FD fish were estimated to be 1.04×10^4 cfu g^{-1}. All of the infected fish exhibited hemorrhage in their tail fins, as a representative clinical symptom. Dissection of the infected organs revealed congestion of liver and enlargement of spleen (Fig. 2).

Detection of virus and pathogenic bacteria in spleen and kidney

DNA and RNA were extracted from the spleens and kidneys of both the FD and UFD groups and used for detection of virus infection. After conversion of RNA to DNA using reverse transcriptase, all of the DNAs were used as templates for amplification of specific virus genes by PCR methodology (Table 1 and 2). Five sets of PCR primers specific for 5 different viruses were used, but only iridovirus specific gene was detected in UFD fish (Fig. 3). The PCR product

Fig. 2. (A) The iridovirus-infected *Oplegnathus fasciatus* showing enlargement of (B) the internal organs and (C) the spleen.

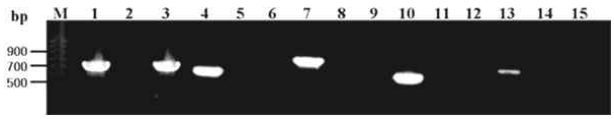

Fig. 3. Detection of the virus infection by PCR amplification. M: Standard marker; 1: Iridovirus DNA positive control; 2: PCR product obtained from spleens and kidneys of FD group using iridovirus DNA primers; 3: PCR product obtained from spleens and kidneys of UFD group using iridovirus DNA primers; 4: Birnavirus DNA positive control; 5: PCR product obtained from spleens and kidneys of FD group using Birnavirus DNA primers; 6: PCR product obtained from spleens and kidneys of UFD group using Birnavirus DNA primers; 7: CP-VNN DNA positive control; 8: PCR product obtained from spleens and kidneys of FD group using CP-VNN DNA primers; 9: PCR product obtained from spleens and kidneys of UFD group using CP-VNN DNA primers; 10: NP-VHS DNA positive control; 11: PCR product obtained from spleens and kidneys of FD group using NP-VHS DNA primers; 12: PCR product obtained from spleens and kidneys of UFD group using NP-VHS DNA primers; 13: GP-HRV DNA positive control; 14: PCR product obtained from spleens and kidneys of FD group using GP-HRV DNA primers; 15: PCR product obtained from spleens and kidneys of UFD group using GP-HRV DNA primers.

from iridovirus specific gene primers was sequenced and compared with the known iridovirus gene resulting in a high degree of sequence identity. No iridovirus gene was detected in any fish from the FD group. Infection by pathogenic bacteria in organs was observed using a commercial detection kit. The bacteria were identified as *Vibrio vulnificus* with 98.1% significance.

Observation of enlarged cell

One of the symptoms of iridovirus infection is morphological change in the cells of internal organs. Microscopic examination of spleens and kidneys of the iridovirus infected fish clearly showed a large numbers of enlarged cells

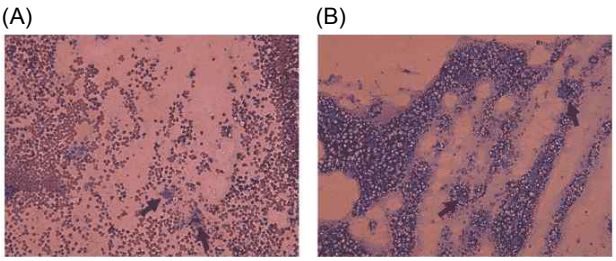

Fig. 4. Enlarged cells of (A) the spleen and (B) kidney from *Oplegnathus fasciatus* (×400).

(Fig. 4). These results were corresponding to enlarged cells of spleen and kidney [11,24].

Discussion

Iridovirus causes large-scale mortality in the striped beakperch, *O. fasciatus* [13,33]. Probiotics have been widely tested for use in aquaculture. Previous reports also show a high survival rate for animals when fed probiotics [10,28,29]. [8] defined probiotics as a live microbial feed supplement. The benefits of probiotics in preventing diseases caused by pathogenic bacteria are well documented [1,14,28,29]. In particular, *Bacillus* species have been reported to be successful for a wide range of invertebrates [12].

The present study is the first report which has used a probiotics, *B. polyfermenticus* KJS-2, to protect *O. fasciatus* from iridovirus and pathogenic bacteria infection. Soybeans (BP2FS) fermented with *B. polyfermenticus* KJS-2 exhibited antibacterial activity against several pathogenic Gram-positive and Gram-negative bacteria (Table 3). In the previous study, Soybeans fermented with *B. polyfermenticus* KJS-2 showed also broad spectrum against most pathogenic bacteria compared to soybeans fermented without *B. polyfermenticus* KJS-2 [17]. BP2FS may possess bioactive materials secreted from *B. polyfermenticus* KJS-2. BP2FS, as a fodder additive, has an extremely powerful effect in reducing mortality of *O. fasciatus* infected with iridovirus and pathogenic bacteria. The protection effect of BP2FS from virus and pathogenic bacteria infection might be due to the highly adhesive feature of *B. polyfermenticus* KJS-2 to the intestinal wall. *B. polyfermenticus* KJS-2 has mannose specific affinity. This paper suggests that BP2FS may be an effective agent in controlling virus and pathogenic bacteria in culture environments. Mortalities of *O. fasciatus* by iridovirus occurred from the common August to early October in Korea as Fig. 1 (c) [15]. Then, there were complex infection by pathogenic bacteria with iridovirus. We performed the challenge test for the prevention of naturally infected-iridovirus and pathogenic bacteria from July to October. The spleen and kidney are the main target organs in an iridovirus-infected fish [33]. We detected iridovirus in spleen and kidney of moribund fish from the UFD group. The tissues from iridovirus-infected *O. fasciatus* had enlarged cells which are typical symptoms in marine and freshwater infected fish [11,34]. The kidney, heart and spleen of virus-infected fish were also infected with *Vibrio*, which are frequently found pathogenic bacteria in iridovirus-infected fish.

Dietary supplementation of BP2FS improved the weight gain, growth rate, disease resistance, life span, and feed conversion ratio of *O. fasciatus*. *B. polyfermenticus* KJS-2 produces digestive enzymes which may be the main reason of body weight gain in *O. fasciatus* [32]. Overall, the FD group appeared healthier and more active than the UFD group. This suggests that BP2FS may be an effective agent in controlling virus and pathogenic bacteria in culture environments. Adhesion of other *Bacillus* and competitive exclusion of pathogens from intestine have already been reported [2,5].

In conclusion, BP2FS reduced mortality of *O. fasciatus* by inhibiting iridovirus and pathogenic bacteria infection. The results of this study suggest that BP2FS may be a potential candidate for an alternative antibiotic and a fodder additive in aquaculture. However, the inhibition mechanism of BP2FS against iridovirus remains to be more comprehensively elucidated.

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초록 : *Bacillus polyfermenticus* KJS-2에서 발효된 콩의 돌돔에 대한 이리도바이러스 및 병원성균에 대한 예방효과

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Bacillus polyfermenticus KJS-2 (*B. polyfermenticus* KJS-2)를 이용하여 발효된 콩(BP2FS)은 8가지의 병원성균에 대해 항균활성을 가졌다. BP2FS는 돌돔의 배양에 있어 사료첨가제로 사용되었다. 돌돔의 배양 시 BP2FS를 투여한 군(6×10^4 cfu g^{-1} 사료)과 투여하지 않은 군으로 나누었으며, 투여한 군은 120일 동안 하루에 두 번씩 투여 하였다. 120일 후에 투여한 군의 돌돔의 몸무게는 67.29±12.62 g이었으며 그렇지 않은 군의 몸무게는 56.56±8.21 g으로 나타났다. 생존율은 투여한 군(80%)과 투여하지 않은 군(40%)이었다. 폐사한 돌돔의 축적된 양은 투여한 군(18.95%)과 투여하지 않은 군(60.98%)이었다. BP2FS를 투여한 군에서 *B. polyfermenticus* KJS-2를 돌돔의 장내에서 검출한 결과 1.04×10^4 cfu g^{-1}으로 나타났다. BP2FS를 투여 하지 않은 군의 돌돔에서는 iridovirus에 감염되었을 경우 나타나는 전형적인 증상인 비장과 간에서 비대세포가 관찰 되었다. 이 결과들은 BP2FS는 돌돔의 이리도바이러스에 대한 감염 시 예방효과를 가져온다 할 수 있다.