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Original Research Article

Characteristics and biological control functions of *Bacillus* sp. PM8313 as a host-associated probiotic in red sea bream (*Pagrus major*) aquaculture

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ABSTRACT

Host-associated probiotics (HAPs) are bacteria originally isolated from rearing water or the host's gastrointestinal tract in order to enhance the host's growth and health. This study investigated the HAP potential of *Bacillus* sp. PM8313, isolated from wild red sea bream (*Pagrus major*), through characterization and feeding trials. Results based on in vitro tests showed that PM8313 is safe, confirming its hemolytic, cytotoxic, and antibiotic resistance. In addition, PM8313 showed advantages as a probiotic with high viability in the gastrointestinal model and a high cell adhesion rate. Whole-genome sequencing demonstrated that PM8313 has a 4,615,871 bp single circular chromosome and a guanine −cytosine content of 45.25%. It also showed the absence of genes encoding virulence factors, such as cytotoxin, enterotoxin, hemolysin, sphingomyelinase, and phospholipase. In the feeding trial, a supplemental diet of 1×10^8 CFU/g PM8313 positively altered the weight gain, digestive enzyme activity, and intestinal microbiota composition of red sea bream. Analysis of nonspecific immune parameters and immune-related gene expression, and a challenge test showed that PM8313 supplementation increases immunity and pathogenic bacteria resistance. Our findings suggest that PM8313 should be considered for application as a novel HAP to red sea bream aquaculture.

1. Introduction

Infectious diseases are the most important constraint in aquaculture as they can cause mass mortality of farmed fish (Raj et al., 2019; Wanna et al., 2021). Although many farmers use antibiotics to control these diseases, some countries have banned the oral administration of synthetic antibiotics to fish used for human consumption, because antibiotic abuse not only creates antibiotic resistance to pathogenic strains but also adversely affects human health in residual antibiotic form (Chen et al., 2015; Hasan et al., 2019). Therefore, nonantibiotics and eco-friendly agents are attracting attention as agents to replace antibiotics and as a key factor in disease management (Farzanfar, 2006).

Probiotics are living microorganisms that when administered in appropriate amounts, provide beneficial effects to the host (Food and Agriculture Organization [FAO]/World Health Organization [WHO], 2001). They maintain the balance of the host's intestinal microbiota, increase immunity, and help digest and absorb nutrients, so they are attracting attention as eco-friendly feed additives to replace antibiotics (Jang et al., 2021a). Probiotics developed for use in aquaculture include *Lactobacillus*, *Lactococcus*, *Bacillus*, and

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Enterococcus species and yeast (Van Doan et al., 2020). However, most of these probiotics are derived from terrestrial sources rather than the environment in which aquatic animals live or the host animal; therefore, they have limited ability to settle within the host's defense system and provide beneficial effects (Gatesoupe et al., 2008; Van Doan et al., 2020). The highest physiological activities of microorganisms are seen in their natural habitats (Ibrahem, 2015), so it may be most efficient to isolate probiotics used in aquaculture from the target fish species or rearing water.

Host-associated probiotics (HAPs) are defined as "bacteria originally isolated from rearing water or the gastrointestinal tract of the host to improve growth and health of the host" (Van Doan et al., 2020). They are established naturally within the host defense system and produce many beneficial substances, such as digestive enzymes and bioactive compounds (Lazado et al., 2015; Sanchez et al., 2012; Gomez et al., 2013; Llewellyn et al., 2014). Therefore, autochthonous bacteria derived from the mucosal layer of aquatic animals are suitable for the development of probiotics for aquaculture (Van Doan et al., 2020).

The red sea bream (*Pagrus major*) is one of the most commercially important fish species for aquaculture in Asia due to its high market value and demand (Dawood et al., 2015, 2020). Research on red sea bream is important for the aquaculture industry, but research on probiotic supplementation for red sea bream is limited, with few studies specifically on HAP development in this species. In addition, one of the most important factors to consider for probiotic development is the safety and characteristics of the bacterial strain used. Thus, this study aims to confirm the characteristics and safety of a HAP candidate strain isolated from healthy red sea bream and to investigate the effect of dietary supplementation on the growth, immunity, and intestinal microbiota of red sea bream.

2. Materials and methods

2.1. Animal ethics

The study was conducted under the guidelines of the Animal Ethics Committee Regulations issued by Dong-Eui University (DEU-A2022-003).

2.2. Bacterial isolation and identification

Wild red sea bream were caught in the South Sea of Korea, and those weighing 100, 300, and 800 g were selected for this study. Bacteria were isolated from the intestine of the wild red sea bream. Briefly, the intestine was homogenized and serially diluted using 0.85% NaCl. The suspension was poured into Luria–Bertani (LB) and De Man, Rogosa and Sharpe (MRS) agar plate (Difco, USA) and incubated at 20–37 °C for 24 h. A single colony was isolated and cultured in liquid medium. Then, it was mixed with 50% glycerol and stored at −80 °C until use in experiments (Jang et al., 2021b). The isolated strain was identified by performing 16S ribosomal RNA gene analysis using the 27F (AGAGT TTGAT CMTGG CTCAG) and 1492R (TACGG YTACC TTGTT ACGAC TT) universal primer sets.

2.3. Probiotic characterization

2.3.1. Hemolysis, toxigenic gene, and cytotoxicity analysis

The safety of the isolated strain was verified through hemolysis, toxigenic gene, and cytotoxicity analysis. Hemolytic activity was determined by incubating bacteria on a blood agar base plate (Kisan Bio, South Korea) at 37 °C for 48 h. For comparison, *B. cereus* with β-hemolysis, purchased from the Korean Collection for Type Culture (KCTC), was used (KCTC 1012 and KCTC 3624). Toxigenic gene analysis was performed using polymerase chain reaction (PCR). *Bacillus* species–specific virulence gene primers were prepared, as previously described (Lee et al., 2019); see Table S1. *B. cereus* was used as a positive control, and PCR amplification was confirmed by electrophoresis using 1% agarose gel. Cytotoxicity was investigated using the Cellrix viability assay kit (Medifab, South Korea), and *B. cereus* was used for comparison.

2.3.2. Cell viability in simulated gastric and intestinal juice

The viability of cells exposed to simulated gastric juice (SGJ) and simulated intestinal juice (SIJ) was evaluated, as previously described (Jang et al., 2019). SGJ contains 2 g/L of NaCl and 3.2 g/L of pepsin powder (pH 2), and SIJ contains 0.3% bile salts (pH 5.9). Cell viability was expressed by calculating the ratio of the number of viable cells to the initial inoculation after 2 and 3 h of the cells exposed to SGJ and SIJ, respectively.

2.3.3. Adhesion to the HT-29 cell line and antibiotic resistance

The human colon adenocarcinoma HT-29 cell line was purchased from the Korea Cell Line Bank. To analyze bacterial adhesion, HT-29 cells were cultured in Roswell Park Memorial Institute-1640 medium (Sigma–Aldrich, USA) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Gibco, USA), 0.1 U/L of penicillin, and 100 mg/L of streptomycin maintained at 37 °C in an atmosphere of 5% CO_2/95% air. An antibiotic-free medium containing bacteria was added to HT-29 cells, which were then incubated at 37 °C for 120 min. After incubation, nonadherent bacteria were removed by washing with phosphate-buffered saline, and adhered bacteria were counted after detachment using 0.05% trypsin–EDTA. The adhesion rate was calculated as the relative ratio of the adhered bacterial colony-forming unit (CFU) to the initial inoculated bacterial CFU.

Antibiotic resistance was expressed by determining the minimum inhibitory concentrations for the eight antibiotics listed by the European Food Safety Authority (EFSA)— chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, streptomycin, tetracycline, and vancomycin—using the E-test–MIC Test Strip (Liofilchem, Italy) (EFSA, 2012).

2.3.4. Availability of prebiotics

Suitable prebiotics were identified by evaluating the availability of various prebiotics (e.g., β-glucan, fructo-oligosaccharide, gluco-oligosaccharide, inulin, and mannan-oligosaccharide). Bacteria were inoculated into glucose-free reconstituted MRS medium in which 2% of each prebiotic was added. Absorbance at 600 nm was measured at 4 h intervals for 24 h to show a growth curve.

2.4. Genome sequencing, assembly, and annotation

Bacteria for use in the study were selected based on probiotic characteristics, and whole-genome sequencing was performed. Total DNA of the selected strain was extracted using the NucleoSpin Microbial DNA Mini kit (Macherey–Nagel, Germany) according to the manufacturer's instructions. The extracted DNA yield was assessed using the Qubit with High sensitivity range kit (Thermo Fisher Scientific, USA). The sequencing library for genomic DNA was prepared using the TruSeq Nano DNA Low Throughput Library Prep kit (Illumina, USA). Sequencing was carried out using the MiSeq illumine system. After sequencing, individual sequence reads were checked for quality control using FastQC v.0.11.8. Illumina Sequencing data were processed to remove low-quality bases and adapter sequences with optimized settings (LEADING, 10; TRAILING, 10; SLIDINGWINDOW, 4:20; MINLEN, 200) using Trimmomatic v0.39. Subsequently, additional phiX control,s were removed from preassembled data. Trimmed sequences were aligned against the phiX genome using bowtie2 v2.3.5.1 with the default options and

filtered out using samtools v1.9. Each of these assembled contigs was annotated using the PATRIC and RAST bacterial databases, and the coding sequence, ribosomal RNA and transfer RNA from their contig sequences were identified.

2.5. Experimental diet preparation

The raw materials for the feed used in the study and their composition ratio were provided by the National Institute of Fisheries Science (NIFS). The basal diet composition is shown in Table 1. The basal control diet without supplementation (MMC) was used. In addition, two other diets, one supplemented with *Bacillus* sp. PM8313 spores (probiotic) at a concentration of 1×10^8 CFU/g diet (MMP) and the other with 0.1% β-glucan + *Bacillus* sp. PM8313 spores (synbiotic) at a concentration of 1×10^8 CFU/g diet (MMS), were prepared. All raw materials were sufficiently mixed to prepare feed using a pellet maker, dried, and stored frozen at −20 °C for use. Feed proximate composition analysis was performed in accordance with the Association of Official Agricultural Chemists standard methods (1995). Moisture content was assessed by drying to a constant weight in an oven at 135 °C. Ash content was determined by complete incineration at 550 °C. Crude protein was measured using Kjeldahl's method (N = 6.25) after acid digestion, while crude lipid was measured after ether extraction with Soxtherm.

2.6. Maintenance of fish and the feeding trial

Red sea bream with an average weight of 35 g were obtained from the Nam-Bu fish farm (Yeosu, Republic of Korea) and acclimatized in 360 L semirecirculating tanks for 1 week. After acclimatization, 180 healthy fish were randomly assigned to 3 groups (20 fish/tank, triplicates). They were fed at 09:00 and 16:00 twice a day up to apparent satiation. Water quality was regularly monitored twice a day, and stable environmental parameters were maintained: temperature, 18.3 ± 0.6 °C; pH, 7.8 ± 0.2; dissolved oxygen, 5.6 ± 0.3 mg/L; salinity, 31.3 ± 0.7 g/kg; and water flow, 1.2 L/min.

Table 1
Composition of the basal experimental diet for red sea bream (*Pagrus major*).

Ingredients, %	Content	Feed proximate composition, %	Content
Fish meal[1]	30.00	Moisture	4.89
Wheat flour	34.08	Crude protein	46.22
Meat meal[2]	24.10	Crude lipid	12.70
Fish oil	6.00	Crude ash	9.16
Squid liver powder	3.00	Supplement for MMP group	
Lecithin	1.00	Probiotics, CFU/g	1×10^8
Mono calcium phosphate	0.20	Supplements for MMS group	
Vitamin C	0.50	Probiotics, CFU/g	1×10^8
Vitamin premix[3]	0.50	β-glucan, %	0.10
Mineral premix[4]	0.50		
Choline	0.12		

MMP = meat meal group supplemented with probiotics; MMS = meat meal group supplemented with synbiotics.
[1] Fish meal: tuna by product meal (Woojin feed Ind. Co. Ltd., South Korea), 15%; sardine fish meal (Cesmec Ltda., Chile), 7.5%; pollock fish meal (Kodiak fish meal Company, USA.), 7.5%.
[2] Meat meal: pork (Hwasong industrial Co. Ltd., South Korea).
[3] Vitamin premix (as mg/kg in diets): ascorbic acid, 300; DL-calcium pantothenate, 150; choline bitate, 3,000; inositol, 150; menadion, 6; niacin, 150; pyridoxine. HCl, 15; rivoflavin, 30; thiamine mononitrate, 15; DL-α-tocopherol acetate, 201; retinyl acetate, 6; biotin, 1.5; folic acid, 5.4; cobalamin, 0.06.
[4] Mineral premix (as mg/kg in diets): NaCl, 437.4; $MgSO_4 \cdot 7H_2O$, 1,379.8; $ZnSO_4 \cdot 7H_2O$, 226.4; Fe-Citrate, 299; $MnSO_4$, 0.016; $FeSO_4$, 0.0378; $CuSO_4$, 0.00033; $Ca(IO)_3$, 0.0006; MgO, 0.00135; $NaSeO_3$, 0.00025.

2.7. Growth performance, feed utilization, and body indices

After the 8-week feeding trial, the weight and length of all fish that were fasted for 24 h were measured. Their growth performance, feed utilization, and organosomatic indices were calculated as follows:

- Weight gain (WG, %) = 100 × (final weight − initial weight)/initial weight
- Specific growth rate (SGR, %/day) = 100 × (ln final weight − ln initial weight)/days
- Feed conversion ratio (FCR) = dry feed intake/(final weight − initial weight)
- Protein efficiency ratio (PER) = (final weight − initial weight)/protein fed
- Condition factor (CF, %) = 100 × body weight/(total body length)^3
- Viscerosomatic index (VSI, %) = 100 × (visceral weight/body weight)
- Hepatosomatic index (HIS, %) = 100 × (liver weight/body weight)

2.8. Analysis of digestive enzymes

Digestive enzyme activity was evaluated using amylase, trypsin, and lipase activity assay kits (BioVision, USA). The anterior midgut of fish ($n = 5$) from each group was collected and homogenized in 4 volumes of assay buffer and then centrifuged at 13,000 × *g* for 10 min. Then, the clear extract was collected and analyzed according to the manufacturer's instructions for each kit.

2.9. Serum biochemical parameter and nonspecific immune analysis

Serum superoxide dismutase (SOD), lysozyme, and myeloperoxidase (MPO) activity was analyzed using the SOD activity colorimetric assay kit (BioVision), the lysozyme detection kit (Sigma–Aldrich), and the MPO colorimetric assay kit (Sigma–Aldrich), respectively, according to the manufacturers' instructions. Respiratory burst (RB) was analyzed using nitro blue tetrazolium (NBT) assay, as previously described (Hasan et al., 2018). Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total glucose, and total cholesterols levels were measured using Mindray commercial kits and a Mindray BS-390 automatic biochemistry analyzer (Mindray Bio-Medical Electronics, China) at the Core-Facility Center, Dong-eui University (Busan, South Korea).

2.10. Intestinal microbiota analysis

After the feeding trial, fecal samples of randomly selected red sea bream were collected, and total DNA of the bacteria was isolated using the FavorPrep Tissue Genomic DNA Extraction Mini Kit (Favorgen Biotech Corp., Taiwan). The V3–V4 region of the isolated total DNA was amplified using primers containing the Illumina overhang adapter sequence:

- Forward primer (50 bp): 5′–TCGTCGGCAG CGTCAGATGT GTATAAGAGA CAGCCTACGG GNGGCWGCAG–3′
- Reverse primer (55 bp): 5′–GTCTCGTGGG CTCGGAGATG TGTATAAGAG ACAGGACTAC HVGGGTATCT AATCC–3′

Next, library quantification, quality control, and sequencing were conducted at the Moagen (Daejeon, South Korea).

2.11. Gene expression analysis

Intestinal samples of randomly selected red sea bream from each group were collected, and RNA was isolated using a Hybrid-R RNA purification kit (GeneAll Biotechnology, South Korea) according to the manufacturer's instructions. Residual DNA was removed using the Riboclear plus kit (GeneAll Biotechnology). The purity and concentration of isolated RNA were evaluated using a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific), and complementary DNA (cDNA) was synthesized using a PrimeScript 1st strand cDNA synthesis kit (Takara, Japan). Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using TB Green Premix Ex Taq (Takara) on a thermal cycler dice Model TP700/760 (Takara, Japan). Gene-specific primers (interleukin [IL]-6, IL-8, nuclear factor kappa B [NF-κB], heat shock protein 70 [HSP70], tumor necrosis factor alpha [TNF-α], growth hormone [GH], and toll-like receptor [TLR]) were prepared using sequences obtained from the National Center for Biotechnology Information; see Table S2. Relative quantification was calculated using thermal cycler dice software V5.0 × with the $2^{-\Delta\Delta CT}$ method and β-actin as a reference gene.

2.12. Challenge with Edwardsiella tarda

A challenge test was performed, as previously described (Khosravi et al., 2015). After 8 weeks, 8 fish from each tank ($n = 24$ fish/group) were anesthetized using 2-phenoxyethanol (500 μL/L) and intraperitoneally injected with 100 μL (1×10^8 CFU/mL) of *E. tarda* (ATCC 15947) purchased from the Korean Collection for Type Cultures (Seoul, South Korea). The number of dead fish was recorded every 6 h, and swabs from tissue samples (gill, spleen, and kidney) were collected and spread on a brain–heart infusion (Difco) agar plate to confirm edwardsiellosis.

2.13. Statistical analysis

The normality and variance homogeneity of the datasets were checked by Shaprio–Wilk and Levene tests, respectively. Before analysis, non-normally distributed data were log-transformed through Kruskal–Wallis test. The statistical significance of the data was analyzed by one-way analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS; IBM, USA), followed by Duncan's multiple range test. A P-value < 0.05 was considered significant.

3. Results

3.1. Bacterial isolation, identification, and general characteristics

Results showed that 16S rRNA sequences of isolated bacteria shared 99.79%, 99.50%, and 99.29% homology with *B. sonorensis* NBRC 101234^T (AYTN01000016), *B. haynesii* NRRL B-41327^T (MRBL01000076), and *B. licheniformis* ATCC 14580^T (AE017333), respectively (Fig. S1). The isolated strain was named *Bacillus* sp. PM8313 (hereafter referred to as PM8313). In general, PM8313 can survive at 15–55 °C and pH 6–8. Optimal growth conditions were identified as 35 ± 5 °C and pH 7 (Table 2).

3.2. Probiotic characterization

3.2.1. Hemolysis, toxigenic gene, and cytotoxicity analysis

B. cereus, which was used as a control with hemolytic activity, formed a clear zone around the colonies and showed hemolytic activity, whereas PM8313 did not show any change, indicating no hemolytic activity. Similarly, *B. cereus* significantly reduced cell viability, whereas PM8313 had no significant effect on cell viability. PCR pattern analysis of seven toxic genes mainly found in *Bacillus* species showed no toxic genes in PM8313 (Fig. S2).

Table 2
General features, minimum information about a genome sequence (MIGS), and probiotics characteristics of *Bacillus* sp. PM8313.

Item	Description
General features	
Classification	Domain Bacteria Phylum Firmicutes Class Bacilli Order Bacillales Family Bacillaceae Genus *Bacillus*
Gram strain	Positive
Cell shape	Rod
Motility	Motile
Sporulation	Endosperm-forming
Temperature range	15–55 °C, optimally at 35 ± 5 °C
Salinity range	NaCl, 0%–5%
pH range	6–8, and optimally at 7
Investigation	
Submitted to INSDC	Accession number CP083580.1
Investigation type	Bacteria
Project name	Genome sequencing of *Bacillus* sp. PM8313
Sequencing	
Sequencing method	Illumina MiSeq
Assembly method	Unicycler, version 0.4.8
Annotation source	RAST server
Genome coverage	297-fold
Genome size	4,615,871 bp
Number of replicons	1
G + C content	45.25%
Protein coding genes	5,007
rRNA	24
tRNA	82
Probiotics characteristic	
Survival in simulated gastric juice	35.11% ± 0.16%
Survival in simulated intestinal juice	43.51% ± 0.19%
Adhesion to HT-29 cell	61.00% ± 0.05%

3.2.2. Cell survival in simulated gastric juice and bile salt

The survival rates of PM8313 in SGJ and SIJ conditions were 35.11% ± 0.16% and 43.51% ± 0.19%, respectively, as shown in Table 2.

3.2.3. Adhesion to HT-29 cells and antibiotic resistance

PM8313 showed an adhesion rate of 61.00% ± 0.05% in HT-29 cells (Table 2). In addition, all the antibiotic resistance tests against the eight antibiotics passed the EFSA criteria (Table S3).

3.2.4. Availability of prebiotics

When investigating the availability of prebiotics to construct a synbiotic combination, PM8313 was able to grow using each of the prebiotic candidates (Fig. S3).

3.3. Genome sequencing, assembly, and annotation

The complete genome of PM8313 was circular and contained 4,615,871 bp with a 45.25% guanine–cytosine (GC) content. In addition, there were 5,007 protein-encoding genes, 24 rRNAs, and 82 tRNAs (Table 2). Fig. 1 shows a circular plot of the genome, including the number of bases, GC content, GC skew, and location of all annotated open reading frames (ORFs) sorted by the clusters of orthologous gene (COG) category and colored. In addition, the outside plots show comparisons with strains representative of various *Bacillus* species (Fig. 1).

3.4. Growth performance, feed utilization, and body indices

Significant changes were observed in WG, SGR, FCR, and FCR parameters in the MMP and MMS groups compared to the MMC (control) group. However, there were no significant differences in any parameters between the MMP and MMS groups. In addition, there were no significant differences in body indices (CF, VSI, and HSI) in all groups compared with the MMC group (Table 3).

3.5. Analysis of digestive enzymes

There was no significant difference in amylase activity in any group. However, trypsin and lipase activity significantly increased in the MMS group (8.89 ± 0.83 mU/g and 58.93 ± 2.58 mU/g, respectively) compared to the MMC group (5.96 ± 1.35 mU/g and 46.63 ± 1.84 mU/g, respectively) (Fig. 2).

3.6. Serum biochemical parameter and nonspecific immune analysis

Results of the nonspecific immune response of each group are shown in Fig. 3. SOD ($P = 0.046$) and lysozyme ($P = 0.041$) activity showed a similar tendency, with significant differences only in the MMS group (75.90% ± 3.47% and 0.77 ± 0.09 U/mL, respectively) compared with the MMC group (65.96% ± 1.68% and 0.61 ± 0.05 U/mL, respectively). RB ($P = 0.008$) significantly increased in both MMP (0.47 ± 0.02) and MMS (0.48 ± 0.01) groups compared with the MMC group (0.43 ± 0.02). There was no significant difference

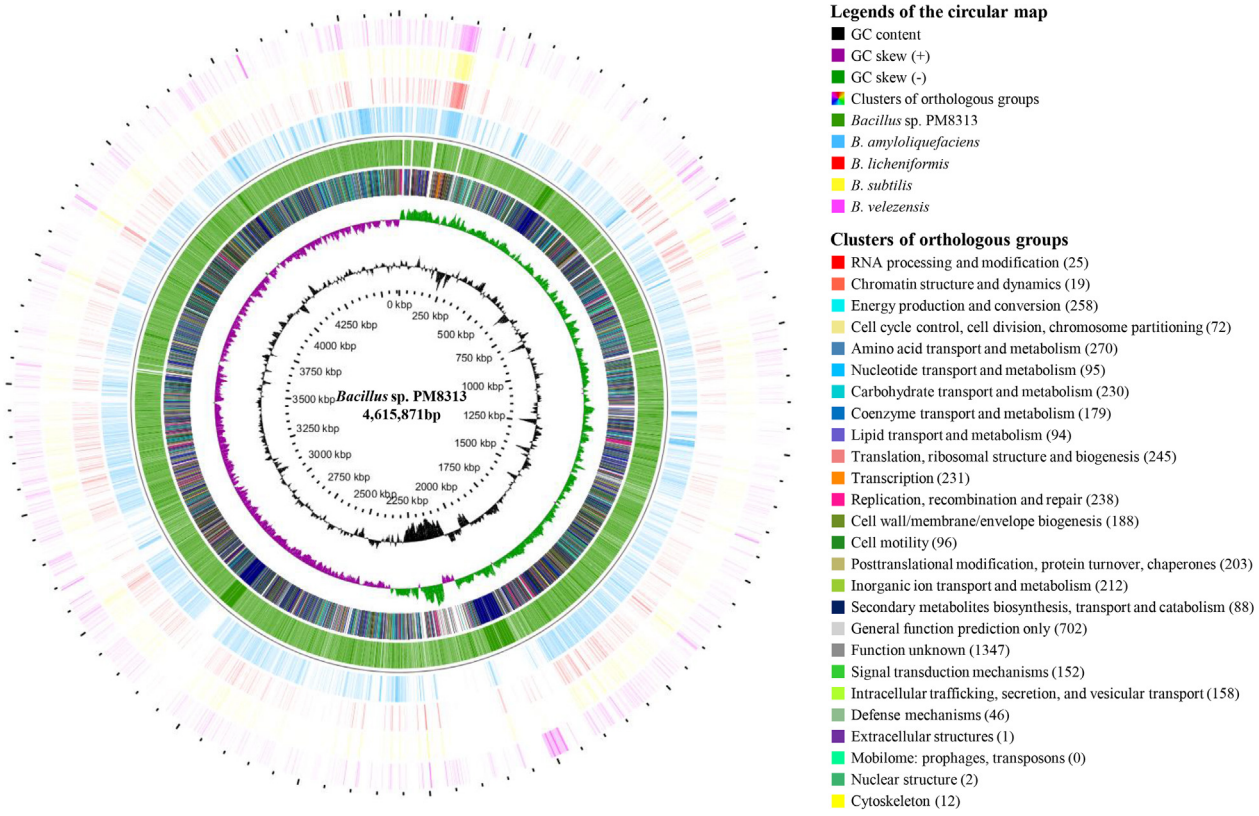

Fig. 1. Circular plot of the comparison between genomes of *Bacillus* sp. PM8313 and *B. amyloliquefaciens* (NC 020272.1), *B. licheniformis* (CP014842.1), *B. subtilis* (NC 000964.3), and *B. velezensis* (CP009679.1). From the center to the outside: the number of bases, GC content, GC skew, location of all annotated ORFs (colored by COG categories). GC = guanine-cytosine; ORFs = open reading frames; COG = clusters of orthologous groups.

Table 3
Growth performance, feed utilization and organosomatic indices of red sea bream supplemented with the experimental feed additives.

Groups	Growth performance, feed utilization and organosomatic parameters						
	WG, %	SGR, %/d	FCR	PER	CF, %	VSI, %	HSI, %
MMC	118.58 ± 2.34[a]	1.40 ± 0.02[a]	1.47 ± 0.04[b]	1.42 ± 0.04[a]	1.68 ± 0.06	2.14 ± 0.12	2.14 ± 0.26
MMP	129.92 ± 6.26[b]	1.49 ± 0.05[b]	1.32 ± 0.01[a]	1.58 ± 0.02[b]	1.73 ± 0.12	2.18 ± 0.14	2.18 ± 0.04
MMS	128.24 ± 3.17[b]	1.47 ± 0.02[b]	1.35 ± 0.02[a]	1.54 ± 0.02[b]	1.71 ± 0.12	2.12 ± 0.21	2.10 ± 0.19

MMC = control; MMP = meat meal group supplemented with probiotics; MMS = meat meal group supplemented with synbiotics.
WG, weight gain = 100 × (final weight − initial weight)/initial weight; SGR, specific growth rate = 100 × (ln final weight − ln initial weight)/days; FCR, feed conversion ratio = dry feed intake/(final weight − initial weight); PER, protein efficiency ratio = (final weight − initial weight)/protein fed; CF, condition factor = 100 × body weight/(total body length)^3; VSI, viscerosomatic index = 100 × (visceral weight/body weight); HSI, hepatosomatic index = 100 × (liver weight/body weight); values are mean ± SD of 3 replicates.
[a,b] Values with different superscript letters within the same column in the table are significantly different ($P < 0.05$). The lack of superscript letters indicates no significant differences ($P > 0.05$).

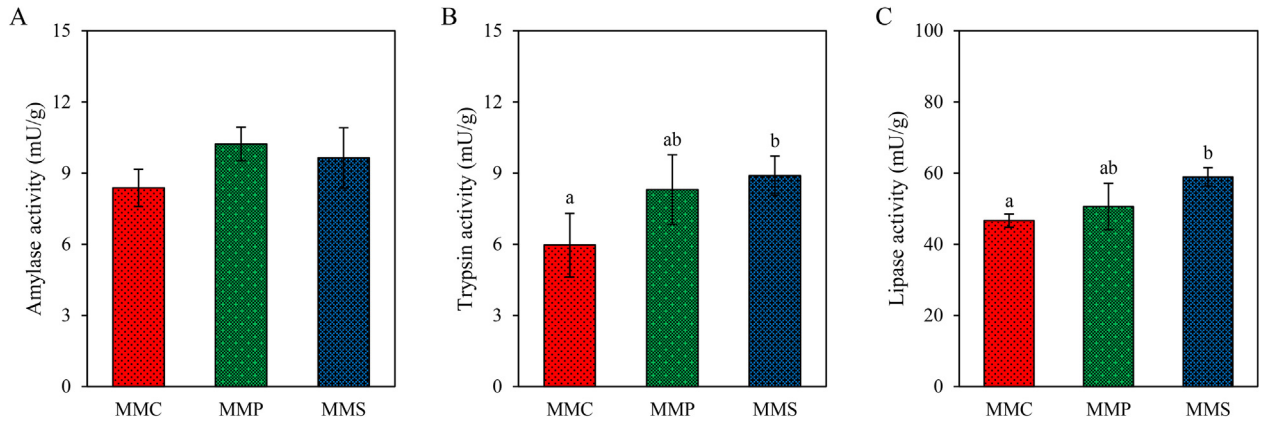

Fig. 2. Comparison of the measured amylase (A), trypsin (B), and lipase (C) activities between the 3 groups. Data represent the mean ± standard deviation; means that do not share the same letter differ significantly ($P < 0.05$, $n = 5$), respectively.

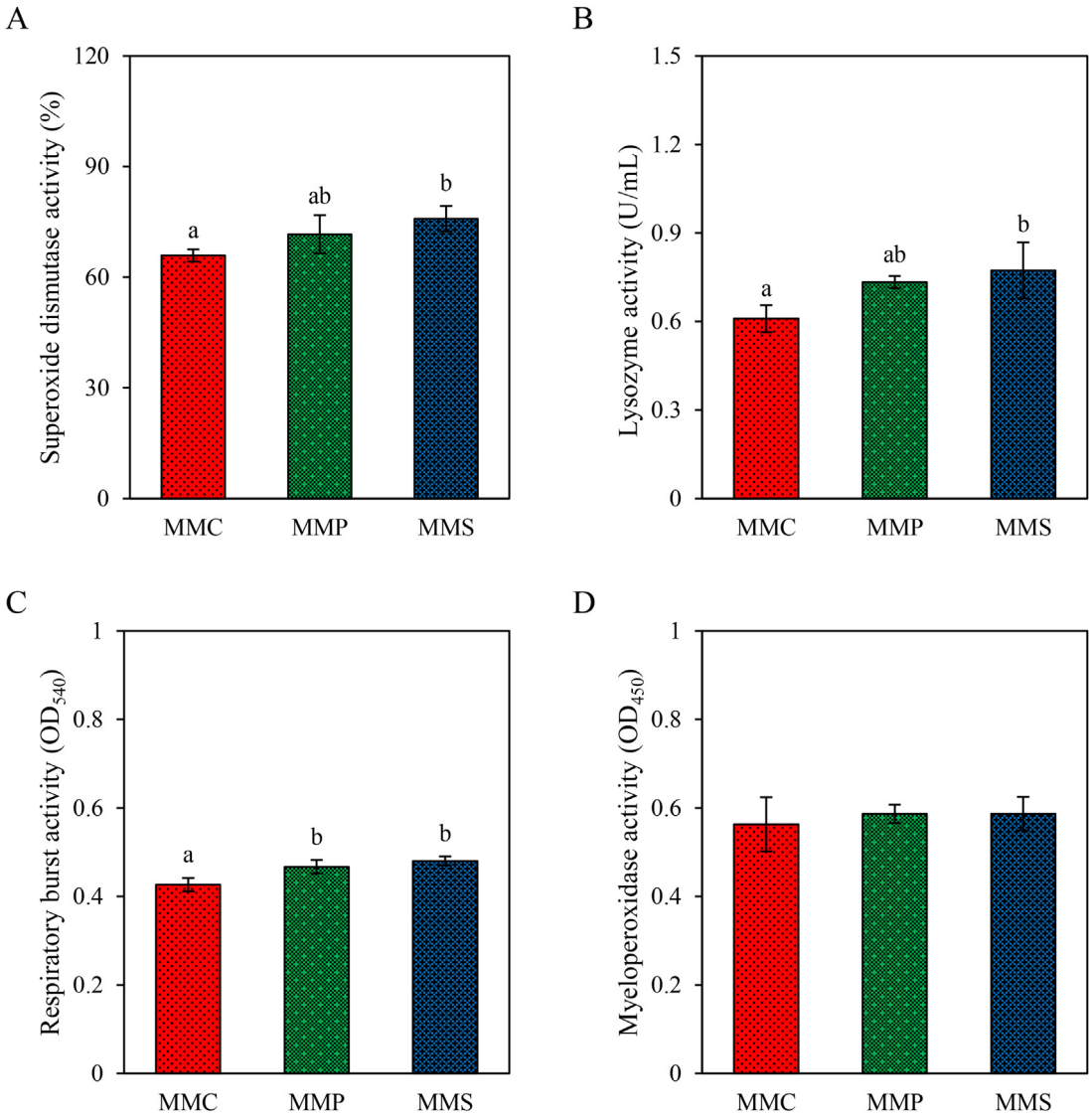

Fig. 3. Serum nonspecific immune parameters (A, superoxide dismutase; B, lysozyme activity; C, respiratory burst activity; D, myeloperoxidase activity) of red sea bream fed the experimental diets. Data represent the mean ± standard deviation of 3 replicates (3 fish/replicate); means that do not share the same letter differ significantly ($P < 0.05$), respectively.

Table 4
Biochemical parameters of red sea bream fed the experimental diets.

Groups	Serum biochemical parameters			
	AST, U/L	ALT, U/L	Total glucose, mg/dL	Total cholesterol, mg/dL
MMC	21.33 ± 2.31	10.31 ± 1.56	50.33 ± 2.52	136.33 ± 8.50
MMP	18.00 ± 2.00	10.07 ± 2.00	49.00 ± 3.00	130.50 ± 4.95
MMS	18.67 ± 3.06	10.33 ± 1.53	50.67 ± 5.86	136.67 ± 2.89

MMC = control; MMP = meat meal group supplemented with probiotics; MMS = meat meal group supplemented with synbiotics; AST = aspartate aminotransferase; ALT = alanine aminotransferase.
Values are mean ± SD of 3 replicates (3 fish/replicate). All values within the same column in the table are not significantly different ($P > 0.05$).

in MPO ($P = 0.757$) in any group. Serum biochemical parameters were not significantly affected by feed additives (Table 4).

3.7. Intestinal microbiota analysis

Significant differences in richness estimates were found in the MMS group compared with the MMC group. The ACE, Chao, and Jackknife estimator values of the MMS group (140 ± 25, 132 ± 21, and 142 ± 25, respectively) increased compared to the MMC (92 ± 20, 84 ± 6, and 84 ± 8, respectively) and MMP (99 ± 2, 96 ± 2, and 102 ± 2, respectively) groups. There was no significant difference between groups in Shannon and Simpson diversity estimate values (Table 5).

Analysis of the β-diversity at the genus level, based on UniFrac metrics using principal coordinate analysis, showed that the MMC and MMS groups were located at relatively long distances, indicating low similarity (Fig. 4).

Comparison of the relative abundance at the phylum level showed that Proteobacteria were the most abundant in all groups. The proportion of Fusobacteria in the MMC group was 1.64% but low in the MMP (0%) and MMS (0.02%) groups. In contrast, the proportion of Firmicutes was relatively high in the MMS (6.94%), followed by MMP (0.68%) and MMC (0.64%) groups (Fig. 5A). At the order level, Vibrionales were most abundant in all groups, followed by Rhizobiales and Fusobacteriales in the MMC group and Bacillales in the MMP and MMS groups (Fig. 5B). *Vibrio* and *Photobacterium* spp. were most abundant at the genus level, although there was a difference in the composition ratio according to the groups. At the genus level, the biggest difference between groups was that the MMC group was rich in *Cetobacterium* (phylum Fusobacteria), whereas the MMP and MMS groups were rich in *Bacillus* (phylum Firmicutes); see Fig. 5C.

Linear discriminant analysis effect size was used to identify significant differences in taxa in the red sea bream intestinal microbiota. The MMS group showed significant differences in *Bacillus* species compared to other groups, and the MMC group showed significant differences in the Fusobacteria phylum and *Lawsonella* species (Fig. 6).

As shown in Fig. 7A, 29 operational taxonomic units (OTUs) were matched in all groups. In addition, 29, 33, and 62 OTUs were uniquely identified in the intestine of the MMC, MMP, and MMS groups, respectively. The relative abundances of the 29 OTUs compared to the MMC group are shown in Fig. 7B. The OTUs commonly increased in the MMP and MMS groups were *Bacillus*, *Bifidobacterium*, *Bosea*, *Enhydrobacter*, *Enterovibrio*, *Nautella*, *Paracoccus*, *Photobacterium*, *Rothia*, *Ruegeria*, and *Tenacibaculum*, while the OTUs commonly decreased were *Acinetobacter*, *Lawsonella*, *Methylobacterium*, *Nocardioides*, *Rahnella*, and *Staphylococcus*.

3.8. Gene expression analysis

Significant differences among the analyzed immune- and growth-related gene expression occurred only in *NF-κB*. The MMS group (3.02 ± 0.46-fold) showed significantly increased *NF-κB* expression compared to the MMC group (Fig. 8).

3.9. Challenge with E. tarda

The cumulative survival of red sea bream against *E. tarda* is shown in Fig. 9. The first mortality occurred 4 days after intraperitoneal injection. The highest survival rate was observed in the MMS group. After 9 days of challenge, all fish in the MMC group

Table 5
Alpha diversity of the intestinal bacterial communities of red sea bream (*Pagrus major*).

Groups	ACE	Chao	Jackknife	Shannon	Simpson
MMC	92 ± 20[a]	84 ± 6[a]	84 ± 8[a]	1.40 ± 0.47	0.46 ± 0.22
MMP	99 ± 2[a]	96 ± 2[a]	102 ± 2[a]	1.40 ± 0.05	0.33 ± 0.04
MMS	140 ± 25[b]	132 ± 21[b]	142 ± 25[b]	1.64 ± 0.85	0.40 ± 0.25

MMC = control; MMP = meat meal group supplemented with probiotics; MMS = meat meal group supplemented with synbiotics.
Values are mean ± SD of 3 replicates (3 fish/replicate).
[a,b] Values with different superscript letters within the same column in the table are significantly different ($P < 0.05$). The lack of superscript letter indicates no significant differences ($P > 0.05$).

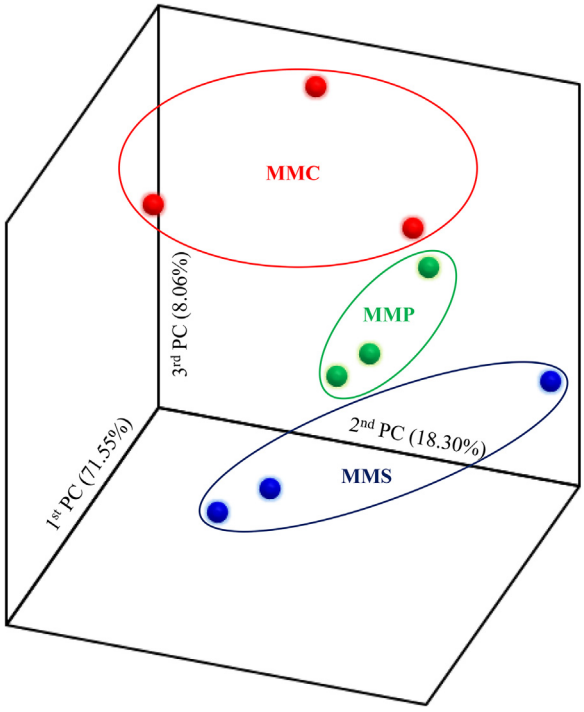

Fig. 4. Principal coordinate analysis (PCoA) based on the weighted unifrac metrics of bacterial operational taxonomic units between the different diets. MMC, basal diet; MMP, diet supplemented with *Bacillus* sp. PM8313 spores at a concentration of 1×10^8 CFU/g diet; MMS, diet supplemented with 0.1% β-glucan + *Bacillus* sp. PM8313 spores at a concentration of 1×10^8 CFU/g diet.

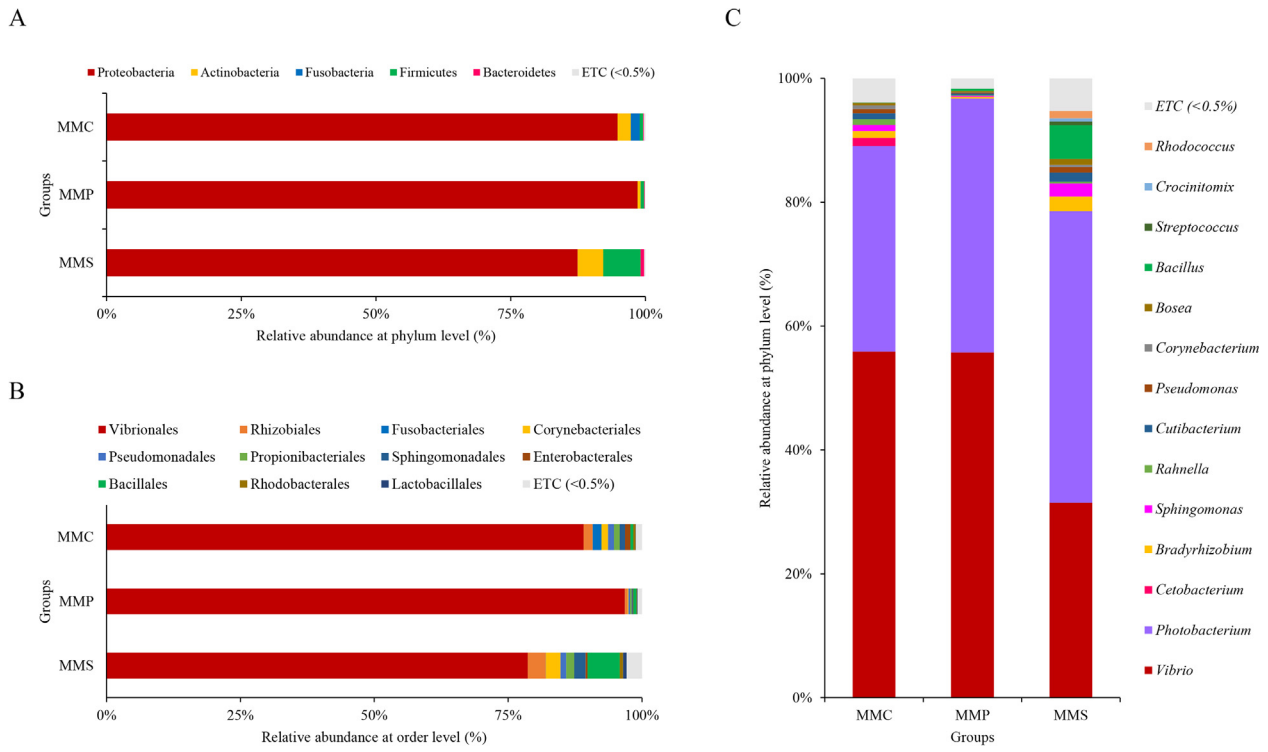

Fig. 5. Composition and relative abundance of intestinal bacterial communities of red sea bream with different diets at the phylum (A), order (B) and genus (C) level. MMC, basal diet; MMP, diet supplemented with *Bacillus* sp. PM8313 spores at a concentration of 1×10^8 CFU/g diet; MMS, diet supplemented with 0.1% β-glucan + *Bacillus* sp. PM8313 spores at a concentration of 1×10^8 CFU/g diet.

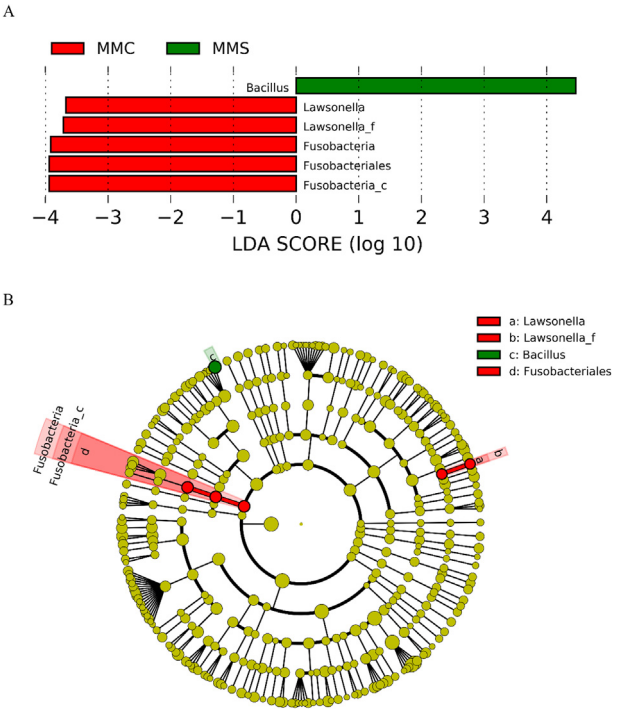

Fig. 6. Linear discriminant analysis effect size analysis of differential abundance of taxa within red sea bream intestinal microbiota following random sampling from each group. (A) Linear discriminant analysis score of abundance of taxa; (B) Cladogram showing differentially abundant taxa among 3 groups from phylum to genus.

died, and the survival rates of the MMP and MMS groups were 66.67% and 86.67%, respectively.

4. Discussion

In this study, HAPs were isolated from the intestine of healthy wild red sea bream, and the isolated strain was identified through 16S rRNA sequencing as a *Bacillus* species. *Bacillus* species are abundant in fish intestines, and they produce natural antimicrobial compounds that antagonize bacterial pathogens, proving beneficial for the health of the host (Santos et al., 2021). Dietary supplementation of *Bacillus* species in aquaculture increases the growth and survival rate of fish (Tarnecki et al., 2019).

PM8313 isolated in this study was viable at 15 °C, which is suitable for providing beneficial effects by settling in the intestines of cold-blooded organisms, such as fish. PM8313 is also a spore-forming strain that can survive in extreme environments. These properties are important advantages for aquaculture feed applications as they facilitate feed integration and long-term storage without loss of characteristics (Hong et al., 2005).

One of the most important characteristics to be considered for probiotic development is the safety of the bacterial strain used (Elshaghabee et al., 2017). Various studies have suggested different methods for probiotic selection (El-Saadony et al., 2021). However, unlike humans or livestock, there are no clear standards for the characteristics and safety of bacterial strains for probiotic development for aquaculture. Consequently, some studies on probiotic development for aquaculture have not verified the source or safety of bacterial strains (Jang et al., 2021a). Some *Bacillus* species can be opportunistic pathogens or toxin producers in fish (Elshaghabee

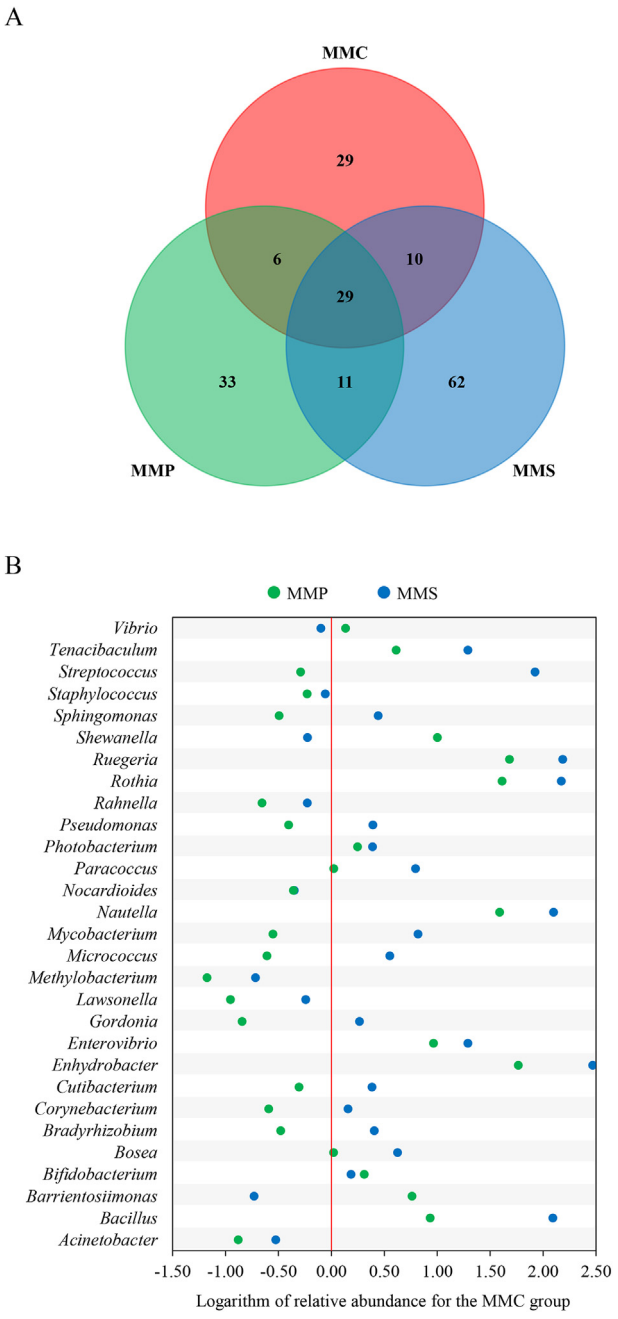

Fig. 7. Venn diagram comparing distribution of OTUs shared by red sea bream fed with different diets (A) and comparison of relative abundance according to MMP of OTUs shared by all groups (B). Numbers represent OTUs correlated in total sequences of each group. OTUs = operational taxonomic unit; MMC = control; MMP = meat meal group supplemented with probiotics; MMS = meat meal group supplemented with synbiotics.

et al., 2017). They can also cause antibiotic resistance (El-Saadony et al., 2021). Therefore, there is a growing need to evaluate the safety of strains and conduct more profound analysis in order to select and identify probiotic candidates (Jang et al., 2021a). In this study, safety was verified according to the probiotic evaluation guidelines of the FAO/WHO (2001) and the EFSA (2012), but standards for safety, plasmid-related antibiotic resistance, and enterotoxin production should be prepared for probiotic development for aquaculture.

Probiotics can be exposed to low pH and bile conditions during digestion and must survive in these environments to reach the host's intestine (Jang et al., 2021b), where they can attach to intestinal epithelial cells and provide beneficial effects to the host (encapsulation). Therefore, high viability in acidic and bile conditions and high adhesion to intestinal epithelial cells are important characteristics considered for probiotic selection (Succi et al., 2005). In general, viability in acidic conditions is assessed using in vitro tests at a pH of 1.5–2 (Jang et al., 2019). In this condition, most lactic acid bacteria have a low survival rate. Therefore, to increase the survival rate, encapsulation using materials such as alginate (Kim et al., 2008), chitosan (Krasaekoopt et al., 2004), poly-γ-glutamic acid (Jang et al., 2019), xanthan gum (Ding et al., 2009), and carrageenan gum (Ding et al., 2009) is also performed. However, *Bacillus* species have high acid resistance because they form spores (Hyronimus et al., 2000). Bile acid produced during digestion reduces bacterial survival by destroying the cell membrane, which is composed mainly of lipids and fatty acids (Succi et al., 2005). Some lactic acid bacteria respond to these stresses by using bile efflux and bile modifications through bile salt hydrolases (Ruiz et al., 2013). Some spore-forming strains have weak resistance to bile acid compared to acid resistance (Hyronimus et al., 2000). The ability to adhere to intestinal epithelial cells is due to cell hydrophobicity and self-aggregation, and a high adhesion rate can increase interaction with cell receptors and enable defense against adhesion of pathogens (Lee et al., 2017; Otero et al., 2004). In this study, PM8313 formed spores and showed high survival rates of 35% and 43% in SGJ and bile conditions, respectively. In addition, its high adhesion rate to HT-29 cells showed that PM8313 is suitable for use as a probiotic.

Previous studies have reported that supplementation of probiotics or synbiotics positively alters the growth performance of rainbow trout (*Oncorhynchus mykiss*) (Soltani et al., 2019), Nile tilapia (*Oreochromis niloticus*) (Xia et al., 2020), rockfish (*Sebastes schlegelii*) (Rahimnejad et al., 2018), and olive flounder (*Paralichthys olivaceus*) (Jang et al., 2021a). Probiotic supplementation can promote WG and improve feed utilization in aquatic animals (Hai, 2015; Van Doan et al., 2020; Zorriehzahra et al., 2016). It also improves host growth performance by improving appetite, increasing digestibility by breaking down indigestible components, increasing vitamin production, and detoxifying compounds in the diet (Giraffa et al., 2010; Van Doan et al., 2020; Zorriehzahra et al., 2016). In particular, since exoenzymes in *Bacillus* species efficiently metabolize various carbohydrates, lipids, and proteins, the activity of these exoenzymes plays an important role in improving the host's growth performance, including WG and feed efficiency (Liu et al., 2017). Therefore, it is widely accepted that digestive enzyme activity levels are useful comparative indicators of a host's feed utilization, digestibility, and growth performance (Suzer et al., 2008). In this study, the WG, SGR, FCR, and PER parameters of the groups supplemented with probiotics (PM8313) and synbiotics (MMP and MMS, respectively) positively changed compared to the control group. Since there were no significant differences in the growth-related gene expression in all groups, it is likely that gene expression does not cause a difference in growth performance. However, trypsin and lipase activity increased in the MMS group. Therefore, this change in enzymatic activity may have affected the change in the growth performance of red sea bream. In fact, in whole-genome sequencing of PM8313, various enzymes responsible for protease and lipase functions were present.

Immune response modulation is one of the most well-known benefits of probiotics (Nayak et al., 2010). Previous studies have evidenced that probiotics can effectively improve the host's innate and adaptive immune responses (Austin et al., 1995; Heyman and Ménard 2002; Isolauri et al., 2002; Taoka et al., 2006a, 2006b; Zhou et al., 2010). The interaction between *Bacillus* spores and

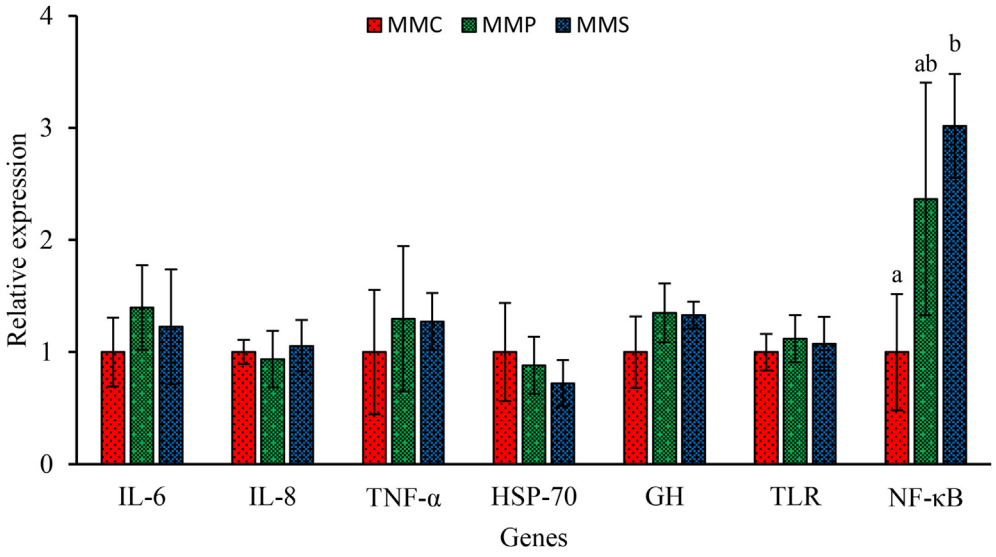

Fig. 8. Profiles of immune- and growth-related gene expression in the intestine of red sea bream. Expression of these genes was measured by RT-qPCR after 8 weeks of feeding for the MMC, MMP, and MMS groups. Levels of gene expression were quantified relative to β-actin transcription. The data are represented as the means ± standard deviation of 3 replicates (3 fish/replicate); means that do not share the same letter differ significantly ($P < 0.05$). MMC = control; MMP = meat meal group supplemented with probiotics; MMS = meat meal group supplemented with synbiotics.

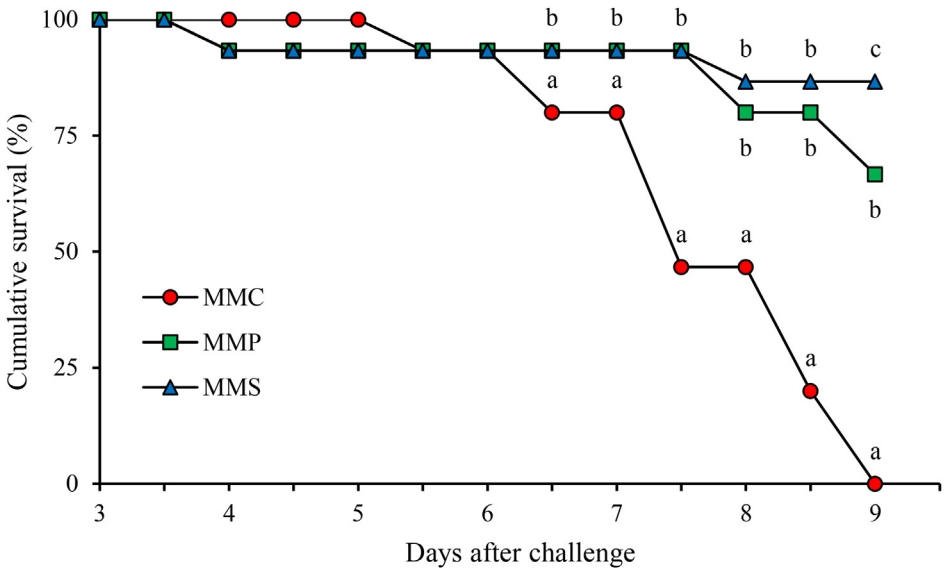

Fig. 9. Cumulative survival rates of red sea bream in each diet challenged by *Edwardsiella tarda* injection (1×10^8 CFU/mL). Means (21 fish/group) were compared at identical times. Means with the same or different letters are not significantly ($P > 0.05$) or are significantly ($P < 0.05$) different, respectively. MMC = control; MMP = meat meal group supplemented with probiotics; MMS = meat meal group supplemented with synbiotics.

macrophages plays an important role in the development of the host's innate immune response (Guo et al., 2016), and supplementation with probiotics *B. coagulans* B16 and *Rhodopseudomonas palustris* G06 increases immune responses, such as SOD activity, respiratory burst activity, MPO activity, and CAT activity, in tilapia (Zhou et al., 2010). This increase in growth performance due to probiotics might be attributed to the improved immune response (Zhou et al., 2010). Similarly, in our study, the MMP group (supplemented with PM8313) showed increased SOD activity, lysozyme activity, and respiratory burst activity compared to the control group. PM8313 supplementation also increased the growth performance of red sea bream. Thus, as shown by Zhou et al. (2010), the immune response may have improved with increased growth performance.

The gut microbiota of fish is altered by various factors, such as habitat, water quality, growth stage, and feeding activity (Jang et al., 2020; 2021), which affects fish metabolism, in turn, influencing nutrient uptake, metabolism pathways, and, ultimately, growth (Ni et al., 2014; Semova et al., 2012; Ye et al., 2011). In this study, PM8313 supplementation increased microbial diversity in the red sea bream gut. In particular, PM8313 colonized the intestine and affected the host's growth, metabolism, and nutrient digestion and absorption. A diverse gut microbiota can maintain fish health by stimulating innate immunity, production of antimicrobial compounds from invading pathogens, and deprivation of the intestinal surface area for pathogenic bacteria to establish themselves (Banerjee et al., 2017). In the MMS group, alterations in intestinal microbial diversity were higher compared to the

MMP group, indicating the effect of prebiotics. This supports our previous findings that prebiotics increase the viability of probiotics in the gastrointestinal tract (Jang et al., 2021b). However, further studies are needed to elucidate the mechanism underlying the effect of the gut microbiota on the growth and immune response of red sea bream. In particular, studies should focus on the genera increased or decreased due to probiotic supplementation in this study.

Cytokines are protein mediators produced by immune cells that contribute to the host's cell growth, differentiation, and defense mechanisms (Peddie et al., 2002). Probiotics directly or indirectly interact with the host's immune cells to regulate the transcription of genes that play important roles in the immune system, such as cytokines (Hasan et al., 2018). In fish immunology, the gene expression profile of cytokines, such as *ILs*, *TNF-α*, and *NF-κB*, indicates activation and involvement in immunological pathways (Hasan et al., 2018, 2019). In this study, probiotic supplementation significantly increased *NF-κB* gene expression. Since *NF-κB* is a key regulator of innate and adaptive immune and inflammatory responses (Iwasaki et al., 2010; Qiu et al., 2020), PM8313 supplementation plays an important role in maintaining the immune balance in red sea bream by regulating *NF-κB* gene expression. Additionally, increased nonspecific immune parameters and upregulated *NF-κB* gene expression enhances immunity, leading to increased protection of red sea bream against *E. tarda* invasion, proliferation, and colonization, resulting in higher survival rates, as seen in this study.

5. Conclusion

PM8313 has potential as a biological control agent in aquaculture. PM8313 isolated from the intestine of wild red sea bream is safe, as shown in in vitro tests confirming the absence of genes with hemolytic and cytotoxic activity. Effects of dietary addition, such as improving growth performance, increasing nonspecific immune activity, regulating the intestinal microbiota, and increasing resistance to pathogenic strains, were also verified. In addition, PM8313 increases resistance to *E. tarda* when used with β-glucan as a synbiotic. Therefore, this new PM8313 supplemental feeding strategy could be utilized in field-level red sea bream aquaculture to increase production and control disease.

Author contributions

Won Je Jang: Conceptualization, Methodology, Investigation, Data curation, Writing original draft, Formal analysis; **Kyung-Bon Lee:** Conceptualization, Methodology, Data curation, Formal analysis; **Su-Jeong Lee:** Methodology, Investigation, Data curation, Formal analysis; **Mi-Hyeon Jeon:** Methodology, Investigation, Data curation; **Sang Woo Hur**, **Seunghan Lee,** and **Bong-Joo Lee:** Conceptualization, Resources; **Jong Min Lee:** Conceptualization, Methodology, Writing - review & editing; **Kang-Woong Kim** and **Eun-Woo Lee:** Conceptualization, Methodology, Resources, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2022.08.011.

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