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Full length article

Probiotic potential of *Bacillus velezensis* JW: Antimicrobial activity against fish pathogenic bacteria and immune enhancement effects on *Carassius auratus*

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ABSTRACT

This study evaluated the probiotic potential of *B. velezensis* JW through experimental and genomic analysis approaches. Strain JW showed antimicrobial activity against a broad range of fish pathogenic bacteria including *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Lactococcus garvieae*, *Streptococcus agalactiae*, and *Vibrio Parahemolyticus*. Fish (*Carassius auratus*) were fed with the diets containing 0 (control), 10^7 , and 10^9 cfu/g of *B. velezensis* JW for 4 weeks. Various immune parameters were examined at 1, 2, 3, and 4 weeks of post-feeding. Results showed that JW supplemented diets significantly increased acid phosphatase (ACP), alkaline phosphatase (AKP), and glutathione peroxidase (GSH-PX) activity. The mRNA expression of immune-related genes in the head kidney of *C. auratus* was measured. Among them, the interferon gamma gene (IFN- γ) and tumor necrosis factor- α (TNF- α) showed higher expression after 3 and 4 weeks of feeding ($P < 0.05$). The expression of interleukin-1 (IL-1) only being significantly upregulated by 10^9 cfu/g of JW after 1 week of feeding ($P < 0.05$). The upregulation of interleukin-4 (IL-4) increased over time from 1st to 4th week. The expression of interleukin-10 (IL-10) and interleukin-12 (IL-12) showed an opposite expression pattern with IL-10 significantly upregulated and IL-12 significantly downregulated by JW containing diets at 2, 3, and 4 weeks of post-feeding ($P < 0.05$). Moreover, fish fed with JW supplemented diets showed significantly improved survival rate after *A. hydrophila* infection. The analysis of the genome of JW revealed several features aiding host health and being relevant to the GIT adaptation. Four bacteriocins, three Polyketide Synthetase (PKS), and five Nonribosomal Peptide-Synthetase (NRPS) gene clusters were identified in the genome. In summary, the above results clearly proved that *B. velezensis* JW has the potential to be developed as a probiotic agent in aquaculture.

1. Introduction

The outbreaks of bacterial disease cause tremendous economic losses to the aquaculture industry, which has become a major constraint in fish farming [1]. Conventional chemotherapy is no longer recommended because the potential threat to human health has become a common concern. The inappropriately used antibiotics remained in the aquaculture products are poised to enter the food supply and eventually taken up by human [2]. Moreover, commensal bacteria associated with aquatic animals can evolve antibiotic resistant phenotype under frequent antibiotic exposure. Those bacteria may serve as reservoirs for resistant genes that could potentially be transferred to pathogenic organisms in humans [3]. One of the alternatives to chemical antibiotics and disinfectants in fish disease control could be the use of probiotic

bacteria as microbial control agents [4].

As defined by the World Health Organization (WHO), probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host [5]. In aquaculture, probiotics aid host health mainly through enzymatic contribution to digestion, inhibition of pathogenic microorganisms, growth promoting factors, and host immune enhancement [6]. Modulation of host immunity is one of the most purported benefits of probiotics consumption [7]. Microorganisms including LAB (lactic acid bacteria) and yeast that widely used for humans or livestock showed similar probiotic effects on fish. For example, *Channa striata* fed with *Lactobacillus acidophilus* and *Saccharomyces cerevisiae* supplemented diets resulted in better growth performance, nutrient digestibility and the expression of immune regulatory genes significantly outperforming those with the control diet

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[8]. Researchers argued that probiotics strains selected from fish intestinal origin may have a greater capacity for competing indigenous harmful microbes and becoming predominant in the enteric environment [9]. Indeed, the gastrointestinal tract (GIT) and fish mucus are most common isolation sources for aquaculture probiotics [10–12].

A variety of microorganisms have been reported as probiotics in aquaculture. The most commonly used probiotic candidates belong to Gram-positive of LAB group or *Bacillus* species family [13]. Regarding Gram-negative probiotics, strains of *Shewanella*, *Aeromonas*, *Vibrio*, *Enterobacter*, and *Pseudomonas* species have been isolated [11]. Compared with other non-spore formers, *Bacillus* spp. has more obviously inherent advantages [14]. The formation of endospores enables them to endure extreme stresses, and offers biological solutions to the formulation and preservation problems when being produced on an industrial scale. Most strains of this species are both aerobic and facultatively anaerobic, which means they can grow in various niches to compete against potential pathogens. Moreover, *Bacillus* has the potential to produce antimicrobial metabolites with an amazing variety of structures. Recent advances in genome sequencing have highlighted the genus *Bacillus* as an unexpected source of antimicrobial compounds [15]. For example, the model gram-positive bacterium *Bacillus subtilis* devotes about 4–5% of the genome to antibiotic production [16]. By using appropriate bioinformatics tools, novel bacteriocin, Polyketide Synthetase (PKS) and Nonribosomal Peptide-Synthetase (NRPS) gene clusters can be identified from a bacterial genome [17].

In the present study, we isolated a potential probiotic strain JW from the GIT of healthy grass carp. This strain was identified as *Bacillus velezensis* by *gyrA* gene sequence comparison. Strain JW has antimicrobial activity against several fish pathogenic bacteria. The humoral factors and the expression of immune-related genes of *C. auratus* were measured after feeding with JW supplemented diets. The whole genome of JW was sequenced and relevant genomic features were analyzed.

2. Materials and methods

2.1. Bacterial isolation and identification

The mid gut of a healthy carp was obtained under sterile conditions. The tissue was rinsed by sterile saline (0.85%) to remove intestinal contents and then homogenized in 1 mL sterile saline (0.85%). Afterwards, the suspension was diluted 10 folds in glass tubes containing 9 mL sterile saline (0.85%). In order to exclude non-spore-forming bacteria, the suspension was heated at 80 °C for 15 min. Then, 0.1 mL suspension was spread on Luria-Bertani (LB) agar plates and incubated at 37 °C for 24 h. Single colonies were re-streaked on LB agar plates twice and the purified strains were stored in 15% (w/v) glycerol broth at –80 °C. For bacterial identification, the 16S rRNA gene was amplified from the chromosomal DNA by PCR with primers 27F (5' to 3': AGAGTTTGATCCTGGCTCAG) and 1492R (5' to 3': CGGTTACCTT GTTACGACTT). The PCR product was gel-purified and sequenced on both strands (Shenggong Biotechnological Ltd., Shanghai, China). The obtained nucleotide sequence was used for a NCBI BLAST to perform the similarity search.

2.2. Antimicrobial assay

Five fish pathogenic bacteria (*A. hydrophila*, *Aeromonas salmonicida*, *Lactococcus garvieae*, *Streptococcus agalactiae*, and *Vibrio parahaemolyticus*), as well as the putative probiotic strain were grown in LB liquid medium at 30 °C, 200 rpm for 18 h. The culture of pathogenic bacteria was diluted to 10^7 cells mL⁻¹ (counted with a hemocytometer) by LB broth, and 200 µL diluted culture was spread on LB agar plate. The plates spread with different pathogenic indicator strains were dried for 30 min on vertical flow clean cabinet. Then a sterile filter paper disk (5 mm diameter) containing 3 µL of the putative probiotic bacterial culture was placed in the center of the plates. After 18 h of incubation at

30 °C, antimicrobial activity was visualized by a clear halo around the inoculation paper.

2.3. Fish and experimental set-up

Juvenile *Carassius auratus* (~25 g) were obtained from Shaanxi Xinmin fish seed field in Heyang (Shaanxi, China). The healthy status of fish was confirmed by the normal physiological and behavioural (movement, appetite) patterns, as well as the absence of disease symptoms and injuries (to fins, gills, eyes, skin). After being transferred to the laboratory, fish were acclimated to the experimental conditions (24 °C) in glass tank equipped with air pump, and fed with the basal diet for 2 weeks before the experiment started. The fish culturing conditions are: 16 h/8 h of light/dark cycle, about 5.60 mg L⁻¹ of dissolved oxygen, 0.015 mg L⁻¹ of nitrites, 0.130 mg L⁻¹ of ammonia, and 7.8 to 8.2 of pH. In order to keep the water quality, 30% water in each tank was changed with aerated tap water every day.

The selected fish were randomly divided into 9 glass tanks (50-L volume with 35 fish per tank) and were fed with different diets: three tanks with non-supplemented control diet, three tanks with JW supplemented diets at 1×10^7 cfu/g, and three tanks with JW supplemented diets at 1×10^9 cfu/g. Thus, each treatment was performed in triplicate and all fish were fed twice per day at 1% of the body weight. The colony-formation unit (cfu) was measured by plating out the various dilutions of the bacterial culture on LB agar plates and counting the colonies.

2.4. Sample collection

Three fish were taken randomly from each tank after 7, 14, 21, and 28 days of feeding. For each sampling day, a total of nine fish were used per treatment for immunological assays. Sampled fish were euthanized by overdose of MS222 (Ethyl 3-aminobenzoate methanesulfonate, Sigma-Aldrich, USA). Blood samples were collected by caudal venipuncture using heparinized syringes. About 1 mL of blood was transferred into an Eppendorf tube and allowed to clot at 4 °C for 4 h. After centrifuged at $3500 \times g$, 4 °C for 10 min, serum of each fish was obtained and stored at –80 °C prior to analysis. The head kidney was sampled for analyzing the expression of genes involved in the immune response. The tissue was collected from each fish and directly immersed in 0.2 mL Trizol reagent (TaKaRa, Japan) until RNA isolation.

2.5. Non-specific immunological measurement

The serum samples collected at 7, 14, 21 and 28 days post-feeding were used for determining the lysozyme (LZY) activity, total acid phosphatase (ACP) and alkaline phosphatase (AKP) activity, and glutathione peroxidase (GSH-PX). The measurement was performed with commercial kits from Nanjing Jiancheng Institute (Nanjing, China) according to the manufacturer's instructions.

2.6. RNA isolation and real-time quantitative PCR

Total RNA was isolated from head kidney with TRIzol reagent (TaKaRa, Japan) method. Briefly, tissue was homogenized in 1 mL TRIzol reagent. After centrifugation at 12g, 4 °C for 5 min, the supernatant was transferred to a new 1.5 mL tube and mixed with 0.5 mL chloroform. The mixture was centrifuged and the aqueous phase was transferred to a new tube. RNA was precipitated by adding 0.5 mL isopropanol. Following centrifugation, the RNA pellet was washed twice with 75% ethanol and dissolved in RNase free water.

One microgram of total RNA was treated with DNase-I (TaKaRa, Japan) to remove genomic DNA, and cDNA was synthesized using a PrimeScript™ RT reagent Kit (TaKaRa, Japan). Real-time quantitative PCR (RT-qPCR) was performed on a CFX96™ Real-time PCR system (Bio-rad, USA). Primer pairs are listed in Table 1 and β -actin gene was

Table 1
Primers used for quantitative real-time PCR.

Genes	Primers (5' to 3')	Accession no.
<i>IFN-γ</i>	Forward: GAAACCTATGGGCGATCAA Reverse: GTAGACACGCTTCAGTCAAACA	EU909368
<i>TNF-α</i>	Forward: CATTCTACGGATGGCATTACTT Reverse: CCTCAGGAATGTGAGTCTTGCAT	EU069817
<i>IL-1β</i>	Forward: GATGCGCTGCTCAGCTTCT Reverse: AGTGGGTGCTACATTAAACCATACG	AJ249137
<i>IL-4</i>	Forward: CGATTGTAGCCGTTACTGGGT Reverse: TGGCAAATGTGTTCTCCG	KX574595
<i>IL-10</i>	Forward: CAAGGAGCTCCGTTCTGCAT Reverse: TCGAGTAATGGTGCCAAAGTCATCA	HQ259106
<i>IL-12</i>	Forward: CTTCAGAAGCAGCTTTGTTGTTG Reverse: CAGTTTTTGAGAGCTCACCAATATC	LN592213.1
<i>β-actin</i>	Forward: GATGCGGAACTGGAAAGGG Reverse: ATGAGGCGAGAGTGGTAGACG	AB039726

included as a reference gene. For each reaction, the total 20 µL volume contains 10 µL of AceQ[®] qPCR master mix (Vazyme, China), 0.8 µL of each primer (10 µM), 1.4 µL of cDNA, and 7 µL of nuclease-free water. The PCR cycling conditions were as follows: one cycle at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. After the PCR reaction, melting curve analysis was performed to exclude the formation of nonspecific products. The Ct value of each gene was recorded and used to quantify gene expression. At least three biological replicates were performed for each sample, and the fold-changes in the relative quantities of the amplified targets were calculated by the $2^{-\Delta\Delta C_t}$ method.

2.7. Challenge test

After sampled for immune parameters, the remaining fish of each group were used to test disease resistance after being challenged with *A. hydrophila*. The pathogenic bacterium was cultured as previously described and the bacterial cells were suspended in phosphate buffer solution to 1×10^6 cell mL⁻¹ (counted with a hemocytometer). The two experimental groups received JW supplemented diets as before. The control group (continued with basal diets feeding) were divided into two groups: a negative control with no *A. hydrophila* treatment but phosphate buffer solution, and a challenge control group treated with *A. hydrophila* injection. Each group (10 fish) with three replicates were intraperitoneally injected with 0.1 mL of *A. hydrophila* suspensions. The protecting effects of strain JW against *A. hydrophila* was evaluated by the relative percent survival (RPS) using the formula: $RPS = 1 - (\text{mortality in immunized group} / \text{mortality in challenge control group}) \times 100$ [18].

2.8. Bacterial genomic DNA isolation, sequencing, and analysis

Strain JW was grown in LB liquid medium to stationary phase. Cells were harvested by centrifuge 1 mL of the culture at $1000 \times g$ for 1 min. The supernatant was discarded and the cell pellet was suspended by 0.2 mL TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.0) buffer. Lysozyme was added at final concentration of $20 \mu\text{g mL}^{-1}$, the suspension was then incubated at 37 °C for 30 min. The genomic DNA was isolated from the lysate by E.Z.N.A.[®] Bacterial DNA Kit (Omega Bio-tek, USA). The DNA quality was checked by a micro volume spectrophotometer ($260/230 > 1.8$, and $260/280 > 2.0$) and 1% agarose electrophoresis gel (with an intact band > 10 kb). Whole-genome sequencing was performed on the Illumina PE150 platform. A-tailed, ligated to paired-end adaptors and PCR amplified with a 350 bp insert was used for the library construction at the Beijing Novogene Bioinformatics Technology Co., Ltd. Illumina PCR adapter reads and low-quality reads were filtered by Trimmomatic (v0.36) using default parameters [19]. The assembly was performed with SPAdes (3.11.1) software [20]. The assembled

genome was annotated with prokaryotic genome annotation pipeline and the RAST server [21]. The Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession PPXP000000000. The version described in this paper is version PPXP010000000. Genome was further analyzed with Anti-SMASH [22] and BAGEL3 [23] for secondary metabolite and bacteriocin biosynthesis gene clusters.

2.9. Statistical analysis

Data were expressed as mean $\pm$ SE of mean (SEM) of three replicates. Student's t-test was used to calculate statistical significance for the results of immune parameters. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Bacterial strain selection and characterization

Strain JW was isolated from the gastrointestinal tract of common carp. The gram-staining and microscopic observation showed it is a rod-shaped gram-positive bacterium. This strain is capable of producing endospores, many cells undergo sporulation during the late stationary phase. On LB agar plate, it forms white and wet colonies with 3–5 mm diameter. When grown on agar plate for more than 3 days, biofilm will be formed as indicated by wrinkled colonies. In order to characterize this strain, partial 16S rRNA sequence was used to performed a BLAST. The results showed that it has 100% identity with several strains including *Bacillus velezensis* Lzh-a42 (CP025308.1), *Bacillus* sp. M1-33 (KX369291.1), *Bacillus subtilis* HMJ-2 (KU317797.1), and *Bacillus amyloliquefaciens* CSL21 (KM091678.1). Thus, 16S rRNA sequence was not enough to discriminate those species since they are phylogenetically close-related. The *gyrA* (DNA gyrase A) gene was used for further identification, which showed 100% similarity with *B. velezensis* Lzh-a42. The neighbor-joining tree (Fig. 1) showed that strain JW formed a distinct cluster with *B. velezensis*.

3.2. The antimicrobial activity and challenge test

As shown in Fig. S1, strain JW has excellent antimicrobial activity against gram-negative fish pathogenic bacteria including *A. hydrophila*, *A. salmonicida*, and *V. parahaemolyticus*. Antagonistic effects were also observed on 2 g-positive pathogens: *L. garvieae* and *S. agalactiae*.

The challenge test revealed that *B. velezensis* JW significantly increase the fish survival rate when challenged with live *A. hydrophila* (Fig. S2). The negative control group exhibited 100% survival rate, while only 30% fish of the challenge control group survived. In comparison, fish fed with diets containing 10^7 cfu/g and 10^9 cfu/g of JW exhibited a survival rate of 67% and 80%, with a relative percent survival (RPS) value of 52.9% and 71.4%, respectively.

3.3. *Bacillus velezensis* JW induces humoral immune responses

In general, fish fed with JW supplemented diets consume the feed comparably well, and there was no noticeable learning period for the fish to get used to the probiotic diets. Moreover, the overall vigour of the fish in JW group is better than that of control. The humoral immune parameters of *C. auratus* after being fed with diets containing 10^7 cfu/g and 10^9 cfu/g of *B. velezensis* JW were measured. A significantly increased alkaline phosphatase (AKP) activity was observed in both 10^7 cfu/g and 10^9 cfu/g groups compared with the control group during the whole feeding trial ($P < 0.05$, Fig. 2B). Fish showed significantly higher acid phosphatase (ACP) activity in 10^7 cfu/g and 10^9 cfu/g groups after first and second week of feeding ($P < 0.05$, Fig. 2C). Moreover, the levels of glutathione peroxidase (GSH-PX) activity in all treatment groups were significantly higher than that in the control group in all sampling time point ($P < 0.05$). Among all the probiotic

Fig. 1. Phylogenetic tree based on *gyrA* gene sequences showing the phylogenetic position of strain JW within the genus *Bacillus*. The *gyrA* gene sequences were aligned using ClustalW tool in MEGA 7.0.26, and phylogenetic analysis was performed using the neighbor-joining method.

groups, 10^9 cfu/g group had a higher activity than the 10^7 cfu/g group (Fig. 2D). Moreover, the serum lysozyme (LYZ) activity was also increased by JW supplemented diets albeit not statistically significant (Fig. 2A).

3.4. *Bacillus velezensis* JW alters the expression of immune-related genes

The relative mRNA expression of immune-related genes in the head kidney of *C. auratus* fed with different levels of JW-containing diets are shown in Fig. 3. The mRNA expression of interferon gamma gene (IFN- γ) showed a higher value for the groups of fish fed with JW diets compared to the control group. A significant difference was observed after 3 and 4 weeks of feeding ($P < 0.05$, Fig. 3A). Likewise, tumor necrosis factor- α (TNF- α) was also recorded significantly higher for fish fed 10^9 cfu/g diets than that of control after 3 and 4 weeks of feeding ($P < 0.05$, Fig. 3B). The mRNA expression of four genes of the interleukin (IL) family were measured. Among them, interleukin-1 (IL-1) only being significantly upregulated by 10^9 cfu/g JW after 1 week of feeding ($P < 0.05$, Fig. 3C). The upregulation of interleukin-4 (IL-4)

increased over time from 1st to 4th week of feeding. Significant difference was observed after 3 and 4 weeks of feeding ($P < 0.05$, Fig. 3D). Compared with control, the mRNA expression of interleukin-10 (IL-10) was significantly higher in both 10^7 cfu/g and 10^9 cfu/g JW feeding groups after 2, 3, and 4 weeks of feeding ($P < 0.05$, Fig. 3E). In contrast, the expression of interleukin-12 (IL-12) was significantly down-regulated by 10^7 cfu/g and 10^9 cfu/g of JW addition after 2, 3, and 4 weeks of feeding ($P < 0.05$, Fig. 3F).

3.5. Probiotic potential of *Bacillus velezensis* JW revealed by genome sequence

The genome of JW strain was sequenced using Illumina PE150 platform and 1,296,923,100 bases were acquired at 8-fold coverage. The pair-end reads were filtered and assembled into 35 contigs with average size of 116,532 and N50 size of 275,576 bp. These contigs were further assembled into 7 scaffolds using reference-guided method conducted by an in-house Python script. The resulting genome sequence has a size of 4,079,992 bp and an overall GC content of 45.9%. The

Fig. 2. (A) serum lysozyme (LYZ) activity, (B) alkaline phosphatase (AKP) activity, (C) acid phosphatase activity, and (D) glutathione peroxidase (GSH-PX) of *C. auratus* after being fed with diets containing 10⁷ cfu/g and 10⁹ cfu/g of *B. velezensis* JW for 4 weeks. Each value represents mean $\pm$ SE (n = 3). Asterisks indicate statistical significance compared with control ($P < 0.05$).

annotation with the NCBI prokaryotic genome annotation pipeline resulted in 4,046 coding DNA sequences (CDSs). Furthermore, 81 RNA were identified, including 72 tRNAs, 4 rRNAs, and 5 non-coding RNAs, respectively.

The FIGfam annotation classes provided by RAST was used to group predicted proteins according to their function. The results showed that 46% of the annotated CDS was assigned to the subsystem and their functions are shown in Fig. 4. Of those CDS, amino acids and derivatives metabolism was the most enriched metabolic category. Notably, branched chain amino acids (BCAA) biosynthesis pathway was presented in the genome. Microbial BCAA metabolism in the rainbow trout gut is reported to have significant influence on the utilization of dietary nitrogen in the yeast-based protein diet [24]. The second largest function group is related to carbohydrates metabolism. This is in accordance with its ability to grow in complex gastrointestinal environment motivated by its capability of metabolizing at least 11 different kinds of sugar. In the genome of JW, there is a complete TCA cycle and electron transport chain that contribute to aerobic respiration. There is also a *narGHJI* operon that support its anaerobic growth using nitrate as an electron acceptor [25]. This capability would be required for JW to grow in anoxic environments.

Bacteriocin, Polyketide Synthetase (PKS) and Nonribosomal Peptide-Synthetase (NRPS) gene clusters are analyzed. As shown in Fig. 5A, JW harbors four bacteriocin synthesis gene clusters including two ribosomally produced and posttranslationally modified peptides (RiPPs), plantazolicin and amylocyclicin; one unmodified bacteriocin

LCI; and a large antimicrobial protein colicin. Moreover, three PKS gene clusters and five NRPS gene clusters were identified in the genome of JW, including an unknown NRPS that possibly synthesizes new antimicrobial compound (Fig. 5B).

4. Discussion

Bacillus spp. have been frequently isolated from healthy fish gastrointestinal tract (GIT) [14,26,27]. Thus, it is believed that they are well-adapted and are more colonizing than transient in GIT. Ran et al. [28] studies the survival and probiotic effects of 21 *Bacillus* strains in the intestine of catfish, their results showed that strains with excellent intestinal persistence conferred significant benefit in reducing catfish mortality when challenged by *Edwardsiella tarda*. *B. subtilis*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, and *Bacillus pumilus* are phylogenetically and phenetically closely-related species which been referred as *B. subtilis* species complex [29]. Members of this group representing a majority of fish and shellfish probiotics. For example, *B. subtilis* strains significantly enhance the weight gain and digestive enzyme activity juvenile white shrimp (*Litopenaeus vannamei*) [30]. *B. amyloliquefaciens* and *B. pumilus* could stimulate growth performance, innate immunity, and stress tolerance of striped catfish [31]. *B. velezensis* was first described in 2005 by Ruiz-Garcia et al. as a surfactant producing bacterium [32]. A recent study revealed that *B. amyloliquefaciens* subsp. *plantarum* is a later hetero-typic synonym of *B. velezensis* [33]. The characterization of *B. subtilis* species group is difficult because they are

Fig. 3. The relative mRNA expression of (A) interferon gamma (IFN- γ), (B) tumor necrosis factor- α (TNF- α), (C) interleukin-1 (IL-1), (D) interleukin-4 (IL-4), (E) interleukin-10 (IL-10), and (F) interleukin-12 (IL-12) genes of *C. auratus* after being fed with diets containing 10^7 cfu/g and 10^9 cfu/g of *B. velezensis* JW for 4 weeks. Each value represents mean $\pm$ SE ($n = 3$). Asterisks indicate statistical significance compared with control ($P < 0.05$).

hardly distinguishable on the basis of traditional phenotypic methods. Phylogenetic analysis of the 16S rRNA gene sequence has insufficient discriminant power due to the highly conserved nature of this gene [34]. Instead, the *gyrA* gene sequences provide a firm framework for the rapid and accurate classification and identification of *B. subtilis* and related taxa [35]. Phylogenetic tree based on the whole *gyrA* gene sequence showed that strain JW allocated within the *B. velezensis* clade (Fig. 1).

LYZ degrades peptidoglycan in the bacterial cell wall leading to rapid killing of Gram-positive organisms. As a barrier for microbial

invasion, LYZ is an important defense molecule of fish innate immune system [36]. In lab conditions, JW only increased the LYZ activity of *C. auratus* insignificantly. We speculated that JW with proper dosage should be able to stimulate LYZ activity since dose-dependent effects were observed. ACP is an enzyme localized within lysosomes. It is important for intracellular digestion of phagocytized antigens and has been used as a marker of macrophage activation in mammalian models and invertebrates [37]. AKP is an extracellular enzyme that hydrolyzes phosphoester bonds in various organic compounds like proteins, lipids, and carbohydrates [38]. More and more studies use ACP and AKP

Fig. 4. The distribution of predicted CDS of *B. velezensis* JW in different categories of metabolic function.

Fig. 5. (A) Bacteriocin, (B) Polyketide Synthetase (PKS) and Nonribosomal Peptide-Synthetase (NRPS) gene clusters identified in the genome of *B. velezensis* JW.

as important markers to evaluate the effects of probiotic bacteria in aquaculture. Li et al. [39] reported that sea cucumber fed with *Lactobacillus plantarum* LL11 supplemented diets showed higher levels of ACP and AKP compared to the control group. In rainbow trout, a significant elevation of the plasma AKP activity was observed when fish were fed with diets containing 10^9 cfu/g *Lactobacillus rhamnosus* for 20 and 30 days [40]. Similarly, *B. velezensis* JW increased the ACP and AKP activity of *C. auratus* in a dose-dependent manner. GSH-PX is involved in fish antioxidant defense system. It plays an important role in removing free radicals and other reactive oxygen species (ROS), accelerating free radical scavenging, and reducing the occurrence of lipid peroxidation [41]. In this study, increased GSH-PX activity were observed in fish treated with continuously 10^7 and 10^9 cfu/g *B. velezensis* JW compared with the control. Similar results have been observed in rockfish (*Sebastes schlegelii*) [42] and *Cyprinus carpio* [43], which demonstrated the probiotic bacteria *Pediococcus acidilactici* and *Lactobacillus delbrueckii* improve the antioxidant capability through the increased GSH-PX activity.

Cytokines produced by immune cells stimulate inflammation at the site of infection, and accelerate the arrival of phagocytic cells to destroy the invading pathogen [44]. Probiotics are able to modulate host immunity by altering the expression of pro-inflammatory cytokines. Our study demonstrated that the diets with *B. velezensis* JW significantly upregulated the expression of IFN- γ and TNF- α of *C. auratus* after 3–4 weeks of feeding. Similar results were reported by Hamdan et al. (2016) who found that the beneficial effects of probiotic *L. plantarum* AH 78 involve the upregulated gene expression of cytokines including IFN- γ in Nile tilapia [45]. The addition of *Shewanella xiamenensis* in the diets of grass carp caused upregulation of cytokines such as IL-1 and TNF- α [11]. The present study also exhibited that *B. velezensis* JW significantly increased the mRNA expression of IL-4 and IL-10, whereas suppressed

the mRNA expression of IL-12 in the head kidney of *C. auratus*. The different modulation effects of JW supplementation on IL-10 and IL-12 activities may be related with the distinct bioactivities of these two cytokines, which has been previous proved in rainbow trout [46]. Intriguingly, Foligné et al. reported that the probiotic *B. subtilis* PB6 induced substantial levels of IL-10 but very low levels of IL-12 on human immunocompetent peripheral blood mononuclear cells [47]. Collectively, our results showed that *B. velezensis* JW modulate the host immunity to a delicate balance: upregulate the expression of cytokines including IFN- γ , TNF- α , IL-4, and IL-10 but downregulate the expression of IL-12.

Genome sequencing has a major contribution on our understanding of the genetic potential and evolution of microorganisms. With the development in sequencing technology, genomic approaches are widely used to get a detailed insight into the evolution and genetic adaptation of bacteria. Although the genome sequences of a number of different probiotic species isolated from the human GIT have been published, the report on the genome sequences of fish probiotic bacteria is rare. Probiotics can display a range of different life-styles associated with host GIT, differing in their time spent free-living outside the host. Such differences in life-style should be reflected in their genome. We showed that *B. velezensis* JW employed a versatile aerobic and anaerobic respiratory and metabolism system to facilitate its survive in the fish GIT. The genomic data will also enable us to know which factors might influence the performance of probiotics, thus allowing a rational approach to strain improvement in the future [48].

GIT host a variety of bacteria with high cell density, which may result in close cell-cell contact between members of the same or different species, promoting both cooperative and antagonistic microbial interactions. Antimicrobials produced by certain bacteria give competitive advantages to the producers over neighboring sensitive

pathogenic or symbiotic strains within this environment [49]. With this background, a search for genes related to bacteriocins and antimicrobial secondary metabolites was carried out with BAGEL3 and antiSMASH. As shown in Fig. 5, we identified four bacteriocins gene clusters, three PKS gene clusters (difficidin, bacillaene, macrolactin), and five NRPS gene clusters (bacilysin, bacillibactin, fengycin, surfactin, and an unknown NRPS). These antimicrobials may enhance the ability of JW to compete against other GIT microbes and which could potentially inhibit pathogenic bacteria. However, further studies are required to fully determine the value of these bacteriocins in aquaculture practice.

In conclusion, *B. velezensis* JW has broad antimicrobial activity against fish pathogenic bacteria. Fish (*C. auratus*) fed with 10^7 and 10^9 cfu/g JW supplemented diets showed significantly improved survival rate after *A. hydrophila* challenging. JW also has immune enhancement effects on *C. auratus*: inducing humoral immune responses and modulating the expression of cytokine genes. Genomic analysis of JW revealed some features aiding host health and being relevant to its GIT adaptation. Moreover, a large number of antimicrobial gene clusters were identified in the genome of JW. These results clearly indicated that *B. velezensis* JW has the potential to be developed as a probiotic agent in aquaculture.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.fsi.2018.04.055>.

References

- [1] M. Bentzon-Tilia, E.C. Sonnenschein, L. Gram, Monitoring and managing microbes in aquaculture - towards a sustainable industry, *Microb. Biotechnol.* 9 (2016) 576–584.
- [2] I. Phillips, M. Casewell, T. Cox, B. De Groot, C. Friis, R. Jones, C. Nightingale, R. Preston, J. Waddell, Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data, *J. Antimicrob. Chemother.* 53 (2004) 28–52.
- [3] T.T.H. Van, J. Chin, T. Chapman, L.T. Tran, P.J. Coloe, Safety of raw meat and shellfish in Vietnam: an analysis of *Escherichia coli* isolations for antibiotic resistance and virulence genes, *Int. J. Food Microbiol.* 124 (2008) 217–223.
- [4] L. Verschuere, G. Rombaut, P. Sorgeloos, W. Verstraete, Probiotic bacteria as biological control agents in aquaculture, *Microbiol. Mol. Biol. Rev.* 64 (2000) 655–671.
- [5] Group JFWW, Guidelines for the Evaluation of Probiotics in Food, London, World Health Organization, ON, Canada: Food and Agriculture Organization, 2002.
- [6] S.S. Giri, V. Sukumaran, M. Oviya, Potential probiotic *Lactobacillus plantarum* VSG3 improves the growth, immunity, and disease resistance of tropical freshwater fish. *Labeo rohita*, *Fish Shellfish Immunol.* 34 (2013) 660–666.
- [7] S.K. Nayak, Probiotics and immunity: a fish perspective, *Fish Shellfish Immunol.* 29 (2010) 2–14.
- [8] M.B. Munir, R. Hashim, Y.H. Chai, T.L. Marsh, S.A.M. Nor, Dietary prebiotics and probiotics influence growth performance, nutrient digestibility and the expression of immune regulatory genes in snakehead (*Channa striata*) fingerlings, *Aquaculture* 460 (2016) 59–68.
- [9] O. Carnevali, M.C. Zamponi, R. Sulpizio, A. Rollo, M. Nardi, C. Orpianesi, S. Silvi, M. Caggiano, A.M. Polzonetti, A. Cresci, Administration of probiotic strain to improve sea bream wellness during development, *Aquacult. Int.* 12 (2004) 377–386.
- [10] N.V. Hai, The use of probiotics in aquaculture, *J. Appl. Microbiol.* 119 (2015) 917–935.
- [11] Z.Q. Wu, C. Jiang, F. Ling, G.X. Wang, Effects of dietary supplementation of intestinal autochthonous bacteria on the innate immunity and disease resistance of grass carp (*Ctenopharyngodon idellus*), *Aquaculture* 438 (2015) 105–114.
- [12] S.T. Tapia-Paniagua, P. Díaz-Rosales, J.M. León-Rubio, I. García de La Banda, C. Lobo, F.J. Alarcón, M. Chabillon, P. Rosas-Ledesma, J.L. Varela, I. Ruiz-Jarabo, S. Arjo, M.A. Esteban, E. Martínez-Manzanares, J.M. Mancera, M.C. Balebona, M.A. Morínigo, Use of the probiotic *Shewanella putrefaciens* Pdp11 on the culture of Senegalese sole (*Solea senegalensis*, Kaup 1858) and gilthead seabream (*Sparus aurata* L.), *Aquacult. Int.* 20 (2012) 1025–1039.
- [13] G. Banerjee, A.K. Ray, The advancement of probiotics research and its application in fish farming industries, *Res. Vet. Sci.* 115 (2017) 66–77.
- [14] R. Meidong, K. Khotchanalekha, S. Doolindachaporn, T. Nagasawa, M. Nakao, K. Sakai, S. Tongpim, Evaluation of probiotic *Bacillus aerius* B81e isolated from healthy hybrid catfish on growth, disease resistance and innate immunity of *Plamang Pangasius bocourti*, *Fish Shellfish Immunol.* 73 (2017) 1–10.
- [15] K.J. Grubbs, R.M. Bleich, M.K.C. Santa, S.E. Allen, S. Farag, T. AgBiome, E.A. Shank, A.A. Bowers, Large-scale bioinformatics analysis of *Bacillus* genomes uncovers conserved roles of natural products in bacterial physiology, *mSystems* 2 (6) (2017).
- [16] T. Stein, *Bacillus subtilis* antibiotics: structures, syntheses and specific functions, *Mol. Microbiol.* 56 (2005) 845–857.
- [17] L. Liu, T. Hao, Z. Xie, G.P. Horsman, Y. Chen, Genome mining unveils widespread natural product biosynthetic capacity in human oral microbe *Streptococcus mutans*, *Sci. Rep.* 6 (2016) 37479.
- [18] D.F. Amend, Potency testing of fish vaccines, *Dev. Biol. Stand.* 49 (1981) 447–454.
- [19] A.M. Bolger, M. Lohse, B. Usadel, Trimmomatic: a flexible trimmer for Illumina sequence data, *Bioinformatics* 30 (2014) 2114–2120.
- [20] A. Bankevich, S. Nurk, D. Antipov, A.A. Gurevich, M. Dvorkin, A.S. Kulikov, V.M. Lesin, S.I. Nikolenko, S. Pham, A.D. Pribelski, SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing, *J. Comput. Biol.* 19 (2012) 455–477.
- [21] R. Overbeek, R. Olson, G.D. Pusch, G.J. Olsen, J.J. Davis, T. Disz, R.A. Edwards, S. Gerdes, B. Parrello, M. Shukla, V. Vonstein, A.R. Wattam, F. Xia, R. Stevens, The seed and the rapid annotation of microbial genomes using subsystems technology (RAST), *Nucleic Acids Res.* 42 (2014) D206–D214.
- [22] M.H. Medema, K. Blin, P. Cimermancic, V. de Jager, P. Zakrzewski, M.A. Fischbach, T. Weber, E. Takano, R. Breitling, antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences, *Nucleic Acids Res.* 39 (2011) W339–W346.
- [23] A. van Heel, A. de Jong, M. Montalbán-López, J. Kok, O.P. Kuipers, BAGEL3: automated identification of genes encoding bacteriocins and (non-)bactericidal post-translationally modified peptides, *Nucleic Acids Res.* 41 (2013) W448–W453.
- [24] O.C. Betiku, C.J. Yeoman, T.G. Gaylord, G.C. Duff, T. Hamerly, B. Bothner, S.S. Block, W.M. Sealey, Differences in amino acid catabolism by gut microbes with/without prebiotics inclusion in GDDY-based diet affect feed utilization in rainbow trout, *Aquaculture* (2017), <http://dx.doi.org/10.1016/j.aquaculture.2017.09.006>.
- [25] G. Unden, S. Becker, J. Bongaerts, J. Schirawski, S. Six, Oxygen regulated gene expression in facultatively anaerobic bacteria, *Antonie Leeuwenhoek* 66 (1994) 3–22.
- [26] S. Lee, K. Katya, Y. Park, S. Won, M. Seong, A. Hamidoghli, S.C. Bai, Comparative evaluation of dietary probiotics *Bacillus subtilis* WB60 and *Lactobacillus plantarum* KCTC3928 on the growth performance, immunological parameters, gut morphology and disease resistance in Japanese eel, *Anguilla japonica*, *Fish Shellfish Immunol.* 61 (2017) 201–210.
- [27] A. Nandi, G. Banerjee, S.K. Dan, K. Ghosh, A.K. Ray, Evaluation of *in vivo* probiotic efficiency of *Bacillus amyloliquefaciens* in labo rohita challenged by pathogenic strain of *Aeromonas hydrophila* MTCC 1739, *Probiotics Antimicrob. Protein* (2017), <http://dx.doi.org/10.1007/s12602-017-9310-x>.
- [28] C. Ran, A. Carrias, M.A. Williams, N. Capps, B.C. Dan, J.C. Newton, J.W. Kloepper, E.L. Ooi, C.L. Browdy, J.S. Terhune, M.R. Liles, Identification of *Bacillus* strains for biological control of catfish pathogens, *PLoS One* 7 (2012) e45793.
- [29] D. Fritze, Taxonomy of the genus *Bacillus* and related genera: the aerobic endospore-forming bacteria, *Phytopathology* 94 (11) (2004) 1245–1248.
- [30] H. Zokaeifar, J.L. Balcasar, C.R. Saad, M.S. Kamarudin, K. Sijam, A. Arshad, N. Nejat, Effects of *Bacillus subtilis* on the growth performance, digestive enzymes, immune gene expression and disease resistance of white shrimp, *Litopenaeus vannamei*, *Fish Shellfish Immunol.* 33 (2012) 683–689.
- [31] H.T.T. Thy, N.N. Tri, O.M. Qu, R. Fotedar, K. Kannika, S. Unajak, N. Areechon, Effects of the dietary supplementation of mixed probiotic spores of *Bacillus amyloliquefaciens* 54A, and *Bacillus pumilus* 47B on growth, innate immunity and stress responses of striped catfish (*Pangasianodon hypophthalmus*), *Fish Shellfish Immunol.* 60 (2017) 391–399.
- [32] C. Ruiz-García, V. Bejar, F. Martínez-Checa, I. Llamas, E. Quesada, *Bacillus velezensis* sp. nov., a surfactant-producing bacterium isolated from the river Velez in Malaga, southern Spain, *Int. J. Syst. Evol. Microbiol.* 55 (2005) 191–195.
- [33] C.A. Dunlap, S.-J. Kim, S.-W. Kwon, A.P. Rooney, *Bacillus velezensis* is not a later heterotypic synonym of *Bacillus amyloliquefaciens*; *Bacillus methylotrophicus*, *Bacillus amyloliquefaciens* subsp. *plantarum* and *Bacillus oryzicola* are later heterotypic synonyms of *Bacillus velezensis* based on phylogenomics, *Int. J. Syst. Evol. Microbiol.* 66 (2016) 1212–1217.
- [34] A.P. Rooney, N.P. Price, C. Ehrhardt, J.L. Swezey, J.D. Bannan, Phylogeny and molecular taxonomy of the *Bacillus subtilis* species complex and description of *Bacillus subtilis* subsp. *inaquosorum* subsp. nov., *Int. J. Syst. Evol. Microbiol.* 59 (2009) 2429–2436.
- [35] J. Chun, K.S. Bae, Phylogenetic analysis of *Bacillus subtilis* and related taxa based on partial *gyrA* gene sequences, *Antonie Leeuwenhoek* 78 (2000) 123–127.
- [36] S. Saurabh, P.K. Sahoo, Lysozyme: an important defence molecule of fish innate immune system, *Aquacult. Res.* 39 (2008) 223–239.
- [37] Z.F. Song, T.X. Wu, L.S. Cai, L.J. Zhang, X.D. Zheng, Effects of dietary supplementation with *Clostridium butyricum* on the growth performance and humoral immune response in *Micthys miyu*, *J. Zhejiang Univ. - Sci. B* 7 (2006) 596–602.
- [38] F. Hassanatbar, H. Ouraji, A. Esmaeili, S. Babaei, Study of the activities of digestive enzymes, amylase and alkaline phosphatase in Kutum Larvae (*Rutilus frisikutum*) fed artemianapilii, *World J. Fish Mar. Sci.* 5 (2013) 266–270.
- [39] C. Li, Y. Ren, S. Jiang, S. Zhou, J. Zhao, R. Wang, Y. Li, Effects of dietary

- supplementation of four strains of lactic acid bacteria on growth, immune-related response and genes expression of the juvenile sea cucumber *Apostichopus japonicus* Selenka, *Fish Shellfish Immunol.* 74 (2017) 69–75.
- [40] A. Panigrahi, V. Kiron, S. Satoh, T. Watanabe, Probiotic bacteria *Lactobacillus rhamnosus* influences the blood profile in rainbow trout *Oncorhynchus mykiss* (Walbaum), *Fish Physiol. Biochem.* 36 (2010) 969–977.
- [41] S. Hussain, W. Slikker Jr., S.F. Ali, Role of metallothionein and other antioxidants in scavenging superoxide radicals and their possible role in neuroprotection, *Neurochem. Int.* 29 (2) (1996) 145–152.
- [42] S. Rahimnejad, F.A. Guardiola, E. Leclercq, E.M. Angeles, M. Castex, E. Sotoudeh, S.M. Lee, Effects of dietary supplementation with *Pediococcus acidilactici* MA18/5M, galactooligosaccharide and their synbiotic on growth, innate immunity and disease resistance of rockfish (*Sebastes schlegelii*), *Aquaculture* 482 (2018) 36–44.
- [43] C.N. Zhang, J.L. Zhang, W.C. Guan, X.F. Zhang, S.H. Guan, Q.H. Zeng, G.F. Cheng, W. Cui, Effects of *Lactobacillus delbrueckii* on immune response, disease resistance against *Aeromonas hydrophila*, antioxidant capability and growth performance of *Cyprinus carpio* Huanghe var, *Fish Shellfish Immunol.* 68 (2017) 84–91.
- [44] H. Kano, O. Mogami, M. Uchida, Oral administration of milk fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1 to DBA/1 mice inhibits secretion of proinflammatory cytokines, *Cytotechnology* 40 (2002) 67–73.
- [45] A.M. Hamdan, A.F. El-Sayed, M.M. Mahmoud, Effects of a novel marine probiotic, *Lactobacillus plantarum* AH 78, on growth performance and immune response of Nile tilapia (*Oreochromis niloticus*), *J. Appl. Microbiol.* 120 (2016) 1061–1073.
- [46] T. Wang, M. Husain, S. Hong, J.W. Holland, Differential expression, modulation and bioactivity of distinct fish IL-12 isoforms: implication towards the evolution of Th1-like immune responses, *Eur. J. Immunol.* 44 (2014) 1541–1551.
- [47] B. Folligné, E. Peys, J. Vandekerckhove, J. Dewulf, J. Breton, B. Pot, Spores from two distinct colony types of the strain *Bacillus subtilis* PB6 substantiate anti-inflammatory probiotic effects in mice, *Clin. Nutr.* 31 (2012) 987–994.
- [48] S. Salminen, J. Nurmi, M. Gueimonde, The genomics of probiotic intestinal microorganisms, *Genome Biol.* 6 (2005) 225.
- [49] A. Dobson, P.D. Cotter, R.P. Ross, C. Hill, Bacteriocin production: a probiotic trait? *Appl. Environ. Microbiol.* 78 (1) (2012) 1–6.