Microbial Pathogenesis 181 (2023) 106161

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Mining the genome of *Bacillus velezensis* FS26 for probiotic markers and secondary metabolites with antimicrobial properties against aquaculture pathogens

Muhamad Firdaus Syahmi Sam-on [a], Shuhaimi Mustafa [a,b,*], Amalia Mohd Hashim [a,b], Mohd Termizi Yusof [a], Shahrizim Zulkifly [c], Ahmad Zuhairi Abdul Malek [a], Muhamad Akhmal Hakim Roslan [d], Mohd Shaufi Mohd Asrore [a]

[a] Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Serdang, 43400, Selangor, Malaysia
[b] Halal Products Research Institute, Universiti Putra Malaysia, Serdang, 43400, Selangor, Malaysia
[c] Department of Biology, Faculty of Science, Universiti Putra Malaysia, Serdang, 43400, Selangor, Malaysia
[d] Halways Sdn Bhd, Jalan Satelit, Putra Science Park, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia

ARTICLE INFO

Keywords:
Bacillus velezensis FS26
Whole genome sequence (WGS)
Secondary metabolites
Aquaculture pathogens
Probiotic marker

ABSTRACT

Bacillus velezensis FS26 is a bacterium from the genus *Bacillus* that has been proven as a potential probiotic in aquaculture with a good antagonistic effect on *Aeromonas* spp. and *Vibrio* spp. Whole-genome sequencing (WGS) allows a comprehensive and in-depth analysis at the molecular level, and it is becoming an increasingly significant technique in aquaculture research. Although numerous probiotic genomes have been sequenced and investigated recently, there are minimal data on *in silico* analysis of *B. velezensis* as a probiotic bacterium isolated from aquaculture sources. Thus, this study aims to analyse the general genome characteristics and probiotic markers from the *B. velezensis* FS26 genome with secondary metabolites predicted against aquaculture pathogens. The *B. velezensis* FS26 genome (GenBank Accession: JAOPEO000000000) assembly proved to be of high quality, with eight contigs containing 3,926,371 bp and an average G + C content of 46.5%. According to antiSMASH analysis, five clusters of secondary metabolites from the *B. velezensis* FS26 genome showed 100% similarity. These clusters include Cluster 2 (bacilysin), Cluster 6 (bacillibactin), Cluster 7 (fengycin), Cluster 8 (bacillaene), and Cluster 9 (macrolactin H), which signify promising antibacterial, antifungal, and anti-cyanobacterial agents against pathogens in aquaculture. The probiotic markers of *B. velezensis* FS26 genome for adhesion capability in the hosts' intestine, as well as the acid and bile salt-tolerant genes, were also detected through the Prokaryotic Genome Annotation System (Prokka) annotation pipeline. These results are in agreement with our previous *in vitro* data, suggesting that the *in silico* investigation facilitates establishing *B. velezensis* FS26 as a beneficial probiotic for use in aquaculture.

1. Introduction

Aquaculture infections are one of the most pressing issues facing the aquaculture industry today and failing to address this issue will lead to national and global food shortages. Immunomodulatory additives, such as antimicrobial peptides, herb extracts, probiotics, prebiotics, and synbiotics, are widely employed in aquatic diets to increase the quality and sustainability of aquaculture production [1,2]. Among them, probiotic seems to be effective in controlling infection and reducing the emergence of antibiotic-resistant bacteria in the environment. *Bacillus* species are among the most widely used and early adopted probiotics in aquaculture, and their use as dietary supplements can boost fish immune systems and disease resistance [3,4]. In this regard, *Bacillus toyoi* is the first probiotic *Bacillus* introduced in aquaculture by Kozasa in the year

* Corresponding author. Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Serdang, 43400, Selangor, Malaysia.
E-mail addresses: firdaussyahmi97@gmail.com (M.F.S. Sam-on), shuhaimi@upm.edu.my (S. Mustafa), amalia@upm.edu.my (A. Mohd Hashim), mohdtermizi@upm.edu.my (M.T. Yusof), shahrizim@upm.edu.my (S. Zulkifly), zuhairimalek@gmail.com (A.Z.A. Malek), akhmaLhalways@gmail.com (M.A.H. Roslan), shaufi@upm.edu.my (M.S. Mohd Asrore).

https://doi.org/10.1016/j.micpath.2023.106161
Received 19 February 2023; Received in revised form 16 May 2023; Accepted 17 May 2023
Available online 18 May 2023

1986 [5].

Another *Bacillus* species, *Bacillus velezensis*, displays high antibacterial potential because its genome contains several coding sequences involved in the biosynthesis of polyketide and peptide antibiotics [6]. Identifying all prospective gene clusters for secondary metabolites in hundreds of newly sequenced genomes has proven exceedingly challenging due to biochemical complexity, the availability of unknown enzymes, and the scattered nature of the needed specialist bioinformatics tools and resources. The antibiotics and secondary metabolite analysis shell (antiSMASH) pipeline, on the other hand, is the first to be capable of identifying biosynthetic loci for the entire range of known secondary metabolite compound classes, including polyketides, non-ribosomal peptides, terpenes, aminoglycosides, aminocoumarins, indolocarbazoles, lantibiotics, bacteriocins, nucleosides and beta-lactam [7]. Even though the antagonistic activity of *B. velezensis* FS26 towards pathogenic *Vibrio* spp. and *Aeromonas* spp. is proven, further study on the identity of the compounds is worth to be evaluated extensively for extended probiotic purposes, especially against different types/species of aquaculture pathogens.

Aside from secondary metabolites, a probiotic marker analysis of newly discovered probiotics is necessary to determine their properties and compare them with the existing commercial probiotics [8]. stated that the use of genotyping is valuable to identify species diversity, invasion, diseases, stress biomarkers, genomic assessment, and evolution in fisheries and aquaculture. Traditionally, researchers have focused on a restricted set of specific DNA fragments to identify genetic markers or variations in the target organism. Due to the rapid development of next-generation sequencing technology, the entire genomes of various strains of probiotic bacteria have been sequenced. This scientific and technological progress has increased our understanding of the relationships between genotypes and functions. It should be highlighted that while generic mechanisms for underlying probiotic effects can be connected to taxonomic categories (genus or species), specific mechanisms are strain-specific [9].

Several investigations on *Bacillus* species and their potential probiotic have been carried out utilising both *in vitro* and *in vivo* settings. Whole-genome sequencing (WGS), on the other hand, can provide additional assistance in the investigation of potential mechanisms of the organism as a probiotic. The combined outcomes of genomic, *in vitro*, and *in vivo* research, according to Khullar et al. [10]; have enhanced the assessment of *Bacillus* species and their probiotic capabilities. In this study, the potential probiotic *B. velezensis* FS26 was used as a bacterium for *in silico* analysis. *B. velezensis* FS26 is a potential probiotic bacterium isolated from the gut of giant freshwater prawn (*Macrobachium rosenbergii*) [11]. Although there have been numerous studies on probiotic bacterial genomes, there is limited *in silico* analysis for *B. velezensis* as a potential probiotic isolated from aquaculture sources. Thus, this study aims to analyse the general genome characteristics and probiotic markers from the *B. velezensis* FS26 genome with secondary metabolites predicted against aquaculture pathogens.

2. Methods

2.1. Collection of Bacillus velezensis FS26

Bacillus velezensis FS26 (16 S rRNA GenBank Accession number: MZ960133) was obtained from the Department of Microbiology, Faculty of Biotechnology, Universiti Putra Malaysia, Selangor, Malaysia. The bacterium was previously isolated by Sam-on et al. [11] from the gut of giant freshwater prawn (*M. rosenbergii*). The bacterial isolate showed potential probiotic characteristics based on *in vitro* evaluation and can be regarded as safe as it does not have γ-hemolytic properties against red blood agar [11].

2.2. Genomic DNA extraction and sequencing

Bacillus velezensis FS26 was cultivated overnight in nutrient broth medium at 30 °C, and bacterial cells were collected by centrifuging the overnight cultures at 12,000 rpm for 5 min. Wizard Genomic DNA Purification Kit (Promega, USA) was used to extract genomic DNA from the collected cells. Prior to sequencing, the DNA was barcoded along with other microbial DNA through the PCR barcoding process using Rapid PCR Barcoding Kit's protocol (SQK-RPB004) (Oxford Nanopore Technologies, UK). The sequencing was conducted on a MinION MK1C sequencer using a R9.4.1 flow cell (Oxford Nanopore Technologies, UK) at Nanyang Technological University, Singapore.

2.3. Base calling, assembly, gene prediction, and functional annotation of Bacillus velezensis FS26 genome

Base calling was done using Guppy v3.2.2 to produce FASTQ files using the base-calling model of dna_r9.4.1_450bps_hac.cfg. Demultiplexing of FASTQ files was performed using the Guppy barcoder. Once demultiplexed, the FASTQ files were assembled using Flye, a long-read assembler to produce contigs (https://doi.org/10.1038/s41587-019-0072-8). The draft sequences from Flye were corrected using Medaka (https://github.com/nanoporetech/medaka). The genome sequence was then annotated using the RAST tool kit (RASTtk) on the PATRIC platform (https://www.patricbrc.org/). The genome sequence for *B. velezensis* FS26 was submitted to GenBank (https://www.ncbi.nlm.nih.gov/GenBank/) and assigned GenBank Accession JAOPEO000000000, BioProject ID PRJNA882923, and BioSample Accession SAMN30955092.

2.4. Average nucleotide identity of Bacillus velezensis FS26 genome

The average nucleotide identity (ANI) of *B. velezensis* FS26 was analysed using JSpeciesWS (https://jspecies.ribohost.com/jspeciesws/#analyse). Fifteen genomes of *B. velezensis* obtained from JSpeciesWS genome DB (database) were used to compare the ANI with the *B. velezensis* FS26 genome. The percentage similarity was represented in a heatmap graph constructed using ClustVis tools (https://biit.cs.ut.ee/clustvis/).

2.5. Genome mining of secondary metabolites of Bacillus velezensis FS26 genome

AntiSMASH bacterial version (https://antismash.secondarymetabolites.org/#!/start) was used to analyse and predict the secondary metabolites of *B. velezensis* FS26 genome. Moreover, the biosynthetic pathway of complete genes for secondary metabolites was evaluated using BlastKOALA (KEGG Orthology and Links Annotation) (https://www.kegg.jp/blastkoala/).

2.6. Genome mining of probiotic markers of Bacillus velezensis FS26 genome

Prokka (https://github.com/tseemann/prokka) was used to annotate the *B. velezensis* FS26 genome. In order to accomplish a rich and reliable annotation of genomic bacterial sequences, Prokka coordinates a range of existing software tools [12]. Probiotic markers from the *B. velezensis* FS26 genome were obtained and analysed based on the annotation.

3. Results and discussion

3.1. Comprehensive Genome Analysis of Bacillus velezensis FS26

The assembled genome for *B. velezensis* FS26 was generated using the Comprehensive Genome Analysis service at the PATRIC website created

by Wattman et al. [13] and annotated using the RASTtk system developed by Brettin et al. [14]. The Comprehensive Genome Analysis service provides a genome that has been constructed, and an overview of the annotated features is provided in Table 1 and Fig. 1. The overall length of the eight contigs in this assembled genome was 3,926,371 base pairs (bp), with an average G + C content of 46.5%. There are 29 ribosomal RNA (rRNA) genes, 75 transfer RNA (tRNA) genes, and 4061 protein coding sequences (CDS) in this genome. This corresponds to the base pair of the *B. velezensis* GY65, LG37, and WLYS23 genomes, which are 3, 915,569 bp, 3,915,568 bp, and 3,915,551 bp, respectively. Furthermore, the average G + C content of *B. velezensis* GY65, LG37, and WLYS23 genomes was 46.5%, which was similar to *B. velezensis* FS26 [15–17].

3.2. Average nucleotide identity of Bacillus velezensis FS26

The ANI approach is commonly used for defining species boundaries and verifying identification. It is also a straightforward metric with great scalability for large data sets [18]. The comparisons of the nucleotide similarity of eleven complete *B. velezensis* genomes and *B. velezensis* FS26 genome were conducted using jSpeciesWS and visualised as the heatmap in Fig. 2. According to Fig. 2, *B. velezensis* LG37, WLYS23, and GY65 showed the highest ANI similarity to *B. velezensis* FS26 at 99.99%. Interestingly, like *B. velezensis* FS26, all three strains were previously isolated from aquaculture sources [15–17]. Moreover, *B. velezensis* KMU01 has the lowest ANI similarity percentage to *B. velezensis* FS26 at 97.42%. *B. velezensis* KMU01 was isolated by Heo et al. [19] from fermented kimchi, a different source from *B. velezensis* FS26. Additionally, the ANI percentage for *B. velezensis* SQR9, FZB42, G341, and 83 was less than 99%, and all of these bacteria were previously isolated from plant sources [20–23]. These findings demonstrate that the ANI of *B. velezensis* genomes is connected to the source of isolation, demonstrating that *B. velezensis* FS26 is substantially similar to *B. velezensis* that adapted to aquaculture. According to the ANI threshold percentage similarity, more than 95% ANI indicated similar species [24], proving the species identification of *B. velezensis* FS26, as all ANI findings for eleven *B. velezensis* genomes with *B. velezensis* FS26 are more than 97% similar.

3.3. Secondary metabolite prediction from Bacillus velezensis FS26 genome

In our previous study [11], *B. velezensis* FS26 showed the ability to secrete antimicrobial substances against pathogenic *Aeromonas hydrophila*, *Aeromonas veronii*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, and *Vibrio campbellii* using agar well diffusion method. The AntiSMASH analysis of the *B. velezensis* FS26 genome was conducted to predict the secondary metabolites potentially associated with these antimicrobial activities. The analysis revealed 12 secondary metabolite gene clusters in Table 2. Cluster 4, Cluster 5, Cluster 6, Cluster 9, and Cluster 10 exhibited 100% similarity to the secondary metabolites fengycin, bacillaene, macrolactin H, bacilysin, and bacillibactin, respectively. Meanwhile, Clusters 1 (difficidin) and 11 (surfactin) showed 93% and 82% similarity, respectively. The remainder exhibited no percentage similarity, but the genes were discovered in the genome nucleotide sequences, which are Clusters 2, 3, 7, and 8.

Table 1
Genome assembly and annotation of *Bacillus velezensis* FS26.

Assembly and annotation	Details
Contigs	8
GC Content	46.5%
Plasmids	0
Contig L50	1
Genome Length	3,926,371 bp
Contig N50	2,068,990
CDS	4061
tRNA	75
rRNA	29
Repeat Regions	25
Partial CDS	0
Miscellaneous RNA	0
Chromosomes	0
Genbank Accession	JAOPEO000000000
BioProject ID	PRJNA882923
BioSample Accession	SAMN30955092

According to Fig. 3, the secondary metabolites predicted on the *B. velezensis* FS26 genome can be classified into three categories. Three clusters of the seven secondary metabolite genes were found to encode the biosynthetic enzymes involved in the synthesis of non-ribosomal lipopeptides, which are produced by large enzyme complexes of non-ribosomal peptide synthetases. These enzymes are fengycin, bacillibactin, and surfactin. Non-ribosomal peptides are molecules that fall within the category of secondary metabolites and serve a range of purposes, such as those of toxic substances, siderophores, pigments, and antibiotics. Like other proteins, their creation is not reliant on the ribosomal machinery [25]. In addition, the secondary metabolites of bacillaene, macrolactin H, and difficidin were synthesized by polyketide synthase (PKs) and bacilysin was synthesized by a ribosome-independent pathway. Table 2 presents the results of the antiSMASH analysis predicting five potential secondary metabolites in the *B. velezensis* FS26 genome that showed 100% similarity to the database, including fengycin, bacillibactin, bacillaene, macrolactin H, and bacilysin.

Fengycin is an antifungal lipopeptide complex generated by many *Bacillus* species, including *Bacillus subtilis* and *B. velezensis* [27–29]. The structure is made up of α-hydroxy fatty acid coupled to a peptide portion made up of ten amino acids, eight of which are structured in a cyclic form (Fig. 3). Fengycin causes the fungal hyphae to be ultrastructurally destroyed. Thus, hyphae treated with fengycin have unconsolidated cytoplasm and cell walls that are gapped and/or detached from the cell membrane [30,31]. No study has been found on the pure fengycin secreted by *Bacillus* species against pathogenic fungi in aquaculture, and most of the reports are related to the phytopathogens in crops. Pathogenic fungi, such as Saprolegnia and Achyla, are reported to be the most prevalent fungus discovered to cause cotton wool disease, which affects the body, fins, and mouth of fish [32,33]. As a result, the fengycin predicted in the *B. velezensis* FS26 genome is a promising antifungal peptide that may be employed to combat cotton wool disease and other pathogenic fungi-caused illnesses in aquaculture.

A non-ribosomal peptide called bacillibactin, discovered in the bacterium *B. subtilis*, serves as a catecholic siderophore in the acquisition of iron, which is essential for the life of the host [34]. Iron intake regulation is required to prevent oxidative damage, which can be aggravated by excess iron in the cell, and most bacteria rely on the DNA-binding protein ferric uptake repressor to control and regulate the expression of iron uptake genes [35]. Previous studies demonstrated that bacillibactin antibiotic detection on marine *Bacillus amyloliquefaciens* MTCC 12,713 had good inhibitory activity against drug-resistant pathogens, such as methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* [36]. These data strengthen the potential of bacillibactin secondary metabolites detected in the *B. velezensis* FS26 genome as broad-spectrum antibacterial agents towards pathogenic bacteria in aquaculture.

Bacillaene is a secondary metabolite of the polyene class that was identified and extracted from the fermentation broth of a *B. subtilis* strain [37]. It is a well-known antibiotic that has mostly gone unstudied due to its notorious volatility. According to the patent submitted by Stannek-göbel et al. [38]; the bacillaene produced by *B. amyloliquefaciens* DSM 33014 showed a significant effect on *V. parahaemolyticus* DSM 10027, a pathogenic bacterium that infects crustaceans. Acute hepatopancreatic necrosis disease in the white leg shrimp, *Litopenaeus vannamei*, is related to the marine Gram-negative bacteria *V. parahaemolyticus*, which has caused significant financial losses in

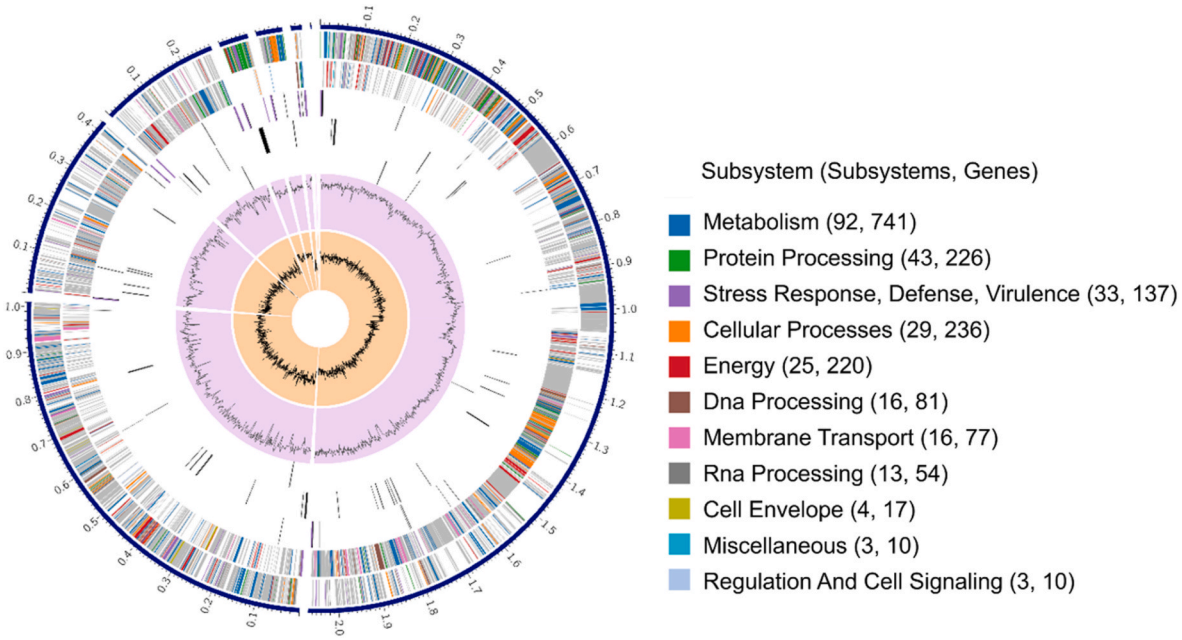

Fig. 1. A circular graphical display of the distribution of the genome annotations for *Bacillus velezensis* FS26 with subsystems and genes frequencies.

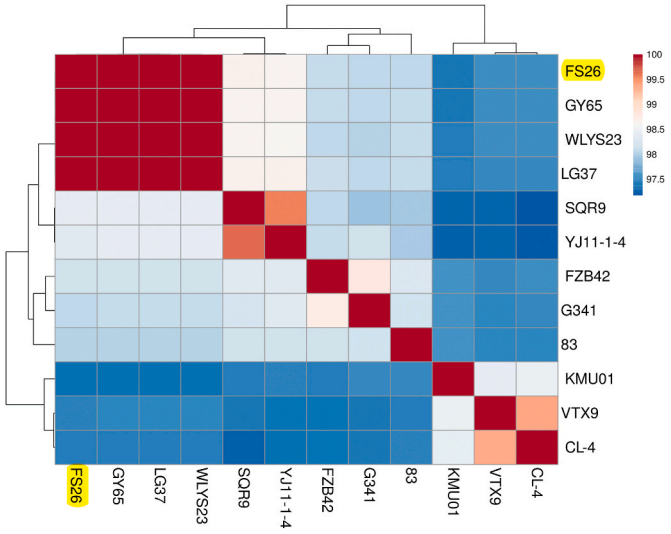

Fig. 2. Heatmap of pairwise genome comparisons between 11 *Bacillus velezensis* genomes and *Bacillus velezensis* FS26 genome based on the Average Nucleotide Identity (ANI) using jSpeciesWS genome database. Heatmap analysis were constructed using ClustVis website (https://biit.cs.ut.ee/clustvis/). *Bacillus velezensis* FS26 is denoted as FS26.

Southeast Asian marine aquaculture [39]. Similar to the study reported by Sam-on et al. [11]; *B. velezensis* FS26 showed a good antagonistic effect on *V. parahaemolyticus* PKK24, which is probably due to the secretion of bacillaene antibiotic that warrants further confirmatory studies.

Macrolactins are remarkable metabolites with antibacterial action against a variety of therapeutically relevant infections. They also have anti-inflammatory, antifungal, antibacterial, and anticancer properties. They are macrolides having 24-membered lactone rings that differ in their chemical structures, except for macrolactin H which contains 22-membrane lactone rings [40,41]. Genes involved in macrolactin production are frequently found on the genomes of *B. velezensis* strains, but not on the genomes of *Bacillus siamensis* or *B. amyloliquefaciens* strains [42]. It was reported that macrolactin G until M (including macrolactin H) showed an antagonistic effect on *S. aureus* IFO 12732 [40]. *S. aureus* is known as the most common pathogen detected in marine seafood worldwide, including fish and shrimp. Consuming aqua products infected with these bacteria and their enterotoxin may lead to human food poisoning [43,44]. Hence, the macrolactin H detected in the *B. velezensis* FS26 genome is a prospective antibacterial agent to solve the foodborne pathogen caused by *S. aureus* in aquatic beverages.

Table 2
List of the putative gene clusters encoding for secondary metabolites by anti-SMASH analysis in *Bacillus velezensis* FS26 genome.

Cluster	Type	From	To	Most similar known cluster	Similarity
1	transAT-PKS	551,750	640,289	difficidin	93%
2	T3PKS	772,752	813,479	–	–
3	terpene	878,817	898,943	–	–
4	NRPS, betalactone, transAT-PKS	927,680	1,061,991	fengycin	100%
5	transAT-PKS, NRPS,T3PKS	1,135,732	1,236,301	bacillaene	100%
6	transAT-PKS	1,455,494	1,543,728	macrolactin H	100%
7	lanthipeptide class-ii	1,710,293	1,732,959	–	–
8	terpene	1,859,675	1,880,415	–	–
9	other	308,655	350,073	bacilysin	100%
10	RiPP-like, NRPS	886,399	938,190	bacillibactin	100%
11	NRPS	202,961	268,369	surfactin	82%

Bacilysin, the simplest peptide antibiotic known, was established in 1946 as an antibiotic generated by a strain of *B. subtilis* that induced partial lysis of *S. aureus* growth culture [26,45,46]. Based on the results in Fig. 4, all six enzymes required for bacilysin biosynthesis were completely detected in the *B. velezensis* FS26 genome. The biosynthesis of bacilysin starts from prephanate to bacilysin together with six enzymes involved, which are prephenate carboxy-lyase (3-[(4 R)-4-hydroxycyclohexa-1,5-dien-1-yl]-2-oxopropanoate-forming) (EC 4.1.1.100), 3-[(4 R)-4-hydroxycyclohexa-1,5-dien-1-yl]-2-oxopropanoate isomerase (EC 5.3.3.19), bacilysin biosynthesis

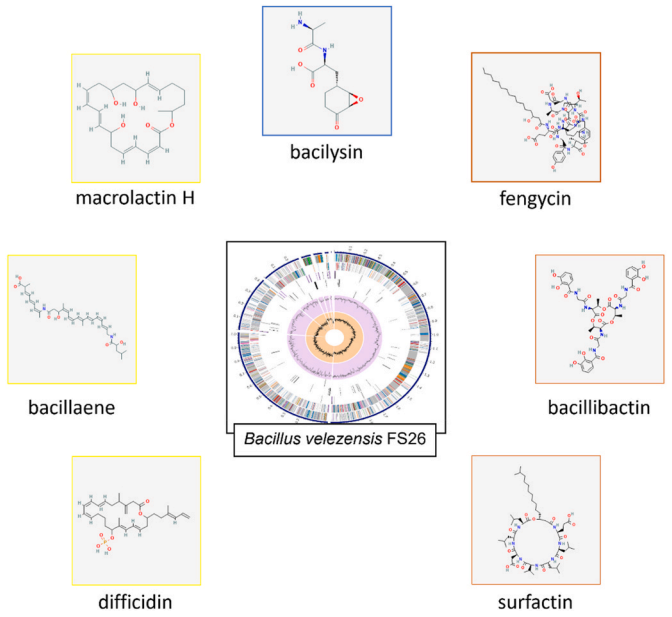

Fig. 3. Classification of antimicrobial peptides predicted by antiSMASH analysis on *Bacillus velezensis* FS26 genome. The compounds with highlighted box in orange are synthesized by non-ribosomal peptide synthetases (NRPSs); yellow colours are synthesized by polyketide synthase (PKSs); blue colour compound bacilysin is synthesized by a ribosome independent pathway. The chemical structure of the secondary metabolites was retrieved from PubChem NCBI [26] pubchem. ncbi.nlm. nih.gov).

oxidoreductase (BacG), bacilysin biosynthesis transaminase (BacF), L-dihydroanticapsin (EC 1.1.1.385), and L-anticapsin (EC 6.3.2.49). The complete bacilysin biosynthesis pathway containing all six genes in the *B. velezensis* FS26 genome is in accordance with the *B. velezensis* FZB42 (previously known as *B. amyloliquefaciens*) genome [47,48]. The bacilysin generated by *B. velezensis* FZB42 exhibits anticyanobacterial activity against the hazardous alga *Microcystis aeruginosa* and can be used as a targeted biocontrol agent, according to a study by Wu et al. [49]. As a result, this information can be utilised to forecast the anticyanobacterial activity of *B. velezensis* FS26 against pathogenic algae in aquaculture, which also remains to be explored.

Table 3 compares the secondary metabolites predicted by the antiSMASH analysis in the *B. velezensis* FS26 genome to other published probiotic bacterial genomes in aquaculture. Numerous secondary metabolites have been predicted in *B. velezensis* strain FTC01, *B. amyloliquefaciens* strain 78-1, and *B. subtilis* strain WS1A, including butirosin A/B, macrolactin H, bacillaene, fengycin, bacillibactin, bacilysin, difficidin, bacilloathiazol A-N, plipastatin, plantazolicin, surfactin, andalusicin A/B, pulcherriminic acid, and 1-carbapen-2-em-3-carboxylic acid, which corresponds to the *B. velezensis* FS26 genome. Moreover, fusaricidin B, ubericin K, and nisin A are anticipated in the genomes of *Pediococcus pentosaceus* strain MR001, *Lacticaseibacillus paracasei* strain DTA93, and *Lactococcus lactis* subsp. lactis strain WFLU12, respectively. The genomes of *Weizmannia coagulans* strain DSM 1 = ATCC 7050, *Lacticaseibacillus rhamnosus* strain TK-F8B, *Bifidobacterium animalis* strain TK-J6A, and *Lactiplantibacillus pentosus* strain MP-10, on the other hand, showed no results for the secondary metabolites predicted based on the antiSMASH analysis.

3.4. Prediction of probiotic marker genes in the Bacillus velezensis FS26 genome

Aside from the secondary metabolites predicted in the *B. velezensis* FS26 genome, probiotic marker genes are also among the important criteria for a good probiotic in aquaculture. The *B. velezensis* FS26 genome demonstrated good adhesion capability in the intestine using auto-aggregation, co-aggregation, and hydrophobicity tests, according to Sam-on et al. [11]. This result correlates to the Prokka annotation results in Table 4, which detected numerous proteins responsible for intestinal adhesion. Lipoprotein signal peptidase (LspA gene) and moonlighting proteins, such as glutamine-binding periplasmic protein (GlnH gene) and elongation factor Tu (Tuf gene), were found in the genome of *B. velezensis* FS26, which are also known as mucus adhesion domain protein (MucBP). This protein is in charge of ligands or effector molecules that contribute to host adhesion, auto-aggregation, and/or co-aggregation with pathogenic bacteria. Furthermore, LspA is correlated with MucBP which was also detected in the *B. velezensis* FS26 genome. These findings are consistent with [50]; who discovered similar MucBPs (LspA, GlnH, and Tuf genes) in probiotic *Lactobacillus pentosus* MP-10. Similarly [51], indicated that LspA was identified in probiotic *Lactobacillus salivarius* UCC118.

An additional important requirement for a successful probiotic in aquaculture is tolerance to acid and bile salts. *B. velezensis* FS26 could endure 0.3% bile salt and acidic conditions for 3 h, as demonstrated by Sam-on et al. [11]. This information is consistent with the Prokka's gene annotation output in Table 4, which identified numerous genes in the *B. velezensis* FS26 genome that are associated with bile salt and acid tolerance. Bile salt can prevent the growth of bacteria by rupturing the bacterial cell membrane [52]. Table 4 shows three proteins identified as bile salt-tolerant genes in the *B. velezensis* FS26 genome, including a chaperone (DnaK), an oligopeptide-binding protein (OppA), and an enolase (Eno). However, no bile salt hydrolase protein (BshA) was found in the genome of *B. velezensis* FS26. These results are in agreement with [53] that detected several bile salt-tolerant genes (DnaK, Eno, and OppA) in probiotic *Lactiplantibacillus plantarum* WCFS1, TL2766, MPL16, CRL681, and CRL1506, but no BshA was detected in all strains.

Probiotic microorganisms must meet crucial standards for acid tolerance to survive in the host's upper gastrointestinal tract [54]. Based on Table 4, 10/33/60 kDa chaperonin, ATP-dependent protease proteolytic subunit, cold shock protein, and TRAP-T-associated universal stress protein were discovered as acid-tolerant proteins in the *B. velezensis* FS26 genome. These findings are in line with that of [10]; in which the genomes of *Bacillus* isolates contained stress proteins conferring acid tolerance, including chaperonin, cold shock protein, and TRAP-T-associated universal stress protein. However, the isolates also had an additional protein that could withstand acidity, known as F1F0 ATP acid-tolerant protein. Aside from that, Nguyen and Kim [55] detected ATP-dependent protease proteolytic subunit as an acid-tolerant protein in the probiotic *L. lactis* WFLU12, similar to that of *B. velezensis* FS26. The prediction of the adhesion-related genes in the host's

Fig. 4. Bacilysin biosynthesis of *Bacillus velezensis* FS26 genome. The result was analysed using BlastKOALA. EC 4.1.1.100 is prephenate carboxy-lyase, EC 5.3.3.19 is 3-[(4 R)-4-hydroxycyclohexa-1,5-dien-1-yl]-2-oxopropanoate isomerase, BacG is bacilysin biosynthesis oxidoreductase, BacF is bacilysin biosynthesis transaminase, EC 1.1.1.385 is L-Dihydroanticapsin and EC 6.3.2.49 is L-anticapsin.

Table 3
Comparison on the secondary metabolites predicted in *Bacillus velezensis* FS26 genome with other published probiotic bacterial genomes using antiSMASH analysis.

Probiotic bacteria	Secondary metabolites/ postbiotics	Accession number
Bacillus velezensis strain FS26	Fengycin, Bacillibactin, Bacillaene, Macrolactin H, Surfactin, Difficidin and Bacilysin	JAOPEO000000000
Bacillus velezensis strain FTC01	Butirosin A/B, Macrolactin H, Bacillaene, Fengycin, Bacillibactin, Bacilysin, Difficidin, Bacilloathiazol A-N, Plipastatin, Plantazolicin, Surfactin	NZ_MAYA00000000
Bacillus amyloliquefaciens strain 78-1	Bacilysin, Difficidin, Bacillaene, Fengycin, Macrolactin H, Butirosin A/B, Andalusicin A/B, Surfactin, Bacillibactin	NZ_JAJBAO000000000
Bacillus subtilis strain WS1A	Pulcherriminic Acid, Bacillibactin, Subtilin, Subtilosin A, Bacilysin, 1-Carbapen-2-Em-3-Carboxylic Acid, Surfactin, Bacillaene, Fengycin, Plipastatin	NZ_JABFHE000000000
Pediococcus pentosaceus strain MR001	Fusaricidin B	NZ_CP047081
Weizmannia coagulans strain DSM 1 = ATCC 7050	No similarity found	NZ_ALAS00000000
Lacticaseibacillus rhamnosus strain TK-F8B	No similarity found	NZ_CP045586
Bifidobacterium animalis strain TK-J6A	No similarity found	NZ_CP045589
Lacticaseibacillus paracasei strain DTA93	Ubericin K	NZ_VTYT00000000
Lactiplantibacillus pentosus strain MP-10	No similarity found	NZ_FLYG00000000
Lactococcus lactis subsp. lactis strain WFLU12	Nisin A	NZ_PKRZ00000000

Table 4
Probiotic marker of *Bacillus velezensis* FS26 using Prokka analysis.

Effect	Annotation	Gene
Adhesion	Putative EAL-domain containing protein	YkuI
	Flagellin	Hag
	Putative glycosyltransferase	EpsD
	putative sugar transferase	EpsL
	Putative acetyltransferase	EpsM
	Putative pyridoxal phosphate-dependent aminotransferase	EpsN
	Sortase D	SrtD
	Lipoprotein signal peptidase	LspA
	ABC transporter glutamine-binding protein	GlnH
	Elongation factor Tu	*tuf*
Bile salt tolerance	Chaperone protein	DnaK
	Oligopeptide-binding protein	OppA
	Enolase	Eno
Acid tolerance	10/33/60 kDa Chaperonin	GroEL, GroES
	ATP-dependent protease proteolytic subunit	Clp
	Cold shock protein	CspB/CspC/ CspD
	TRAP-T-associated universal stress protein	TeaD

intestinal tract, as well as the acid and bile salt-tolerant genes as the probiotic markers in the *B. velezensis* FS26 genome, is supported in the previous *in vitro* study by Sam-on et al. [11].

4. Conclusion

In conclusion, the *in silico* prediction by several bioinformatic tools against the genome of *B. velezensis* FS26 indicates that the predictions are consistent with the previous *in vitro* data by Sam-on et al. [11]. Fengycin, bacillibactin, bacillaene, macrolactin H, and bacilysin were anticipated by the antiSMASH analysis in the *B. velezensis* FS26 genome with 100% similarity to the available database. In addition, *B. velezensis* FS26 genome mining identified numerous genes that function as probiotic markers in terms of host intestinal adhesion, as well as acid and bile salt tolerance. Further research on *in vivo* applications is required to demonstrate the efficacy and safety of these probiotic potential bacteria in a real-aquaculture environment.

Supplementary materials

No supplementary materials in this study.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Muhamad Firdaus Syahmi Sam-on: Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Shuhaimi Mustafa:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Conceptualization. **Amalia Mohd Hashim:** Writing – review & editing, Validation, Supervision, Software, Resources, Data curation. **Mohd Termizi Yusof:** Writing – review & editing, Supervision, Resources. **Shahrizim Zulkifly:** Writing – review & editing, Visualization, Validation. **Ahmad Zuhairi Abdul Malek:** Software, Formal analysis. **Muhamad Akhmal Hakim Roslan:** Writing – review & editing. **Mohd Shaufi Mohd Asrore:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

The authors would like to express their gratitude to the Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Halal Products Research Institute, Universiti Putra Malaysia and Federico Lauro's laboratory at Nanyang Technological University, Singapore for providing facilities for genomic library preparation and sequencing.

References

[1] N. Akhter, B. Wu, A.M. Memon, M. Mohsin, Probiotics and prebiotics associated with aquaculture: a review, Fish Shellfish Immunol. 45 (2) (2015) 733–741.
[2] D. Matulić, J. Barišić, I. Aničić, T. Tomljanović, R. Safner, T. Treer, R. Čož-Rakovac, Growth, health aspects and histopathology of brown bullhead (*Ameiurus nebulosus* L.): replacing fishmeal with soybean meal and brewer's yeast, Sci. Rep. 10 (1) (2020) 1–16.
[3] A. Irianto, B. Austin, Probiotics in aquaculture, J. Fish. Dis. 25 (11) (2002) 633–642.

[4] F.K. Kuebutornye, E.D. Abarike, Y. Lu, V. Hlordzi, M.E. Sakyi, G. Afriyie, C.X. Xie, Mechanisms and the role of probiotic *Bacillus* in mitigating fish pathogens in aquaculture, Fish Physiol. Biochem. 46 (3) (2020) 819–841.

[5] M. Kozasa, Toyocerin (*Bacillus toyoi*) as growth promotor for animal feeding, Microbiol. Aliments, Nutr. 4 (1986) 121–135.

[6] J.Q. Pereira, A.C. Ritter, S. Cibulski, A. Brandelli, Functional genome annotation depicts probiotic properties of *Bacillus velezensis* FTC01, Gene 713 (2019), 143971.

[7] M.H. Medema, K. Blin, P. Cimermancic, V. De Jager, P. Zakrzewski, M. A. Fischbach, R. Breitling, antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences, Nucleic Acids Res. 39 (2) (2011) 339–346.

[8] Y.H. Li, H.P. Wang, Advances of genotyping-by-sequencing in fisheries and aquaculture, Rev. Fish Biol. Fish. 27 (3) (2017) 535–559.

[9] C. Hill, F. Guarner, G. Reid, G.R. Gibson, D.J. Merenstein, B. Pot, M.E. Sanders, Expert consensus document: the International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic, Nat. Rev. Gastroenterol. Hepatol. 11 (2014) 506–514.

[10] G. Khullar, R. Det-udom, P. Prombutar, C. Prakitchaiwattana, Probiogenomic analysis and safety assessment of *Bacillus* isolates using Omics approach in combination with *in-vitro*, Lebensm. Wiss. Technol. 159 (2022), 113216.

[11] M.F.S. Sam-on, S. Mustafa, M.T. Yusof, A.M. Hashim, S. Abbasiliasi, S. Zulkifly, M. A.H. Roslan, Evaluation of three *Bacillus* spp. isolated from the gut of giant freshwater prawn as potential probiotics against pathogens causing Vibriosis and Aeromonosis, Microb. Pathog. 164 (2022), 105417.

[12] T. Seemann, Prokka: rapid prokaryotic genome annotation, Bioinformatics 30 (14) (2014) 2068–2069.

[13] A.R. Wattam, J.J. Davis, R. Assaf, S. Boisvert, T. Brettin, C. Bun, R.L. Stevens, Improvements to PATRIC, the all-bacterial bioinformatics database and analysis resource center, Nucleic Acids Res. 45 (D1) (2017) D535–D542.

[14] T. Brettin, J.J. Davis, T. Disz, R.A. Edwards, S. Gerdes, G.J. Olsen, F. Xia, RASTtk: a modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes, Sci. Rep. 5 (1) (2015) 1–6.

[15] G. Liu, Y. Deng, H. Ma, J. Jiang, J. Feng, Z. Guo, Antibacterial characterization of *Bacillus velezensis* LG37 and mining of genes related to biosynthesis of antibacterial substances, Isr. J. Aquac. Bamidgeh 74 (2022) 1–12.

[16] D.F. Zhang, X.L. Xiong, Y.J. Wang, Y.X. Gao, Y. Ren, Q. Wang, C.B. Shi, *Bacillus velezensis* WLYS23 strain possesses antagonistic activity against hybrid snakehead bacterial pathogens, J. Appl. Microbiol. 131 (6) (2021) 3056–3068.

[17] J. Wang, D. Zhang, Y. Wang, Z. Liu, L. Liu, C. Shi, Probiotic effects of the *Bacillus velezensis* GY65 strain in the Mandarin fish, Siniperca chuatsi, Aquaculture Reports 21 (2021), 100902.

[18] N.J. Varghese, S. Mukherjee, N. Ivanova, K.T. Konstantinidis, K. Mavrommatis, N. C. Kyrpides, A. Pati, Microbial species delineation using whole genome sequences, Nucleic Acids Res. 43 (14) (2015) 6761–6771.

[19] S. Heo, J.H. Kim, M.S. Kwak, M.H. Sung, D.W. Jeong, Functional annotation genome unravels potential probiotic *Bacillus velezensis* strain KMU01 from traditional Korean fermented kimchi, Foods 10 (3) (2021) 563.

[20] Q. Xiong, D. Liu, H. Zhang, X. Dong, G. Zhang, Y. Liu, R. Zhang, Quorum sensing signal autoinducer-2 promotes root colonization of *Bacillus velezensis* SQR9 by affecting biofilm formation and motility, Appl. Microbiol. Biotechnol. 104 (16) (2020) 7177–7185.

[21] B. Fan, C. Wang, X. Song, X. Ding, L. Wu, H. Wu, R. Borriss, *Bacillus velezensis* FZB42 in 2018: the gram-positive model strain for plant growth promotion and biocontrol, Front. Microbiol. 9 (2018) 2491.

[22] S.M. Lim, M.Y. Yoon, G.J. Choi, Y.H. Choi, K.S. Jang, T.S. Shin, J.C. Kim, Diffusible and volatile antifungal compounds produced by an antagonistic *Bacillus velezensis* G341 against various phytopathogenic fungi, Plant Pathol. J. 33 (5) (2017) 488.

[23] K.A. Balderas-Ruíz, P. Bustos, R.I. Santamaria, V. González, S.A. Cristiano-Fajardo, S. Barrera-Ortíz, L. Serrano-Carreón, *Bacillus velezensis* 83 a bacterial strain from mango phyllosphere, useful for biological control and plant growth promotion, Amb. Express 10 (1) (2020) 1–19.

[24] C. Jain, L.M. Rodriguez-R, A.M. Phillippy, K.T. Konstantinidis, S. Aluru, High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries, Nat. Commun. 9 (1) (2018) 1–8.

[25] M.A. Martínez-Núñez, Nonribosomal peptides synthetases and their applications in industry, Sustain. Chem. Process. 4 (1) (2016) 1–8.

[26] E.P. Abraham, D. Callow, K. Gilliver, Adaptation of *Staphylococcus aureus* to growth in the presence of certain antibiotics, Nature 158 (4023) (1946) 818–821.

[27] N. Vanittanakom, W. Loeffler, U. Koch, G. Jung, Fengycin-a novel antifungal lipopeptide antibiotic produced by Bacillus subtilis F-29-3, J. Antibiot. 39 (7) (1986) 888–901.

[28] M. Deleu, M. Paquot, T. Nylander, Effect of fengycin, a lipopeptide produced by *Bacillus subtilis*, on model biomembranes, Biophys. J. 94 (7) (2008) 2667–2679.

[29] A.M. Emam, C.A. Dunlap, Genomic and phenotypic characterization of *Bacillus velezensis* AMB-y1; a potential probiotic to control pathogens in aquaculture, Antonie Leeuwenhoek 113 (12) (2020) 2041–2052.

[30] L.M. González-Jaramillo, F.J. Aranda, J.A. Teruel, V. Villegas-Escobar, A. Ortiz, Antimycotic activity of fengycin C biosurfactant and its interaction with phosphatidylcholine model membranes, Colloids Surf. B Biointerfaces 156 (2017) 114–122.

[31] D. Zhang, R. Qiang, Z. Zhou, Y. Pan, S. Yu, W. Yuan, Z. Yang, Biocontrol and action mechanism of Bacillus subtilis lipopeptides' fengycins against *Alternaria solani* in potato as assessed by a transcriptome analysis, Front. Microbiol. 13 (2022).

[32] U.A. Zahura, M.B.R. Chowdhury, M.A.R. Faruk, Fungal infection in freshwater fishes of Mymensingh Bangladesh, Indian J. Fish. 51 (1) (2004) 61–68.

[33] P. Sarkar, V.S. Raju, G. Kuppusamy, M.A. Rahman, P. Elumalai, R. Harikrishnan, J. Arockiaraj, Pathogenic fungi affecting fishes through their virulence molecules, Aquaculture 548 (2022), 737553.

[34] M. Zhou, F. Liu, X. Yang, J. Jin, X. Dong, K.W. Zeng, D. Yang, Bacillibactin and bacillomycin analogues with cytotoxicities against human cancer cell lines from marine *Bacillus* sp. PKU-MA00093 and PKU-MA00092, Mar. Drugs 16 (1) (2018) 22.

[35] W. Raza, Q. Hussain, Q. Shen, Production, regulation and transportation of bacillibactin in *Bacillus subtilis*, J. Chem. Soc. Pakistan 34 (2012) 996–1002.

[36] K. Chakraborty, V.K. Kizhakkekalam, M. Joy, R.D. Chakraborty, Bacillibactin class of siderophore antibiotics from a marine symbiotic *Bacillus* as promising antibacterial agents, Appl. Microbiol. Biotechnol. 106 (1) (2022) 329–340.

[37] P.S. Patel, S. HuANG, S. Fisher, D. Pirnik, C. Aklonis, L. Dean, F. Mayerl, Bacillaene, a novel inhibitor of procaryotic protein synthesis produced by *Bacillus subtilis*: production, taxonomy, isolation, physico-chemical characterization and biological activity, J. Antibiot. 48 (9) (1995) 997–1003.

[38] Lorena Stannek-göbel, Stefan Pelzer, Thomas Berngruber, Claudia Borgmeier, Stella Molck, Compositions containing bacillaene producing bacteria or preparations thereof, U.S. Patent Application 17/429 (2022), 905, filed June 2.

[39] S.M. Foo, W.W.H. Eng, Y.P. Lee, K. Gui, H.M. Gan, New sequence types of *Vibrio parahaemolyticus* isolated from a Malaysian aquaculture pond, as revealed by whole-genome sequencing, Genome Announc. 5 (19) (2017), e00302-e00317.

[40] T. Nagao, K. Adachi, M. Sakai, M. Nishijima, H. Sano, Novel macrolactins as antibiotic lactones from a marine bacterium, J. Antibiot. 54 (4) (2001) 333–339.

[41] A. Ortiz, E. Sansinenea, Macrolactin antibiotics: amazing natural products, Mini Rev. Med. Chem. 20 (7) (2020) 584–600.

[42] B. Fan, J. Blom, H.P. Klenk, R. Borriss, *Bacillus amyloliquefaciens, Bacillus velezensis*, and *Bacillus siamensis* form an "operational group *B. amyloliquefaciens*" within the *B. subtilis* species complex, Front. Microbiol. 8 (2017) 22.

[43] N. Arfatahery, A. Mirshafiey, T.P. Abedimohtasab, M. Zeinolabedinizamani, Study of the prevalence of *Staphylococcus aureus* in marine and farmed shrimps in Iran aiming the future development of a prophylactic vaccine, Procedia in Vaccinology 9 (2015) 44–49.

[44] N. Arfatahery, A. Davoodabadi, T. Abedimohtasab, Characterization of toxin genes and antimicrobial susceptibility of *Staphylococcus aureus* isolates in fishery products in Iran, Sci. Rep. 6 (1) (2016) 1–7.

[45] M. Rajavel, A. Mitra, B. Gopal, Role of *Bacillus subtilis* BacB in the synthesis of bacilysin, J. Biol. Chem. 284 (46) (2009) 31882–31892.

[46] G. Özcengiz, İ. Öğülür, Biochemistry, genetics and regulation of bacilysin biosynthesis and its significance more than an antibiotic, New biotechnology 32 (6) (2015) 612–619.

[47] X.H. Chen, A. Koumoutsi, R. Scholz, A. Eisenreich, K. Schneider, I. Heinemeyer, R. Borriss, Comparative analysis of the complete genome sequence of the plant growth–promoting bacterium *Bacillus amyloliquefaciens* FZB42, Nat. Biotechnol. 25 (9) (2007) 1007–1014.

[48] KEGG, *Bacillus Velezensis* FZB42. BlastKOALA, 2007. Retrieved from on 13th September 2022 from, https://www.genome.jp/kegg-bin/show/organism?org=bay.

[49] L. Wu, H. Wu, L. Chen, S. Xie, H. Zang, R. Borriss, X. Gao, Bacilysin from *Bacillus amyloliquefaciens* FZB42 has specific bactericidal activity against harmful algal bloom species, Appl. Environ. Microbiol. 80 (24) (2014) 7512–7520.

[50] H. Abriouel, B. Pérez Montoro, C.S. Casimiro-Soriguer, A.J. Pérez Pulido, C. W. Knapp, N. Caballero Gómez, N. Benomar, Insight into potential probiotic markers predicted in *Lactobacillus pentosus* MP-10 genome sequence, Front. Microbiol. 8 (2017) 891.

[51] J.P. van Pijkeren, C. Canchaya, K.A. Ryan, Y. Li, M.J. Claesson, B. Sheil, P. W. O'Toole, Comparative and functional analysis of sortase-dependent proteins in the predicted secretome of *Lactobacillus salivarius* UCC118, Appl. Environ. Microbiol. 72 (6) (2006) 4143–4153.

[52] Y. Wang, H. Zhang, L. Zhang, W. Liu, Y. Zhang, X. Zhang, T. Sun, *In vitro* assessment of probiotic properties of Bacillus isolated from naturally fermented congee from Inner Mongolia of China, World J. Microbiol. Biotechnol. 26 (8) (2010) 1369–1377.

[53] L. Albarracin, F. Raya Tonetti, K. Fukuyama, Y. Suda, B. Zhou, A.A. Baillo, J. Villena, Genomic characterization of *Lactiplantibacillus plantarum* strains possessing differential antiviral immunomodulatory activities, Bacteria 1 (3) (2022) 136–160.

[54] M. Bull, S. Plummer, J. Marchesi, E. Mahenthiralingam, The life history of *Lactobacillus acidophilus* as a probiotic: a tale of revisionary taxonomy, misidentification and commercial success, FEMS Microbiol. Lett. 349 (2) (2013) 77–87.

[55] T.L. Nguyen, D.H. Kim, Genome-wide comparison reveals a probiotic strain *Lactococcus lactis* WFLU12 isolated from the gastrointestinal tract of olive flounder (*Paralichthys olivaceus*) harboring genes supporting probiotic action, Mar. Drugs 16 (5) (2018) 140.