

Article

In Vitro Probiotic Characterization and Whole-Genome-Based Safety Screening of *Bacillus* spp. Isolated from the Gastrointestinal Tract of *Betta splendens* Regan

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Abstract

Spore-forming *Bacillus* probiotics are widely used in aquaculture, but host-associated strains from Siamese fighting fish (*Betta splendens* Regan) remain uncharacterized. Ten *Bacillus* isolates were recovered from the gastrointestinal tract of farmed *B. splendens* by heat selection and were identified by biochemical testing and 16S rRNA gene sequencing. In vitro assays covered growth under acid, bile salts and gastrointestinal enzymes, hydrophobicity, auto-aggregation, β -galactosidase activity, antibiotic inhibition zones and antimicrobial activity. BM5 showed the highest relative growth at pH 3 and with gastrointestinal enzymes, while BM2 had the highest in 0.3% bile salts and β -galactosidase activity ($p < 0.05$). BF2 and BM2 were strongly hydrophobic. Measurable inhibition zones were recorded for all isolate–antibiotic combinations except BF3 with vancomycin. Notably, only BF1 inhibited *Pseudomonas aeruginosa*. BF1 and BM2 were selected for whole-genome sequencing. Both genomes harbored intrinsic chromosomal resistance genes characteristic of the *B. pumilus* group (*blaBPU-1*-like and *cat86*), neither of which was flanked by an insertion sequence. Homologues of 35 virulence-associated genes, including *hlyIII* and *inhA*, were identified. Neither acquired resistance determinants nor genes encoding enterotoxins or emetic toxins were detected in the database searches. However, these database-dependent findings are only a preliminary screen and do not establish safety; in vivo evaluation is warranted.

Keywords: aquaculture; *Betta splendens* gut bacteria; *Bacillus*; probiotics; gut microbiota; whole-genome sequencing (WGS)

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1. Introduction

Betta splendens Regan, the Siamese fighting fish, is a small air-breathing freshwater teleost native to the Mekong basin and one of the most widely traded ornamental fish worldwide. As a carnivorous species, it has a relatively simple digestive tract. In commercial production, fish are reared at high density and, from an early age, in small individual

containers, conditions under which bacterial disease can spread rapidly [1–3]. Thailand is a leading exporter of ornamental fish: *Betta splendens* ranks first among ornamental species in both export volume and value, contributing approximately 400 million THB (40% of total ornamental fish exports) and holding an 11% share of the global market [4]. This trade sits within a national aquaculture sector producing approximately 0.9 million tonnes annually [5].

The rapid growth of aquaculture production has raised concerns, particularly about bacterial infections that affect fish health and product quality. Antibiotics are widely used in aquaculture for treatment, prophylaxis, and metaphylaxis. However, inadequate regulation and enforcement in many producing countries, together with the lack of antibiotics developed specifically for aquaculture, have raised concerns about antimicrobial misuse and resistance [6–10]. Consequently, probiotics have gained increasing attention as potential alternatives to antibiotics for disease prevention in aquaculture [11–13].

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host [14]. Several bacteria have been used as probiotics [15–17], among which *Bacillus* species are suitable candidates because they grow under both aerobic and facultative anaerobic conditions, produce antimicrobial compounds active against pathogens [11,18,19], and form spores that withstand the heat and stress of feed preparation [20–23]. *Bacillus* probiotics have been isolated from the environment and the digestive tracts of humans, terrestrial animals, and aquaculture animals, with carp and other farmed freshwater fish (e.g., *Labeo rohita*, *Labeo calbasu*, *Oreochromis niloticus*, *Onychorhynchus mykiss*) being common sources [24–29].

Probiotic candidates are traditionally selected using in vitro assays that simulate key probiotic functions. These include tolerance to acid and bile salts, adhesion to mucus or epithelial cells, and production of antimicrobial substances [30,31]. To complement these functional assays, genomics-based assessment of probiotic candidates has been increasingly adopted [32]. Because probiotics are intended as alternatives to antibiotics, candidate strains themselves must not carry acquired resistance determinants. Phenotypic antibiotic susceptibility testing, combined with genomic screening for resistance and virulence genes, has therefore become a standard validation requirement; both approaches were applied here.

Despite this interest, the gastrointestinal (GI) tracts of many fish species remain unexplored. The gut microbiota of *B. splendens* has been described only at the community level [33], and to our knowledge no *Bacillus* isolate from this host has been characterized phenotypically or genomically. Whether this fish carries strains suitable as autochthonous probiotics is therefore unknown.

In this study, we obtained ten heat-selected *Bacillus* isolates from the gastrointestinal tract of *B. splendens*, characterized them using in vitro probiotic assays benchmarked against *B. subtilis* TISTR1248, and screened the complete genomes of the two leading candidates for resistance genes, virulence factors and biosynthetic gene clusters. To our knowledge, this is the first study to combine phenotypic probiotic screening with complete-genome safety assessment of *Bacillus* isolated from *B. splendens*.

2. Materials and Methods

2.1. Isolation of Heat-Selected *Bacillus* spp.

The *Bacillus* strains were recovered using a previously described method with some modifications [34,35]. Briefly, archived digestive tract samples of six four-month-old *B. splendens* were obtained from [33] under animal license AR05/63. The fish were reared as described in the original study [33]. Guts were pooled into two batches according to sex, homogenized with a tissue grinder and suspended in 1 mL of normal saline (0.85% NaCl) by vigorous mixing. The homogenates were incubated in a water bath at 80 °C for 5 min

and 100 µL aliquots were spread onto each of six nutrient agar (NA) plates. The plates were incubated at 37 °C for up to three days. Ten colonies, five from the male and five from the female batch, were randomly selected and purified by streaking onto individual nutrient agar (NA) plates under sterile conditions. All isolates were morphologically characterized using a light microscope and stored at −80 °C in nutrient broth (NB) supplemented with 20% (*v/v*) glycerol (final concentration).

2.2. DNA Extraction and Molecular Analysis

Genomic DNA was extracted from pure colonies using a commercial silica column kit (iNtRON Biotechnology Inc., Seongnam-si, Republic of Korea) according to the manufacturer's specifications. An approximately 400 bp fragment of the 16S rRNA gene was amplified by polymerase chain reaction (PCR) with the universal primer pair Uni338F forward (5'-ACTCCTACGGGAGGCAGCAG-3') and Uni797R reverse (5'-GGACTAC-CAGGGTATCTATCCTGTT-3') [36]. Amplicons were purified using a commercial DNA purification kit (iNtRON Biotechnology Inc., Seongnam-si, Republic of Korea) and sequenced in both forward and reverse directions using the amplification primers (Bionics Co., Ltd., Seoul, Republic of Korea). Kits, reagents, thermal cycling conditions and the sequencing provider are detailed in Supplementary Methods S1. Sequences were compared against the GenBank database using BLAST (<https://blast.ncbi.nlm.nih.gov/>) to assess sequence similarity, and the ten sequences were submitted to NCBI under accession numbers OR186679-OR186687 and OR186704.

2.3. Biochemical Characterization and Identification of *Bacillus* Isolates

Pure colonies were characterized biochemically with a commercial *Bacillus* identification kit (HiMedia Laboratories Pvt. Ltd., Thane, India), following the manufacturer's instructions. The isolates were examined using sterile media for malonate, Voges-Proskauer, citrate, o-nitrophenyl-β-D-galactopyranoside (ONPG), nitrate reduction, catalase, arginine and five carbohydrate utilization tests: sucrose, mannitol, glucose, arabinose and trehalose. Biochemical profiles were compared with those of the *Bacillus* reference strains supplied with the kit. The kit, its manufacturer, and the reference strains are detailed in Supplementary Methods S2.

2.4. Characterization of Probiotic Properties

The probiotic properties of all ten isolates were investigated in vitro as outlined below. The turbidity of the cultures corresponded to 10⁷–10⁸ CFU/mL and was assessed using the 0.5 McFarland standard as a reference prior to use in the probiotic property assays. Growth in the acid, bile salt, pepsin, pancreatin and lysozyme assays was quantified as optical density relative to an untreated control. These values describe changes in turbidity only; they do not distinguish vegetative cells from spores, or viable from non-viable cells, and are therefore reported throughout as relative growth rather than survival. "Tolerance" is used operationally to denote maintenance of relative growth under the stated condition.

2.4.1. Hemolytic Activity and Biofilm Formation Test (Congo Red Agar Method)

Hemolytic activity, as an indicator of potential pathogenicity, was examined on blood agar supplemented with 5% (*w/v*) sheep blood following a previously described procedure [37]. Plates were incubated at 37 °C for 24–48 h to detect patterns of hemolytic activity: α-hemolysis, β-hemolysis or γ-hemolysis (no hemolysis). To examine biofilm formation, all isolates were streaked on Mueller–Hinton agar (HiMedia, Thane, India) containing 0.8 g/L of Congo red dye and incubated at 37 °C for 48 h. Black colonies indicated biofilm formation, while red denoted no biofilm [38]. Details of media and reagents are provided in Supplementary Methods S3.

2.4.2. Acid (pH) and Bile Salt Tolerance Assays

Acid (pH) tolerance was assessed using a modified version of the method described by [39,40]. Briefly, overnight cultures of each isolate were centrifuged ($12,000\times g$, 5 min, $4\text{ }^{\circ}\text{C}$) and resuspended in nutrient broth (NB) at pH 1.0, 2.0, 3.0 and 5.0 (adjusted with 1 M HCl or 1 M NaOH), while pH 7.0 was used as a control. Culture density was recorded as optical density at 600 nm after incubation at $37\text{ }^{\circ}\text{C}$ for 3 h. Relative growth was calculated following the method of [41], as described in Supplementary Equation S1.

Tolerance of the isolates to bile salts was assessed as previously described [39,42], with slight modifications. An inoculum suspension (2% *v/v*) of each isolate was added to NB containing different concentrations of bile salt (0.3, 0.5, and 1% *w/v*) and incubated at $37\text{ }^{\circ}\text{C}$ for 24 h. Culture density was estimated by optical density at 560 nm and compared with the control (isolate inoculum without bile salt). Relative growth was calculated following the method of [41], as described in Supplementary Equation S2.

2.4.3. Pepsin Tolerance Assay

The tolerance of isolates to pepsin was evaluated in nutrient broth (NB) supplemented with pepsin (3 mg/mL) using a previously described method [39,43,44], with slight modifications. Briefly, overnight cultures of isolates were centrifuged at $12,000\times g$ for 5 min at $4\text{ }^{\circ}\text{C}$. The cells were washed twice with phosphate buffer (pH 7 ± 0.2) and were suspended in NB containing pepsin at a pH of 1.0, 2.0 or 3.0 and incubated at $37\text{ }^{\circ}\text{C}$ for 3 h. NB without pepsin at pH 7.0 was used as the control. Growth of the isolates was monitored by measuring optical density at 600 nm after the incubation period. The growth relative to the untreated control was calculated as in Supplementary Equation S3.

2.4.4. Pancreatin Tolerance Assay

Pancreatin tolerance was determined according to the method described by [39,43,44], with some modifications. Cells grown overnight were centrifuged at $12,000\times g$ for 5 min at $4\text{ }^{\circ}\text{C}$ and washed twice with PBS (phosphate-buffered saline, pH 7.3 ± 0.2). The cells were resuspended in NB supplemented with pancreatin (1 mg/mL) at pH 8.0 and incubated at $37\text{ }^{\circ}\text{C}$ for 3 h. NB without pancreatin at pH 7.0 was used as the control. Isolates were monitored for growth by measuring optical density at 600 nm after the incubation period. The growth relative to the untreated control was calculated as in Supplementary Equation S4.

2.4.5. Lysozyme Activity

Lysozyme response was assessed according to a previously reported method [45] with a slight modification. A 0.3 mL aliquot of overnight culture was inoculated into 10 mL of NB and incubated at $37\text{ }^{\circ}\text{C}$ for 1 h. A total of 1 mL of lysozyme (100 $\mu\text{g/mL}$) was then added to the growing cultures. Growth of the isolates was monitored at 0, 1, 2, 3, and 4 h by measuring optical density at 600 nm (OD_{600}). Isolates were described by the shape of their OD_{600} growth curve relative to the lysozyme-free control as follows: growth unaffected (U), the curve was indistinguishable from the control; growth reduced (R_0), the rate of increase in turbidity was lower than the control; and growth arrested (A), no further increase in turbidity was recorded after lysozyme addition. These descriptors refer to turbidity only and are not susceptibility categories.

2.4.6. β -Galactosidase Activity

β -galactosidase activity was determined according to the method of [42] with some modifications. Briefly, the isolates were grown overnight in NB, and cells were centrifuged at $12,000\times g$ for 5 min at 4 °C. The pellet was washed twice with 60 mM $\text{Na}_2\text{HPO}_4\cdot 7\text{H}_2\text{O}$ /40 mM NaH_2PO_4 buffer (pH 7.0), permeabilized with 50 μL of toluene/acetone (1:9 *v/v*) solution, and mixed vigorously for 7 min. A total of 100 μL of the permeabilized cell suspension was placed in a tube, and 900 μL of phosphate buffer and 200 μL of *o*-nitrophenyl- β -D-galactopyranoside (ONPG) (4 mg/mL) were added. The mixture was incubated at 37 °C for 15 min in a water bath and then 0.5 mL of 1 M Na_2CO_3 was added to each tube to stop the reaction. The OD was recorded at 420 and 560 nm for each tube. The β -galactosidase activity was expressed as Miller units and calculated as described in Supplementary Equation S5.

2.4.7. Exopolysaccharide (EPS) Production

The ability of isolates to produce exopolysaccharide (EPS) was evaluated on NA supplemented with 100 g/L of sucrose, as described by [46] with a slight modification. Overnight cultures of each isolate were streaked on NA plates and incubated at 37 °C for 24 h. Colonies above 1.5 mm in diameter were considered positive for slimy product formation.

2.4.8. Proteolytic Activity

The proteolytic activity was measured following [46] with slight modifications. Briefly, 10 μL of each culture was applied to sterile Whatman paper disks, which were then placed on the surface of NA supplemented with 5% skim milk, and incubated for 24 h at 37 °C. A clear zone around the disk was considered proteolytic activity. The clear zone was measured along two perpendicular axes, *x* (A) and *y* (B).

2.4.9. Lipolytic Activity

The lipolytic activity was determined according to the method described previously [46] with minor modifications. A 10 μL aliquot of each overnight culture was applied to sterile Whatman paper disks. Inoculated disks were placed on the surface of NA supplemented with Tween-20 (1, 3, 5%) and incubated at 25 °C for 72 h. The formation of an opaque area was considered positive for lipolytic activity. The opaque area was measured along two perpendicular axes, *x* (A) and *y* (B).

2.4.10. Auto-Aggregation Assay

Specific cell–cell interactions were determined using a modified auto-aggregation assay [47,48]. The selected isolates were grown in nutrient broth (NB) at 37 °C for 24 h. Overnight cultures were harvested by centrifugation, washed twice with phosphate-buffered saline (PBS) and resuspended in 2 mL of PBS. The bacterial suspensions were then incubated at 37 °C. An aliquot (1 mL) was carefully removed from the upper layer of the suspension, and its absorbance was measured at 0 h (A_0), 3 h, 6 h and 24 h (A_t) of incubation using a UV/visible spectrophotometer at 600 nm. Auto-aggregation was calculated as a percentage of the initial optical density as described in Supplementary Equation S6.

2.4.11. Cell Surface Hydrophobicity

Cell surface hydrophobicity of isolates was measured according to the method described by [49] with some modifications. Overnight cultures were collected by centrifugation at $12,000\times g$ for 5 min at 4 °C and washed twice with PBS buffer (pH 7) and resuspended in the same buffer to an OD_{600} of approximately 0.5 (corresponding to 10^7 – 10^8 CFU/mL, as described above). The cell suspension was transferred to a new test tube and

toluene (1 mL) was added. The tube was vigorously mixed for 3 min and incubated at 37 °C for 1 h for phase separation. The aqueous phase was carefully removed, and cell surface hydrophobicity was determined by measuring the optical density at 600 nm before and after the addition of toluene. Hydrophobicity was calculated as described in Supplementary Equation S7. Isolates showing values below 50% were considered hydrophilic, whereas those showing values of 50% or higher were considered hydrophobic.

2.4.12. Antibiotic Inhibition Zone Assay

Antibiotic inhibition zones were measured by disk diffusion, following [43,44]. A 100 µL aliquot of each isolate's culture was spread onto NA; antibiotic disks were placed on the plates and incubated for 24–48 h at 37 °C. The disk diffusion method was applied to measure inhibition zones for clinically important antibiotics, which included ampicillin (10 µg), vancomycin (10 µg), streptomycin (10 µg), and chloramphenicol (30 µg). The antibiotic activity was visualized as a zone of inhibition around the antibiotic disks. The disk diffusion method followed CLSI M02 guidelines for methodology only; interpretive breakpoints were not applied, as CLSI M45 MIC criteria and EFSA FEEDAP microbiological cut-offs for *Bacillus* spp. are not directly applicable to disk diffusion zone diameters. Inhibition zone diameters were measured along two perpendicular axes (A and B).

2.4.13. Antimicrobial Activity

Antimicrobial activity of the isolates against three bacterial pathogens, namely *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* was determined by the standard well diffusion method [50] with a slight modification. Pathogen strains were obtained from the laboratory culture collection; specific strain designations were not recorded. Overnight grown pathogenic bacteria were spread on NA plates. Wells were cut into the agar, and each was filled with 100 µL of whole overnight culture from one isolate. Plates were incubated at 37 °C for 24–48 h. The diameter of the inhibition zone was measured and recorded as positive when the clear zone measured ≥ 2 mm in diameter. The diameter of the zone of inhibition was measured with axis x (A) and axis y (B).

2.5. Genome Sequencing and Bioinformatic Analysis

Two isolates, BF1 and BM2, were selected for genome sequencing based on their complementary probiotic properties. BM2 exhibited superior bile salt tolerance at 0.3%, β -galactosidase activity and 24 h auto-aggregation and ranked second in hydrophobicity and relative growth at pH 5.0. BF1 was the sole isolate displaying antagonistic activity against *P. aeruginosa*. Together, the two strains also represented both the male and female batches. DNA libraries were performed using the Native Barcoding Kit 24 V14 (SQK-NBD114.24; Oxford Nanopore Technologies (Oxford, UK) according to the manufacturer's instructions. Barcoded samples were pooled and loaded onto an R10.4.1 (FLO-MIN114) flow cell. Sequencing was carried out on an ONT MinION Mk1D platform, following the standard ONT ligation sequencing protocol. Basecalling was performed using Dorado (v1.3.0) with the super-high-accuracy (SUP) model to generate a FASTQ file [51]. All software tools were executed with default parameters unless otherwise noted. Subsequently, NanoPlot (v1.42.0) was used to visualize sequencing metrics and assess data quality prior to downstream analysis De Coster [52]. Raw reads were processed to remove adapter sequences using Porechop (v0.2.4) [53]. Reads shorter than 1000 bp or with a mean Phred quality score below 12 were filtered out using NanoFilt (v2.6.0) [52]. De novo assembly of the filtered long reads was performed with Flye (v2.9) with an estimated genome size of 4.3 Mb, followed by three rounds of polishing using Medaka (v1.8.0) [54]. Genome assembly quality was assessed with QUAST (v5.2.0) [55]. Genome completeness estimation was performed through the identification of Benchmarking Universal Single-Copy Orthologs

(BUSCO v6.0.0) [56], using the bacillales_odb10 lineage dataset, which contains 450 BUSCO markers. Genome annotation was subsequently performed using Prokka (v1.14.6) [57]. Average nucleotide identity (ANI) values between the BF1 and BM2 genomes and 41 complete circular chromosomes of *B. safensis* were computed and visualized using ANI-clustermap (v2.0.1) (<https://github.com/moshi4/ANIclustermap>, accessed on 10 January 2026). Functional annotation and pathway mapping were performed using the KEGG Automatic Annotation Server (KAAS) [58]. Genes were assigned KEGG Orthology (KO) identifiers and mapped to metabolic pathways using the bi-directional best hit (BBH) method; the server was accessed on 25 December 2025. Finally, circular genome maps were generated using GenoVi (v0.4.3) [59]. Antimicrobial resistance (AMR) genes were identified using ABRicate (v1.0.1) [60] with default thresholds of $\geq 80\%$ nucleotide identity and $\geq 80\%$ coverage. Screening was performed against multiple reference databases, including ResFinder, CARD, and NCBI. The reference databases were updated or retrieved on 1 September 2026. Identified hits were classified as intrinsic or acquired on the basis of their chromosomal location and reported distribution within the *B. pumilus* group; this classification is inferential and was not confirmed experimentally. To examine the genomic context of the resistance determinants, insertion sequences were predicted with ISEScan v1.7.3 using default parameters [61]. An insertion sequence was considered to flank a resistance gene when located within 10 kb of that gene on the same replicon [62,63]. Other classes of mobile genetic element, including plasmids, integrative conjugative elements and integrons, were not specifically screened. Additionally, putative virulence factors were predicted by performing BLAST searches against the Virulence Factor Database (VFDB) [64] with default thresholds of $\geq 90\%$ nucleotide identity and $\geq 80\%$ coverage. The VFDB reference set available for *Bacillus* is derived largely from the *B. cereus* group; hits are therefore reported as homologues of virulence-associated genes and are not evidence of a virulent phenotype. The phylogenomic tree was constructed using conserved house-keeping genes identified by autoMLST2 with 1000 bootstrap replicates (<https://automlst2.cs.uni-tuebingen.de/index>, accessed on 10 January 2026) [65] and visualized using iTOL v7 (<https://itol.embl.de/>, accessed on 10 January 2026). The selected genes were aligned using MAFFT, and the concatenated alignment was used for phylogenetic reconstruction with IQ-TREE. Genome-based taxonomic relationships were additionally assessed using the Type (Strain) Genome Server (TYGS; <https://tygs.dsmz.de/>, accessed on 10 January 2026), which uses Genome BLAST Distance Phylogeny (GBDP) and digital DNA–DNA hybridization (dDDH) for genome-based species delineation [66]. Putative secondary metabolite biosynthetic gene clusters (BGCs) in the genomes of *Bacillus* spp. were predicted using antiSMASH (v8.0.4) with default parameters and relaxed stringency [67]. Additionally, putative bacteriocins were identified using BAGEL4 online web server [68]. Gene-cluster comparisons were performed using the CAGECAT bioinformatics clinker tool with a minimum alignment sequence identity of 80% [69]. The complete genome sequences of isolate BF1 and BM2 are available in NCBI GenBank under BioProject number PRJNA1459368 and BioSample accession numbers SAMN57545553 and SAMN57545851.

2.6. Statistical Analysis

All experiments were performed in three independent biological replicates ($n = 3$). Results are presented as mean $\pm$ standard deviation (SD). One-way ANOVA followed by Dunnett's test was used to compare each isolate with the control (*B. subtilis* TISTR1248). Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. *Bacillus* Identification and Screening for Pathogenic Activity

All ten isolates were recovered from the digestive tracts of *B. splendens*. Colonies were white and creamy with a jelly-like surface on NA plates and no pigmentation. Gram staining revealed that all isolates were Gram-positive rods. Isolates were non-motile and grew aerobically. All ten isolates exhibited γ -hemolysis and did not form biofilms on Congo red agar.

3.2. Biochemical Characterization of the Isolates

A commercial *Bacillus* identification kit was used to identify *Bacillus* species among all ten strains (Table 1). The kit and its manufacturer are detailed in Supplementary Methods S2. Almost all isolates were positive for citrate utilization, nitrate reduction, catalase activity and arginine dihydrolase. Additionally, three isolates (BF2, BF5, and BM1) hydrolyzed ONPG, while glucose and trehalose were utilized by BF4 and BM2, respectively. Other substrates, including malonate, Voges–Proskauer’s, sucrose, mannitol, and arabinose, were not utilized by any of the isolates. Notably, the ONPG result for the *B. subtilis* TISTR1248 control differed from that reported for *B. subtilis* in the kit’s identification index: the control was negative for ONPG, whereas *B. subtilis* is typically positive for ONPG. This discrepancy may reflect strain-specific biochemical variation within *B. subtilis* or differences in assay conditions. The arginine result for the control (negative) was consistent with the reference profile. Therefore, the biochemical profiles reported here should be interpreted as comparative data among isolates rather than as definitive species-level identification.

Table 1. Biochemical tests used to identify the ten *Bacillus* strains. +, positive; −, negative; D, delayed reaction; NR, not reported.

Tests		Identification Index of Various <i>Bacillus</i> Species											
		Malonate	Voges Proskauer’s	Citrate	ONPG	Nitrate Reduction	Catalase	Arginine	Sucrose	Mannitol	Glucose	Arabinose	Trehalose
Isolates	BF1	−	−	+	−	+	+	+	−	−	−	−	−
	BF2	−	−	+	+	+	+	+	−	−	−	−	−
	BF3	−	−	−	−	+	+	+	−	−	−	−	−
	BF4	−	−	+	−	+	+	+	−	−	+	−	−
	BF5	−	−	+	+	+	+	+	−	−	−	−	−
	BM1	−	−	+	+	−	+	−	−	−	−	−	−
	BM2	−	−	−	−	−	+	−	−	−	−	−	+
	BM3	−	−	+	−	+	+	+	−	−	−	−	−
	BM4	−	−	−	−	+	+	−	−	−	−	−	−
	BM5	−	−	+	−	+	+	+	−	−	−	−	−
Control	<i>B. subtilis</i> TISTR1248	−	−	+	−	+	+	−	−	−	+	−	+
Standard references	<i>B. cereus</i>	−	+	+	−	D	+	D	D	−	+	−	+
	<i>B. pumilus</i>	−	+	+	+	−	+	−	+	+	+	+	+
	<i>B. subtilis</i>	−	+	+	+	+	+	−	+	+	+	+	+
	<i>B. coagulans</i>	−	D	−	D	−	+	D	D	−	+	D	+

<i>B. atrophaeus</i>	–	+	+	NR	+	+	NR	+	+	+	+	+
<i>B. megaterium</i>	+	–	+	+	D	+	–	+	+	+	+	+
<i>B. thuringiensis</i>	NR	+	+	–	+	+	+	D	–	+	–	+

3.3. Identification by 16S rRNA Gene Sequencing

Sequencing of the 16S rRNA gene identified all selected isolates as *Bacillus* spp., with *B. pumilus* as the top hit in BLAST searches using the new sequences as queries. The probiotic potential of all isolates was characterized and compared with the control *B. subtilis* TISTR1248 (Thailand Institute of Scientific and Technological Research; TISTR). The results are presented in the following sections.

3.4. In Vitro Assessments

3.4.1. Relative Growth Under Acidic Conditions and Bile Salt Exposure

The relative growth (%) of all isolates varied considerably with pH, with mean values of 6.89 ± 4.05 , 17.69 ± 5.72 , 29.77 ± 9.38 and $58.02 \pm 14.38\%$ at pH 1.0, 2.0, 3.0 and pH 5.0 in NB medium, respectively. At pH 1.0, isolates BF2, BF3, BF4, BF5, BM2, and BM4 displayed significantly higher relative growth than *B. subtilis* TISTR1248; readings were not available for BF1, BM1, BM3, and BM5. At pH 2.0, all isolates except BF1, BF3, BM3 and BM4 displayed significantly higher relative growth than the control, whereas at pH 3.0, all isolates except BF1 and BF3 displayed significantly higher relative growth than the control. At pH 5.0, all isolates except BF3 and BM4 displayed significantly higher relative growth than the control. Relative growth of all isolates was assessed at bile salt concentrations of 0.3%, 0.5% and 1% (Table 2). The growth of the isolates was 36.07 ± 15.96 , 28.49 ± 12.81 , and $25.84 \pm 11.59\%$ at 0.3, 0.5, and 1% bile salt, respectively. Relative growth at 0.3% bile salt was highest in BM2 and lowest in BF5; at 0.5% and 1%, it was highest in BF2 and lowest in BF5. All isolates also maintained measurable relative growth at 0.5% and 1% bile salt. When compared with *B. subtilis* TISTR1248 and the other strains, the relative growth (%) of BF2 and BM2 was significantly higher at 0.3% bile salt ($p < 0.05$). Additionally, BF2, BM2 and BM3 showed significantly higher relative growth at 0.5% bile salt, while BF2 and BM2 showed significantly higher relative growth than *B. subtilis* TISTR1248 at 1% bile salt ($p < 0.05$).

Table 2. Relative growth (OD-based) of the ten isolates and the reference strain under acidic conditions and in the presence of bile salts. Results are shown as mean $\pm$ SD ($n = 3$). Values are optical density relative to the untreated control (OD₆₀₀ for acid, OD₅₆₀ for bile salts) and describe relative turbidity; they are not viable cell counts. * Significant difference compared with *B. subtilis* TISTR1248 at $p < 0.05$, with values higher than those of the control. ^a n.a., not available; readings could not be obtained. Mean values were calculated from the ten isolates only, excluding the reference strain *B. subtilis* TISTR1248. At pH 1.0, mean and SD were calculated from six isolates (BF2, BF3, BF4, BF5, BM2, BM4) because readings were not available for BF1, BM1, BM3, and BM5.

Probiotic Properties Test		Relative Growth (%) Under Acidic Conditions				Relative Growth (%) in the Presence of Bile Salts (w/v)		
		pH 1.0	pH 2.0	pH 3.0	pH 5.0	0.3%	0.5%	1%
Control	<i>B. subtilis</i> TISTR1248	1.37 ± 0.13	13.51 ± 0.06	15.67 ± 0.75	39.05 ± 0.18	56.28 ± 0.16	37.15 ± 0.08	38.00 ± 0.35
Isolates	BF1	n.a. ^a	11.12 ± 0.90	16.97 ± 0.85	44.31 ± 1.00 *	28.80 ± 0.59	27.68 ± 0.58	27.50 ± 0.60
	BF2	9.95 ± 0.08 *	15.52 ± 0.03 *	27.09 ± 0.15 *	63.37 ± 0.09 *	58.10 ± 0.52 *	49.34 ± 0.39 *	45.80 ± 0.52 *

BF3	3.59 ± 0.05 *	12.72 ± 0.15	16.12 ± 0.16	39.49 ± 0.41	20.41 ± 0.03	14.44 ± 0.01	13.67 ± 0.06
BF4	12.95 ± 0.13 *	25.08 ± 0.11 *	37.69 ± 0.31 *	72.66 ± 0.16 *	27.07 ± 0.14	16.94 ± 0.32	11.30 ± 0.07
BF5	8.93 ± 0.02 *	19.81 ± 0.16 *	31.08 ± 0.23 *	64.46 ± 0.33 *	16.13 ± 0.51	12.44 ± 0.62	10.65 ± 0.06
BM1	n.a. ^a	19.70 ± 0.24 *	39.13 ± 0.72 *	47.33 ± 0.46 *	25.49 ± 0.35	22.24 ± 0.30	21.80 ± 0.74
BM2	2.30 ± 0.02 *	16.71 ± 0.07 *	32.10 ± 0.24 *	76.87 ± 0.08 *	69.98 ± 0.51 *	47.01 ± 0.33 *	39.16 ± 0.25 *
BM3	n.a. ^a	12.33 ± 0.29	29.27 ± 1.08 *	54.57 ± 0.24 *	43.64 ± 0.59	38.45 ± 0.28 *	34.31 ± 0.35
BM4	3.63 ± 0.16 *	14.44 ± 0.65	22.60 ± 1.08 *	39.87 ± 1.55	25.00 ± 0.63	21.88 ± 0.60	22.81 ± 0.44
BM5	n.a. ^a	29.48 ± 0.33 *	45.67 ± 0.99 *	77.23 ± 0.85 *	46.07 ± 1.41	34.49 ± 0.23	31.43 ± 0.84
Mean of all isolates	6.89 ± 4.05	17.69 ± 5.72	29.77 ± 9.38	58.02 ± 14.38	36.07 ± 15.96	28.49 ± 12.81	25.84 ± 11.59

3.4.2. Relative Growth in the Presence of Gastrointestinal Enzymes and Lysozyme

The percentage relative growth of the ten isolates after exposure to pepsin ranged from 7.74 to 20.88% at pH 1.0, 9.08 to 24.88% at pH 2.0, and 14.71 to 38.12% at pH 3.0 (Table 3). At pH 3.0, relative growth was highest in BM5 and lowest in BM4. Among all isolates, BF1, BF2, BF4, BF5, BM1, BM3 and BM5 had significantly higher relative growth than *B. subtilis* TISTR1248 at pH 3.0 ($p < 0.05$). Moreover, all isolates showed measurable relative growth in the presence of pancreatin at pH 8.0, with a mean relative growth of $22.27 \pm 7.97\%$. Isolate BM5 had the highest relative growth (41.89%), while BM4 had the lowest (12.53%). All isolates showed significantly higher relative growth with pancreatin than *B. subtilis* TISTR1248, except for BF2, BF3, BM3, and BM4 at pH 8.0 ($p < 0.05$). In the lysozyme assay, six isolates showed growth arrest after lysozyme addition, two showed reduced growth, and two showed curves indistinguishable from their controls. *B. subtilis* TISTR1248 and isolates BF2 and BF4 showed unaffected growth curves (Table 3).

Table 3. Relative growth (OD-based) of the ten isolates and the reference strain in the presence of pepsin and pancreatin, and growth response to lysozyme. Results are presented as mean ± SD of triplicates ($n = 3$). * Significant difference compared with *B. subtilis* TISTR1248 at $p < 0.05$, with values higher than those of the control. Isolates were described by the shape of their OD₆₀₀ growth curve as growth unaffected (U), growth reduced (R₀), or growth arrested (A), as defined in Section 2.4.5. These descriptors refer to turbidity only and are not susceptibility categories. All values are OD-derived relative growth and do not represent survival or viable cell numbers.

Probiotic Properties Test		Relative Growth (%) with Pepsin			Relative Growth (%) with Pancreatin	Lysozyme Activity (100 µg Lysozyme/mL)
		pH 1.0	pH 2.0	pH 3.0	pH 8.0	
Control	<i>B. subtilis</i> TISTR1248	12.88 ± 0.13	15.97 ± 0.11	17.58 ± 0.04	18.85 ± 0.09	U (growth unaffected)
Isolates	BF1	13.29 ± 0.38	14.01 ± 0.55	19.39 ± 0.76 *	22.14 ± 0.64 *	A (growth arrested)
	BF2	11.38 ± 0.03	12.79 ± 0.20	18.39 ± 0.30 *	18.42 ± 0.10	U (growth unaffected)
	BF3	10.39 ± 0.14	11.53 ± 0.12	16.09 ± 0.32	16.23 ± 0.20	A (growth arrested)
	BF4	18.22 ± 0.18 *	19.72 ± 0.60 *	36.88 ± 0.49 *	29.56 ± 0.47 *	U (growth unaffected)
	BF5	15.14 ± 0.01 *	16.07 ± 0.10	22.01 ± 0.28 *	19.42 ± 0.10 *	A (growth arrested)
	BM1	19.31 ± 0.31 *	19.32 ± 0.27 *	25.17 ± 0.37 *	25.13 ± 0.33 *	R ₀ (growth reduced)

BM2	13.13 ± 0.24	14.52 ± 1.21	18.04 ± 0.65	19.55 ± 0.04 *	A (growth arrested)
BM3	8.28 ± 0.15	13.00 ± 0.44	19.37 ± 0.20 *	17.81 ± 0.05	A (growth arrested)
BM4	7.74 ± 0.35	9.08 ± 0.32	14.71 ± 0.54	12.53 ± 0.74	A (growth arrested)
BM5	20.88 ± 0.29 *	24.88 ± 0.48 *	38.12 ± 0.21 *	41.89 ± 0.41 *	R ₀ (growth reduced)
Mean of all isolates	13.78 ± 4.44	15.49 ± 4.35	22.82 ± 7.51	22.27 ± 7.97	

3.4.3. Exopolysaccharide Production and Enzyme Activities

All isolates produced slime (EPS) on sucrose-supplemented NA with colony diameters above 1.5 mm. All isolates had proteolytic activity, with clear-zone diameters of 17.07 ± 3.26 mm (A) and 16.87 ± 3.23 mm (B). The highest proteolytic activity was observed in BF3 and BM4, with clear zones of 20.00 ± 0.00 mm (A) and 20.33 ± 0.06 mm (B), respectively. The lowest activity was observed in BM5, with clear zones of 10.67 ± 0.06 mm (A) and 10.67 ± 0.06 mm (B). BF3 and BM4 had significantly higher proteolytic activity than *B. subtilis* TISTR1248 ($p < 0.05$). All isolates had lipolytic activity at 1% Tween 20 (Table 4). Five isolates had lipolytic activity at 3% Tween 20. None of the isolates had lipolytic activity at 5% Tween 20. Isolates BF3, BF5, BM2, and BM4 showed significantly higher lipolytic activity than *B. subtilis* TISTR1248 at 1% Tween 20. β -galactosidase activity ranged from 514.97 to 1103.21 Miller units. The highest value was obtained from isolate BM2 (1103 Miller units), while BF1 showed the lowest activity. Only BM2 showed significantly higher activity than *B. subtilis* TISTR1248 ($p < 0.05$).

Table 4. Proteolytic, lipolytic and β -galactosidase activity of the ten isolates characterized in this study. Data are presented as mean ± SD ($n = 3$). ND = not detected. * Significant difference compared with *B. subtilis* TISTR1248 at $p < 0.05$, with values higher than the control.

Probiotic Properties Test		Proteolytic Activity		Lipolytic Activity (mm)						β-Galactosidase (Miller Unit)
		(mm)		1% Tween 20		3% Tween 20		5% Tween 20		
		A	B	A	B	A	B	A	B	
Control	<i>B. subtilis</i> TISTR1248	19.67 ± 0.15	18.33 ± 0.12	17.67 ± 0.35	17.67 ± 0.35	ND	ND	ND	ND	1021.91 ± 1.00
Isolates	BF1	13.00 ± 0.17	13.00 ± 0.17	17.67 ± 0.12	17.67 ± 0.12	ND	ND	ND	ND	514.97 ± 0.00
	BF2	19.00 ± 0.10	19.00 ± 0.10	16.67 ± 0.38	16.67 ± 0.38	13.50 ± 0.07	13.50 ± 0.07	ND	ND	557.30 ± 0.58
	BF3	20.00 ± 0.00 *	20.00 ± 0.00 *	21.00 ± 0.10 *	21.00 ± 0.10 *	ND	ND	ND	ND	786.30 ± 0.58
	BF4	15.00 ± 0.10	15.33 ± 0.15	18.00 ± 0.17	18.00 ± 0.17	19.30 ± 0.06	19.30 ± 0.06	ND	ND	622.96 ± 1.00
	BF5	18.00 ± 0.10	17.67 ± 0.12	21.00 ± 0.17 *	21.00 ± 0.17 *	14.00 ± 0.00	14.00 ± 0.00	ND	ND	624.30 ± 1.15
	BM1	19.67 ± 0.12	18.33 ± 0.06	16.00 ± 0.36	16.00 ± 0.36	ND	ND	ND	ND	883.60 ± 3.51
	BM2	19.33 ± 0.06	19.33 ± 0.06	19.33 ± 0.12 *	19.33 ± 0.12 *	14.00 ± 0.00	14.00 ± 0.00	ND	ND	1103.21 ± 0.58 *
	BM3	16.00 ± 0.01	15.00 ± 0.10	17.33 ± 0.06	17.33 ± 0.06	ND	ND	ND	ND	611.30 ± 0.58
	BM4	20.00 ± 0.00 *	20.33 ± 0.06 *	20.00 ± 0.10 *	20.00 ± 0.10 *	18.00 ± 0.26	18.00 ± 0.26	ND	ND	629.96 ± 2.65
	BM5	10.67 ± 0.06	10.67 ± 0.06	15.67 ± 0.12	15.67 ± 0.12	ND	ND	ND	ND	516.65 ± 2.08

3.4.4. Hydrophobicity and Auto-Aggregation Assays

Auto-aggregation rates ranged from 4.35% to 41.28% at 3 h, 12.18% to 45.17% at 6 h, and 46.02% to 75.83% at 24 h after incubation (Table 5). Among the isolates, BM2 had the highest auto-aggregation rate (75.83%), while BM5 showed the lowest rate (46.02%) at 24 h. The control, *B. subtilis* TISTR1248, showed an auto-aggregation rate of 77.42% at 24 h. All isolates, except BM3, BM4, and BM5, had auto-aggregation rates above 50% after 24 h of incubation. Cell surface hydrophobicity ranged from 55.19% to 69.05%. BF2 exhibited the highest hydrophobicity and BM5 the lowest. Isolates BF2, BF5, BM1, BM2, and BM4 had significantly higher hydrophobicity than *B. subtilis* TISTR1248 ($p < 0.05$).

Table 5. Cell surface hydrophobicity and auto-aggregation of the ten isolates and the reference strain. * Significant difference compared with *B. subtilis* TISTR1248 at $p < 0.05$. Values are expressed as mean $\pm$ SD ($n = 3$).

Probiotic Properties Test		Hydrophobicity (%)	Auto-Aggregation (%)		
			3 h After Incubation	6 h After Incubation	24 h After Incubation
Control	<i>B. subtilis</i> TISTR1248	63.47 $\pm$ 0.10	15.12 $\pm$ 0.28	24.46 $\pm$ 0.13	77.42 $\pm$ 0.17
Isolates	BF1	64.36 $\pm$ 0.64	16.14 $\pm$ 0.95	34.37 $\pm$ 0.30 *	56.17 $\pm$ 0.16
	BF2	69.05 $\pm$ 0.30 *	28.95 $\pm$ 0.20 *	36.11 $\pm$ 0.07 *	73.10 $\pm$ 0.18
	BF3	59.14 $\pm$ 0.56	41.28 $\pm$ 0.25 *	40.79 $\pm$ 0.07 *	59.76 $\pm$ 0.05
	BF4	62.55 $\pm$ 0.18	23.92 $\pm$ 0.22 *	12.18 $\pm$ 0.80	66.40 $\pm$ 0.24
	BF5	66.51 $\pm$ 0.25 *	28.99 $\pm$ 0.49 *	45.17 $\pm$ 0.26 *	74.69 $\pm$ 0.08
	BM1	66.90 $\pm$ 0.14 *	10.46 $\pm$ 0.20	19.06 $\pm$ 0.09	54.89 $\pm$ 0.05
	BM2	68.57 $\pm$ 0.59 *	17.97 $\pm$ 0.26 *	32.85 $\pm$ 0.22 *	75.83 $\pm$ 0.24
	BM3	56.80 $\pm$ 1.34	4.35 $\pm$ 0.22	16.03 $\pm$ 0.11	48.51 $\pm$ 0.06
	BM4	65.71 $\pm$ 0.47 *	11.23 $\pm$ 0.15	29.21 $\pm$ 0.26 *	46.75 $\pm$ 0.55
	BM5	55.19 $\pm$ 0.44	10.98 $\pm$ 0.02	17.20 $\pm$ 0.29	46.02 $\pm$ 0.24

3.4.5. Antibiotic Inhibition Zones

Measurable inhibition zones were recorded around the ampicillin, chloramphenicol and streptomycin disks for all ten isolates, and around the vancomycin disk for all isolates except BF3 (Table 6). Inhibition zones for ampicillin, chloramphenicol, streptomycin, and vancomycin were 28.89 $\pm$ 8.53 (A) and 28.82 $\pm$ 8.44 (B) mm, 24.84 $\pm$ 2.78 (A) and 25.11 $\pm$ 2.62 (B) mm, 23.96 $\pm$ 1.76 (A) and 24.38 $\pm$ 1.98 (B) mm, and 21.72 $\pm$ 2.85 (A) and 21.82 $\pm$ 2.60 (B) mm, respectively. For vancomycin, no inhibition zone was detected for BF3, and the mean was therefore calculated from the remaining nine isolates. Isolate BM4 showed smaller inhibition zones than *B. subtilis* TISTR1248 for all four antibiotics tested.

Table 6. Inhibition zone diameters (mm) for the ten isolates and the reference strain. Values are expressed as mean $\pm$ SD of three independent replicates ($n = 3$). The inhibition zone diameter was measured in two perpendicular directions (A and B), and the mean values are shown. * Significant difference compared with *B. subtilis* TISTR1248 at $p < 0.05$, with values higher than the control. Zone diameters are reported as raw measurements. No susceptibility or resistance categories were assigned, because CLSI M45 and EFSA FEEDAP criteria for *Bacillus* spp. are MIC-based and are not applicable to disk-diffusion zone diameters. The absence of a measurable zone (BF3, vancomycin) is reported as an observation and is not interpreted as resistance.

Probiotic Properties Test		Inhibition Zone (mm)							
		Ampicillin (10 μ g/disk)		Chloramphenicol (30 μ g/disk)		Streptomycin (10 μ g/disk)		Vancomycin (10 μ g/disk)	
		A	B	A	B	A	B	A	B

Control	<i>B. subtilis</i>	24.47 ±	26.40 ±	28.19 ±	27.83 ±	23.33 ±	24.00 ±	23.23 ±	26.53 ±
	TISTR1248	0.14	0.16	0.08	0.08	0.03	0.05	0.09	0.32
Isolates	BF1	43.83 ± 0.34 *	43.33 ± 0.40 *	21.32 ± 0.03	22.92 ± 0.14	24.89 ± 0.08	24.63 ± 0.06	24.83 ± 0.10	24.00 ± 0.05
	BF2	21.43 ± 0.04	21.17 ± 0.08	26.00 ± 0.10	27.00 ± 0.13	25.32 ± 0.03 *	25.67 ± 0.08 *	22.10 ± 0.07	22.67 ± 0.03
	BF3	22.17 ± 0.16	22.10 ± 0.01	29.18 ± 0.08 *	29.33 ± 0.08 *	19.89 ± 0.05	20.02 ± 0.09	No zone	No zone
	BF4	22.33 ± 0.08	22.53 ± 0.23	29.17 ± 0.08 *	28.72 ± 0.11 *	22.84 ± 0.06	22.66 ± 0.03	21.40 ± 0.11	21.47 ± 0.13
	BF5	21.90 ± 0.05	22.60 ± 0.07	24.96 ± 0.08	25.37 ± 0.06	24.75 ± 0.02 *	25.39 ± 0.10 *	23.67 ± 0.13	22.17 ± 0.25
	BM1	34.83 ± 0.14 *	35.23 ± 0.16 *	25.08 ± 0.08	24.66 ± 0.08	25.56 ± 0.04 *	26.04 ± 0.01 *	22.20 ± 0.08	22.47 ± 0.05
	BM2	25.27 ± 0.11	24.37 ± 0.06	24.63 ± 0.04	24.67 ± 0.04	25.04 ± 0.01 *	25.37 ± 0.01 *	19.93 ± 0.02	20.27 ± 0.05
	BM3	36.57 ± 0.02 *	36.83 ± 0.24 *	24.33 ± 0.06	24.59 ± 0.11	24.85 ± 0.08 *	26.03 ± 0.11 *	25.00 ± 0.09	25.57 ± 0.09
	BM4	21.50 ± 0.18	21.67 ± 0.08	21.08 ± 0.09	21.42 ± 0.04	22.35 ± 0.03	22.72 ± 0.02	15.67 ± 0.08	16.47 ± 0.05
	BM5	39.07 ± 0.34 *	38.37 ± 0.36 *	22.67 ± 0.10	22.42 ± 0.04	24.13 ± 0.02 *	25.27 ± 0.09 *	20.67 ± 0.08	21.33 ± 0.08

3.4.6. Screening for Antimicrobial Activity

Isolate BF1 inhibited the growth of *Pseudomonas aeruginosa*, with inhibition-zone diameters of 24.00 ± 0.53 (A) and 17.33 ± 0.64 (B) mm. None of the other isolates inhibited the growth of any of the pathogenic bacteria tested (Table 7).

Table 7. Antimicrobial activity of selected isolates. The diameter of the inhibition zone was measured in two perpendicular directions (A and B), and the values are presented as the mean $\pm$ SD of triplicates. ND = not detected.

Probiotic Properties Test		Inhibition Zone (mm)					
		<i>Escherichia coli</i>		<i>Pseudomonas aeruginosa</i>		<i>Staphylococcus aureus</i>	
		A	B	A	B	A	B
Control	<i>B. subtilis</i> TISTR1248	ND	ND	ND	ND	ND	ND
Isolates	BF1	ND	ND	24.00 ± 0.53	17.33 ± 0.64	ND	ND
	BF2	ND	ND	ND	ND	ND	ND
	BF3	ND	ND	ND	ND	ND	ND
	BF4	ND	ND	ND	ND	ND	ND
	BF5	ND	ND	ND	ND	ND	ND
	BM1	ND	ND	ND	ND	ND	ND
	BM2	ND	ND	ND	ND	ND	ND
	BM3	ND	ND	ND	ND	ND	ND
	BM4	ND	ND	ND	ND	ND	ND
	BM5	ND	ND	ND	ND	ND	ND

3.5. Whole-Genome Analysis

Genomic DNA from isolates BF1 and BM2 was sequenced using Oxford Nanopore Technologies (ONT), yielding 538 $\times$ and 465 $\times$ coverage, respectively. The complete sequenced genomes of isolates BF1 and BM2 each consisted of one circular chromosome (BF1, 3,784,823 bp; BM2, 3,799,432 bp), with average GC contents of 41.67% and 41.58%,

respectively (Figure 1A,B). Genome completeness was estimated using Benchmarking Universal Single-Copy Orthologs (BUSCO v6.0.0) with the bacillales_odb10 dataset, which contains 450 BUSCO markers. Both genomes showed more than 95% completeness (BF1, 96.0%; BM2, 95.3%). Genome annotation revealed 3937 CDSs, 24 rRNA genes, and 81 tRNA genes for BF1, and 3941 CDSs, 24 rRNA genes, and 82 tRNA genes for BM2. The circular genome maps illustrate the complete genome architectures of the two *Bacillus* strains analyzed in this study. Both genomes displayed a highly conserved chromosomal organization, with coding sequences evenly distributed across the chromosome (Figure 1A,B). Functional annotation based on Clusters of Orthologous Groups (COG) classification showed that a large proportion of genes were assigned to categories related to core metabolic and cellular functions, including amino acid transport and metabolism (E), carbohydrate transport and metabolism (G), energy production and conversion (C), transcription (K), and translation (J) (Figure 1C,D). Comparative analysis of COG category distributions between the two genomes revealed broadly similar functional profiles, with only minor variations in gene counts across specific categories, likely reflecting strain-level specialization.

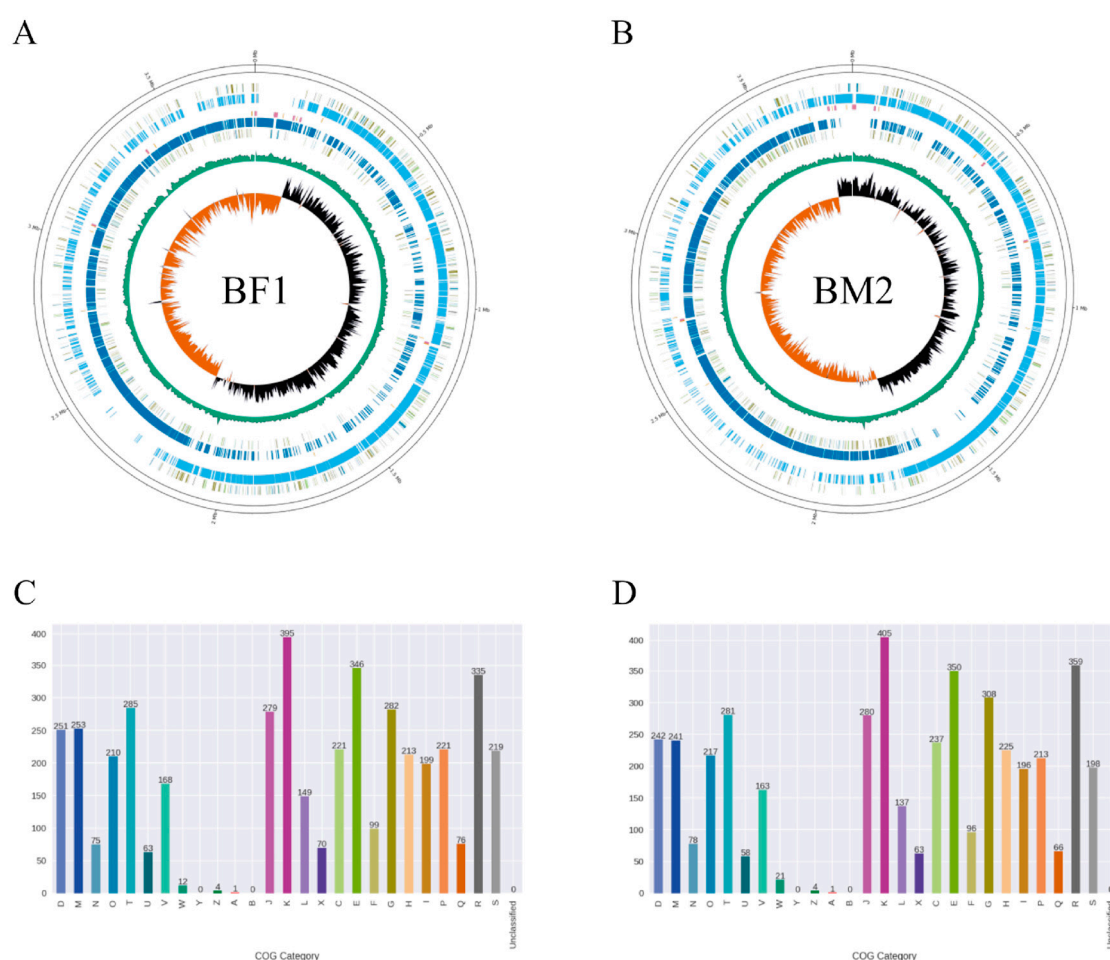


Figure 1. Complete genome maps and clusters of orthologous group (COG) functional classifications of *Bacillus* strains BF1 and BM2 isolated from the digestive tract of Siamese fighting fish. (A) Circular genome map of strain BF1. (B) Circular genome map of strain BM2. (C) COG functional categories of BF1. (D) COG functional categories of BM2. Each strain possessed a single circular chromosome: BF1 (3,784,823 bp; GC content 41.67%) and BM2 (3,799,432 bp; GC content 41.58%).

3.5.1. Phylogenetic Analysis of *Bacillus* spp. Isolates

To determine the taxonomic placement of BF1 and BM2, a genome-scale phylogenetic analysis was performed. The phylogenetic tree, constructed from concatenated single-copy core genes, demonstrated that both isolates clustered within the genus *Bacillus* (Figure 2A,B). The results showed that isolates BF1 and BM2 clustered with *B. safensis* within the *B. pumilus* group. Furthermore, comparison of the whole-genomes of BF1 and BM2 with 41 other complete circular genomes of *B. safensis* revealed ANI values $\geq 96\%$ with *B. safensis* (Supplementary Figure S1).

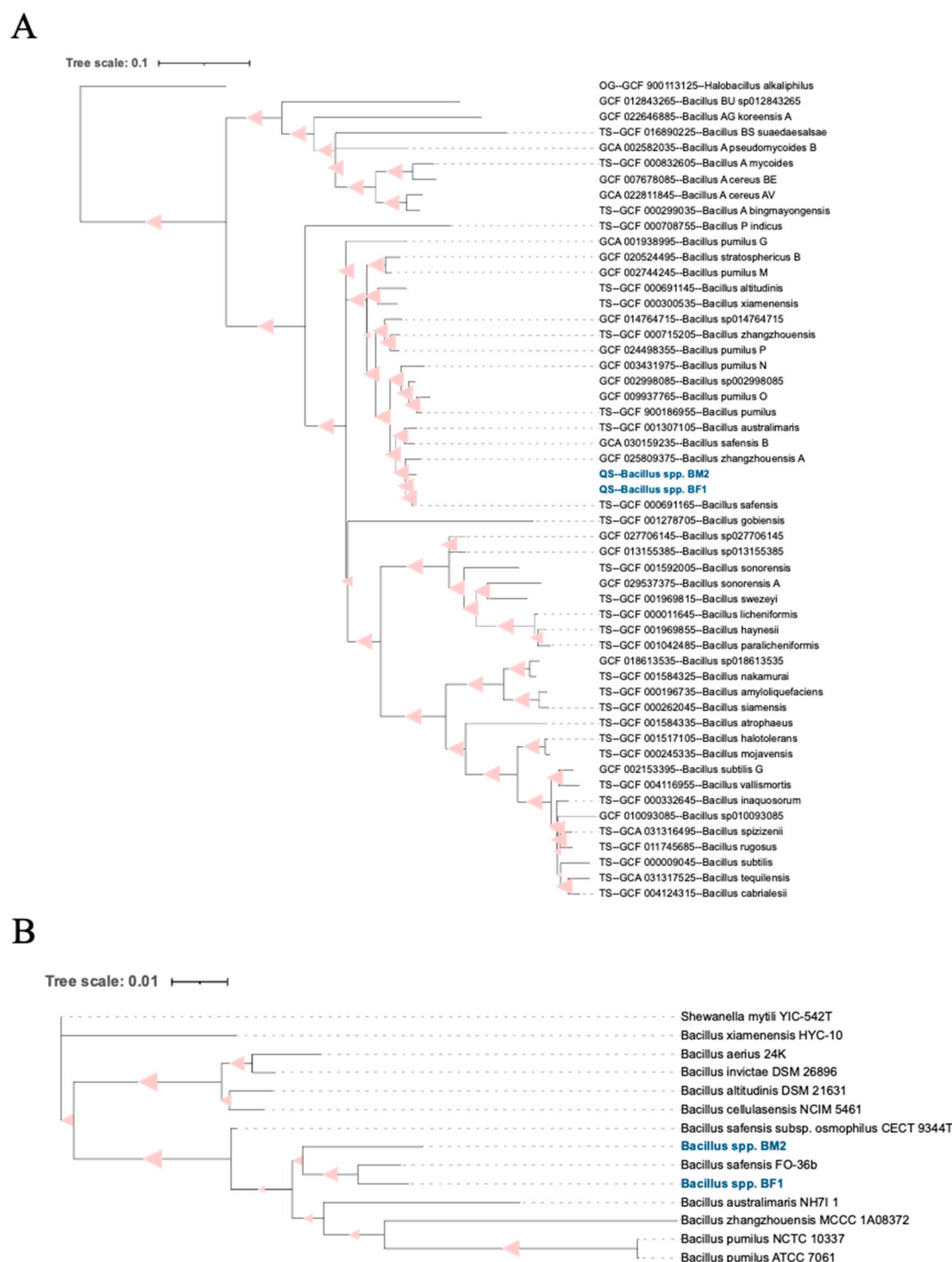


Figure 2. Genome-scale phylogenetic analysis of *Bacillus* strains BF1 and BM2. (A) Phylogenetic tree based on concatenated single-copy core genes using autoMLST2 showing the placement of strains

BF1 and BM2 within the genus *Bacillus*. **(B)** Phylogenetic tree of the isolates inferred using the Type Strain Genome Server (TYGS), which compares whole-genome sequences against reference type strains, showing the relationship of strains BF1 and BM2 with closely related *Bacillus* species, with *Shewanella mytili* YIC-542T as an outgroup. Bootstrap values are indicated at the nodes, and strains BF1 and BM2 were closely related to *B. safensis*. Pink triangles indicate collapsed nodes. Blue text highlights the strains analyzed in this study (BF1 and BM2).

3.5.2. Secondary Metabolite Biosynthetic Gene Clusters (BGCs) of *B. safensis* and the Other Species

Potential BGCs involved in secondary metabolite production were identified using antiSMASH v8.0.4 [64]. Additionally, putative bacteriocins and RiPPs were predicted using the BAGEL4 web server [65]. The results revealed the presence of multiple conserved BGCs associated with secondary metabolite production, including non-ribosomal peptide synthetase (NRPS), non-ribosomal peptide metallophore (NRP-metallophore), betalactone, terpene, ribosomally synthesized and post-translationally modified peptide (RiPP), type III PKS (T3PKS), (thio) azol(in)e-containing peptide, and siderophore clusters. Comparative analysis demonstrated that both strains harbored conserved BGCs related to bacteriocin, siderophore, and RiPP biosynthesis. Notably, genes within the *dhb* gene cluster (*dhbA-dhbF*) were detected; these genes are associated with siderophore-mediated iron acquisition and environmental adaptation (Figure 3A). Additionally, both strains harbored gene clusters encoding the ferric-bacillibactin ABC transport system, including the substrate-binding protein *FeuA*, which mediates uptake of the iron-chelating complex ferric-bacillibactin. The genomes also included the transcriptional regulator *Btr* involved in transcriptional activation of the *feuABC-ybbA* operon, as characterized in *B. subtilis*. As illustrated in Figure 3B, a bacilysin-associated gene cluster including core biosynthetic genes (*bacABCDE*) was identified in both genomes. In addition, genome mining revealed a BGC with high homology to plantazolicin-like clusters previously characterized in *B. pumilus*, *B. velezensis*, and *Corynebacterium urealyticum*. Notably, this cluster was identified exclusively within the genome of strain BF1 (Figure 3C). Furthermore, a conserved pumilarin-like gene cluster was observed in BF1 and BM2 and across closely related *Bacillus* species, including *B. safensis*, *B. pumilus*, and *B. altitudinis* (Figure 3D). This cluster exhibited strong synteny and high sequence similarity (approximately 97–100% identity) between BF1 and BM2, as well as with reference genomes.

3.5.3. Preliminary Genomic Safety Screening and Functional Genes

For the *Bacillus* probiotic candidates, we identified key genes associated with survival and function (probiotic mechanisms), as well as genes associated with safety. These included genes involved in acid tolerance, bile salt resistance, adhesion and colonization, antimicrobial activity, metabolism and nutrition, antioxidant activity, transferable antibiotic resistance, virulence factors, and toxin production. As part of the safety assessment, antimicrobial resistance genes were screened using ABRicate v1.0.1, which detected a class D beta-lactamase sequence (*blaBPU-1*-like, 88.85% nucleotide identity) and a type A-6 chloramphenicol acetyltransferase sequence (*cat86*). Both are chromosomally located and correspond to determinants reported as intrinsic to the *B. pumilus* group. No acquired antimicrobial resistance genes were detected in the databases screened; this does not exclude determinants that are absent from those databases. ISEScan v1.7.3 detected no insertion sequences within 10 kb of either resistance determinant in the BF1 or BM2 genome; the nearest elements lay between 154 kb and 1079 kb away (Supplementary Table S1). This is consistent with a chromosomal location, although it does not by itself establish that the determinants are intrinsic.

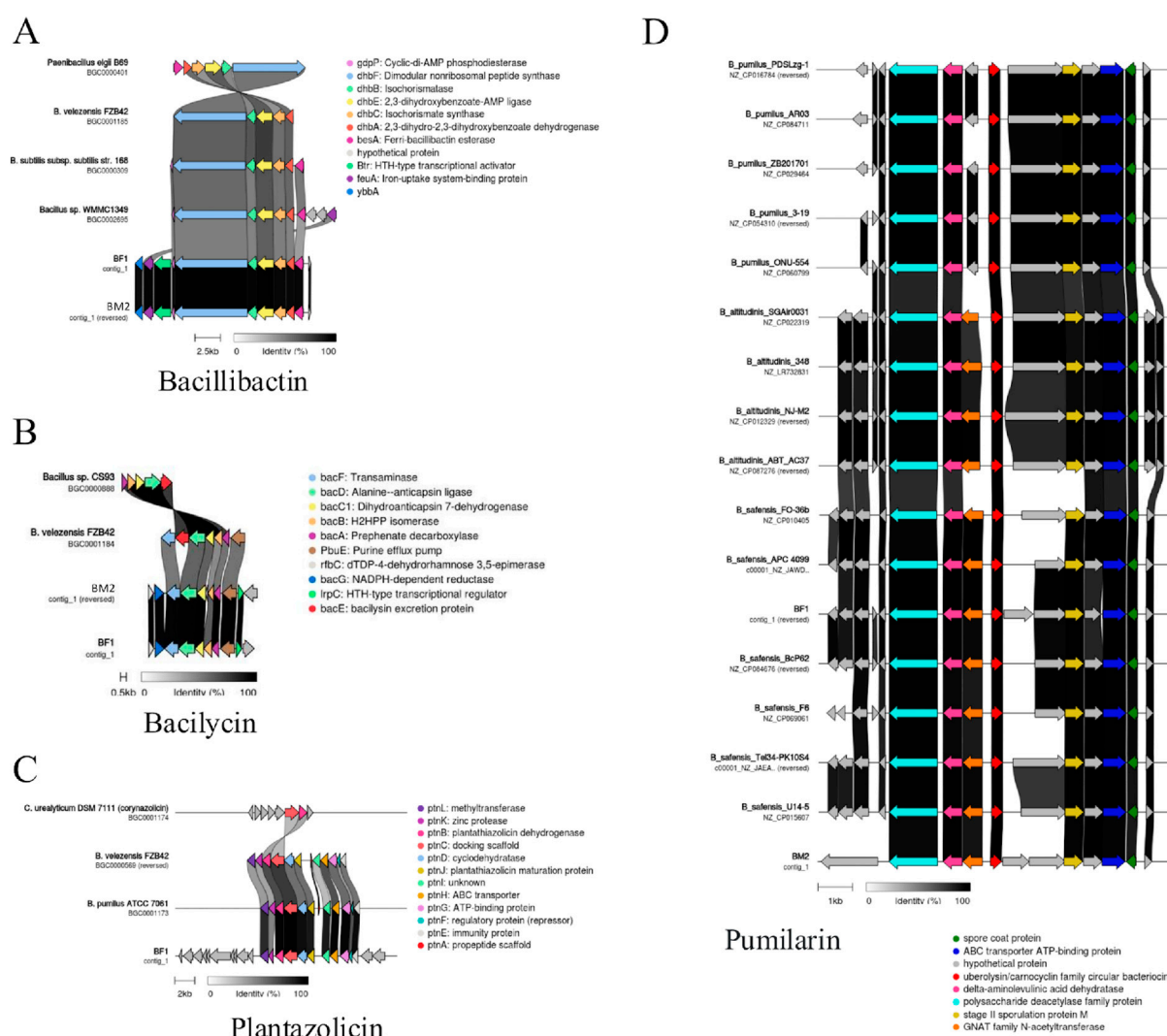


Figure 3. Comparative analysis of biosynthetic gene clusters associated with secondary metabolite biosynthesis in *Bacillus* strains BF1 and BM2. (A) Bacillibactin biosynthetic gene cluster. (B) Bacilycin biosynthetic gene cluster. (C) Plantazolicin-like biosynthetic gene cluster identified only in strain BF1. (D) Pumilarin-like biosynthetic gene cluster in strains BF1 and BM2 and related to *B. safensis*. Sequence identity is shown from 0% to 100%.

The absence of a flanking insertion sequence reduces, but does not exclude, the possibility of horizontal transfer: other classes of mobile element were not screened, and transferability was not tested experimentally. In addition, we screened the genomes of BF1 and BM2 against the Virulence Factor Database (VFDB). This homology-based analysis identified homologues of 35 of the 75 virulence-associated genes examined (Supplementary Table S2). Detection of a homolog indicates sequence similarity only and does not demonstrate expression, function or a contribution to pathogenicity. Because the reference set is derived largely from the *B. cereus* group, the partial overlap of the BF1 and BM2 profiles with that group reflects shared genus-level genes rather than a virulent phenotype. Both candidates harbored a gene encoding immune inhibitor A metalloproteinase (*inhA*; KEGG KO: K09607). However, they lacked genes for hemolysin II (*hlyII*) and the tripartite non-hemolytic enterotoxin (*nheA*, *nheB*, *nheC*), which are common in virulent *B. cereus* strains. By contrast, both candidates harbored *hlyIII*, encoding hemolysin III. This gene is common in *Bacillus* species and is not regarded as a primary virulence determinant of the *B. cereus* group. The *hlyIII* locus was nonetheless not functionally tested in this study, and the γ -hemolytic phenotype observed on sheep blood agar was recorded under a

single set of conditions. When compared with *B. anthracis*, the candidates possessed a near-complete poly- γ -glutamate capsule biosynthesis locus (KEGG annotation confirmed *capA* [K07282], *capB* [K01932], and *capC* [K22116]), although they lacked *capE*. The absence of *capE* does not by itself exclude γ -PGA production, and capsule formation was not assessed phenotypically. Notably, γ -PGA synthesis via *capABC/pgsBCA* is widely distributed among *Bacillus* species, including non-pathogenic strains such as *B. subtilis* and *B. licheniformis*, where the polymer is typically released rather than forming a surface-anchored capsule. Crucially, they did not harbor the genes for anthrax toxin components (*cya*, *lef*, *pagA*) or their key transcriptional regulators (*atxA*, *acpA*, *acpB*). Finally, screening for genes characteristic of *B. thuringiensis* confirmed the absence of the major insecticidal toxin determinants (*cry*, *cyt*, *vip*). Regarding stress tolerance, genomic analysis revealed that both isolates lacked the canonical acid- and bile-tolerance genes, namely those encoding glutamate decarboxylase (*gadB*; KO: K01580) and bile salt hydrolase (*bsh*; KO: K01442). Notably, domains corresponding to a bile acid:Na⁺ symporter (*yocS*; BASS family; KO: K03453) were identified in both genomes. Analysis of genetic determinants for adhesion indicated the presence of key systems in both isolates, including the sortase system (*srtA*; KO: K07284) and the autoinducer-2 synthesis gene for quorum sensing (*luxS*; KO: K07173). In contrast, genes encoding mucus-binding proteins (*mub*; KO: K20276) and the exopolysaccharide biosynthesis cluster (*epsABCD*; KO: K11935-K11938) were absent. The genomes also encoded a repertoire of genes associated with beneficial metabolite production. These included genes encoding the terminal steps of butyrate formation (*ptb*, K00634; *buk*, K00929), enzymes for folate, riboflavin and thiamine biosynthesis (*folC*, K11754; *ribA*, K01497; and *thiE*, K00788), beta-galactosidase for lactose digestion (*bgaB*; KO: K12308), and surfactin synthetase (*srfA*; KO: K15654–K15656), which produces the antimicrobial lipopeptide surfactin, alongside lactate dehydrogenase (*ldh*; KO: K00016). Furthermore, both isolates possessed genes encoding oxidative stress defense enzymes, including superoxide dismutase (*sod*; KO: K04564) and catalase (*kat*; KO: K03781). Both genomes carried *srfAB* (KO: K15655), a gene with a predicted role in biosurfactant production.

4. Discussion

Evaluation of bacterial strains as probiotics requires substantial in vitro investigation. Performing in vitro experiments that simulate gastrointestinal (GI) tract conditions and screening for potential risks and benefits to the host are the first steps when assessing a probiotic candidate. In the present investigation, ten *Bacillus* isolates were obtained from the digestive tract of farmed *B. splendens*, and their probiotic properties were characterized. An effective probiotic strain should be non-pathogenic to the host and tolerant to GI tract conditions. Probiotic strains should also be able to attach to the mucosa or enteric epithelial cells, thereby supporting intestinal colonization. These potential probiotic properties are important for providing beneficial effects to the host. In the hemolysis and biofilm-formation assays, the isolates did not exhibit hemolytic or biofilm-forming phenotypes [70,71]. However, these in vitro findings do not confirm in vivo safety, and further evaluation is required before these isolates can be considered safe candidates. Biochemical profiles of the ten isolates and the control differed from those of the reference *Bacillus* strains, and species-level identification could not be achieved from biochemical testing alone.

Survival of gastrointestinal transit and colonization of the intestine are essential probiotic attributes, and both depend on tolerance to low pH and bile salts [72]. Resistance to pH 1.0 to 3.0 and 0.3% bile salt has been widely used as a standardized proxy for mammalian gastric and intestinal conditions. These conditions follow protocols developed for lactic acid bacteria and are retained here for comparability with the probiotic literature. However, they do not reproduce the gastrointestinal environment of *B. splendens*, which, as a small carnivorous teleost, has a short digestive tract, comparatively brief gastric

transit and intestinal pH values reported to be less acidic than those of the mammalian stomach [73–75]. Recovery of the isolates from that gut nevertheless provides complementary evidence of their compatibility with the host environment. All assays were performed at 37 °C, whereas *B. splendens* is typically reared at 24–28 °C. This temperature follows the convention for *Bacillus* characterization but does not reflect the conditions under which the host is reared. In the present study, the selected isolates showed limited relative growth at pH 3.0 but maintained relative growth at pH 5.0, in agreement with previous studies of *Bacillus* strains [76–78]. The limited acid tolerance at pH 3.0 may reflect the natural habitat of these isolates, which were recovered from the fish intestine where the pH typically ranges from 6.5 to 7.6, a condition closer to neutrality than to the highly acidic gastric environment [73–75]. The 0.3% bile salt concentration is considered important when evaluating bile-tolerant probiotics. Only BF2 and BM2 exceeded the *B. subtilis* TISTR1248 control (58.10% and 69.98%, respectively, versus 56.28%); they were also the only strains with significantly higher relative growth at every bile salt concentration tested. The higher bile tolerance observed in BF2 and BM2 is consistent with their intestinal origin, as the fish intestine is continuously exposed to bile salts, which may have selected for bile-resistant strains. These findings suggest that BF2 and BM2 may have potential as probiotic candidates [42,79]. Nevertheless, these findings are based on in vitro assays and may not fully reflect the complex gastrointestinal environment, warranting further in vivo investigation in the target fish species. Tolerance to pepsin, pancreatic enzymes, and lysozyme is also a key factor in the survival of probiotics once they enter the host. These enzymes are involved in nutrient digestion in the GI tract. Based on previous studies, strains retaining more than 50% of control growth after enzyme exposure are considered promising probiotic candidates [38]. Because that threshold was originally defined using viable counts, the comparison with our OD-based values is indicative only. In the present study, all isolates showed relative growth below 50%, yet almost all exceeded the control after exposure to pepsin and pancreatin. These isolates therefore show some tolerance to gastrointestinal enzymes, although none met the 50% criterion. In addition, several isolates maintained measurable relative growth after lysozyme exposure at different incubation periods. In particular, BM5 and BF4 showed the highest relative growth in the presence of pepsin and pancreatin, BF4 additionally showed a lysozyme growth curve indistinguishable from its control; these strains therefore merit further evaluation [45]. It should be noted that tolerance in the acid, bile salt, and gastrointestinal enzyme assays was inferred from optical density relative to untreated controls. These values therefore represent relative growth rather than absolute survival; confirmation by plate counts or viability staining is addressed among the limitations below.

Digestive enzyme activity is one of the recommended attributes when evaluating a probiotic strain. *Bacillus* produces proteolytic and lipolytic enzymes that hydrolyze dietary proteins and lipids, releasing nutrients the host can use. This factor is important for digestive processes, nutrient utilization, and the growth rate of fish [80,81]. In the present study, all isolates produced proteolytic and lipolytic enzymes. Moreover, all isolates showed β -galactosidase activity exceeding 500 Miller units, which is similar to values reported for well-known lactic acid bacteria [42]. This enzyme hydrolyses lactose and galactooligosaccharides (GOS), releasing galactose and glucose, which the gut microbiota can ferment to short-chain fatty acids that benefit the host. Interestingly, although BM2 and the reference strain were ONPG-negative in the identification kit (Table 1), they showed the two highest β -galactosidase activities in the Miller assay. This discrepancy may reflect differences in assay sensitivity and substrate accessibility: the ONPG test in the identification kit detects membrane-associated β -galactosidase activity under kit-specific conditions, whereas the Miller assay measures total enzyme activity in permeabilized cells. Exopolysaccharide (EPS) production is another property frequently assessed in probiotic

bacteria, including lactic acid bacteria (LAB), because it may contribute to host health and nutrition. All isolates and the reference strain produced slime, indicating EPS production, in agreement with previous findings [82]. EPS produced by probiotic bacteria has been shown to have immunomodulatory, antioxidant, and anti-tumorigenic activities, thereby adding another potential layer of health benefits to probiotics. Colonization of the intestinal wall is also considered a desirable property of probiotic bacteria. Hydrophobicity and auto-aggregation are among the main criteria used for selecting probiotics. Bacteria with higher hydrophobicity can bind more effectively to epithelial cells, thereby providing a colonization advantage in the intestinal tract. All isolates exceeded 50% hydrophobicity, and most showed strong auto-aggregation. Our observations are similar to those of previous studies on *Bacillus* species isolated from different food sources [24,78,83]. In vivo, this property could contribute to competition with pathogens for adhesion sites, which remains to be tested. Therefore, these selected isolates can be considered candidate strains warranting further safety and efficacy assessment [84,85]. Because probiotics should not contribute to the spread of antibiotic resistance among intestinal or food-borne bacteria, the isolates were screened by disk diffusion. Measurable inhibition zones were observed for all isolates with ampicillin, chloramphenicol and streptomycin, and for all isolates except BF3 with vancomycin. For BF1 and BM2, in which *blaBPU-1*-like and *cat86* sequences were detected, zone diameters were of the same order as those of *B. subtilis* TISTR1248. However, in the absence of validated interpretive criteria these data cannot establish whether the two genes are expressed or whether they confer resistance. Because the CLSI M45 and EFSA FEEDAP criteria for *Bacillus* are MIC-based and not applicable to disc diffusion zone diameters, MIC determination under these criteria would be required to assess expression and resistance; zone diameters are therefore reported without susceptibility categories, consistent with previous work on *Bacillus* probiotics [71]. BF3 was not sequenced and the basis for the absence of a measurable vancomycin zone was not determined; BF3 was not selected as a candidate strain. Antimicrobial activity against pathogens is another important attribute to consider when selecting potential probiotic strains for maintaining a healthy microbial balance in the GI tract. Only one isolate, BF1, showed antagonistic activity against the pathogens tested. However, the absence of inhibition under standard in vitro conditions does not exclude the possibility of in vivo activity, as the gut environment may trigger the expression of otherwise silent biosynthetic gene clusters. Alternatively, the beneficial effects of these isolates may arise from other mechanisms, such as competitive exclusion, mucus adhesion, or host immune modulation, all of which warrant further investigation.

Additionally, partial 16S rDNA gene sequencing was employed to identify the isolates. BLAST analysis showed 100% identity between the ~400 bp fragments and sequences from members of the genus *Bacillus*, with the top matches corresponding to *B. pumilus*. Species-level identification could not be achieved for any isolate based on this marker alone. Collectively, these findings indicate that the ten isolates obtained from the gastrointestinal tract of Betta fish belong to the genus *Bacillus*. All strains were isolated from the GI tract of *B. splendens*, suggesting that they are host-associated strains. Host-associated strains are often preferred as probiotic candidates because they may be better adapted to the host gut. These strains may have originated from the surrounding water. However, because each fish was housed in a separate container, an association with the fish microbiota is more likely. These results are consistent with recent studies identifying host microbiota as an important source of probiotic candidates [86–89]. Moreover, the successful application of host-associated *Lactobacillus* and *Bacillus* spp. as probiotics in aquaculture has been previously reported [89]. Subsequent whole-genome sequencing of the two selected isolates enabled a more precise taxonomic classification and comprehensive genomic characterization. BM2 showed the highest relative growth at 0.3% bile salt and

the highest β -galactosidase activity, whereas BF1 showed stronger antimicrobial activity. Based on genome analysis, isolates BF1 and BM2 were identified as *B. safensis*. In addition, several genes associated with probiotic function were identified, supporting the results obtained from the in vitro assays. Safety is a critical consideration in the evaluation of probiotic candidates. Whole-genome analysis revealed the presence of hemolysin III, *blaBPU-1*-like, and *cat86* in both isolates. The isolates were non-hemolytic and did not form biofilms, but neither this phenotypic result nor the absence of major toxin genes establishes safety on its own; both constitute preliminary screening that requires confirmation in the host (Supplementary Figure S2A,B).

Detection of hemolysin-related genes should be interpreted together with phenotypic data rather than in isolation; it cannot, however, be discounted, and the *hlyIII* homolog detected in both genomes warrants functional follow-up. Both *blaBPU-1*-like and *cat86* sequences are chromosomally located and correspond to determinants reported as intrinsic to the *B. pumilus* group [90,91], and no acquired antimicrobial resistance genes were detected in the databases screened. Probiotic bacteria must survive passage through the gastrointestinal tract, which requires tolerance to acid and bile salts. BF1 and BM2 lacked the canonical acid tolerance gene *gadB* (glutamate decarboxylase) and the bile salt hydrolase gene *bsh*, both of which are well-established contributors to stress tolerance in lactobacilli and other probiotics [92–97]. The absence of these genes suggests that BF1 and BM2 may employ alternative mechanisms for surviving gastrointestinal transit. In this study, both probiotic candidates showed the presence of *yocS*, which encodes a sodium symporter (BASS family) for bile acids, offering a potential alternative mechanism for bile salt resistance. This finding is similar to results from previous studies on *Bacillus* species [98,99]. The presence of *yocS* offers one possible explanation for the relative growth maintained in the in vitro bile salt assays despite the absence of *bsh*; this remains to be tested directly. Regarding adhesion, the presence of *srtA* (sortase A) and *luxS* (autoinducer-2 synthase) indicates that BF1 and BM2 possess genetic systems supporting adhesion and intercellular communication. Sortase A anchors LPXTG-motif proteins to the cell wall, and these surface proteins have been correlated with adhesion and biofilm formation in several probiotic species [100–102]. However, the absence of *mub* (mucus-binding proteins) and the *epsABCD* exopolysaccharide biosynthesis cluster suggests that BF1 and BM2 lack the canonical, high-affinity mucus adhesion machinery found in some well-characterized probiotics, such as *Limosilactobacillus reuteri* and *Lactobacillus acidophilus* [103,104]. Although all isolates produced EPS in the plate assay, genome mining did not identify the canonical *epsABCD* cluster typically associated with EPS biosynthesis. This apparent discrepancy suggests that these specific *Bacillus* strains may utilize alternative, non-canonical biosynthetic pathways or novel gene clusters for EPS production. Future transcriptomic analyses or deeper genomic annotations are required to identify the genes responsible for this EPS phenotype. Regarding beneficial metabolite production, the presence of genes encoding the terminal steps of butyrate formation (*ptb* and *buk*) is particularly noteworthy. Butyrate is a short-chain fatty acid with well-established benefits for gut health, including serving as an energy source for intestinal epithelial cells, reinforcing the intestinal barrier, and exerting anti-inflammatory effects. The presence of folate, riboflavin and thiamine biosynthesis genes (*folC*, *ribA*, *thiE*) further supports the potential for vitamin production. Vitamin biosynthesis is a common probiotic attribute that may contribute to the micronutrient supply of the host, particularly under intensive rearing with formulated feeds. The beta-galactosidase gene (*bgalB*) may facilitate carbohydrate metabolism, which could be beneficial for improving digestion and nutrient absorption in hosts [105]. Moreover, the presence of genes encoding superoxide dismutase (*sod*) and catalase (*kat*) suggests that BF1 and BM2 can cope with oxidative stress encountered in the gastrointestinal tract. These enzymes protect bacterial cells from reactive oxygen species generated by host

immune responses and may also contribute to reducing oxidative stress in the host intestinal environment [101,106,107]. The ability to mitigate oxidative stress is increasingly recognized as an important probiotic trait, particularly in inflamed gut conditions. The *blaBPU-1*-like and *cat86* genes are among the most frequently reported determinants in *Bacillus* spp. [108,109]. Expression of the resistance and virulence genes detected here was not measured, and the disc-diffusion data do not establish that they are silent. The nearest insertion sequences lay 154 kb to 1079 kb from the two resistance determinants, two to three orders of magnitude beyond the span of any characterized composite transposon, so the genomic context provides no evidence of a mobilizable unit. This does not exclude horizontal transfer, which would require conjugation or transformation experiments to assess, and other classes of mobile element were outside the scope of the present screen. The genomic analysis therefore identifies features that require targeted follow-up rather than evidence that risk is absent. Finally, the observation of antibacterial activity, coupled with the presence of *srfAB* (a component of the surfactin synthetase operon), suggests that antimicrobial lipopeptide production may contribute to the probiotic functionality of BF1 and BM2. Surfactin and related lipopeptides produced by *Bacillus* species are known to exhibit antimicrobial activity against a range of bacterial pathogens, including both Gram-positive and Gram-negative organisms [110]. However, only BF1 exhibited strong antagonistic activity against *P. aeruginosa* in vitro. This genotype–phenotype mismatch in BM2 may reflect the presence of silent or cryptic biosynthetic gene clusters. The expression of such clusters is tightly regulated and often requires specific environmental triggers, nutritional conditions, or microbial cross-talk that were not present under our standard in vitro assay conditions. Alternatively, variation in promoter regions or regulatory genes in BM2 may limit expression of these clusters.

Genomic analysis using antiSMASH and BAGEL4 revealed several antimicrobial biosynthetic pathways. Both genomes carried the *dhb* cluster, which directs the synthesis of the catecholic siderophore bacillibactin, a structural analog of the enterobactin produced by Gram-negative bacteria [111,112]. By sequestering iron, bacillibactin may promote the competitive exclusion of other microorganisms [113]. Both genomes also carried the *bacABCDE* cluster responsible for bacilysin, a broad-spectrum dipeptide antibiotic [114]. After uptake by target cells, bacilysin is hydrolyzed to release anticapsin, which inhibits glucosamine-6-phosphate synthase and thereby blocks cell wall precursor synthesis, leading to cell lysis [115]. Additionally, characteristic genes encoding cyclodehydratases, dehydrogenases, and other modification enzymes typically involved in the biosynthesis of thiazole- or oxazole-modified microcins (TOMMs) were identified [116]. The presence of this specialized biosynthetic cluster suggests the potential for the production of plantazolicin-like compounds. These metabolites are known for their ultranarrow-spectrum bactericidal activity toward closely related bacterial taxa, including *B. anthracis* [117,118]. Furthermore, the identification of ribosomally synthesized and post-translationally modified peptides (RiPPs), such as plantazolicin-like compounds, indicates the potential for selective modulation of the gut microbiome through targeted antibacterial activity. The detection of a conserved pumilarin-like gene cluster not only supports the strains' taxonomic affiliation with the *B. pumilus* group but also suggests the presence of a specialized defense mechanism that may contribute to niche competition and intestinal colonization. The gene organization of this cluster was consistent with a previously reported pumilarin-associated cluster in *B. safensis* genomes, comprising genes encoding precursor peptides, modification enzymes, ABC transporters, and immunity-related proteins, which are characteristic features of circular or RiPP-type bacteriocin biosynthesis systems.

Experimental evidence from *B. safensis* APC 4099 strains has demonstrated that such clusters can be functionally active, producing detectable antimicrobial compounds, including pumilarin and related peptides [119]. Overall, the complete genome maps and

functional composition of these *Bacillus* isolates indicate a conserved core genome together with metabolic and regulatory features linked to environmental adaptation and secondary metabolite production, supporting their potential for antimicrobial biosynthesis. The integrated probiogenomic analysis provides a molecular basis for the in vitro results and is consistent with the antimicrobial traits observed in vitro for *B. safensis* BF1. Any conclusion about its suitability as a probiotic remains provisional until safety and efficacy are demonstrated in the host.

5. Conclusions

The present study showed that ten *Bacillus* isolates from the gut of *B. splendens* possessed several probiotic-associated properties, including maintenance of relative growth under bile salt exposure, cell surface hydrophobicity, auto-aggregation and the production of digestive enzymes. Measurable inhibition zones were recorded for all isolate–antibiotic combinations except BF3 with vancomycin, for which no zone was detected; these zone diameters are descriptive and were not translated into susceptibility categories. Isolate BF1 exhibited antagonistic activity against *Pseudomonas aeruginosa*. Based on the phenotypic and genomic results, *B. safensis* BF1 and BM2 are candidate strains that warrant further safety and efficacy assessment in aquaculture systems. *B. safensis* BF1 showed the strongest antimicrobial activity, whereas *B. safensis* BM2 showed the highest relative growth at 0.3% bile salt, together with the highest β -galactosidase activity and 24 h auto-aggregation. Overall, this study suggests that gut-associated *Bacillus* strains from *B. splendens* may be a source of probiotic candidates for sustainable fish production. Several limitations should be noted. Tolerance assays were based on optical density and therefore describe relative growth rather than viable cell survival; confirmation by plate counts or viability staining is required, particularly as these spore-forming isolates may retain turbidity through spore persistence rather than vegetative growth. The safety evaluation was performed in silico and in vitro and has not been validated in a live host. Database-dependent screening detects only determinants represented in the reference databases used, and detection of a homolog does not establish expression. The genomic-context analysis was limited to insertion sequences; plasmids, integrative conjugative elements and integrons were not specifically screened, so the mobilization potential of the two resistance determinants has not been fully characterized. The regulatory status of *B. safensis* (for example, its current standing under the EFSA Qualified Presumption of Safety list, which should be verified at the time of application) would also need to be confirmed before any feed use. The assay conditions follow protocols developed for lactic acid bacteria and do not reproduce the gastrointestinal environment or rearing temperature of *B. splendens*. Genomes were obtained for only two of the ten isolates; other isolates with strong individual traits, such as BM5 for acid and gastrointestinal enzyme tolerance, remain candidates for future genomic analysis. All isolates derive from a single farm in Chiang Rai, Thailand, from pooled gut material of six fish that were housed individually from two months of age [33]. In addition, the pathogen strains used in the antimicrobial assays were obtained from the laboratory culture collection, and specific strain designations were not recorded; the antimicrobial result for BF1 therefore rests on an unidentified *P. aeruginosa* strain. Future studies should include in vivo testing to assess safety, colonization ability, host benefits, and disease protection effects under aquaculture conditions.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/sci8100298/s1>, Figure S1. Average nucleotide identity (ANI) analysis of *Bacillus* strains BF1 and BM2 with 41 complete genomes of *B. safensis*. The heatmap shows pairwise ANI values (%), with the color scale ranging from 96% (blue) to 100% (red). Rows and columns represent individual genomes. Strains BF1 and BM2 exhibited ANI values of $\geq 96\%$ with *B.*

safensis. Figure S2. Phenotypic characterization of *Bacillus* isolates using hemolysis and biofilm formation assays. (A) Hemolysis activity on blood agar. (B) Biofilm formation on Congo red agar. Strains BM1 to BM5, BF1 to BF5, and *B. subtilis* TISTR1248 are shown. In the top row of panels A and B, the order from left to right is BM1, BM2, BM3, BM4, BM5, and *B. subtilis* TISTR 1248. In the bottom row, the order is BF1, BF2, BF3, BF4, BF5, and *B. subtilis* TISTR 1248. All isolates showed γ -hemolysis on sheep blood agar and produced red colonies on Congo red agar. A *hlyIII* homolog was nevertheless detected in the genomes of BF1 and BM2, illustrating that the presence of a hemolysin-associated gene is not predictive of a hemolytic phenotype under these conditions. Table S1. Safety-related genes detected in the genomes of *Bacillus* isolates BF1 and BM2, with their genomic coordinates and the genomic context of the two resistance determinants. Table S2. Virulence-associated genes identified in *Bacillus* strains BF1 and BM2 by screening against the Virulence Factor Database (VFDB). A total of 35 homologues were identified among 75 virulence factors examined. Methods S1. DNA extraction, PCR amplification, and sequencing details. Methods S2. *Bacillus* identification kit and manufacturer details. Methods S3. Hemolytic activity and biofilm formation test. Equation S1. Relative growth calculation for acid (pH) tolerance assay. Equation S2. Relative growth calculation for bile salt tolerance assay. Equation S3. Relative growth calculation for pepsin resistance assay. Equation S4. Relative growth calculation for pancreatin resistance assay. Equation S5. β -galactosidase activity calculation (Miller units). Equation S6. Auto-aggregation percentage calculation. Equation S7. Cell surface hydrophobicity calculation.

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Institutional Review Board Statement: The gastrointestinal samples used in this study were obtained from a previous study [33] conducted under animal license AR05/63, which was approved by the Institutional Animal Care and Use Committee (IACUC) of Mae Fah Luang University. The present study used only archived samples and did not involve direct experimentation on live animals.

Informed Consent Statement: Not applicable.

Data Availability Statement: The 16S rRNA gene sequences generated in this study have been deposited in GenBank under the accession numbers OR186679–OR186687 and OR186704. The complete genome sequences of *B. safensis* BF1 and BM2 are available in NCBI GenBank under BioProject number PRJNA1459368 and BioSample accession numbers SAMN57545553 and SAMN57545851. All data are publicly available at <https://www.ncbi.nlm.nih.gov/genbank/> (accessed on 4 October 2026).

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