

doi: 10.30827/ars.v66i2.31858

Artículos originales

Evaluation of the Probiotic Potential of *Bacillus velezensis* SNR14-4 Strain from Nile Tilapia Gills Using Genomic and In Vitro Approach

Evaluación del potencial probiótico de la cepa *Bacillus velezensis* SNR14-4 de branquias de tilapia del Nilo mediante un enfoque genómico e in vitro

Niveditha Dinesh¹  0000-0009-8515-3092

Sethu Madhavan¹  0009-0004-3007-4761

Rashid Muhammed N R¹  0009-0005-6205-2599

Deepa John²  0000-0001-9065-7089

Sini Hariharan³  0000-0002-7006-8209

Kottayath G Nevin¹  0000-0002-0844-9342

¹Department of Marine Bioscience, Faculty of Ocean Science and Technology, Kerala University of Fisheries and Ocean Studies, Panangad PO, Kochi-682506, India.

²Centre for Bioactive Substances from Marine Organisms, Kerala University of Fisheries and Ocean Studies, Panangad PO, Kochi-682506, India.

³Department of Biochemistry, Government College Kariavattom, Thiruvananthapuram-695581, Kerala, India

Correspondence

Kottayath G Nevin
nevinkg@kufos.ac.in

Received: 03.11.2024

Accepted: 12.01.2025

Published: 18.03.2025

Acknowledgment

The authors are grateful to the Sophisticated Analytical Instrument Facility (SAIF), IIT Mumbai, for helping with the HRLC MS-QTOF analysis.

Funding

No funding was received for the work

Conflict of interest

The authors declare no conflict of interest.

Resumen

Introducción: La presente investigación evaluó una nueva cepa de *Bacillus velezensis* SNR14-4, aislada de las branquias de tilapia del Nilo con la intención de considerarla como un contendiente probiótico prometedor.

Métodos: Inicialmente, se llevó a cabo un análisis extenso del genoma del aislado particular empleando herramientas bioinformáticas para anticipar sus características y potenciales atributos probióticos. El genoma total de SNR14-4, reconocido como *B. velezensis* mediante ARNr 16S y secuenciación del genoma completo y análisis filogenético, está compuesto por un cromosoma circular singular con un tamaño de genoma de 4,1 Mb, una longitud total de 4.183.910 pb y una longitud media de 4183910 pb. contenido de guanina-citosina (GC) del 46,52 %. Se adquirieron conocimientos valiosos utilizando AntiSMASH para detectar grupos de genes biosintéticos de metabolitos secundarios, y se logró la anotación de genes funcionales relevantes para los rasgos probióticos utilizando RASTtk y PROKKA. La ausencia de elementos de virulencia, determinada mediante análisis genómico, facilitó una exploración *in vitro* específica.

Resultados: SNR14-4 mostró características probióticas notables y demostró eficacia antimicrobiana contra patógenos comunes de los peces. El análisis HR-LCMS QTOF del extracto microbiano reveló varios compuestos antimicrobianos potentes sintetizados por la cepa.

Conclusiones: *B. velezensis* SNR14-4 se muestra prometedor como candidato a probiótico, ya sea como punto de fuente único o como parte de consorcios de probióticos formados por cepas similares.

Palabras clave: Probióticos; Acuicultura; Antimicrobiano; secuencia del genoma

Abstract

Introduction: The current investigation assessed a novel strain of *Bacillus velezensis* SNR14-4, isolated from the gills of Nile tilapia intending to consider it as a promising probiotic contender.

Methods: Initially, an extensive analysis of the genome of the particular isolate was carried out employing bioinformatics tools to anticipate its characteristics and potential probiotic attributes. The total genome of SNR14-4, recognized as *B. velezensis* via 16S rRNA and whole-genome sequencing and phylogenetic analysis, is composed of a singular circular chromosome with a genome size of 4.1 Mb, a total length of 4183910 bp, and an average guanine-cytosine (GC) content of 46.52 %. Valuable insights were acquired utilizing AntiSMASH to detect secondary metabolite biosynthetic gene clusters, and functional gene annotation relevant to probiotic traits was accomplished by utilizing RASTtk and PROKKA. The absence of virulence elements, ascertained via genomic analysis, facilitated a targeted *in vitro* exploration.

Results: SNR14-4 displayed notable probiotic characteristics and exhibited antimicrobial efficacy against common fish pathogens. HR-LCMS QTOF analysis of the microbial extract unveiled several potent antimicrobial compounds synthesized by the strain.

Conclusions: *B. velezensis* SNR14-4 showcases promise as a probiotic candidate, either as a single point of source or as a part of probiotic consortia made of similar strains.

Keywords: Fish immunity; Probiotics; Aquaculture; Antimicrobial; Genome sequence

Highlights

A novel strain of *Bacillus velezensis* SNR14-4 was identified from Nile tilapia which showed no virulence factors. SNR14-4 showed probiotic features and antimicrobial activity *in vitro* against prevalent fish pathogens and also shown to produce many bioactive components. Genetic analysis of SNR14-4 showed that the stain could be a potential probiotic candidate that might play a valuable role if it is to be incorporated in fish/animal feed or combined with other candidates as a probiotic consortium.

As one of the top ten fishes consumed on a worldwide scale, Tilapia culture requires shielding from bacterial infections that can lead to economic losses for farmers. The most common bacterial pathogens infecting fish, especially tilapia, *Vibrio parahaemolyticus*, *Listeria monocytogenes*, and *Flavobacterium psychrophilum*, are identified and defended by the use of antibiotics when they lead to infection. Although antibiotics have made it easier to treat these pathogenic diseases in fish, the birth of new antibiotic-resistant mutants is a serious threat to tilapia aquaculture and worldwide fish production.

New microbes with potential antibiotic and probiotic characters are required due to the increased resistance of bacteria. This study provides the new strain with all these properties.

This strain has the potential probiotic properties that might play a valuable role if it is to be incorporated in fish/animal feed or combined with other candidates as a probiotic consortium.

Introduction

As one of the most common species of fish, *Oreochromis niloticus*, commonly known as Nile tilapia, has contributed to more than 80 % of the edible fish population in the past few years⁽¹⁾. As one of the top ten fishes consumed on a worldwide scale, Tilapia culture requires shielding from bacterial infections that can lead to economic losses for farmers⁽²⁾. The most common bacterial pathogens infecting fish, especially tilapia, *Vibrio parahaemolyticus*, *Listeria monocytogenes*, and *Flavobacterium psychrophilum*, are identified and defended by the use of antibiotics when they lead to infection^(3,4). Although antibiotics have made it easier to treat these pathogenic diseases in fish, the birth of new antibiotic-resistant mutants is a serious threat to tilapia aquaculture and worldwide fish production⁽⁵⁾.

Probiotics have proven to be a good defense against the rising concern of using antibiotics leading to antibiotic-resistant mutants⁽⁶⁾. The current paper has been successful in revealing the probiotic features of a newly discovered strain of *B. velezensis*. Previously, the strain's candidacy in xenobiotic degradation was revealed using 'gene-before-lab' approach and it also addressed the existence of cryptic gene clusters⁽⁷⁾. The production of antimicrobial compounds essentially does not guarantee that these compounds are good candidates for use as probiotic supplements. *Bacillus spp.*, a broad class of organisms, has long been known to produce many nontoxic compounds that can be effectively used in probiotics⁽⁸⁾. Numerous probiotic microbes have been used in aquaculture for many years⁽⁹⁾, and *Bacillus* species are known for their large number of secondary metabolites that can act against fish pathogens by regulating water quality and the gut microbiota⁽¹⁰⁾. Mining natural sources such as water, soil, and air, for novel strains, could be strenuous and unpredictable due to the incompatibility of the isolates with fish *in vivo*.

Bacillus species, namely, those members of the operational group *Bacillus amyloliquefaciens*⁽¹¹⁾, with a keen focus on *B. velezensis* of this group, have proven to be a treasure house of secondary metabolites that can be applied to numerous industrial sectors⁽¹²⁾. Many researchers have previously explored the potential of *B. velezensis*, and their work has contributed to the discovery of many novel bioactive secondary metabolites that can be used as probiotics in animals, including fish^(8,13-16). The majority of the probiotics used today are counterproductive, as most are originally obtained from non-fish candidates. The need for more effective, naturally adaptive probiotic candidates of fish origin has to have opted for prolonged and fully efficient immunity against fish pathogens. The proximity of antimicrobial-producing microbes in the mouth, gut, or gills can assure resistance against pathogens. As many pathogens can enter through the gills or mouth, it is necessary to protect fish of commercial value by supplementing feeds that contain probiotic isolates⁽¹⁷⁾.

Methods

Characterization of the isolate

The sample collection, screening, isolation, and cultural and morphological characterization of the isolate were done previously⁽⁷⁾. Three healthy *Oreochromis niloticus* (Nile tilapia) were procured from the hatchery of Kerala University of Fisheries and Ocean Studies, located in Kochi, Kerala, India. The health status of the fish was ascertained to be optimal, devoid of any viral, bacterial, or fungal infections. Carefully, the gills of the fish were dissected and positioned onto sterile Petri dishes. A quantity of five grams of gill tissue was precisely measured, followed by rinsing with an equivalent amount of Phosphate-buffered saline (PBS) to cleanse the gills thoroughly. This rinsing procedure was repeated thrice to guarantee the elimination of all impurities and potential contaminants. Subsequently, the

gills were homogenized utilizing a sterile mortar and pestle in 3 ml of PBS. A milliliter of the homogenized specimen was then transferred to a sterile 10 ml screw cap tube along with 9 ml of distilled water to achieve a 10⁻¹ dilution. The solution was subjected to further serial dilutions up to 10⁻⁴. Following this, 100 µl of the solution was inoculated onto nutrient agar plates and placed in an incubator at 37 °C for 24 hours. The predominant bacterial strain was subcultured multiple times to attain a pure culture, initially identified *via* microscopic examination and Gram staining.

Biochemical characteristics

The strain was tested for biochemical characteristics, and most of the protocols for the procedure have been adopted from previously conducted research on other strains of *Bacillus genus*^(18,19,20,21). The biochemical assays involved were the indole test, methyl red test, Voges-Proskauer test, citrate test, urease test, catalase test, hemolysis, starch hydrolysis, and spore formation. Each test was performed in triplicate.

DNA isolation and 16S rRNA sequencing

The DNA isolation was carried out using the protocol described by Green and Sambrook, 2107⁽²²⁾. Briefly, cells were lysed using sodium dodecyl sulfate and proteinase K. The DNA was then extracted with phenol:chloroform, and precipitated with isopropanol. The 16S rRNA gene sequence analysis was carried out to accurately identify the species and the strain that have been isolated^(23,24). PCR amplification was performed using a PCR master mix (2X) from Emerald, with purified and spooled-out isolated DNA. PCR was initiated by denaturation at 94 °C for 5 mins, followed by 35 cycles of denaturation at 94 °C, annealing at 50 °C for 30 s, extension at 72 °C for 2 mins, and a final extension at 72 °C by holding the reaction mixture for 7 mins. The PCR products were analyzed by 1 % agarose gel electrophoresis. The product was subsequently sequenced using the universal bacterial primers forward primer 27F (AGAGTTTGATCCTG GCTCAG) and reverse primer 1492R (GGTTACCTGTGTTACGACTT) through polymerase chain reaction (PCR). The 16S rRNA sequence homology was compared with 16S rRNA sequences of other *B. velezensis* strains available in the National Centre for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST). Similarity analysis was carried out to specifically identify the strain isolated. For further clarity, a phylogenetic tree was constructed using MEGA 11 software to establish an evolutionary relationship. The initially identified sequence was deposited in NCBI.

Gene-before-lab Approach: Whole-genome sequencing combined with de novo assembly

Whole-genome sequencing of the isolated strains was performed with the aid of Medgenome Labs Ltd., Bangalore, India. The quality of the raw data was assessed using the fastq-mcf tool (version – 1.04.8030), and a 2.2 GB file was generated. The sample was sequenced using NovaSeq after the removal of all human contaminants using the BWA-MEM tool (version 0.7.12). De novo assembly was performed using the SPAdes assembler (version. 3.11.1). ORF prediction followed by annotation of the WGS data was carried out using Prodigal software. Reference guide assembly was carried out, and consensus genome FASTA sequences were developed from the aligned bam files using the SAMtools and BCFTools versions. The identity of the isolate was evaluated *via* the genome-based taxonomic classification tool: Type Strain Genome Server (TYGS) run against nine other *Bacillus* members⁽²⁵⁾. The tool was accessed through (<https://tygs.dsmz.de/>). Additionally, the strain identity was evaluated by using the Genome-to-Genome Distance Calculator (GGDC) software⁽²⁶⁾. PubMLSt web service accessed through <https://pubmlst.org/species-id/> was used to compare multiple housekeeping genes that are conserved across related species⁽²⁷⁾. Average Nucleotide Identity (ANI) was calculated using JSpeciesWS accessed *via* <https://jspecies.ribohost.com/jspeciesws/>⁽²⁸⁾.

Functional gene annotation, genome mining for Probiotic marker genes

The consensus genome FASTA file was also used for gene annotation and gene function prediction using several different web-based and online gene annotation pipelines, such as Prokka in Proksee (<https://proksee.ca/>), BAKTA (<https://bakta.computational.bio/>)⁽²⁹⁾, and DFAST (<https://dfast.ddbj.nig.ac.jp/dfc/>)⁽³⁰⁾. The results were further cross-checked against the comprehensive database of the Bac

terial and Viral Bioinformatics Resource Center (BV-BRC) (<https://www.bv-brc.org/>)⁽³¹⁾. The Virulence Factor Database (VFDB) was used to analyze the presence of any virulence genes in the present isolate. The non-virulence properties were further evaluated by using Virulence Finder 2.0⁽³²⁾, AntiSMASH 7.0 was employed for the rapid and thorough genome-wide mining for secondary metabolite-synthesizing gene clusters and gene clusters encoding antibiotics⁽³³⁾. The false prediction level was minimized by manually BLASTing the coding sequences of the biosynthetic gene clusters from AntiSMASH against Pfam⁽³⁴⁾.

Many *Bacillus* strains are known to be good probiotics and are used in feeds and food^(35,36). The probiotic marker genes were manually detected by citing previous studies on probiotic *Bacillus* spp., and the specific genes were searched in both BV-BRC/PATRIC, Prokka, and Protein Data Bank (PDB)^(37,38,39). The sequences were further confirmed using BLASTp against amino acid sequences in the NCBI database. Functional pathways were confirmed by the *Kyoto Encyclopedia of Genes and Genomes* (KEGG) database.

Antibiotic susceptibility and safety of the strain

The isolated strain was tested against several commercial antibiotics^(40,41) purchased from HiMedia. *In silico* analysis was centered on the Resistance GeneIdentifier (RGI) (<https://card.mcmaster.ca/analyze/rgi/>)⁽⁴²⁾. ResFinder 4.5.0 was used to double-check the results of RGI^(43,44). ResFinder was accessed through <http://genepi.food.dtu.dk/resfinder>. The culture (24 hours) was swabbed using sterile cotton swabs on Mueller–Hinton agar plates with proper dilution for uniform growth. The antibiotic discs were carefully placed gently over the swabbed MHA plates and incubated overnight at 37 °C. The results were read by measuring the zone produced, in mm. Pathogen Finder 1.1 was used to evaluate the pathogenicity of the isolate towards the human host⁽⁴⁵⁾.

Bile tolerance, acid tolerance, and high-temperature resistance of the isolate

Genomic analysis data on stress-tolerant genes of probiotic relevance generated from PATRIC, PROKKA, and NCBI BLAST was used as a guiding principle for the *in vitro* evaluation. The evaluation of bile tolerance was carried out using a 24-h-old culture of an isolate that was inoculated in Luria Bertani broth supplemented with 0.30 % (w/v) bile salts at 30 °C and incubated for 0, 1, 2, 3, or 4 h^(41,46). After each hour of incubation, the viable cell count was calculated by streaking the incubated samples onto NA plates. Acid tolerance was determined by inoculating the isolate SNR14-4 into Luria Bertani broth with the pH adjusted to 2, 3, 4, or 5⁽³⁶⁾.

Antimicrobial activity

Based on AntiSMASH evaluation, antibacterial activity was evaluated by checking the activities against *L. monocytogenes*, *Fl. psychrophilum*, *V. parahaemolyticus*. The pathogenic strains were all previously isolated from infected fish hosts^(8,16,47). The antibacterial activity was evaluated initially by the well diffusion method using a cell-free supernatant of the *Bacillus* isolate SNR14-4. Minimal activity was observed against *Flavobacterium* and *V. parahaemolyticus*, and therefore, the crude cell extract was subjected to solvent extraction. The inoculum concentration was set to 1×10^{12} CFU/ml. The cell-free extract of SNR14-4 was further subjected to solvent extraction using different solvents, such as hexane, petroleum ether, and ethyl acetate. 200 ml of CFE were mixed with 200 ml of each solvent in a separating funnel and kept overnight for mass transfer of antimicrobial compounds. The respective fractions were then separated and evaporated using a rotary evaporator. The evaporated residue was dissolved in 0.5 % DMSO. The activity of each fraction was evaluated by the well diffusion method. Fifty microliters of the SNR14-4 sample were added to one well, 50 μ l of 0.5 % DMSO was used as a control, and streptomycin was used as a positive control. To increase the reliability of the results obtained, nutrient media without the inoculate was subjected to the same treatment as carried out with the inoculated media. Solvent extraction of the uninoculated media was carried out using hexane, petroleum ether, and ethyl acetate. The uninoculated media was fractioned and evaporated and used as the negative control. The antimicrobial activity was seen as clear zones, whose diameter was measured in mm. The antibacterial activity was evaluated in triplicate.

Autoaggregation test

The autoaggregation test was carried out using previous reports on similar strains with some modifications⁽³⁷⁾. A 24-hour-old culture of SNR14-4 in 10 ml of Luria media was incubated at 30°C, and the culture was centrifuged at 5000 × g for 15 mins. The pellet was washed with PBS, and after two washes, the pellet was resuspended in PBS at pH 7.2. The absorbance was measured at 620 nm after 1, 2, 3, 4, and 5 h of incubation. The autoaggregation of *B. velezensis* SNR14-4 was calculated by the following formula: autoaggregation % = $(1 - [A_t/A_0]) \times 100$, where A_t denotes the absorbance at time t = 0, 1, 2, 3 and 4 h and A_0 denotes the absorbance at time = 0 h.

Hydrophobicity assay

Hydrophobicity was measured by measuring the adhesion of the strains to organic solvents. For the determination of cell surface hydrophobicity, a protocol previously carried out for probiotic strains with slight modifications⁽⁴⁸⁾. One ml of 24-hour culture was mixed with 100 µl of xylene. The mixture was vortexed for 1 min. After the phases were separated, the aqueous part was removed, the optical density was measured at 480 nm, and the hydrophobicity was calculated using the following equation: Hydrophobicity % = $(H_0 - H_1)/H_0 \times 100$ %, where H is the optical density at 630 nm, H_0 is the initial optical density and H_1 is the final optical density.

Hemolytic activity

The activity of the isolated Bacillus strain was determined on agar plates supplemented with 5% human blood. The isolate was streaked onto agar plates from a 24-hour-old culture. Hemolysis activity was recorded as the extent of the hemolytic zone. Green zones represent α-hemolysis, and the plates were incubated at 37°C for 24-36 hours, clear zones represent β-hemolysis, and no zones represent no hemolysis.

HR-LCMS/MS-Q-TOF analysis

The ethyl acetate fraction that showed prominent activity was analyzed to detect the active component that exhibited antimicrobial activity using HR-LCMS-QTOF (Agilent Technologies, USA). Mass Hunter (Agilent) software was used for data acquisition. A ZORBAX Eclipse Plus – C18 (150 × 2.1 mm, 5 microns, Agilent) column was used with the following gradient solvent system (A: water; B: methanol 95:5), and sample profiling was carried out with a flow rate of 0.3 ml/min.

In silico ADME and toxicity analysis

The *in silico* prediction of the toxicity and ADME parameters, pharmacokinetic properties, druglike nature, and the medicinal chemistry friendliness of the molecules identified through HRLC-MS Qtof was evaluated using Swiss ADME software (<http://www.swissadme.ch/>) and protox-3.0 (<https://tox.charite.de/protox3/>) software.

Statistical analysis

All the tests were conducted in triplicate. The mean values of the observations taken from each replicate were used for statistical analysis. The mean values were statistically analyzed by the Web Agri Stat Package 2.0 (ICAR Goa), and the mean differences were compared at 5 % significance.

Results

Identification of the organism

A total of three isolates were recovered from the homogenized gill fraction. Only one of the three isolates showed appreciable and prominent growth. Hence, this strain was chosen as the primary focus of the present study. After streaking on nutrient agar, the isolated strains formed single colonies that were white, opaque, wrinkled, and dry. The edges were irregular, and the colony as a whole was flat and

rough in appearance with a lobate margin. When picked with an inoculating loop, the colony was sticky and dense. Microscopic observation revealed that the strain was a gram-positive rod, small in cell size, and showed positive results for endospore formation, exhibiting the ability to sporulate. Observations of cell morphology and culture characteristics revealed that the strain was a *Bacillus* species. The isolate showed positive results for methyl red, Voges-Proskauer, urease, starch hydrolysis, and catalase. The citrate utilization test, indole test, and oxidase test were negative. The experiment was carried out in triplicate and all generated the same result⁽⁷⁾.

Molecular identification of the strain as *B. velezensis* SNR14-4

The 16S rRNA sequencing results were compared with other 16S rRNA sequences available in NCBI using BLAST. The isolated strain showed 99% similarity to *B. velezensis*. The phylogenetic tree constructed using MEGA 11 software (Fig 1) shows the clade distance score, which confirmed that the organism was a novel strain of *B. velezensis*.

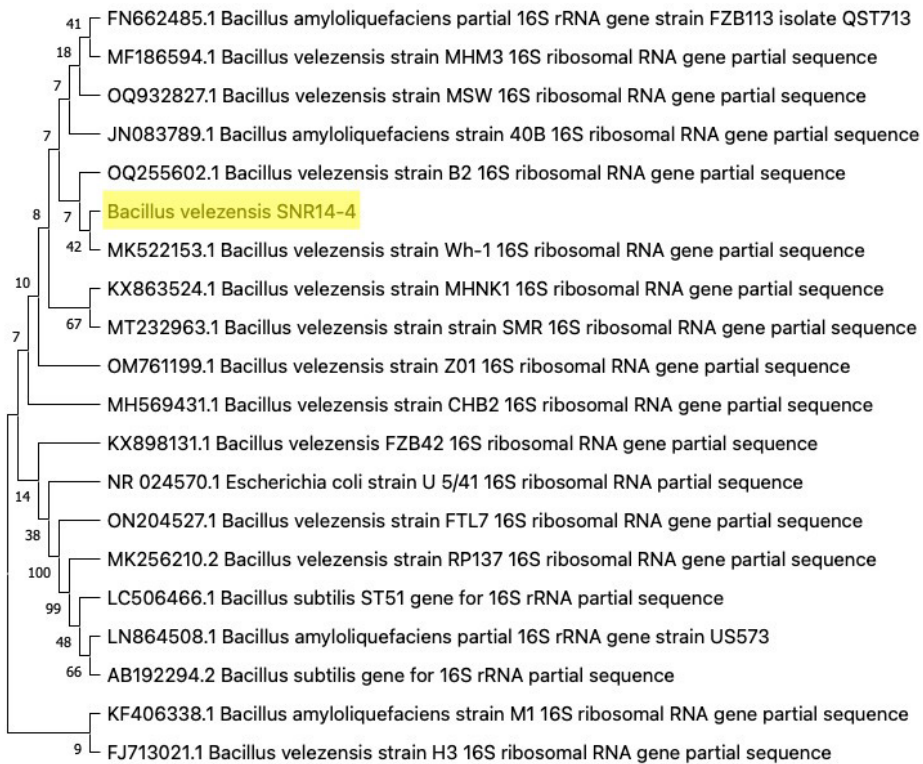


Figure 1. Phylogeny of the isolated strain. Phylogenetic tree constructed by the neighbor-joining method using MEGA 11 software, showing the relatedness of *B. velezensis* SNR14-4 to other *B. species* (Madhavan et al., 2024).

A detailed phylogenetic analysis was done on the strain previously while exploring the genomic potential of the strain by the same authors and the obtained 16S rRNA sequence was submitted to GenBank under the designation *B. velezensis* SNR14-4 (accession number: OR226766)⁽⁷⁾.

Insights into the complete genome assembly of *B. velezensis* SNR14-4

De novo assembly was completed using the SPAdes assembler (v3.11.1), and the assembly was executed with a k-mer size of 55 using the de-Brujin graph method, which revealed that the number of scaffolds (≥ 0 _bp) was 343, the N50 was 166965 bp, the N75 was 117388 bp, the L50 was 8 bp, and the L75 was 16 bp. The assembly created a 4.1 Mb file with a single circular chromosome and 1 contig with a genome length of 41,83,910 bp, with an average guanine-cytosine (GC) content of 46.52 %, which is consistent with the GC ratio of other *B. velezensis* variants⁽⁷⁾. The sample was aligned to the reference genome using NCBI BLAST, and the closest match was with *B. velezensis* strain *SGAir0473*, with an alignment percentage of 93.64 %. The strain was proposed as a novel. The sequence was then named *B. velezensis* strain *SNR14-4* and deposited in the SRA-NCBI database with Bioproject accession ID: PRJNA994302 (<https://www.ncbi.nlm.nih.gov/sra/PRJNA994302>). 16S rRNA sequencing analysis showed that the strain was *B. velezensis*. 16S rRNA sequencing analysis showed that the strain was *B. velezensis*. The strain's identity was confirmed using additional tools such as PubMLST, TYGS, JSpeciesWS and GGDC. TYGC analysis of the isolate against nine members of *Bacillus* genus showed that *B. velezensis* with NRRL B-41580 showed more similarity. GGDC data generated based on TYGC evaluation by comparing with several sample strains also showed that the strain is *B. velezensis* (Table 1).

Table 1. Genome-to-Genome Distance Calculation of different reference genome against Sample

Query genome	Reference genome	DDH	Model C. I	Distance	Prob.DDH \geq 70 %
Formula 1 (HSP length/total length)					
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> F3A	87.20	83.6 - 90.1	0.0959	96.12
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> ATR2	90.8	87.7 - 93.2	0.0765	97.56
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> FZB42	85.6	81.9 - 88.6	0.1040	95.31
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> CYS06	86.1	82.4 - 89.1	0.1017	95.16
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> B8	87.6	84.1 - 90.4	0.0224	91.2
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> R-71003	83.7	79.9 - 86.9	0.1135	94.15
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> LF 01	86	82.3 - 89	0.1022	95.51
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> CPA1-1	87.6	84 - 90.4	0.0939	96.3
Formula 2 (identities/HSP length)					
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> F3A	81	78.1 - 83.6	0.0222	91.33
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> ATR2	90.5	88.3 - 92.4	0.0115	95.97
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> FZB42	85	82.3 - 87.4	0.0176	93.72
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> CYS06	80.8	77.9 - 83.4	0.0224	91.2
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> B8	83.4	80.6 - 85.9	0.0194	92.87
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> R-71003	83.6	80.8 - 86.1	0.0192	92.98
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> LF 01	80.3	77.4 - 82.9	0.023	90.82
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> CPA1-1	83.4	80.6 - 85.9	0.0194	92.86
Formula 3 (identities/total length)					
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> F3A	89	86.1 - 91.3	0.1160	99.27
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> ATR2	93.2	90.9 - 95	0.0871	99.73
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> FZB42	88.4	85.5 - 90.8	0.1198	99.16
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> CYS06	88.1	85.1 - 90.	0.1218	99.11
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> B8	89.7	86.9 - 91.9	0.1114	99.38
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> R-71003	86.7	83.6 - 89.2	0.1305	98.79
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> LF 01	87.9	84.9 - 90.4	0.1228	99.07
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> CPA1-1	89.7	86.9 - 91.9	0.1115	99.37

DDH- DNA-DNA Hybridization: HSP - High-Scoring Segment Pair

The process of calculating distances involves two steps: (i) comparing two genomes using the BLAST program to retrieve HSPs/MUMs, and (ii) utilizing three different formulas to estimate the distance from

the set of HSPs/MUMs. The distances are converted to DDH-analogous values. The likelihoods that DDH is $\geq 70\%$ and $\geq 79\%$ are reported using logistic regression (a particular kind of GLM). The primary purpose of GGDC is to determine the species' in silico relatedness.

PubMLST, and JSpeciesWS analysis also further confirmed the identity of the species as *B. velezensis*. The comparative G+C percentage content of SNR 14-4 with other *velezensis* species showed slight difference (Table 2).

Table 2. Percentage of G+C content difference of reference to *B. velezensis* SNR14-4

Query genome	Reference genome	Difference in % G+C
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> F3A	0.02
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> ATR2	0.18
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> FZB42	0.05
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> CYS06	0.07
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> B8	0.02
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> R-71003	0.39
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> LF 01	0.04
<i>B. velezensis</i> SNR14-4	<i>B. velezensis</i> CPA1-1	0.03

>1 within a single species; ≤ 1 between distinct species; (G+C : Guanine + Cytosine)

Gene annotation and functional gene prediction show the absence of virulence factors

The RASTtk bioinformatic tool in BV-BRC produced more detailed analytical data on the genome of the isolates, and the resulting contig data showed a total of 4163 CDSs, 205 repeat regions, 84 tRNAs, and 27 rRNAs. The data were cross-reviewed with other gene annotation pipelines, such as DFAST and BAKTA⁽⁷⁾. VFDB analysis through PATRIC showed that no potent virulence factors were present in the isolated strain which was confirmed through Virulence Finder2.0 that showed no hits. The absence of virulence factors hints at the safety of the strain for use as a probiotic. Moreover, there were 50 drug targets and 197 transporters in the overall genome of the isolate.

Genome mining revealed several probiotic markers in *B. velezensis* SNR14-4

Genome analysis using AntiSMASH revealed the presence of different antibiotic-, antifungal-, and bacteriocin-encoding gene clusters in SNR14-4. The results are summarized in Table 3.

Table 3. AntiSMASH analysis of SNR14-4 for secondary metabolites and biosynthesis-related gene clusters.

Region	Type	Nucleotide length		Probable similarity to known cluster	Similarity (%)
		From	To		
Region 1	NRPS, transAT-PKS, betalactone	108,443	242,587	Fengycin (NRP)	92
Region 2	Terpene	271,311	291,438	-	-
Region 3	T3PKS	373,617	413,965	-	-
Region 4	transAT-PKS	542,626	634,999	Diffricidin (Polyketide)	100
Region 5	NRP-metallophore, NRPS, RIPP-like	1,275,489	1,327,282	Bacillibactin (NRP)	100
Region 6	NRPS	1,607,518	1,673,902	-	-
Region 7	Other	1,868,903	1,910,321	Bacilysin (Other)	100
Region 8	NRPS	2,522,641	2,587,693	Surfactin (NRP:Lipo-peptide8)	100

Region 9	PKS-like	3,157,783	3,199,027	butirosin A/butirosin saccharide	7
Region 10	Terpene	3,281,765	3,302,505	-	-
Region 11	TransAT-PKS	3,606,557	3,694,385	Macrolactin H (Polyketide)	100
Region 12	TransAT-PKS, T3PKS, NRPS	3,914,441	4,017,072	Bacillaene (Polyketide+NRP)	100

NRPS: nonribosomal peptide synthetase; T3PKS: Type III polyketide synthases; AT-PKS: Transacyltransferase polyketide synthases; RiPP: Ribosomally synthesized and posttranslationally modified peptides.

The presence of diffidin, fengycin, bacillaene, bacilysin, bacillibactin, and macrolactin H biosynthesis-related gene clusters may indicate the probiotic characteristics of the strain. The genes that impart probiotic characteristics are summarized in Table 4.

Table 4. Marker genes of SNR14-4 exhibiting probiotic properties generated by genomic mining using PATRIC, NCBI and Prokka

Probiotic Gene Clusters and Functions	Coverage percentage	Similarity percentage
Antimicrobial Production Genes		
Surfactin (srfAA)	99	97
Bacillomycin (bmyD)	100	97.48
Fengycin (fenA)	100	98.61
Iturin (ItuD (ituD), ItuA (ituA), ItuB (ituB), and ItuC (ituC))	84	97.14
Difficin (dfnF)	100	97.99
Macrolactin (mlnI)	100	98.35
Bacillibactin(dhbE)	95	67.92
Bacilysin (bacA)	100	99.72
Bacillaene (bael)	100	99.6
Bacillaene (baeS)	99	97.84
Stress Resistance Genes		
Sigma factor B(sigB)	100	99.13
GroEL/ES	79	78.01
Adhesion and Biofilm Formation		
Biofilm matrix protein (tasA)	100	99.24
Immune System Modulation		
Spo0A	98	68.01

The genes were curated from previously reported works on *Bacillus*, and the genes were manually searched in the annotated assembly using PATRIC, NCBI, and Prokka^(18,19). All results align with previously reported research on probiotic strains. The presence of these genes is beneficial for targeting and confirming the probiotic characteristics and safety of the novel *B. velezensis* strain. The manual search for probiotic genes and BLAST analysis of the genome showed that the isolate's genome contained genes that encode for Iturin, an antifungal lipopeptide that can disturb the plasma membrane of fungal pathogens mediated by oxidative stress as well as by interfering with glycolysis, gluconeogenesis and the tricarboxylic acid cycle⁽²⁰⁾. AntiSMASH analysis does not reveal these antimicrobial genes. These genes could be inactive or non-functional but their presence in the genome could address the existence of cryptic genes. They may be activated under the right circumstances.

SNR14-4 shows antibiotic susceptibility

The isolates were susceptible to the following antibiotics: clarithromycin (CLR), streptomycin (S), chloramphenicol (C), tetracycline (TE), kanamycin (K), vancomycin (VA), doxycycline hydrochloride (DO), erythromycin (E), levofloxacin (LE), and ampicillin (A/S). Clarithromycin and ampicillin had the greatest effects on the growth of the isolates. Comparatively, the isolate showed less sensitivity to vancomycin and tetracycline. The diameter of each disc formed against the isolated strain is summarized in Table 5.

Table 5. Antibiotic susceptibility of the isolate of SNR14-4

Antibiotic used	Zone Diameter
Clarithromycin	37 ± 0.2 mm
Streptomycin	34 ± 0.2 mm
Chloramphenicol	30 ± 0.1 mm
Vancomycin	28 ± 0.1 mm
Doxycycline hydrochloride	30 ± 0.1 mm
Tetracycline	28 ± 0.1 mm
Kanamycin	29 ± 0.1 mm
Erythromycin	30 ± 0.1 mm
Levofloxacin	35 ± 0.1 mm
Ampicillin	37 ± 0.1 mm

The values are the means ± SDs of three independent estimations

The genome was evaluated for antibiotic-resistant genes (ARGs) using ResFinder and CARD RGI, which showed the absence of significant virulence factors. Even though RGI reported the presence of *clbA*, *tet(45)*, and *Bcl*-resistant genes the *in vitro* analysis showed no resistance, hence these genes could either be cryptic or non-functional. Pathogen Finder showed that the isolate is not harmful to humans.

SNR14-4 is tolerant to pH, and bile

Strain *SNR14-4* showed appreciable resistance to low pH values of 2 and 3, making the isolate valuable for being incorporated into fish feed to survive acidic conditions within the gut of the fish (Fig 2)^(49,50).

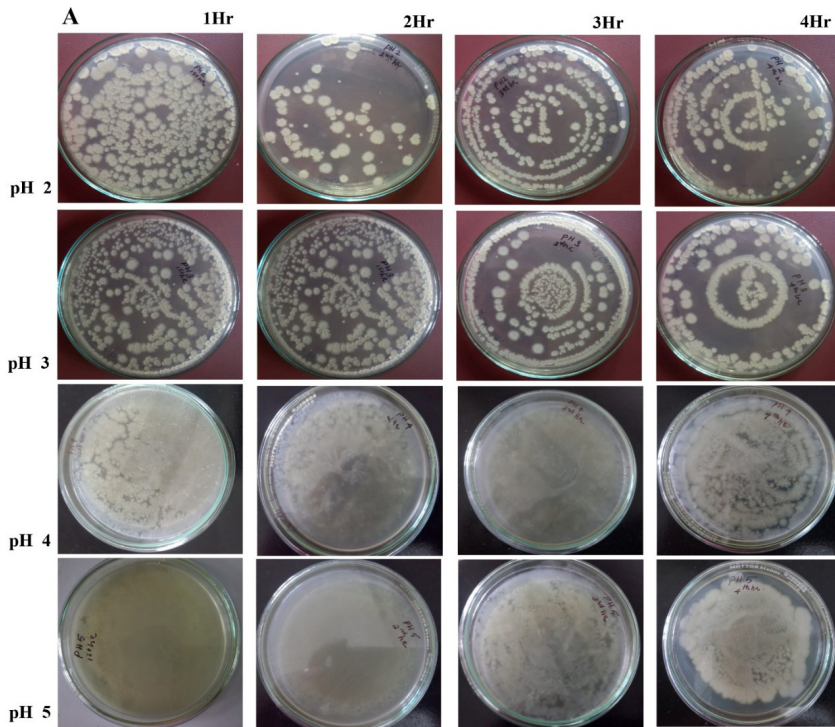


Figure 2. Acid tolerance of SNR14-4 in the pH range of 2-5. A: Growth pattern of SNR14-4 at different pH values: pH 2-, pH 3, pH 4, and pH 5 tolerant colonies from the first to fourth hours of incubation to fourth hours of incubation, M-P: pH 5-tolerant colonies from the first to fourth hours of incubation. The experiment was conducted in triplicate.

A bile tolerance assay was carried out using varying concentrations of bile salt in Luria Bertani, with a maximum concentration of 0.3 %. The maximum exposure time was 4 hours, and all plates incubated showed more than 50 % survivability. The experiment was carried out in triplicate (Fig 3).

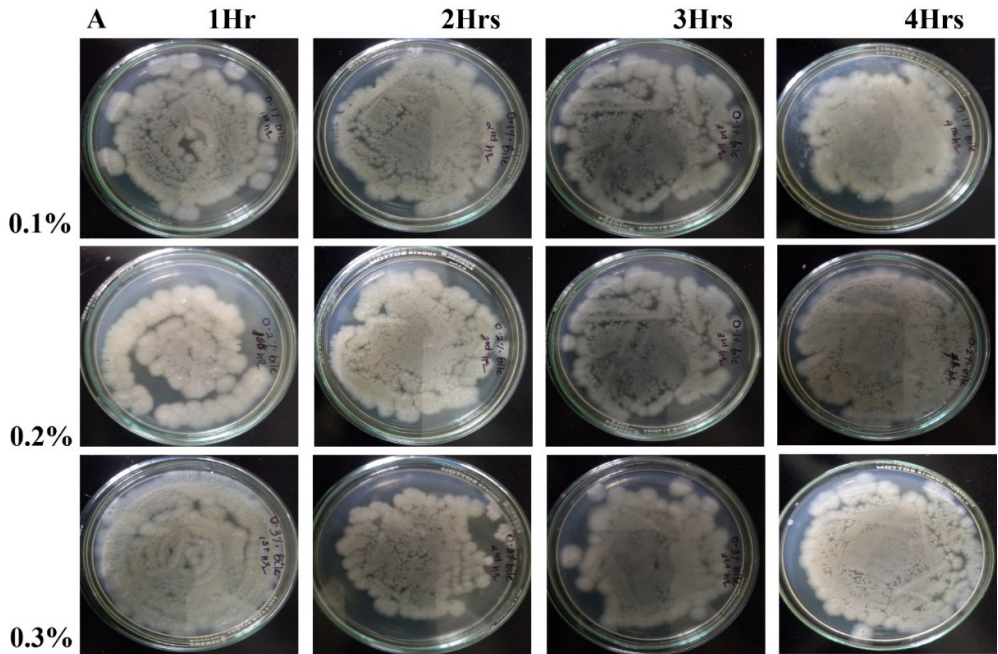


Figure 3. Bile tolerance of SNR14-4 to varying concentrations of 0.1-0.3 % bile for incubation periods of 1 to 4 hrs. The experiment was conducted in triplicate.

Tolerance to acid and bile has proven to be advantageous to the isolate, as it can effectively colonize the guts of the fish under *in vivo* conditions. The results were found to be consistent with the analysis of PATRIC and NCBI.

SNR14-4 shows promising *in vitro* antimicrobial activity

The isolated strain could produce antimicrobial activity against the prominent pathogenic *Flavobacterium* spp., *Listeria*, and *Vibrio* spp. Among the different solvent fractions, the ethyl acetate fraction exhibited antimicrobial activity. The control of DMSO (0.5 %) showed no clear zone, and the positive control of streptomycin was used to compare the diameter of the clear zone produced by the isolate. The diameters, summarized in Table 6, show that the isolate has good potential for use as a substitute for synthetic or commonly available antibiotics, with the greatest activity against *V. parahaemolyticus* (18 mm zone) and the least activity against *Flavobacterium* (14 mm zone).

Table 6. Antibacterial activity of the isolate of SNR14-4

Pathogen	Control (mm)	EA _f (mm)
<i>Flavobacterium</i> spp.	33 ± 0.1 mm	15 ± 0.1 mm
<i>Vibrio parahaemolyticus</i>	26 ± 0.1 mm	18 ± 0.1 mm
<i>Listeria monocytogenes</i>	19 ± 0.1 mm	17 ± 0.1 mm

EA_f: Ethyl acetate Fraction; The values are the means ± SDs of three independent estimations.

The uninoculated media that was treated as same as the inoculated media showed no activity. The analysis of antibiotic isolation and purification from the ethyl acetate fraction is the scope of further research. The results generated were parallel to those predicted by the gene-before-lab approach.

SNR14-4 showed promising probiotic characteristics; autoaggregation, hydrophobicity, and hemolytic activity

The autoaggregation assay of the isolated strain SNR14-4 revealed 85.7 % autoaggregation at the first hour (T_1), 84.2 % at the second hour, and 85.3 %, 85.5 %, and 85.9 % at 3 h, 4 h and 5 h, respectively. The hemolytic assay carried out using 5% human blood showed no hemolytic zones, as observed for alpha hemolysis. Strains with alpha and gamma hemolytic activity were previously used as probiotic strains (data not shown). A strain isolated from fish gills has proven to be a good candidate for use as a probiotic because of its α -hemolysis property⁽⁵¹⁾

Preliminary phytochemical analysis and HR-LCMS-QT reveals several bioactive compounds from SNR14-4

Preliminary phytochemical analysis revealed the presence of several components in the ethyl acetate fraction of *viz.* Coumarins, terpenoids, flavonoids, polyphenolic compounds, etc. Although the total ion chromatogram of the HRLC MS-QTOF analysis of ethyl acetate showed several peaks, a few compounds were identified. The identified compounds were saponins (cyclopassifloic acid C), alkaloids (guanvoline and dihydroaspidospermatine), biosurfactants (surfactin), aliphatic nitro compounds (miserotoxin) and aspergillilic acid (Table 7).

Table 7. Biologically active compounds identified using HRLCMS-QTOF

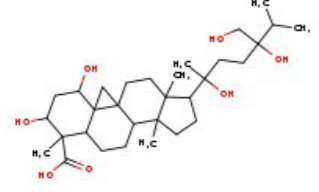
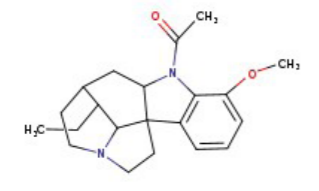
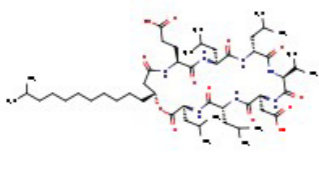
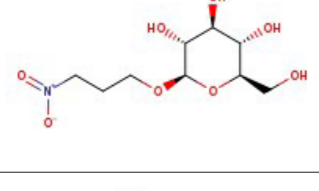
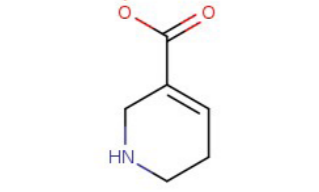
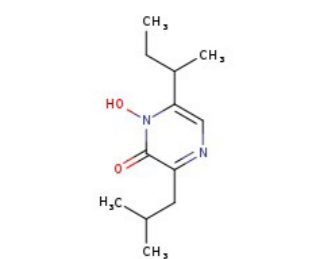
Sl No	RT	Mode Compound	Mass	Formula
1.	13.803 -ve	Cyclopassifloic acid C	536.3691	Ca1 H2O7
2.	23.463 -ve	14,19-Dihydroaspidospermatine	340.2129	Ca1 H28 N2 O2
3.	24.972 -ve	Surfactin	1035.6881	Cs3 H93 N7 O13
4.	1.629 +ve	Miserotoxin	267.0954	Co H17 N O8
5.	3.338 +ve	Guvacoline	141.0799	Cr H11 N O2
6.	7.727 +ve	Aspergillilic acid	224.1527	Cu2 H20 N2 O2

RT; Retention time

Compounds showed no toxicity with acceptable ADME properties

The compounds produced by SNR14-4 was found to be non-toxic without any mutagenicity. 14,19-Dihydroaspidospermatine and miserotoxin were predicted to be insoluble. Except cyclopassifloic acid C all other compounds satisfied the Lipinski rule for a potential drug (Table 8).

Table 8. *In silico* ADME and toxicity properties of the compounds produced by SNR14-4

Compound	Structure	Lipophilicity Log P _{o/w}	Water Solubility Log S (SILICOS-IT)	GI absorption	BBB permeant	Druglikeness (Lipinski)	Mutagenicity; Predicted Toxicity class; LD ₅₀
Cyclopassilic acid C		3.66	Soluble	Low	No	No; 2 violations: MW>500, NHorOH>5	No;6; 9800mg/kg
14,19-Dihydroaspido-spermatine		2.88	Moderately soluble	High	Yes	Yes; 0 violation	No; 4; 325mg/kg
Surfactin		4.83	Insoluble	Low	No	No; 3 violations: MW>500, NorO>10, NHorOH>5	No;4; 1190mg/kg
Miserotoxin		-2.25	Insoluble	Low	No	Yes; 0 violation	No; 6; 23000mg/kg
Guvacoline		0.56	Soluble	High	No	Yes; 0 violation	No;4; 750mg/kg
Aspergillic acid		2.14	Soluble	High	Yes	Yes; 0 violation	No; 4; 600mg/kg

ADME : Absorption, Distribution, Metabolism, and Excretion; GI : Gastrointestinal ; BBB : Brain-blood barrier

Discussion

With decades of research and scientists stating *Bacillus spp.* as the most useful and diverse hub of bioactive secondary metabolites, the present study added a new member to the list. *B. velezensis* SNR14-4 was isolated from Tilapia gills, and relevant assays that define this bacterium as a probiotic were carried out. The present study aimed to introduce a novel and targeted approach for unlocking various characteristics of an organism through a “gene-before-lab” approach. When the approach was employed to analyze the probiotic characteristics of *B. velezensis* SNR14-4, the *in vitro* analysis results were consistent with the bioinformatics predictions. The gene-to-lab approach was beneficial for reducing the *in vitro* analysis time, and 100% of the probiotic test results were confirmed. The strain was culturally, morphologically, and biochemically identified as a member of *Bacillus*. There is a strong genetic similarity of 99% with members of *B. amyloliquefaciens* and *B. velezensis*.^(52,53) Several disputes have been claimed over the identity of specific members of the *Operational Group Bacillus amyloliquefaciens* (OGBa) (which requires techniques such as 16S rRNA sequencing, phylogenetic tree construction, genome to genome calculation and DNA-DNA hybridization value⁽⁵⁴⁾). All these techniques pointed out that the current strain shared most similarity with *B. velezensis*.⁽⁵⁵⁾ Given the close identity morphological and biochemical characterization gives limited information about the strain, but interestingly the strain was urease-positive and amylase-positive. As most *B. velezensis* strains are urease-negative, the urease-positive nature denotes the strain similarity to *B. velezensis* isolated from plant rhizospheres as well as from other soil sources⁽⁵⁶⁾.

Although many *B. velezensis* strains have been isolated, a complete evaluation of the entire gene pool has rarely been reported^(57,58). This is the first genome-guided study in the *B. velezensis* strain to explore the probiotic features. Genome mining and bioinformatics-guided analysis of the whole genome of the isolate revealed the presence of probiotic markers, and these findings were consistent with previously reported work in Nile Tilapia^(51,59). The genetic markers were observed to be functional through *in vitro* evaluation, and their products were confirmed through HR-LCMS analysis. Genome-guided evaluation of strains can also be considered a primary step in building a consortium as a strategic laboratory, and *in vivo* protocols can be designed over time. *Bacillus velezensis* TS5⁽⁶⁰⁾, *Bacillus velezensis* R-71003⁽⁴⁹⁾, *Bacillus velezensis* LF01⁽⁶¹⁾, *Bacillus velezensis* CYS06⁽⁶²⁾, *Bacillus velezensis* CPA1-1⁽⁶³⁾ are some of the strains that has been extensively studied for its probiotic effects, some on fish, with a focus on the genetic information. Considering the undeniable genetic similarity, it can be argued that the current strain can also harbour the same capability. This hypothesis has been proven with the progress of the evaluation.

The work carried out previously on the *Bacillus* species was anchored around the usefulness of the Virulence Finder tool in evaluating the safety of isolates^(60,64). The result obtained using this tool showed that the present strain is safe and free from virulent genetic factors and is not pathogenic to humans, indicating that if fish supplemented with the strain as probiotic feed probably poses no threat to humans. The sporulation ability explains the stress tolerance of the isolate at higher temperatures⁽⁸⁾, bile tolerance and acid tolerance were vital to determine the ability of the isolate to survive in the gastrointestinal tract of Nile tilapia as well as other commercially cultured fish species.

Given the history of using *Bacillus* species as potentially useful probiotics⁽⁶⁵⁾, the present strain also showed hydrophobicity and autoaggregation properties⁽⁶⁶⁾, which, from the viewpoint of a probiotic candidate, are essential, as binding to the mucus epithelium may facilitate the proliferation of probiotic bacteria and lead to better immunity⁽⁶⁷⁾. The research has covered all necessary *in vitro* assays that are required to determine the safety of the strain for incorporation into fish, especially into tilapia, as the strain has no pathogenic traits. Being α -hemolytic as it produces no clear zones but does show a greenish-brown discoloration, results in incomplete hemolysis. The addition of alpha- and gamma-hemolytic strains to fish is safe, as shown in previous research, hence the addition of the present strain along with *in vivo*-proven probiotic strain could amplify the probiotic effect. The autoaggregation values were calculated using previously published works on *Bacillus* as well as probiotics^(67,68). The higher values of autoaggregation show that the strain has better potential to colonize the fish gut and stimulate immune responses, as well as establish better immunity. Hydrophobicity is an important prop-

erty of probiotic bacteria, and the current isolate has shown 78 % hydrophobicity⁽⁶⁹⁾. Hydrophobicity is predicted based on the MATH formula, which corresponds to van der Waals forces and electrostatic interactions between microbes and organic solvents and indirectly reflects the efficiency of binding or adhering to the gut tissue of the fish⁽⁶⁷⁾.

A potential fish probiotic candidate should show good activity against fish-pathogen by producing antibiotics. The evaluation of the present strain in comparison to other similar strains proposes to be a substitute for normally available antibiotics and boost aquaculture as never before if incorporated with feed or as a part of a consortium⁽⁷⁰⁻⁷³⁾. The *in silico* analysis has guided the research to be time-efficient and resource-saving. It also addresses the presence of cryptic genes present in the genome. With further study of the genome characters, it is possible to configure an *in vitro* condition that can activate the cryptic gene clusters and possibly generate novel bioactive compounds.

The ethyl acetate fraction actively contains a large number of strong antibacterial and fungicidal chemicals, according to the HR-LCMS-QTOF analytical results. These substances could be the cause of the inhibitory effects on fish pathogenicity. The chemicals of interest identified are alkaloids, dihydroaspido-spermatine, which inhibits TNF- α ^(74,75), guvacolinean, which has some cytotoxic activity⁽⁷⁶⁾, cyclopassifloic acid C, which belongs to the class saponins⁽⁷⁷⁾, miserotoxin, a nitro compound⁽⁷⁸⁾ with proven antibacterial activity, and aspergillid acid⁽⁷⁹⁾, which is most widely used as an antibiotic and an anti-fungal agent. Surfactin, an extremely potent cyclic lipopeptide biosurfactant made by several *Bacillus* strains, shows haemolytic, antiviral, antibacterial, and anticancer effects, particularly on Ehrlich ascites, breast and colon cancers, leukemia and hepatoma^(80,81). The presence of these compounds in the active fraction explains the effect of the novel strain against the selected pathogens. These fungicidal compounds, as well as biocontrol agents against plant fungi, also illustrate the immense potential of *SNR14-4* against fungal pathogens in tilapia (data unpublished). Through a comprehensive approach starting from 'gene-before-lab', *in vitro* analysis, and HR-LCMS analysis, *B. velezensis* *SNR14-4* can be considered a safe, useful, and effective member of the *Bacillus* genus for use as a probiotic candidate. The extent of further exploration of the industrial potential of this novel strain remains the scope of future research.

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