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Probiotic potential of *Bacillus velezensis* STPB10 sourced from the gut microbiota of a hillstream fish *Schizothorax richardsonii* (Gray, 1832) for aquaculture applications

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The study focused on the evaluation of the probiotic potency of *Bacillus velenzensis* STPB10, isolated from the digestive tract of the hillstream fish Snow trout (*Schizothorax richardsonii)*. Ten *B. velezensis* strains were identified through colony morphology, biochemical analysis, and 16 S rRNA methods. The representative laboratory strain STPB10, was subjected to various in vitro assessments including tolerance to pH, bile salt resistance, NaCl, temperature, biofilm, endospore formation, antibiotic susceptibility and antagonistic activity to determine its potential as a probiotic strain. *B. velezensis* STPB10 exhibited positive results for catalase, glucose, arginine, lysine, and endospore tests, while it yielded negative results for oxidative/fermentative, DNase, and urease tests. This test isolate was γ-hemolytic in nature and demonstrated growth across a temperature range of 15 to 35 °C. Moreover, it exhibited survival rates of 31, 39.7, 47, 63, and 75% at pH values of 2, 2.5, 3, 3.5, and 4, respectively, after 6 h of incubation. At a bile salt concentration of 0.3%, the bacterium exhibited a survivability of more than 99%. A strong biofilm production by *B. velezensis* STPB10 was detected in tryptone soya broth enriched with 0.45% glucose ($p < 0.05$). It also exhibited significantly greater adhesion to intestinal mucus (63.67%). Following exposure of the intestinal mucus to *B. velezensis* STPB10, the adhesion of *A. hydrophila*, *A. veronii*, *(A) salmonicida* and *V. anguillarum* to the mucus was notably reduced. It was susceptible to several antibiotics and produced an antagonistic effect against pathogenic bacteria *Aeromonas salmonicida*, *Aeromonas veronii*, *Vibrio anguillarum*, and *Aeromonas hydrophila* isolated from diseased fish. The pathogenicity of *(B) velezensis* STPB10 through intraperitoneal injection and immersion challenge at cell concentrations of 10^8 and 10^9 CFU mL^{-1} revealed that the strain did not produce any pathogenic risk to common carp. These findings highlight the resilience and adaptability of *B. velezensis* STPB10 as a candidate probiotic in aquaculture.

Keywords Probiotics, *Bacillus velezensis*, Hill streams fishes, *Schizothorax richardsonii*

Notably, freshwater aquaculture comprises 77% of edible aquaculture production, with the majority of farmed finfish originating from freshwater environments. However, freshwater sources make up only 0.3% of the earth's surface water, and they are globally threatened by various factors, including overfishing, pollution, habitat degradation, and climate change[1]. The intensification of aquaculture, characterized by high-density cultivation, has led to environmental challenges. Organic waste discharges deplete oxygen and produce toxic metabolites, while the practice can also disrupt recreational water use. Additionally, water resources are often inefficiently reused in extensive systems, leading to increased disease incidence and declining health in aquatic species[2]. Probiotic bacteria have emerged as a pivotal component in modern aquaculture and fisheries, offering sustainable alternatives to traditional chemical treatments. These beneficial microorganisms, when introduced into aquatic environments, contribute to enhanced health, growth, and disease resistance in cultured species[3]. The incorporation of probiotics also plays a crucial role in maintaining water quality within aquaculture systems. Certain probiotic strains aid in the decomposition of organic matter, reduction of harmful substances

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like ammonia and nitrite, and the prevention of pathogenic microbial proliferation, thereby promoting a healthier aquatic environment[3]. Probiotics derived from fish gut microbiota can protect against pathogens, aid in digestion, and boost the immune response[4]. Microorganisms like *Lactobacillus*, *Bacillus*, *Enterococcus*, and *Vibrio* act as probiotics in aquaculture[5], exerting positive effects by maintaining gut microbiota balance, enhancing growth performance, and overall improving the gut ecosystem. The knowledge of fish gut bacterial flora and their potential as probiotics can contribute to sustainable and healthy aquaculture practices[6]. They are the live microorganisms that when consumed in adequate amounts, confer health benefits on the host. They can work through various mechanisms, including inhibitory substance production, competition with pathogens, and immune system stimulation[3]. By understanding the gut microbiota of fish and selecting suitable probiotics, it's possible to improve fish health, growth performance, and overall productivity in aquaculture practices[7]. Probiotics have shown to protect various fish species, including *Labeo rohita*, *Oreochromis niloticus*, *Epinephelus bruneus*, *Oncorhynchus mykiss*, *Cyprinus carpio* and *Dicentrarchus labrax* from infections caused by pathogenic microorganisms[8-14].

Bacillus strains hold promise as potential probiotics and biological control agents. Initially *Bacillus velezensis* was suggested as a heterotypic synonymous with *B. amyloliquefaciens*[15]. *Bacillus velezensis* possesses broad-spectrum antibacterial properties, making it an important biocontrol agent against a wide range of plant and animal diseases[16]. Subsequently, other research highlighted the remarkable ability of *B. velezensis* to combat pathogens by generating various antimicrobial compounds like bacillomycin, surfactin, fengicins, and amylocyclicin[17]. It has shown strong efficacy in controlling plant diseases such as potato scab, lotus root rot, and Fusarium head blight in wheat (New references). *B. velezensis*, discovered in marine recirculation aquaculture systems (RASs), exhibited antagonistic effects against *Aeromonas salmonicida* and *Aeromonas hydrophila*[16]. In addition, *B. velezensis* is effective against several fish infections, including *Aeromonas hydrophila* in crucian carp (*Carassius auratus*), *Streptococcus agalactiae* in Nile tilapia (*Oreochromis niloticus*), *Vibrio harveyi* in hybrid grouper, and *(A) hydrophila* in grass carp[16]. The studies have unveiled the structures of antimicrobial compounds; Bacilysin, Bacillibactin, Bacillaene, Fengycin, Macrolactin H, and Surfactin) from *(B) velezensis*[18], indicating its potential as a probiotic for rainbow trout, *Oncorhynchus mykiss*[19] and other fishes. Genome analysis has verified that *B. velezensis* contains an abundance of genes associated with producing antimicrobial compounds and engaging in D-galacturonate metabolism[20]. Disease and fish health management in aquaculture should prioritize harmless, preventive, and eco-friendly methods due to the limitations in the usage of antibiotics and vaccines. Thus, application of *B. velezensis* as probiotics in fish models can contribute positively to the practices of aquaculture.

In the Kumaon region of Uttarakhand, where freshwater aquaculture plays a significant role, the isolation and characterization of probiotic bacteria from fish gut microbiota in local streams hold great promise for the aquaculture industry. The present study describes an assessment of the probiotic potential of *Bacillus velezensis* STPB10 isolated and characterized from the gut microbiota of a hillstream fish, snow trout, *Schizothorax richardsonii*.

Materials and methods

Collection of fish specimens

Fifty ($n = 50$) *Schizothorax richardsoni*, were collected from the Kosi River in the Kumaon Region of Almora (Latitude 29°30'00" to 29°50'30" North and Longitude 79°30'00" to 79°50'00" East) and transported to the Wet Laboratory at the Directorate of Coldwater Fisheries Bhimtal, Nainital, India in oxygenated polythene bags.

Processing of the gut sample for isolation of B. velezensis

Upon arrival, the fish were first anesthetized and their body surfaces were sterilized with 70% ethanol. Subsequently, they were euthanized using ethyl-3-aminobenzoate methanesulfonate (MS-222 Himedia, India) at a concentration of 300 mg L^{-1}. The gut samples were collected under sterile conditions from *Schizothorax richardsoni* and processed on tryptic soya agar (TSA, HI MEDIA) plates for the isolation of *B. velezensis* along with other gut microbiota (Fig. 1). The culture plates were incubated at 28 °C for 24 to 48 h to promote bacterial growth for subsequent analyses. Following incubation, the morphological characteristics of the bacterial colonies on tryptic soy agar (TSA) were documented. Representative colonies were selected, re-streaked on TSA to obtain pure cultures, and maintained for further characterization. The isolated bacterial strain was designated as laboratory strain STPB10.

Identification of B. velezensis: biochemical and molecular characterization (16 S rRNA gene)

The biochemical identification process for the bacterial isolates was conducted based on established methods and protocols[21]. These tests included determining the Gram staining, KOH test, catalase, and Simon citrate as well as several hydrolysis reactions (starch, aesculin, DNase, skimmed milk, and gelatinase). Additionally, tests for urease, nitrate reduction, indole production, amino acid metabolism (arginine, lysine), and sugar fermentation (sucrose, glucose, fructose, and sorbitol) were carried out for further biochemical analysis.

Genomic DNA extracted using the Wizard Genomic DNA Purification Kit (Promega, USA) underwent PCR with universal primers UFF2 (5' AGAGTTTGATCCTGGCTCAG 3') and URF2 (5' ACG GGC GGT GTG TTC 3'), amplifying a 1400 bp 16 S rDNA sequence[22]. After that, the PCR product was sent for sequencing by Sanger's sequencing method with the help of an ABI Big Dye Terminator Cycle Sequencing kit v3.1 and ABI 3730 XL (Applied Biosystems). The partial forward and reverse sequences of the 16 S rRNA gene were assembled using CLC Genomics Workbench software (version 11.0.1). The resulting consensus sequence was then analyzed using the Basic Local Alignment Search Tool (BLAST) available through the National Center for Biotechnology Information (NCBI) to determine sequence similarity with other *Bacillus* species.

Fig. 1. Collection of gut samples from *Schizothorax richharsonii*.

Construction of phylogenetic tree and analysis

The closely related *Bacillus* sequences of the query sequence under the same genus were retrieved from NCBI along with type strains and some Indian isolates and multiple alignments were done by Clustal W[23]. The aligned sequences were used to infer the evolutionary history according to the neighbour-joining algorithm[24] using Molecular Evolutionary Genetic Analysis (MEGA X)[25]. The evolutionary distances were calculated by the Maximum Composite Likelihood method[26] and the bootstrap test for 1000 replicates was analyzed to estimate the stability of groupings[27]. The phylogenetic tree construction and its analysis revealed the identification of test bacteria to *B. velezensis*, designated with laboratory strain no STPB10.

In vitro assessment of probiotic potency

Biofilm assay

The quantification of biofilm was performed as described by Hassan et al., 2011[28] with some modifications. In 96-well flat-bottom polystyrene microtiter plates, 30 µL of *B. velezensis* at a concentration of 10^8 CFU mL^{-1} was inoculated with 200 µL of growth media (Tryptone soya broth) with and without glucose (0.45%). The plates were then incubated at 28 °C upto 72 h. After incubation, the planktonic cells were eliminated through two washes with phosphate buffer saline (PBS) (pH 7.4). For staining, 150 µL of 0.1% crystal violet was added to each well, and the plates were incubated for 1 h at 28 °C. Subsequently, they were washed with PBS (pH 7.4). The stain retained by adherent bacteria was then resolved by adding 200 µL of 95% ethanol and allowing it to stand at 4 °C for 1 h. The quantification of biofilm formation was performed by measuring the optical density at 590 nm. The cut-off value (ODc) was determined as the mean OD of the negative control. Based on OD measurements, strains were categorized as follows: no biofilm producers (OD ≤ ODc), weak producers (ODc < OD ≤ 2×ODc), moderate producers (2×ODc < OD ≤ 4×ODc), and strong biofilm producers (OD > 4×ODc) (Borger et al. 2012).

Hemolytic activity

Hemolytic activity testing was performed following the method described by Carrillo et al., 1996[29] with slight modifications. To perform the hemolytic activity, a 24 h grown culture of *B. velezensis* STPB10 was inoculated onto a blood agar plate, typically using sheep blood. After inoculation at 28 °C, the plates were incubated overnight to allow the bacteria to grow. The resulting hemolysis was then observed, and the type of hemolysis was determined based on the hemolytic activity produced.

Staining of bacterial spore

The Schaeffer-Fulton method was employed for the staining of bacterial spores, involving the growth of the isolate STPB10 on tryptone soya agar (TSA) at 35 °C for 24 h under aerobic conditions. The bacterial culture was heat-fixed and subjected to staining with malachite green, resulting in the endospores acquiring a distinctive green color. Samples were observed under oil immersion (100X) using a light microscope (Leica DM 3000 LED) for the detailed examination.

Physiological parameters for the growth of B. velezensis STPB10

Temperature

Cultures were exposed to varying temperatures (15 °C, 25 °C, and 35 °C) in tryptic soy broth (TSB). Following 24 to 48 h of incubation, growth was evaluated visually. Turbid growth was considered indicative of a positive result.

Salt tolerance test

The salt tolerance test assessed the test bacterial growth at various salt concentrations (0.5, 2, 4, 6, and 8% NaCl). *B. velezensis* STPB10 was streaked on nutrient agar containing different salt concentrations and incubated at 28 °C for 24 to 48 h, and results were observed. Positive results indicated growth at different salt concentrations.

pH tolerance

pH tolerance activity was performed according to the method described by Millette et al., 2008[30] with slight modifications. *B. velezensis* STPB10 was initially cultured overnight in TSB under aerobic conditions at 35 °C. Subsequently, 1 mL aliquot of culture at log 7 CFU mL^{-1} was inoculated into 10 mL of TSB to establish the initial inoculum of log 6 CFU mL^{-1}. Following this, the cultures were centrifuged and the resulting pellets were subjected to washing twice in the phosphate buffer (pH 7.2). The bacterial pellets were then re-suspended in 5 mL of sterile TSB and the pH was adjusted to the various levels, ranging from 2.0 to 4.0, mimicking gastric conditions. The test culture was incubated in tubes at 37 °C for 0, 3, and 6 h and after incubation, the culture was neutralized using sterile phosphate-buffered saline (PBS). For quantification, 100 µL aliquots and their dilutions were plated on tryptic soy agar and incubated at 37 °C for 48 h. Bacterial colonies on the agar plates were counted, and the survival rate was calculated as a percentage using the formula:

$$\text{Survival Rate } (\%) = (\log \text{CFU } N_1)/(\log \text{CFU } N_0) \times 100$$

where N_1 represents the viable count after incubation, and N_0 is the initial viable count.

Bile salt activity

The strain ability to tolerate bile salts was assessed using a modified method based on the protocol described[31]. The overnight grown *B. velezensis* STPB10 culture with a density of log 6 CFU mL^{-1} was centrifuged and suspended in tryptic soya broth containing bile salts and incubated at 35 °C for 24 h. After the incubation, the culture was plated onto tryptic soya agar plates for colony count and viability assessment. The tube containing tryptic soya broth without the addition of bile salt was kept in control. The Log values of the viable count; log CFU N_1 and log CFU N_0 were calculated for both the bile-exposed bacterial culture and the control, respectively. The survival rate (%) was determined using the formula as stated below:

$$\text{Survival Rate } (\%) = (\log \text{CFU } N_1)/(\log \text{CFU } N_0) \times 100$$

where N_1 is the viable count after bile exposure, and N_0 is the initial viable count before exposure.

Adhesion mucus assays

Intestinal mucus was isolated from healthy common carp (*Cyprinus carpio*) ($n = 30$) with an average body weight of 25 ± 5 g. The fish were starved for 48 h before dissection. Following dissection, the gut was removed, homogenized in PBS and centrifuged twice at 12,000 g for 5 min at 4 °C. The resulting supernatant was collected, and protein concentrations were adjusted to 0.5–1 mg/mL using the Bradford Protein Assay Kit. The mucus solution was then sterilized under UV light exposure for 30 min and stored at −20 °C. The mucus binding to plates was verified[32]. For the adhesion assay, probiotic bacterial strains were stained with SYTO 9 green fluorescent nucleic acid dye (2 µL per 10^8 CFU). Each well of a 96-well plate was coated by adding 25 µL of mucus sample and 75 µL of a coating buffer pH 9.6 (16.8 g of Sodium hydrogen carbonate and 21.2 g of Sodium carbonate). The plate was incubated overnight at 4 °C, followed by washing with PBS. A 25 µL aliquot of a 10^8 CFU mL^{-1} solution of fluorescently labelled bacteria was then added to each well, and the plate was incubated in the dark at room temperature for 30 min. After washing, 50 µL of PBS was added to each well, and fluorescence was read at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The percentage adhesion

to intestinal mucus was quantified[32,33]. Adhesion was quantified as the percentage of fluorescence from the bacteria bound to the mucus relative to the fluorescence of the initial bacterial suspension added to each well.

Antagonistic activity detection

To assess the antagonistic activity of acid-bile-tolerant *B. velezensis* STPB10 against laboratory fish pathogens; *Aeromonas hydrophila* RTMCX1 (JX390650), *Aeromonas veronii* GCAFBLC228 (MK245984), *Aeromonas salmonicida* MHJM250 (MN900593), and *Vibrio anguillarum* MHJL248 (MN900589), the slightly modified agar-well diffusion method was followed[34]. For the preparation of the bacterial cell suspension, STPB10 strain was cultured in tryptone soya broth at 28 °C for 24 h. After the incubation, the cells of STPB10 strain were harvested by centrifugation at 5000 rpm for 10 min. The harvested live cells were then reconstituted in sterile phosphate buffer saline (PBS, pH 7.4) to adjust the final cell concentrations around 10^8 CFU mL^{-1}. The indicator bacterial pathogens, *A. hydrophila* RTMCX1 (JX390650), *A. veronii* GCAFBLC228 (MK245984), *A. salmonicida* MHJM250 (MN900593), and *V. anguillarum* MHJL248 (MN900589), were inoculated onto MHA (Mueller-Hinton agar) plates with a volume of 200 µL. The cell density for each bacterial pathogen was maintained at 10^8 CFU mL^{-1}. Wells of 6.0 mm diameter were cut into MHA agar plates aseptically and allowed to fill with 100 µL of STPB10 live culture. Then MHA plates were incubated at 28 °C for 24 h. After incubation, the plates are examined for zones of inhibition (ZOI) around the wells, with a ZOI of 1 mm or more considered positive. The measurement of the zone of inhibition was carried out using a standard caliper. The measurements were taken from the center of the well to the edge of the clear zone in multiple directions, and the average diameter was calculated to represent the zone of inhibition. This method ensured consistency and provided a reasonable approximation even when the zones were not perfectly circular. The basis for using this approach lies in established microbiological techniques for measuring irregular inhibition zones, as referenced in CLSI (Clinical and Laboratory Standards Institute) guidelines, where averaging measurements across different axes is considered acceptable for non-uniform inhibition zones. While circular zones allow for direct diameter measurements, irregular zones require a more careful estimation using multiple directional readings to maintain accuracy and comparability.

The experiment was conducted in triplicates for each bacterial pathogen.

Assessment of antimicrobial susceptibility

For testing the antimicrobial susceptibility, the disc diffusion assay was done according to the CLSI guidelines[35,36]. A single colony of the isolate was transferred into a test tube comprising 5 mL of TSB medium and incubated at 28 °C in a shaker incubator. After the incubation, the isolates were measured to get 0.5 McFarland standard turbidity (1.5×10^8 CFU mL^{-1}). With a sterile cotton swab, the culture was evenly spread on Muller Hinton (MH) agar plate and allowed to dry for 5 min. Later the antibiotics disc of tetracycline, ampicillin, erythromycin, streptomycin, and kanamycin were placed at centers and pressed down to ensure complete contact with the agar surface. The plates were kept for incubation for 24 h at 35 °C. The zone of inhibition (diameter) was measured to grade the test isolate as sensitive, intermediate, and antibiotic-resistant. The antibiotics were selected based on the gram-positive characteristics of the test bacterium, prior studies on *B. velezensis*, and the global approval of certain antibiotics for use in aquaculture practices.

Challenge study

Preparation of probiotic bacterial suspension of *B. velezensis* bacterial strain STPB10

Freshly grown culture of STPB10 was inoculated into 100 mL of nutrient broth and incubated for 24 h in a shaking incubator to get the required CFU count. The culture was then collected in a sterile 15 mL screw cap conical bottom plastic tube (Tarson, India) and centrifuged at 5000 rpm for 5 min (Sorovel, Thermo Scientific). After decanting the supernatant, the bacterial pellet was suspended in 0.85% PBS and centrifuged at 5000 rpm for 5 min for washing. This washing step was repeated twice to ensure the complete removal of broth contents from the culture. Following thorough washing, the pellet was reconstituted in 100 mL of 0.85% PBS and adjusted using a McFarland densitometer unit, DEN-1 (Grant-bio, England) to bacterial suspensions of 3.0×10^8 CFU mL^{-1} and 3.0×10^9 CFU mL^{-1} for the challenge study.

Experimental frameworkExperimental framework

Intraperitoneal administration of B. velezensis STPB10

The fingerlings ($n = 500$) of common carp, *Cyprinus carpio* with average weight and length of 24.5 ± 1.0 g and 110. 5 ± 0.5 mm respectively, were collected from the cage rearing unit of Bhimtal Lake, Uttarakhand. Subsequently, the experimental fish ($n = 270$) were randomly distributed into 9 medium-sized aquariums (100 L capacity each) with each aquarium housing 30 common carp fingerlings. The fish were then allowed to acclimatized to the Wet laboratory conditions for a week. During the adaptation period, the fish were fed twice daily, at 8:00 am and 5:00 pm. Both the control and two treatment groups of common carp were established in triplicates. Each experimental group received single dose of intraperitoneal injection of 100 µL of *B. velezensis* STPB10 at concentrations of 3.0×10^8 CFU mL^{-1} and 3.0×10^9 CFU mL^{-1} separately, while the control groups were injected with 100 µL of sterile phosphate buffer saline (PBS). The clinical symptoms and mortality of the fish were observed for a period of 30 days after the challenge. After the experiment, the liver, spleen, and kidney tissues from both experimental and control groups of common carp were aseptically collected, homogenized, and plated on tryptic soy agar for the isolation of *B. velezensis* STPB10.

Immersion challenge

In a separate experiment aimed at investigating the pathogenicity of the test bacterium, 90 common carp fingerlings were randomly allocated into control and treatment groups, with each group replicated three times

across nine glass aquariums (each with a capacity of 30 L), housing 10 fish per aquarium. The two treatment groups were subjected to immersion challenges with two different cell concentrations of *B. velezensis* STPB10, adjusted to 3.0×10^8 CFU mL^{-1} and 3.0×10^9 CFU mL^{-1} of bacterial suspensions that were directly added to the aquaria containing 30 L of water, while the control groups received PBS. These concentrations were prepared in smaller volumes (stock suspensions) and then added to each aquarium to achieve the target final concentrations uniformly throughout in the total volume of water per tank. The fish were monitored over a 30-day period post-challenge for any signs of clinical symptoms or mortality. Water quality was carefully maintained throughout the experimental period by partial water exchange (approximately 10–15%) every 48 h and continuous aeration to ensure optimal dissolved oxygen levels. Additionally, uneaten feed and debris were removed regularly to minimize organic load and maintain water hygiene.

In both the challenge studies; intraperitoneal injection and immersion challenge, the survivability of the challenged common carp fingerlings was recorded daily throughout the experimental period.

Statistical analysis

The data were analysed statistically using Student's t-test. Statistical significance was determined at a two-tailed level ($p < 0.05$) and analyzed with the SPSS Statistics program version 19.0 (SPSS, Inc., Chicago, IL, USA). In the figures, numerical data and bars are displayed as mean values with their corresponding standard deviations.

Results

Water quality parameters

The water quality parameters measured during the collection of *Schizothorax* specimens indicated favorable conditions within the sampling river. The average values recorded were as follows; water temperature of 24.2 °C, dissolved oxygen concentration (DO) 7.56 mg L^{-1}, pH 7.0, total dissolved solids 49 mg L^{-1}, and ammonia nitrogen (TAN) 0.01 mg L^{-1}, fell within the optimal range.

Candidate probiotic, B. velezensis

From the digestive tract of 50 hill stream fish, *Schizothorax richardsonii*, 85 bacterial isolates were recovered. Among them, ten ($n = 10$) isolated were characterized as *B. velezensis* strains. Since the phenotypic and genotypic characteristics of all 10 *B. velezensis* isolates were identical, one representative strain, designated STPB10, was selected for testing its probiotic characteristics, including colony morphology, biochemical analysis, and 16 S rRNA gene sequencing.

The other bacterial species identified from the gut contents of *S. richardsonii* included *Bacillus altitudinis*, *B. amyloliquefaciens*, *Exiguobacterium undae*, *Enterobacter lauduigi*, *E. cloacae*, *E. mori*, *Plesiomonas shigelloides*, *Cronobacter sakazakii*, *Citrobacter freundii*, *Staphylococcus epidermidis*, *Aeromonas veronii*, and *Deefgea chitinilytica*.

Morphological, biochemical and physiological characteristics

The chosen representative isolate, STPB10 exhibited round, long rod-shaped white, dry colony morphology and endospore-forming in the nature. It was Gram-positive, and tested positive biochemically for catalase, skimmed milk, arginine, lysine, and urease, and produced gas by utilization of glucose. It was negative for KOH and citrate and nitrate utilization.

Molecular identification and phylogenetic analysis

The isolate of *B. velezensis* STPB10 was identified using 16 S rRNA gene sequencing, and the sequence data was submitted to NCBI with the accession number PP447245. Phylogenetic analysis was conducted using the maximum likelihood method. The evolutionary lineage was deduced through the application of the Maximum Likelihood method and the Kimura 2-parameter model. The tree displaying the highest log likelihood (−5559.40) was presented, with the percentage indicating the frequency of associated taxa clustering together beneath the branches. Initial trees for the heuristic search were generated using the Neighbor-Joining method based on pairwise distances estimated through the Maximum Composite Likelihood (MCL) approach. The scale-drawn tree exhibited branch lengths, representing the number of substitutions per site. This analysis encompassed 23 nucleotide sequences, including codon positions 1 st + 2nd + 3rd + Noncoding, totaling 1596 positions in the final dataset. The evolutionary assessments were executed using MEGA X. Comparing 16 S rRNA gene sequences revealed that *B. velezensis* STPB10 (NCBI PP447245) had a 100% match with NR075005 *B. velezensis* FZB42 16 S and NR116240 *B. velezensis* CBMB205, followed by 99.90% match with NR116022 *B. amyloliquefaciens* BCRC 11,601 (Fig. 2).

Probiotic characteristics of Bacillus velezensis, STPB10

Biofilm formation

Isolate *B. velezensis* STPB10 showed biofilm production in both TSB without glucose and TSB supplemented with 0.45% glucose. However, the significant biofilm production by *B. velezensis* STPB10 was observed in TSB supplemented with 0.45% glucose ($p < 0.05$). The highest OD values; 1.09 and 2.74 were observed at 48 h, whereas at 72 h, the values increased to 1.65 and 3.10 in TSB without glucose and TSB with glucose (0.45%), respectively. Although the OD values at 24 h were below 1, the strain still qualified as a strong biofilm producer (Fig. 3).

Hemolysis and DNase activity

The test isolate *B. velezensis* STPB10 was found γ-hemolytic in nature. It also demonstrated negative DNase activity in the present study.

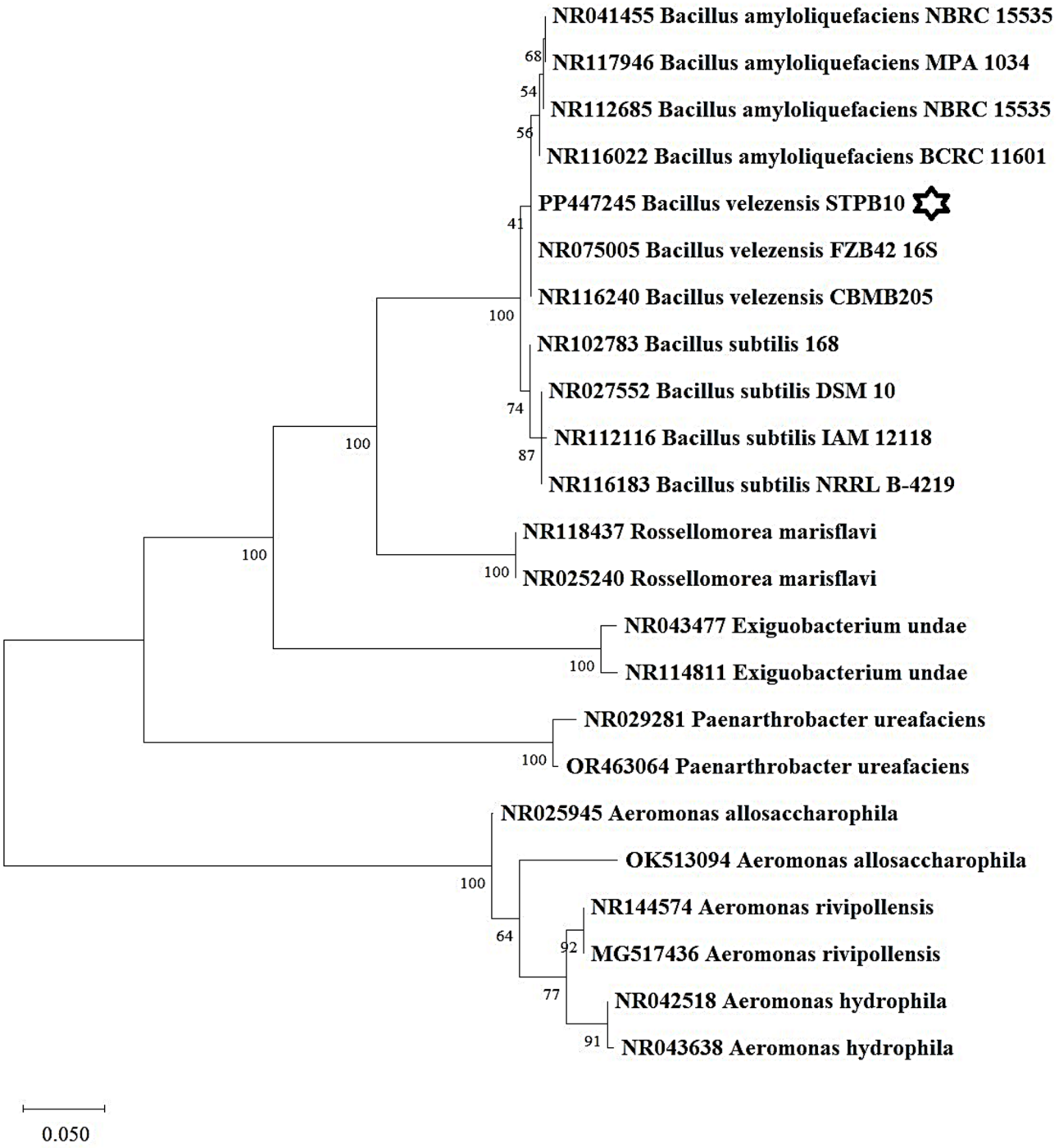

Fig. 2. Phylogenetic analysis of *B. velezensis* STPB10 (NCBI PP447245) demonstrated the evolutionary lineage of the test bacterium with other *Bacillus* species. Species under the genus *Aeromonas*, *Paenarthrobacter*, *Rossellomorea*, and *Exiguobacterium* are taken as outgroup bacteria for the construction of the phylogenetic tree.

Results of physiological parameters versus growth of B. velezensis STPB10

In the study, the probiotic characteristics of *B. velezensis* STPB10 demonstrated significant resilience to environmental stressors.

Tolerance to pH

In the evaluation of low pH tolerance, *B. velezensis* STPB10 showed its ability to survive in different low pH gradients. It exhibited survival rates of 31, 39.7, and 47% at pH values of 2, 2.5, and 3 respectively, post 3 and 6 h

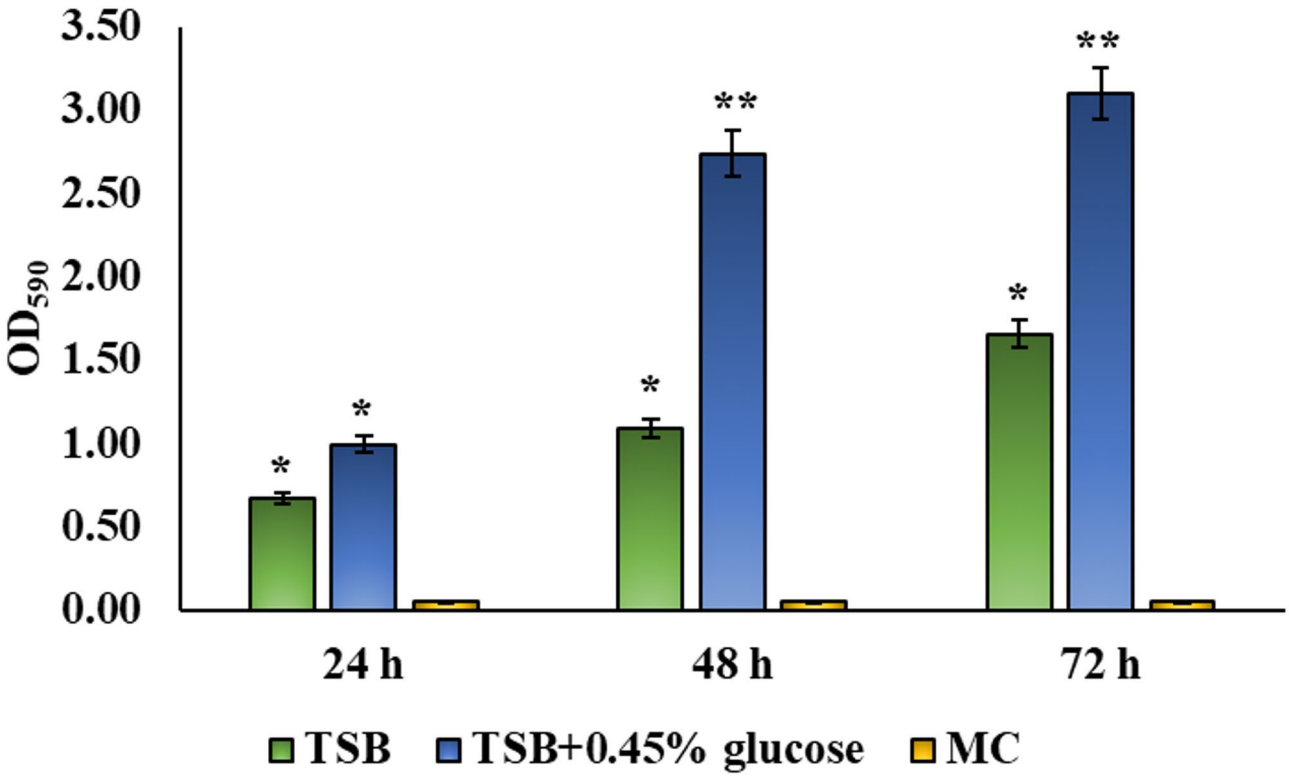

Fig. 3. Biofilm formation of *B. velezensis* STPB10 using the microtitre plate assay. After incubation at 28 °C for 24, 48 and 72 h in TSB media with and without glucose, the data are presented as mean ± standard deviations. The cut-off (ODc) was defined as the mean OD value of the negative control. Based on the OD values, strains were classified as non-biofilm producers (OD ≤ ODc), weak biofilm producers (ODc < OD ≤ 2×ODc), moderate biofilm producers (2×ODc < OD ≤ 4×ODc), or strong biofilm producers (OD > 4×ODc). TSB: Tryptone soya broth; MC: Media control.

Isolate	pH					Bile salt 0.3%
STPB10 survival rates (%)	2.0	2.5	3.0	3.5	4.0	
	31	39.7	47	63	75	99

Table 1. Acid and bile tolerance patterns of *B. velezensis* STPB10 at different pH values and bile salt (0.3%).

incubation period. At higher pH of 3.5 and 4, the test bacterial isolate showed remarkable tolerance, consistently sustaining survival rates of exceeding 63 and 75% respectively, post-3 and 6 h incubation period (Table 1).

Tolerance to 0.3% bile salt

The test for bile salt tolerance, *B. velezensis* STPB10 showed a remarkable growth rate, exceeding 90% survival rates, in 0.3% bile salt, indicating its ability to grow in the presence of bile salt (Table 1).

Tolerance to different NaCl concentrations

The candidate probiotic STPB10 demonstrated significant tolerance to different NaCl concentrations (0.5, 2, and 4% salt) with increasing growth rate. The test isolate STPB10 also showed its growth at higher salt concentrations of 6, and 8% NaCl, but with decreasing growth rate (Fig. 4).

Tolerance to temperature gradients

The isolate STPB10 showed temperature-dependent growth at 15, 25, 30, and 35°C, with a maximum growth rate of 90% at 35 °C (Fig. 5), which indicated that the test bacterium requires higher temperature conditions for optimal viability.

Adhesion to intestinal mucus

B. velezensis STPB10 strain showed significantly higher adhesion to intestinal mucus (63.67%) compared to BSA (18%) and polystyrene (22.67%) ($p < 0.05$) (Fig. 6). After exposure of the intestinal mucus to *B. velezensis*

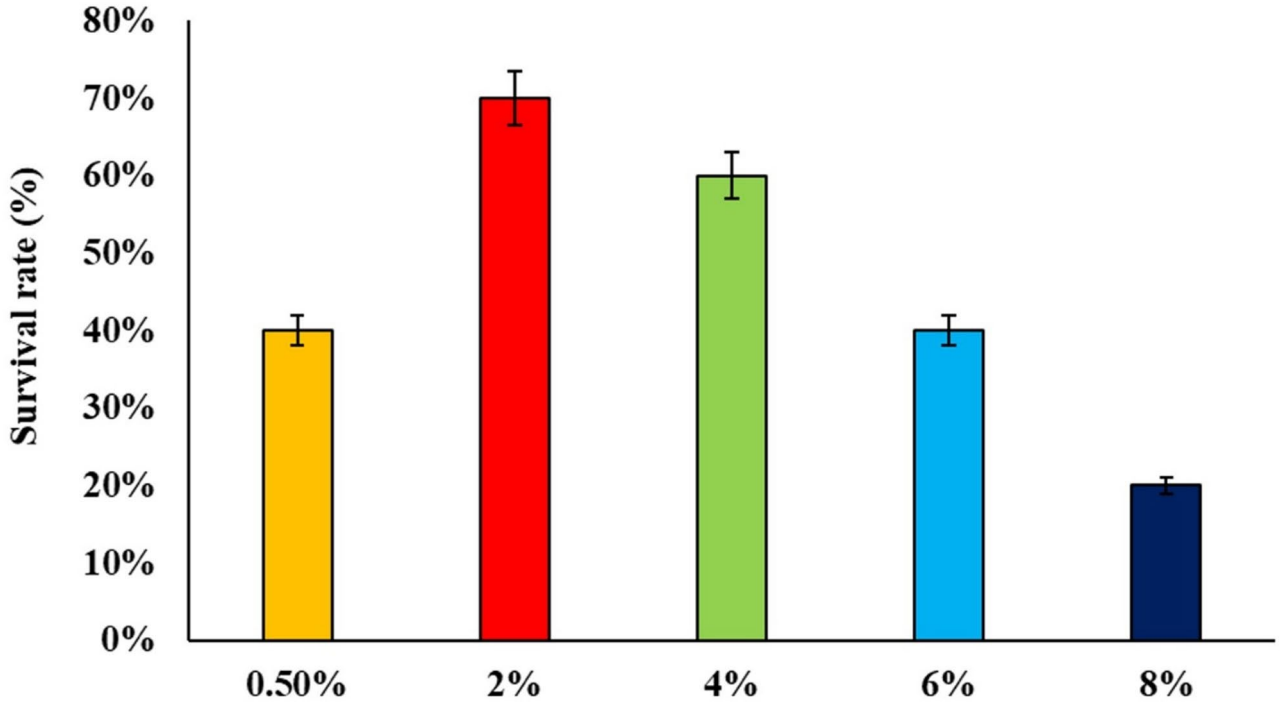

Fig. 4. Salt tolerance of isolate *B. velezensis* STPB10. The test isolate could grow at different NaCl concentrations; 0.5, 2, 4, 6, and 8%.

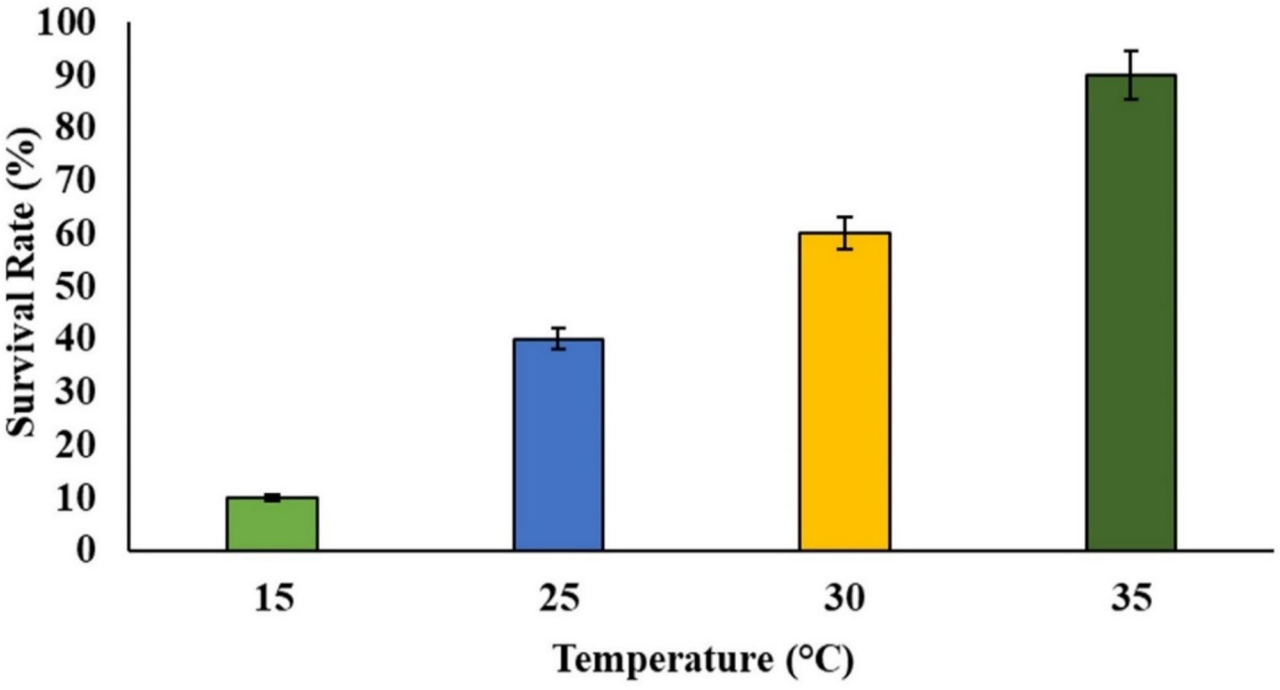

Fig. 5. Temperature tolerance of *B. velezensis* STPB10. The results are the mean values of three replicates. The horizontal bars represent the different temperature levels; 15, 25, 30, and 35 °C, and the vertical bars represent the percentage survival.

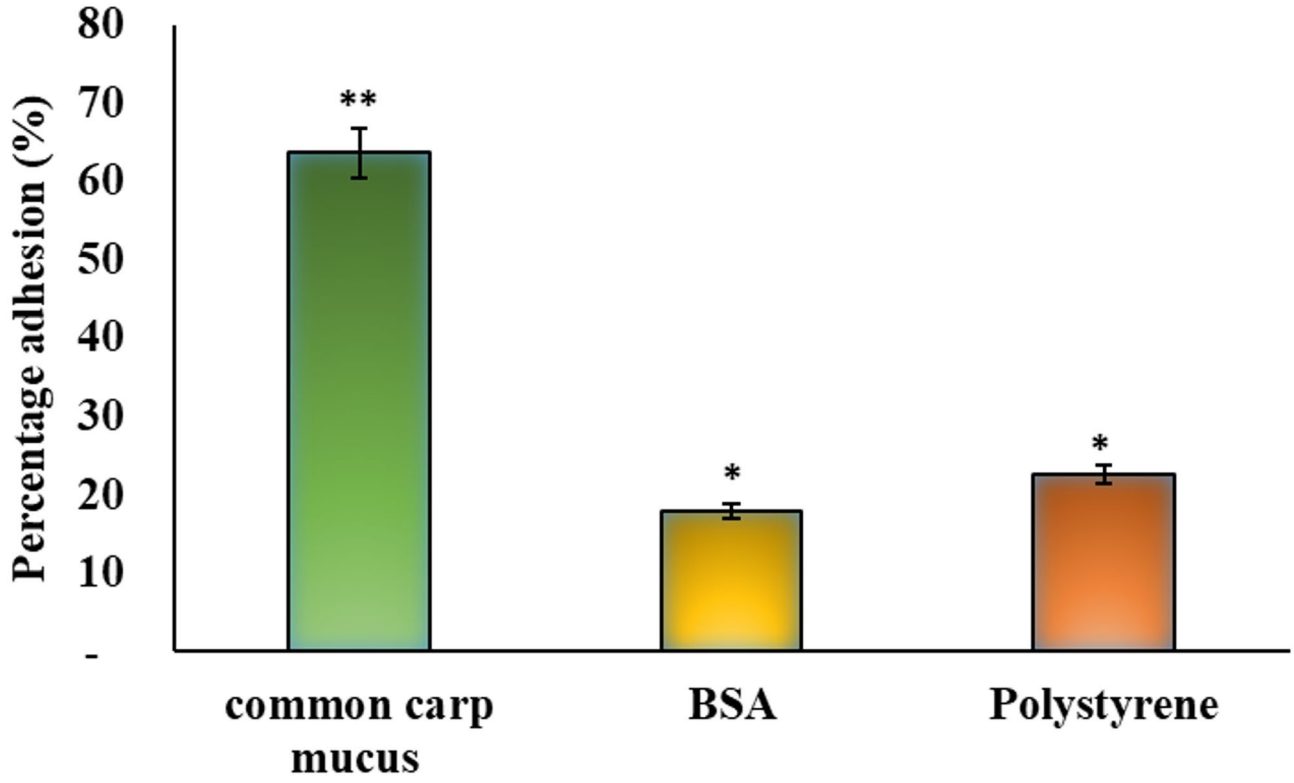

Fig. 6. Percentage adhesion of *Bacillus velezensis* STPB10 strain to common carp intestinal mucus, BSA, and polystyrene. Data presented in the percentage of absorbance of fluorescently stained bacteria ± SD. *Significant is determined at $p < 0.05$.

STPB10, the adhesion capacity of *A. hydrophila*, *A. veronii*, *A. salmonicida*, and *V. anguillarum* to the mucus was remarkably reduced by 26.72%, 45.87%, 58.59%, and 57.14% respectively (Fig. 7).

Antagonistic activities

The antagonistic activity showed the variable effectiveness of *B. velezensis* STPB10 in inhibiting the growth of laboratory bacterial pathogens isolated from the fish. The test bacterium STPB10 displayed the highest antagonistic activity against *A. salmonicida* with an inhibition zone diameter of 30 mm. Effectively in contrast, STPB10 exhibited the lowest antagonistic activity against *A. hydrophila* with a zone diameter of 15 mm. The antagonistic activities against *A. veronii* and *V. anguillarum* were 26 and 22 mm, respectively (Fig. 8).

Antibiogram of *B. velezensis* STPB10

The antibiotic susceptibility results showed that the test bacterium was sensitive to all the approved aquaculture antibiotic tetracycline (37 mm), ampicillin (26 mm), erythromycin (26 mm), streptomycin (35 mm), and kanamycin (27 mm).

Results of challenge assays

In both intraperitoneal and immersion challenge tests, the outcomes revealed no mortalities and observable clinical indications for disease progression among the experimental fish throughout the experimental phase. Additionally, there was no isolation of any *Bacillus* strain from the experimental fish, suggesting that the *B. velezensis* STPB10 strain demonstrates commendable biosafety concerning common carp. Furthermore, the challenged fish exhibited no discernible stress-related traits.

Discussion

In recent years, the use of *Bacillus* species as biological control agents has gathered significant attention due to their versatility and effectiveness. In aquaculture, probiotics such as *Bacillus* spp., *Lactobacillus* spp., and *Saccharomyces* sp. are widely utilized to enhance fish health and productivity[37,38]. Among these, *B. velezensis* is particularly noteworthy for its ability to produce potent bioactive molecules, volatile organic compounds (VOCs) and antimicrobial substances, including bacillomycin, surfactins, phengicins, amylocycine, and lipopeptides, which demonstrate strong antagonistic activity against pathogens[14,39]. Moreover, studies have revealed that *B. velezensis* consumption supports the regulation of the innate immune system and mitigates the pathogenic effects of *(A) veronii* in crucian carp[40]. The present study is focused on screening of probiotic potency of *(B) velezensis* STPB10 isolated from gut of a hillstream fish, which could be beneficial for various applications, including food and the health of the fish in aquaculture. The probiotic candidate, *B. velezensis* STPB10 has been characterized

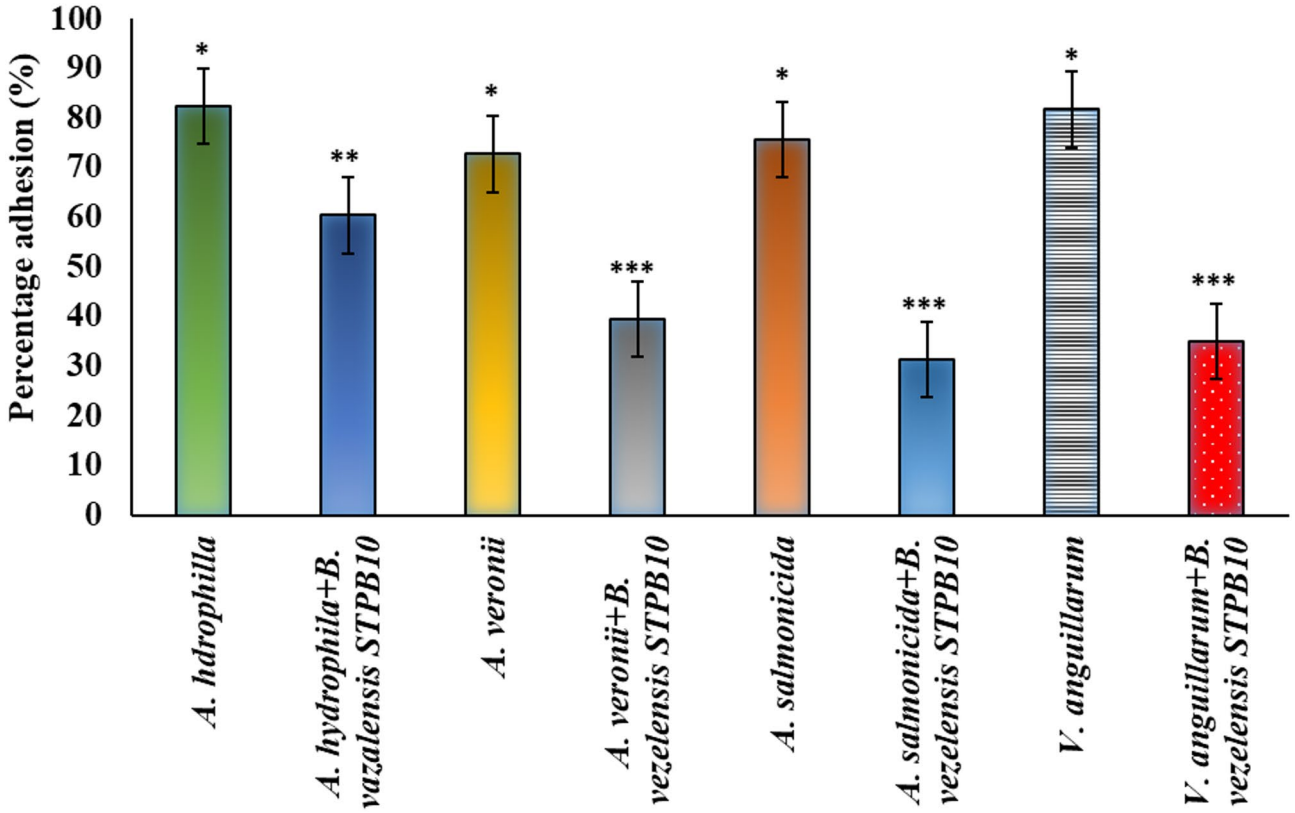

Fig. 7. Percentage adhesion of *A. hydrophila*, *A. veronii*, *(A) salmonicida*, and *V. anguillarum* to common carp intestinal mucus following exposure to *(B) velezensis* STPB10. Data presented in percentage of absorbance of fluorescently stained bacteria ± SD. *Significant is determined at $p < 0.05$.

based on the colony morphology, physiological traits, and biochemical features. These characteristics were employed to differentiate it from other bacterial isolates under the same genus, *Bacillus*. The identification of the test isolate is further established through partial 16 S rRNA gene sequencing and phylogenetic analysis, which revealed the closely relatedness of isolate STPB10 to *Bacillus velezensis* with a similarity index of 99.7%.

Bacillus species can be administered to fish either orally or through water, where they contribute to improved overall body condition and enhanced gastrointestinal (GI) microbial populations[41,42,43] In the present study, the isolated bacterium STPB10 exhibited Gram-positive, rod-shaped characteristics with a cream-white colony morphology. Variations in texture were observed across different strains, ranging from a viscous creamy appearance within the first 24 h to a dry, white, wrinkled surface thereafter, along with differences in elevation and size. These features align closely with the typical morphological traits of *B. velezensis* strains, known for being aerobic, Gram-positive, rod-shaped bacteria capable of producing endospores[39,44,45]. This supports the identification of STPB10 as a member of the *Bacillus* genus and highlights its potential applicability in aquaculture.

Different species of *Bacillus* are considered non-pathogenic and non-toxic with high survival rates, making them excellent candidates for probiotic applications[14]. The test isolate *B. velezensis* STPB10 was found to be γ-hemolytic and it also showed negative DNase activity, indicating that it can be safely used as a probiotic[46,47,48]. It is also found that some probiotic bacteria like *Lactococcus*, *Lactobacillus*, and *Streptococcus thermophilus*, can exhibit α-hemolytic activity also. These bacteria are known for their ability to ferment sugars and produce lactic acid, and in some cases, they may produce enzymes that can partially lyse RBCs, leading to the greenish zone around the colonies. While alpha-hemolysis is often associated with non-pathogenic or less virulent bacteria, it can still occur in probiotics. It is simply a characteristic of some strains of bacteria. Probiotic strains that exhibit alpha-hemolysis may be considered safe and beneficial for their application in aquaculture.

In the study, the impact of bile and pH on the growth and survival of *B. velezensis* STPB10 was assessed to simulate the passage of bacteria through the gastrointestinal tract[14]. In the bile salt tolerance test, *B. velezensis* STPB10 exhibited remarkable growth, with survival rates exceeding 90% in the presence of 0.3% bile salt, demonstrating its ability to thrive in bile salt conditions. In a similar study, it is reported that *B. velezensis* D-18 has been able to survive a 1.5-h exposure to 10% seabass bile[14]. STPB10 also demonstrated versatile growth and survival across a wide pH range, with optimal growth observed at pH levels 4.0, 3.5, and 3.0. Interestingly, growth was also recorded at an acidic pH of 2.5. This aligns with previous researches conducted, which identified a broad pH range (4.0–10.0) where *Bacillus* could thrive[14,49]. The adaptation of microbes to extreme pH environments, such as those found in soda lakes, highlights their exceptional resilience. This phenomenon has been extensively explored, reporting the highest growth of *B. velezensis* strains; B20 and B30 at pH levels of

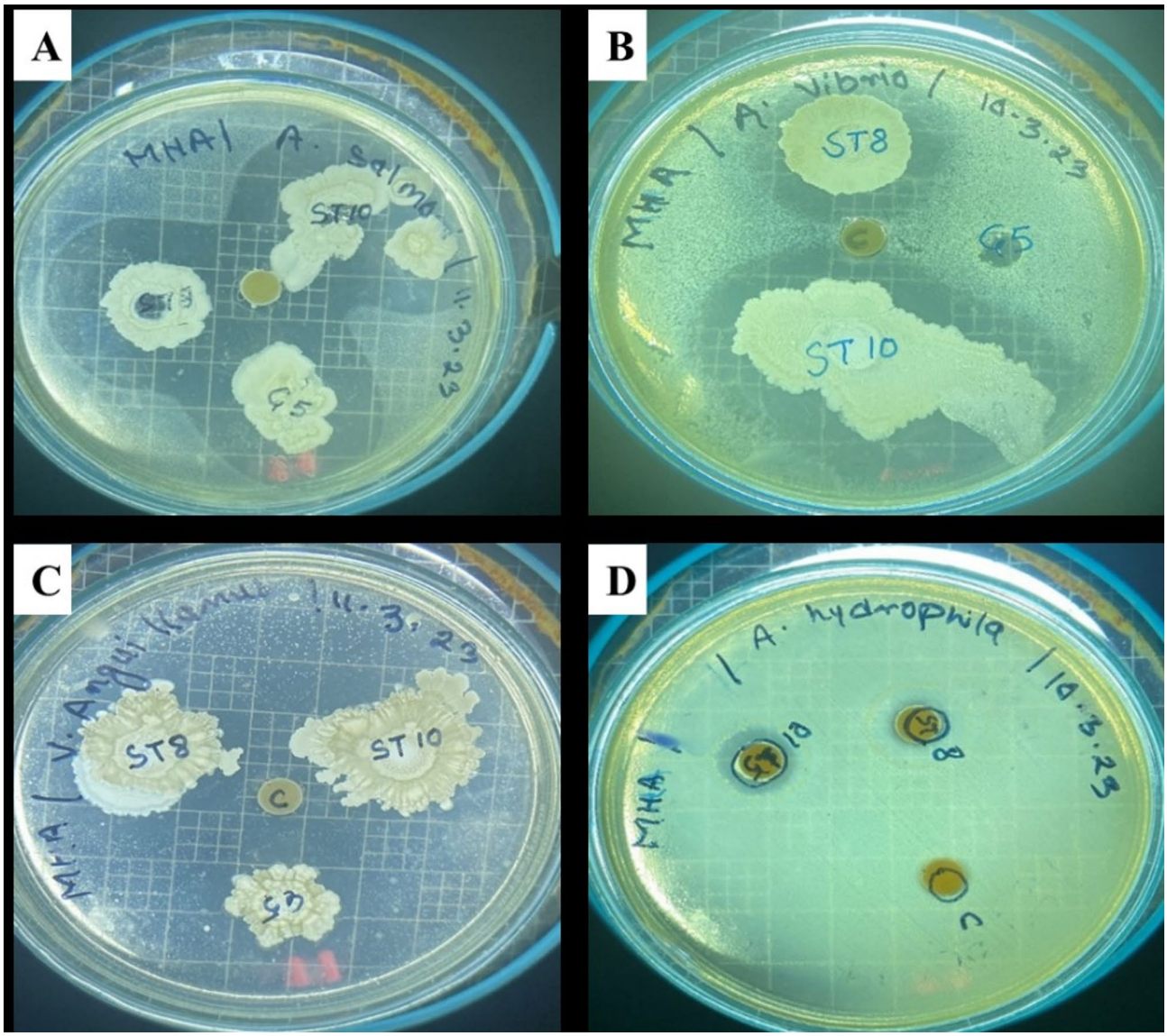

Fig. 8. Antagonistic activity of *B. velezensis* STPB10. Antagonistic activity against (A) *A. salmonicida* with a zone size of 30 mm, (B) *A. veronii* with 26 mm, (C) *V. anguillarum* with a zone size 22 mm, and (D) *A. hydrophila* with a zone size 15 mm.

7.0, 8.5, and 10.0[39]. Alkaliphiles, such as these strains, use secondary proton uptake via membrane-associated antiporters to maintain an internal pH greater than 7.5, even when the external pH is different. This process often involves sodium-ion pumps that help lower the internal pH by transporting hydrogen ions into the cells[50]. The findings highlighted diverse bacterial growth patterns across varying pH levels, supporting the current results, reinforcing the ability of *Bacillus* species to grow in a wide pH range[51].

Salinity levels in aquaculture systems can vary, especially in brackish water aquaculture or systems where salinity adjustments are made. Testing probiotics under different salinity conditions provides insight into their adaptability and potential success in diverse aquaculture settings. This ensures that probiotics can be applied effectively across various freshwater systems without negative impacts on water quality or fish health. Moreover, probiotic bacterium has specific environmental requirements for optimal growth. While some strains are more adaptable to varying salinity levels, others may not survive or grow efficiently in aquaculture environments if the salinity is not within their tolerance range. The isolate STPB10 exhibited notable tolerance to varying salinity levels, with optimal growth at 2% NaCl and minimal growth at 8% NaCl, while maximum growth was recorded at 0.5% NaCl. These results classify STPB10 as halotolerant, given its ability to grow across all tested salt concentrations. This finding aligns with the studies on *B. velezensis*, which have demonstrated similar salinity tolerance[52]. Similarly, isolates B20 and B30 of *B. velezensis* showed salinity tolerance, with maximum growth at 0.0 M NaCl, followed by 0.5 M, and minimal growth at 2.0 M NaCl[39]. Isolate B30 was classified as halophilic due to its ability to grow at 2.0 M NaCl, while isolate B20 was identified as halotolerant, growing across all concentrations[39]. These findings underscore the adaptive strategies of *Bacillus* isolates to thrive under adverse

conditions, supporting their potential applications in environments with fluctuating salinity levels, consistent with prior reports on *Bacillus* spp[53].

The study demonstrated that STPB10 exhibiting significant growth across a broad temperature range, with an optimal temperature of 35 °C. Similarly, it was reported that *B. velezensis* strains; B20 and B30 were capable of growing across a wide range of temperatures. It was also noted that bacterial isolates obtained from the gut of freshwater fish, *Schizothorax richardsonii* generally thrived at lower temperatures compared to isolates from sediment samples collected at the same locations[39]. The ability of bioactive bacteria to grow under varied temperature conditions highlights their adaptability and resilience. This adaptability is particularly valuable for probiotic applications in aquaculture, as it enables these microbes to function effectively across different environmental temperatures. Additionally, while the optimal growth temperature for the isolates was 35 °C, some strains were capable of surviving at temperatures as high as 60 °C, further demonstrating their thermal tolerance[39]. This adaptability to thrive across a wide temperature range enhances the potential of these microbes for biocontrol applications and enables them to perform effectively in environments with fluctuating temperatures. These findings are consistent with the studies that have documented the temperature resilience of bacterial isolates from Lake Bogoria[54-57].

Pathogenic bacteria often need to attach to host mucus to initiate infection. However, this adhesion does not always indicate virulence. For example, probiotics are chosen for their ability to bind to mucus, aiding in beneficial colonization without causing disease[58]. Nevertheless, for most pathogens, binding to mucus is a critical first step in disease development[59,60,61]. In our study, *B. velezensis* STPB10 showed strong adhesion (63.67%) to fish intestinal mucus, significantly more than to BSA or polystyrene. This suggests that its binding is likely facilitated by physical interactions, electrostatic forces, and surface structures like lipoteichoic acids and lectin-coated appendages[62]. In adhesion assays, similar findings have been reported with other probiotic strain *Vagococcus fluvialis* demonstrating stronger adhesion to intestinal mucus (42.56%) compared to bovine serum albumin (BSA) or polystyrene[32]. *B. velezensis* D-18 strain also showed greater adhesion to intestinal mucus (60.33%) than BSA or polystyrene[14]. We also tested the ability of *B. velezensis* STPB10 to block pathogens from binding to mucus and found notable decrease in mucus adhesion by *A. hydrophila*, *A. veronii*, *(A) salmonicida*, and *V. anguillarum* by 26.72, 45.87, 58.59, and 57.14%, respectively. The adhesion capacity of *V. anguillarum* to intestinal mucus was significantly reduced by 60% following exposure to *(B) velezensis* D-18[14]. Similarly, in competitive adhesion assays, *V. anguillarum* adhesion to mucus was reduced by 54.54% after exposure to the probiotic strain *V. fluvialis*[32]. *A. hydrophila* strain A38 exhibited strong adhesion to mucus glycoproteins across all tested size fractions[33]. Infection studies have shown that more virulent bacterial strains tend to display higher adhesion compared to less virulent strains, as observed in *Yersinia enterocolitica*[63,64] and *Pseudomonas cepacian*[65].

For safety assessment, it is important to evaluate the antibiotic susceptibility of the test bacterium in accordance with the 2002 FAO/WHO guideline[47]. In the study, STPB10 exhibited susceptibility to erythromycin, ampicillin, streptomycin, tetracycline, and kanamycin. This aligns with findings, where MIC results of *Bacillus* spp. indicated sensitivity to chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, tetracycline, and vancomycin based on European food safety and authority (EFSA) breakpoint values[66]. According to the 2018 EFSA criteria, 123 strains of *B. velezensis* isolated from fermented soybean food were sensitive to above said antibiotics, with 8 strains exhibiting resistance to streptomycin[67]. Streptomycin MIC distribution showed a continuous profile, distinct from acquired resistance patterns[68]. Epidemiological cut-off values (ECOFF) for 26 *B. velezensis* strains have been established[69], distinguishing wild-type from resistant strains. Furthermore, referring to the 2021 EUCAST breakpoint values, it is found that the most of *B. velezensis* isolates were susceptible to clindamycin, erythromycin and vancomycin. Additionally, the genome analysis research indicated that a significant proportion of *B. velezensis* strains (96.7%) carry the potential tetracycline resistance gene (tetL). This implies that *B. velezensis* poses minimal risk concerning antibiotic resistance[16,66]. These findings highlight the favourable biosafety profile of *B. velezensis* STPB10 strain. Consequently, *B. velezensis* may not pose a substantial risk regarding antibiotic resistance in the context of food fermentation, human and use in aquaculture.

The gastrointestinal tract (GIT) hosts a diverse and densely populated microbial community, where high cell density facilitates close cell-to-cell interactions among members of the same or different species. These interactions can foster both cooperative and antagonistic relationships, shaping the microbial ecosystem within the GIT[70]. *B. velezensis* is known to produce a wide range of antimicrobial compounds effective against various fish pathogens, including genera such as *Aeromonas*, *Edwardsiella*, *Streptococcus*, *Vibrio*, *Nocardia*, and *Salmonella*[40,71,72]. *B. velezensis* CYS06, which showed efficacy against *Aeromonas* spp., *Edwardsiella ictaluri*, and *Flavobacterium columnare*, demonstrated the broad-spectrum antagonistic properties[16]. In the current study, *B. velezensis* STPB10 was tested for antagonistic activity against four fish pathogens previously isolated and identified in the laboratory. The isolate exhibited significant inhibition zones, ranging from 15 to 30 mm in diameter, against *A. salmonicida* (30 mm), *A. veronii* (26 mm), *V. anguillarum* (22 mm), and *(A) hydrophila* (15 mm). These results indicate its potent antagonistic potential. The antimicrobial efficacy of *(B) velezensis* can be attributed to the production of secondary metabolites such as polyketides, lipopeptides, and antimicrobial peptides, which inhibit the growth of both bacteria and fungi[18]. Specific compounds, including bacillibactin, fengycin, bacilysin, surfactin, difficidin, and mersacidin, demonstrate strong antagonistic effects against pathogens[73]. *B. velezensis* strain AMB-y1 revealed traits supporting probiotic properties[74]. Comparative genomic studies between AMB-y1, STPB10, and other strains could provide insights into mechanisms that enable *B. velezensis* to outcompete pathogens in the gastrointestinal tract of fish[16]. Furthermore, *B. velezensis* exhibits antagonistic activity against pathogens such as *Listeria monocytogenes*, *Micrococcus flavus*, *Bacillus cereus*, and various fungal species, alongside inhibitory effects on Gram-positive bacteria[75]. Its broad antimicrobial spectrum, including activity against animal pathogens like *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella* spp, reinforces its potential as a biocontrol agent and probiotic[76].

Probiotics develop complex biofilm communities that provide significant advantages for microbial populations in coping with diverse abiotic and biotic challenges[77]. Successful colonization depends on the targeted adhesion of probiotics to specific epithelial surfaces, such as the intestinal mucosa. This adhesion not only stabilizes their presence but also inhibits pathogenic bacteria through competitive exclusion or steric hindrance, while potentially activating the host immune response[78]. Evaluation of biofilm formation in different media provides valuable insights into how bacteria adapt to environmental stresses and colonize diverse niches. In this study, we demonstrate that *B. velezensis* STPB10 is capable of forming strong biofilms in vitro. Many species of the genus *Bacillus*, particularly certain strains of *B. subtilis*, are excellent candidates for forming a protective biofilm by incorporating probiotic bacteria during their growth[79]. Biofilm formation by probiotic bacteria is regarded as a beneficial trait as it supports colonization and extends their persistence on the host mucosa, thereby preventing the colonization of pathogenic bacteria[80]. The modified growth medium TSB supplemented with 0.45% glucose significantly promoted biofilm formation in the present study. A similar finding was observed with a different modified medium, where MRS medium also supported significant biofilm formation by *B. subtilis*[79]. Biofilms formed by *lactobacilli* are commonly found in various natural environments, with the gastrointestinal tract being a key target for probiotic activity[81]. *Lactobacillus rhamnosus* GG (ATCC 53103) is one of the most clinically studied probiotic strains[82]. It can adhere to the human intestinal mucosa and persist for over a week after oral ingestion by healthy adults. This strain also forms biofilms in vitro on abiotic surfaces like polystyrene, a process strongly influenced by the culture medium and gastrointestinal conditions such as low pH, high osmolarity, or the presence of bile or mucin[82]. *L. rhamnosus* GG forms biofilms in Man Rogosa Sharpe (MRS) medium without glucose, and mucin presence enhances its biofilm formation by 20%, while low pH reduces its biofilm capacity[81].

In the study, the challenge assay was conducted to evaluate the safety profile of *B. velezensis* STPB10. The assessment of the pathogenicity of test bacterium via intraperitoneal injection and immersion challenge at cell concentrations of 10^8 and 10^9 CFU mL^{-1} indicated that the strain poses no threat of pathogenicity to common carp. Similarly, it has been noted that the intraperitoneal administration of *B. velezensis* CYS06 at a concentration of 1.0×10^9 CFU mL^{-1} did not result in any mortality or show any clinical signs of disease progression in challenged grass carp[16]. The inability to recover *B. velezensis* STPB10 from the internal organs (liver, spleen, and kidney) indicates that it does not translocate into or colonize vital tissues, supporting its non-invasive and non-pathogenic nature- an essential prerequisite for safe probiotic use in aquaculture. Thereby, suggesting the safety of *B. velezensis* for fish.

Conclusion

B. velezensis strain STPB10, isolated from the digestive tract of *S. richardsonii*, exhibits promising probiotic characteristics, making it a potential candidate for applications in aquaculture. It has shown tolerance to pH, bile, NaCl and temperature, as well as antagonistic activity against fish pathogens, emphasizing its potential to improve the health and growth of aquatic organisms. The present findings can contribute to the significance of the application of *B. velezensis* in addressing the challenges of fish health management in aquaculture, fostering the overall well-being of aquatic species.

Data availability

Data is provided within the manuscript. Sequence data that support the findings of this study have been deposited in the National Centre for Biotechnology Information (NCBI) as Bacillus velezensis STPB10 (NCBI PP447245).

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Author contributions

Manju Arya: Performed research and prepared the initial draft. Neetu Shahi and Ila Bisht: Conceived the study,

analysed the data and supervised the work. Nityanand Pandey: Analyzed the data. Sumanta Kumar Mallik: Designed the study, analyzed the data, prepared final draft and edited the manuscript. The edited version of the article is approved by all the co-authors.

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Declarations

Ethics approval and consent to participate
The experiment adhered to the ARRIVE guidelines for animal research (Kilkenny et al. 2010). Prior to starting the experimental trial, approval was obtained from the Institutional Animal Care and Use Committee (IACUC) and was conducted in compliance with the Institutional Biosafety Committee (IBSC), Department of Biotechnology (DBT), and the Ministry of Science and Technology, Government of India, in accordance with the regulations outlined in the "Manufacture, Use/Import/Export and Storage of Hazardous Microorganisms/Genetically Engineered Organisms or Cells, 1989 (Rules 1989)" under the Environment (Protection) Act, 1986. Throughout the trial, the care, handling, and use of animals followed the protocols established by the Institutional Animal Care and Use Committee (IACUC) at the ICAR-Directorate of Coldwater Fisheries Research.

Competing interests
The authors declare no competing interests.

Conflict of interest
The authors declare that they do not have any competing interests in related to finance and personal relationships that could have appeared to influence the present study.

Additional information
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