

Probiotic Traits and Anti-Biofilm Potential of *Bacillus subtilis* JS03 From Freshwater Sponge *Ephydatia fluviatilis* Against Clinical Pathogens

Yaser Arafath

Pondicherry University

Mubashira Mannil

Muslim Educational Society College

Aifa Fathima

Pondicherry University

Aswathi Bharathan

Pondicherry University

S Gokila

Pondicherry University

George Sehgal Kiran

Pondicherry University

Saqib Hassan

Sathyabama Institute of Science and Technology

Joseph Selvin

josephselvinss@gmail.com

Pondicherry University

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Abstract

Probiotics have been used for centuries to stimulate the immune system and serve as a promising alternative to antibiotics. This study investigated whether *Bacillus subtilis* JS03, isolated from the freshwater sponge *Ephydatia fluviatilis*, possesses significant probiotic characteristics. The isolate was identified through Gram staining, colony morphology, biochemical tests, and 16S rDNA sequencing, and screened for amylase, cellulase, gelatinase, protease, and lipase production, all yielding positive results. Probiotic characterization included survival at varying pH (1–5), bile salt concentrations (0.15–3%), sodium chloride concentrations (1–5%), and temperatures (40–80°C), alongside evaluation of auto-aggregation, hydrophobicity, and hemolytic activity. The isolate demonstrated moderate acid tolerance, with 80.76% survival at pH 3–4, 96.55% survival at 3% bile salt (3.5×10^7 CFU/mL), relative growth OD of 0.617 at 1% NaCl, and 88.4% viability at 60°C for 30 min. *B. subtilis* JS03 exhibited strong antibacterial activity against *S. aureus* MTCC 737 (20 mm inhibition zone) and other clinical pathogens, including Coagulase-negative *Staphylococcus* spp., MSSA, and *Enterococcus faecalis*. Antimicrobial susceptibility testing revealed sensitivity to norfloxacin, ciprofloxacin, chloramphenicol, meropenem, and imipenem, with resistance to penicillin, ampicillin, tetracycline, and streptomycin. Non-hemolytic activity and absence of lecithinase production indicated no major pathogenic traits. Overall, *B. subtilis* JS03 demonstrates robust stress tolerance and significant antimicrobial activity, supporting its strong potential as a probiotic candidate.

Introduction

The gut is composed of complex bacterial groups that help the host maintain gut pH, integrity of the gut lining, energy levels, and immune system development [1]. A good probiotic must possess certain characteristics, such as tolerance to low pH and high temperatures, before it can be used as a commercial product [2]. Commonly used probiotics include *Lactobacillus* spp., *Bifidobacterium* spp., *Lactococcus* spp., *Streptococcus* spp., *Enterococcus* spp., *Bacillus* spp., and *Saccharomyces cerevisiae* [3]. In particular, *Bacillus* spp. possess anticancer and antioxidant properties, and most of them produce vitamins and antibacterial compounds, making them excellent sources of probiotics [4]. Some well-known probiotic *Bacillus* spp. include *Bacillus cereus* [5], *Bacillus clausii* [6], *Bacillus coagulans* [7], *Bacillus licheniformis* [8], *Bacillus polyfermenticus* [9], *Bacillus pumilus* [10], and *Bacillus subtilis* [11]. *B. subtilis* is an aerobic, gram-positive, soil-inhabiting bacterium that synthesizes numerous heterologous proteins [12]. *Bacillus* is ubiquitously distributed in nature. Previous studies have shown that *Bacillus* species have been isolated from various sources, such as air, water, the human gut, the animal gut, vegetables, and food [13, 14].

Bacillus spp. produce spores that can withstand extreme conditions, providing them with a clear advantage over common probiotics such as *Lactobacillus* and *Bifidobacterium*. Commonly used probiotic strains for human consumption include *B. clausii* (Enterogermina®, Sanofi), *B. subtilis*, and *B. licheniformis* (Biosporin, Biopharma). The probiotic effects were found to be strain-specific. *Bacillus* spp. have been isolated from various environments, such as plants [15], air [16], soil [17, 18], food [19],

humans [20], and the marine environment [21–23]. The genus *Bacillus* spp. serves as a multifunctional probiotic that prevents pathogen growth and enhances nutrient assimilation in the host gut [24]. Some of the commonly expressed enzymes are amylases, xylanases and β -galactosidase [25], cellulases [26], and alkaline serine proteases [27]. *Bacillus* exhibits high metabolic activity in the gut by synthesizing antimicrobials (numerous new antibiotics are discovered every year). [28] Studies have shown that 5% of the *B. subtilis* genome is involved in synthesizing diverse antibiotics [29]. Certain *Bacillus* isolates produce surfactin and iturin, which have antimicrobial properties. [29]. These lipopeptides possess antifungal, antiviral, antitumor, anticoagulant, and immune response-stimulating activities in the host. [30]. *B. subtilis* synthesizes numerous enzymes that can be used in molecular biology, the pharmaceutical industry, and fermentation [31]. Some *Bacillus* spp. secrete enterotoxins [32]. Safety assessments of *B. subtilis* must be conducted in terms of toxin secretion, antibiotic resistance, and biogenic amine synthesis. [4].

Freshwater sponges are of ecological, evolutionary, and microbiological significance, making them a subject of increasing research interest [33]. Most sponge microbiome studies have been conducted on marine sponges, rather than freshwater sponges [34]. Freshwater sponges are filter feeders that harbor numerous microbes for nutritional and metabolic support [35]. *Bacillus* spp. associated with sponges contribute to the production of sponge-specific metabolites and novel bioactive compounds [36, 37]. *B. subtilis* is known for producing a wide range of proteins and enzymes that assist it in overcoming varying environmental conditions [12]. The fermentative cycle for *B. subtilis* is typically 48 h, whereas that for *Saccharomyces cerevisiae* is approximately 180 h [37].

E. fluviatilis is known to harbor diverse *Pseudomonas* species (*Pseudomonas jessenii*, *Pseudomonas protegens*, and *Pseudomonas oryzihabitans*) [38]. The unique nature of freshwater sponges, when combined with molecular findings from selected bacterial communities, makes them a valuable research model. [39]. In shellfish aquaculture, probiotic supplements include *Lactobacillus*, *Enterococcus*, *Bacillus*, *Aeromonas*, *Alteromonas*, *Arthrobacter*, *Bifidobacterium*, *Clostridium*, *Microbacterium*, *Paenibacillus*, *Phaeobacter*, *Pseudoalteromonas*, *Pseudomonas*, *Rhodospiridium*, *Roseobacter*, *Streptomyces*, and *Vibrio*. [40]. Studies have shown that incorporating *Bacillus* spp. improves the growth, performance, digestive enzyme activity, and immune response of white leg shrimp (*Litopenaeus vannamei*) [41]. Probiotics improve growth and resistance to diseases, and enrich the microbiota in aquaculture settings. [42]. Sponge-associated microbes constitute 40% of the total sponge biomass, where they assist the host against invading pathogens and predators (defense mechanisms), provide important nutrients (nitrogen fixation and photosynthesis), and synthesize important secondary metabolites (antimicrobials) [43, 44].

While *Bacillus* species are recognized probiotics, detailed studies specifically characterizing novel *Bacillus* probiotic strains isolated from unexplored ecological niches like freshwater sponges remain limited [45]. They may help in the survival of transient microbiota by favoring their growth and combating undesirable microorganisms. Certain strains have been developed as part of the diet for animal feeds [46]. *In vitro* probiotic characterization of *Bacillus* species isolated from freshwater sponge *E. fluviatilis* is notably rare and remains unexplored [38, 47, 48]. In addition, *Bacillus* spp. possesses several advantages

over *Bifidobacteria* and *Lactobacilli*, such as spore-forming ability and survival in desiccated form; this endospore-forming capability enables survival in harsh GI conditions, such as low pH, elevated temperatures, high bile salt concentrations, nutrient starvation, and desiccation [49]. *Bacillus* spp. are a good source of enzymes, such as amylase and protease [50, 51]. Comparatively, there are very few *Bacillus* probiotics available on the market compared to *Bifidobacterium* and *Lactobacillus* spp. Each *B. subtilis* strain has a specific mode of action against pathogens, which can be direct or indirect. In addition, *B. subtilis*-based probiotics help strengthen the intestinal barrier and enhance the immune response. They also aid in cross-feeding (the selective synthesis of metabolites that feed the gut microbiota). Given the limited research and the scarcity of characterization on probiotics in freshwater sponges, the present study aimed to determine if *B. subtilis* JS03, isolated from *E. fluviatilis*, shows robust probiotic characteristics, such as tolerance to high stress and the absence of pathogenic traits, qualify it as a safe probiotic candidate.

Materials and methods

Sample Collection

Samples of the freshwater sponge *E. fluviatilis* were collected from Pimpalas Lake, Bhiwandi, Maharashtra (19°15'33.6" N 73°04'12.3" E) in February 2021. The collected samples were washed thrice with sterile double-distilled water to remove external debris and transported to the laboratory in a sample transportation box. Sponge samples were processed to isolate sponge-associated microorganisms. [52].

Characterization of Sponge Samples

The modified nitric acid method was used for spicule preparation, as reported by [53]. A 2–3 mm piece of the sponge sample was excised and washed with ethanol. The sponge samples were then allowed to air-dry completely. Dried sponge samples were placed into a clean test tube containing 2 mL 65% nitric acid and incubated at 37 °C for 18 h until digestion was complete. After digestion, the acid was completely aspirated, and the digested sponge sample was washed with sterile distilled water until no acid remained. The digested spicules were suspended in 96% ethanol. Canada Balsam was added to the slide, which was then heat-fixed. The spicules were examined under a high-power objective using an EVOS M7000 Imaging System (Thermo Fisher). Taxonomic identification and spicule morphology were compared with the findings of [53–55].

Isolation of *Bacillus* spp.

The sponge sample was subjected to 10-fold serial dilution using sterile phosphate-buffered saline (PBS) and aseptically spread onto Nutrient agar (NA), R2 agar (Reasoners 2A agar), and Yeast Extract and Malt Extract (YEME) using the spread plate technique. The plates were incubated at 30 °C for 24–72 h. After incubation, typical *Bacillus*-type colonies were stored with 30% glycerol (v/v) at -80°C.

Molecular Identification and Phylogenetic Tree Construction

Genomic DNA from the *Bacillus* isolate was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The quality of the extracted DNA was assessed using a Nanodrop spectrophotometer (Thermo Fisher Scientific) at 260/280 and 260/230 nm. Quantification was performed using a Qubit fluorometer. For the PCR reaction, a 25 µL 16S rRNA mixture was prepared, consisting of 12.5 µL Taq 2x master mix, 0.4 µL of primer, 8.9 µL of distilled water, and 2.8 µL of template DNA. Universal primers 27 F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-AGAGTTTGATCCTGGATGCTCAG-3') were used to amplify the target genes. Initially, the denaturation step was performed at 94 °C for 3 min, followed by 30 cycles consisting of denaturation at 94 °C (1 min), annealing at 52 °C (2 min), and extension at 72 °C for 2 min. The reaction was terminated by a final extension step at 72 °C (2 min). PCR was performed using a thermal cycler (Bio-Rad, USA). PCR products (3 µL) were subjected to electrophoresis on a 1% (w/v) agarose gel in the 1XTAE buffer to check the integrity of the PCR product. The isolated sequences were trimmed using BioEdit V 7.2.5, and uncovered regions were removed. [56]. EzBioCloud and NCBI-BLAST were used to gather information on *Bacillus* spp. sequences that were similar to our target sequence. An intraspecific and interspecific phylogenetic tree was constructed using MEGA X to rule out the closest evolutionary bacterial species using the neighbor-joining method and an unweighted pair group method with an arithmetic mean (UPGMA) with 1000 bootstrap values. [57, 58].

Biochemical and Initial Identification

Discrete large colonies with irregular edges (typical *Bacillus* colonies) were sub-cultured in fresh media to obtain a pure isolate. Characterization of the isolated strains included the morphology of the culture and physiological and biochemical features, following standard protocols. [59] [60].

Exopolysaccharide Production

Exopolysaccharide (EPS) production was evaluated using a previously described method. [61]. An 18-hour-old culture was streaked onto a (0.08%) Congo red plate containing (Brain Heart Infusion Agar (37 g/l), Sucrose (50 g/l), Congo red (0.8 g/l), Agar (10 g/l)) (Congo red was prepared separately). After incubation at 37 °C for 24 h, ropy red colonies were selected as 36 h strains.

Cellulolytic Activity

Cellulolytic activity was assessed using modified Luria-Bertani medium (LB) supplemented with 5 g/L of carboxymethylcellulose. [62]. And incubated at 37 °C for 24 h. Cellulolytic activity was revealed by flooding the plate with 10 mL of Congo Red dye in 0.1 M Tris-HCl buffer (pH 8.0) for 30 min, followed by washing with 0.5 M NaCl solution. The results were interpreted based on the appearance of clear zones around colonies.

Hemolytic Activity

The *Bacillus* isolate was streaked onto 5% sheep blood agar (Medbold Biologics, Bangalore) and incubated at 37 °C for 24–36 h. The plates were then observed for hemolytic activity[63]. Hemolysis was categorized into three types: α-hemolysis and partial hydrolysis of red blood cells; β-hemolysis, complete hydrolysis of red blood cells; and γ-hemolysis, no hemolysis. [64]. *S. aureus* ATCC 25923 was used as a positive control for β-hemolysis.

Bacterial Adhesion to Hydrocarbon (BATH) and Auto-aggregation Tests

Auto-aggregation and bacterial adhesion to hydrocarbons (BATH) were assessed [65]. Hydrocarbon xylene was used in the BATH test. An overnight culture (1 mL) was centrifuged at 8,000 × g for 15 min at 4°C. The samples were then rinsed twice with phosphate-buffered saline (PBS: 140 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.2). The pellet was resuspended in the same buffer, and the absorbance was measured at 600 nm. An equal volume of xylene was added, vortexed for 3 min, and incubated at room temperature (27°C). The aqueous phase was collected separately, and the absorbance was measured at 600 nm. The following formula was used to calculate the adhesion percentage: [65]

$$\text{Adhesion \%} = [(A_0 - A) / A_0] \times 100$$

A₀— Absorbance before extraction with organic solvents

A—Absorbance After Extraction with Organic Solvents

Auto-aggregation was performed using 10 mL of the test culture from the stationary phase, incubated at 15 °C for 3 and 24 h. After incubation, 1 mL of the upper suspension was transferred, and the optical density (OD) was measured at 600 nm. The following formula was used to calculate the auto-aggregation [65]

$$\text{Auto-aggregation \%} = 1 - [\text{OD of upper suspension} / \text{OD of total culture}] \times 100$$

Probiotic characterization

Acid tolerance assessment

An acid tolerance test was performed as described. A 1% (v/v) overnight culture of *B. subtilis* JS03 was inoculated into PBS adjusted to pH 1, 2, 3, 4, and 5 and incubated at 30°C. The survival of the microorganisms was evaluated by measuring the optical density at 600 nm and enumerating viable cells using the spread plate method. At constant time intervals (3, 6, 12, and 24 h), 0.1 mL of each sample was plated onto nutrient agar to determine CFU/mL. All experiments were performed in triplicate.

Survival (%) was calculated using the following formula:

$$\text{Survival (\%)} = (N_t/N_0) \times 100$$

where N_0 represents the initial viable count, and N_t represents the viable count after exposure to acidic conditions [66].

Bile Tolerance Assessment

Bile tolerance was assessed as described by [67]. Overnight-grown (37 °C, 150 rpm) test cultures were inoculated (1% v/v) into Nutrient Broth (NB) supplemented with 0.15%, 0.30%, 0.60%, 1.25%, and 3% ox bile (w/v). Before and after the incubation period, the cell density was measured at 600 nm. The medium without bile salts served as the control, and the rate of inhibition was calculated using the following formula: The time to reach the log phase at various bile concentrations was assessed. [68]. Enumeration was performed using the spread plate method on NA at 3, 6, 12, and 24 h. The experiments were performed in triplicate to assess bile tolerance.

$$C_{inh} = \frac{(\text{Tt}-\text{T0}) \text{ control} - (\text{Tt}-\text{T0}) \text{ treatment}}{(\text{Tt}-\text{T0}) \text{ control}}$$

T₀: The OD600 at time zero

Tt: The OD600 after 3, 6, 12, 24 h incubation

A. C_{inh} (inhibitory) value of less than 0.4 was used when selecting a suitable probiotic candidate to be considered significant[69].

Salt Tolerance Assessment

NB was supplemented with 1%, 2%, 3%, 4%, and 5% sodium chloride, followed by an inoculation of 1% (v/v) of the overnight test culture (37 °C, 150 rpm) into the medium and incubated at 37 °C, followed by enumeration. Bacterial cell density before and after the incubation period was measured at 600 nm at 3, 6, 12, and 24 h.

Temperature Tolerance Assessment

Temperature tolerance assessment was carried out in NB with 1% (v/v) overnight (37°C, 150 rpm) *Bacillus* culture and incubated at varying temperatures of 40, 60°C, and 80°C. Cell viability at various temperatures was assessed at 600 nm, and enumeration was determined (log₁₀ CFU/mL) at 3, 6, 12, and 24 h using the spread plate technique on NA.

Gastrointestinal Tract (GI) Tolerance Test

The viability of the probiotic strain was tested in the presence of simulated GIT conditions to assess its *in vitro* digestion capability. Pepsin (Sigma-Aldrich) at a concentration of 3 g/L was prepared in phosphate-buffered saline (PBS) at a pH of 3.0 to mimic the gastric fluid conditions. A culture concentration of 10^7 was inoculated into the prepared simulated gastric juice. After 1 h of incubation at 37 °C, 1 mL of the culture was mixed with 9 ml of the prepared simulated gastric juice. The mixture was incubated at 37 °C for 2 h. The viable cells were then cultured onto freshly prepared nutrient agar (NA) at varying time points, starting from 1, 2, and 3 h. Finally the number of colonies were calculated based on the log CFU [70].

Growth Curve and Incubation Temperature

One hundred microliters of *B. subtilis* JS03 culture was inoculated into NB (99.9 mL) (Himedia) and incubated at 37 °C for 24 h at 150 rpm (activation phase). Then, 1 mL of overnight culture broth of *B. subtilis* JS03 was taken and inoculated into 99 mL of fresh NB, LB broth (Luria Bertani broth), and BHIB (Brain Heart Infusion Broth), and incubated at 37°C for 120 h at 150 rpm. Every 24 h, 200 µL of the bacterial culture was transferred into a sterile 96-well plate (polystyrene), and optical density was measured at 600 nm (OD_{600}) (BIOTEK, Synergy HXT multimode reader). Each reading was performed in triplicate. After 120 h of incubation, a complete growth curve was drawn, and a graph was plotted using OD_{600} values at each time point. *B. subtilis* JS03 culture incubation optimization was performed by inoculating 100 µL of culture onto NB (99.9 mL), followed by incubation at 10, 20, 30, and 40°C. Bacterial biomass was measured by OD_{600} at 3, 6, 12, and 24 h (in triplicate). The relative bacterial growth was then determined.

Biofilm Formation Test

Biofilm formation was assessed using the crystal violet (CV) assay, as previously described [71, 72]. An overnight culture with (10^7 - 10^8) CFU/mL was added to NB in a 96-well plate and incubated for 6, 12, 24, 36, and 48 h at 37°C. The culture broths were discarded, and the attached cells were washed three times with sterile PBS. The adherent cells were fixed using 250 µL of 100% methanol for 15 min. After methanol treatment, the plates were air-dried, and 250 µl of 2% (w/v) CV was added and left for 5 min. The CV-treated wells were washed three times with sterile PBS. The crystal violet residue was dissolved in 250 µl of 33% glacial acetic acid [73]. The optical density was measured at 595 nm using a Synergy HTX plate reader. *P. aeruginosa* was used as a positive control for this experiment.

Biofilm Degradation Ability

Biofilm degradation ability of *B. subtilis* JS03 was tested against biofilm-forming pathogens, such as *S. aureus*, MSSA, *K. pneumoniae*, *E. coli*, *Listeria monocytogenes*, and *Bacillus cereus*. The pathogens were grown overnight and inoculated into a 12-well plate, then incubated for 24 h at 37 °C. The contents of the wells were discarded after incubation, then 150 µl of concentrated cell-free supernatant of *B. subtilis*

JS03 was added and kept for 5 h. Later, the wells were washed, and acridine orange staining was performed. The stained biofilms were observed using a fluorescence microscope (EVOS M7000 [74]).

Biofilm Inhibition Ability

Bacterial cultures were grown overnight and then adjusted to 1×10^4 CFU/mL using sterile NB. Then, 100 μ l of the cell-free supernatant of *B. subtilis* JS03 and 100 μ l of CFU-adjusted bacterial cultures were added to a sterile 96-well polystyrene microtiter plate and incubated at 37 °C for 24 h to initiate the biofilm inhibition test. After incubation, the contents were discarded. Using sterile PBS (0.1 M, pH 7.2), the adherent cells were rinsed twice and left to air dry. The OD was measured at 595 nm after the CV assay (Synergy HTX, microtiter plate reader USA). The blank consisted of a well without supernatant added, and the blank consisted of only sterile NB. The experiment was repeated thrice.

$$\text{Percentage biofilm inhibition} = \frac{\text{OD}_{\text{control}} - \text{OD}_{\text{treated}}}{\text{OD}_{\text{control}}} \times 100$$

Pre-coated Experiment

Pre-coated experiments were performed as previously described [75]. 200 μ l of. Biofilm-forming *B. subtilis* JS03 (200 μ L) was seeded into a sterile 96-well plate and incubated for 24 h at 37 °C. The specific volume was chosen to cover the bottom and sides of the wells. After incubation, the contents of the wells were discarded, followed by washing the plates with 100 μ l PBS (0.1 M, pH 7.2) to remove any unattached supernatant. Then, 16 h-grown pathogenic cultures such as *S. aureus*, MSSA, *L. monocytogenes*, *K. pneumoniae*, *E. coli*, and *B. cereus* were taken, and CFU/mL was adjusted to (1×10^7 CFU/mL) using sterile NB. The pathogens were added to the previously seeded wells. The microtiter plates were incubated at 37 °C for 24 h. After the incubation, the contents were discarded and washed twice with sterile PBS (0.1 M, pH 7.2). Finally, biofilm formation was assessed using a crystal violet assay. The reduction percentage was calculated using the following formula:

$$\% \text{ microbial adhesion} = 1 - (\text{ODc}/\text{ODo}) \times 100$$

ODc = OD value of the well with supernatant and pathogenic bacteria

ODo = OD representing the value of the well with only pathogenic cultures

This assay specifically focuses on the adhesion of pathogenic bacteria to the well and the percentage reduction in the presence of probiotics. Analysis was carried out in triplicate, and the mean optical density was determined.

Determination of Antibacterial Activity

The antimicrobial activity of *B. subtilis* JS03 was evaluated using the agar cross-streak method on Mueller-Hinton Agar (MHA) against a panel of clinically relevant gram-positive and gram-negative pathogens, including *S. aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *coagulase-negative*

Staphylococcus spp, *E. faecalis*, Methicillin-sensitive *S. aureus*, Methicillin-Resistant *S. aureus*, and *Pseudomonas aeruginosa*, to assess its broad-spectrum inhibitory potential. *B. subtilis* JS03 was inoculated in a straight line onto MHA and incubated at 37 °C for 24 h. After 24 h, the previously mentioned pathogens were streaked perpendicular to *B. subtilis* JS03 and incubated at 37 °C for 24 h. Secondary screening was performed using the crude supernatant (50 µL) of the *Bacillus* culture, which was placed inside an agar well on a freshly made lawn of test cultures (*S. aureus* MTCC 737, *K. pneumoniae* MTCC 3384, *E. coli* MTCC 728, and *P. aeruginosa* MTCC 2295) and incubated at 37 °C for 18 h. The zone of inhibition was represented as a clear halo around the well, and the activity was measured by diameter (in mm) [76].

Antibiotic Susceptibility Test

B. subtilis JS03 was tested against commonly consumed clinically significant antibiotics such as Co-trimoxazole (COT) 25 µg, Nalidixic acid (NA) 30 µg, Ceftriaxone (CTR) 10 µg, Ciprofloxacin (CIP) 10 µg, Norfloxacin (NX) 10 mcg, Penicillin (P) 10 units, Chloramphenicol (C) 30 mcg, Ampicillin (AMP) 10 mcg, AmoxCalv (AMC) 30 mcg, Meropenem (MRP) 10 mcg, Colistin (CL) 10 mcg, tetracycline (TE) 30 mcg, Cefotaxime (CTX) 30 mcg, Ceftazidime (CAZ) 30 mcg, Streptomycin (S) 10 mcg, Trimethoprim (TR) 5 mcg, Imipenem (IE) 10/750 mcg) were checked by disk diffusion method. Antibiotic disks were placed on freshly prepared Mueller-Hinton agar medium and incubated overnight at 37°C. The diameter of the zone was measured using a digital caliper [77]. The results were interpreted based on three categories: resistant (no zone), intermediate (12–17 mm), and sensitive (≥ 18 mm).

Statistical Analysis of Data

The experiments were performed in triplicate. Statistical analyses were performed using one-way and two-way analysis of variance (ANOVA) with GraphPad Prism 9.0.0 (Windows). This analysis compared various groups to assess the statistical significance of *B. subtilis* JS03 across various conditions (adhesion, aggregation, growth curve, bile, pH, temperature, and sodium chloride concentrations) to determine whether any significant differences existed among the various treatments ($p < 0.05$). The significance levels were graded as follows: $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). The multiple comparison Tukey test, sidaks multiple comparison test and Geisser-Greenhouse correction were also used. All values are expressed as mean with 95% CI.

Results

Morphological identification of sponge samples

Fragments of the sponge samples were examined using a live imaging microscope. The sponge tissue appeared green, whereas the dried specimens appeared dirty and white. The skeletal arrangement is a network of compact tissue organization that is thinner and without branches. Megasccleres were needle-like and slightly curved, while microsccleres were absent. At 40x magnification, the spicules were

visualized. Spicule morphology is unique to species at the clade level. The class Desmospongiae is characterized by spicules with monaxonic or tetraxonic symmetry. [53, 55].

Isolation of Sponge-Associated Bacteria

In this study, 41 bacterial isolates were obtained using the conventional culture method and examined based on their morphological characteristics (**Table 1**). Among the 41 isolates, 29 were gram-negative, and 12 were gram-positive (identified based on gram staining, colony morphology, virulence screening, and enzyme screening). Isolate Y1 showed a typical *Bacillus*-type colony with a large, greyish, fuzzy color and irregular edges (**Fig. 2**). Gram staining exhibited a straight rod with endospores, catalase positivity, and aerobic nature, with other biochemical characteristics, as shown in Table 1. This confirmed that the isolated bacterial strain belonged to *Bacillus* [78]. The screened culture was then subjected to 16S rRNA sequencing using the Sanger sequencing method.

Table 1
Characteristics of *B. subtilis* JS03.

Biochemical Test	Test	<i>B. subtilis</i> JS03
	Indole test	Negative
	Methyl red test	Negative
	Voges-Proskauer test	Positive
	Citrate test	Positive
	Catalase test	Positive
Enzymatic Test	Hemolytic activity	γ hemolysis
	Amylase	Positive
	Cellulase	Positive
	Gelatinase	Positive
	Protease	Positive
	Lipase	Positive

The 16s rRNA sequence of the bacterial isolate was fed into the BLAST search engine against the collection of GenBank Nucleotide, and a phylogenetic tree was constructed to determine the taxonomical assignments. BLAST results indicated that the isolated bacteria had 98–99% identity with *Bacillus halotolerans* OdE1 and *B. subtilis* JS03 (GenBank accession no. PP936059 PP936059). The

phylogenetic tree represents the closeness of the sequence available in the database, and the sequence has been submitted to the NCBI GenBank (**Fig. 3**

Enzyme activity and exopolysaccharide production.

An enzyme activity study demonstrated that the strain *B. subtilis* JS03 exhibited positive results for protease (15 mm), lipase (22 mm), casein hydrolysis (10 mm), gelatinase (20 mm), cellulase (18 mm), and γ -hemolysis of blood agar (hemolysis is a prominent characteristic of a pathogen), and no opalescence was observed around the colonies in the egg yolk agar medium (presence of opalescence indicates a pathogenic trait). On Congo red agar, the black color around the colonies indicated that the bacterial colony formed a biofilm (Fig. 4).

Probiotic characterization

Acid and Bile Tolerance

Probiotic microorganisms must survive at a pH of 3.0 or below 3.0, and the ingested food must pass through a period of 2–3 h. The incubation periods were 3, 6, 12, and 24 h. *B. subtilis* JS03 exhibited the lowest survival rate at pH 1.0, 12.20% (24 h) ($p < 0.0082^{**}$) and at pH 3.0 and 4.0, the survival percentage, calculated from CFU counts, increased significantly ($p < 0.0032^{**}$) to 72.74% (24 h) and 80.76% (24 h) ($p < 0.0001^{**}$), respectively (Fig. 5A and **Supplementary Figure S2 A-E**). The control group (vegetative cells). *B. subtilis* JS03 was tested with varied concentrations of ox bile in the medium (0.15%, 0.30%, 0.60%, 1.25%, and 3%) at time intervals of 3, 6, 12, and 24 h. *B. subtilis* JS03 showed resistance to bile salt concentrations (0.15%, 0.30%, 0.60%, 1.25%, and 3%) after 3 h of incubation. The results were found to be 63.22% ($p < 0.002^{***}$), 76.72% ($p < 0.0001^{****}$), 90.23% ($p < 0.0005^{***}$), 89.22% ($p < 0.0001^{****}$), 96.55% ($p < 0.0001^{****}$). After 3 h, the cell viability gradually decreased ($< 50\%$). Lowest viability was observed at 24 h (27.53%, 32.99%, 38.29%, 40.46%, and 41.57%). From the above data, we conclude that *B. subtilis* JS03 exhibited strong initial tolerance to bile salts during the first 3 h of exposure; however, prolonged incubation resulted in a gradual decline in viability. (Fig. 5B and **Supplementary Figure S2 F-J**). *B. subtilis* JS03 maintained high viability under gastrointestinal stress. At pH 3.0, the strain demonstrated 37.68% survival after 12 h (3.8×10^7 CFU/mL) and 72.74% after 24 h (7.3×10^7 CFU/mL). At pH 2.0, survival dropped to 12.0% by 6 h (1.2×10^6 CFU/mL), whereas minimal viability was recorded at pH 1.0 (0.059×10^6 CFU/mL). In bile tolerance assays, the strain retained 7.6×10^7 CFU/mL at 0.3% ox bile at 24 h, 6.2×10^7 CFU/mL at 1.25% by 24 h, and survived up to 3.0% bile (3.5×10^7 CFU/mL).

Salt and Temperature Tolerance

Tolerance to various salt concentrations (1%, 2%, 3%, 4%, and 5%) was assessed *in vitro* at time intervals of 3, 6, 12, and 24 h. At a 1% NaCl concentration, the relative growth of *B. subtilis* JS03 after 24 h was 0.617, which was higher than at 2, 3, 4, and 5% up to 24 h (Fig. 5C and **Supplementary Figure S3 A-E**). *B.*

subtilis JS03 also exhibited better tolerance at higher concentrations (2–5%). Temperature tolerance indicated that *B. subtilis* JS03 had a better survival rate at 40 °C (98.2%) ($p < 0.0001^{****}$) for 30 min, followed by 60 °C (88.4%) ($p < 0.0001^{****}$), and at 80 °C, the survival rate decreased (15.04%) (Fig. 5D and Supplementary Figure S3 F-H). At 40°C, *B. subtilis* JS03 achieved its highest growth (1.1×10^8 CFU/mL), with good viability at 60°C (30 min) (6.4×10^7 CFU/mL). Growth declined sharply at 80°C (30 min) ($< 1.0 \times 10^5$ CFU/mL) but remained detectable, suggesting moderate thermal stability. At a refrigeration temperature (10°C), the strain maintained 2.3×10^6 CFU/mL, indicating its potential for cold-chain storage. The results were significant ($P < 0.0001$) based on the Brown-Forsythe and Welch ANOVA tests.

Bacterial Adhesion to Hydrocarbons (BATH) and Auto-aggregation Tests

The hydrophobicity of the bacterial cell surface of *B. subtilis* JS03 was evaluated using the Bacterial Adhesion to Hydrocarbons (BATH) assay, which measures the adhesion percentage of bacteria in the hydrocarbon phase. The experiment was repeated four times, yielding adhesion percentages of 34.182% (1 h), 40.552% (2 h), 53.290% (3 h), and 66.454% (4 h) within the significant range of ($p < 0.0001^{***}$). These values indicate varying degrees of hydrophobicity and adhesion. The highest adhesion percentage recorded was 66.454% (4 h), demonstrating the strongest hydrophobicity and adhesion capability compared with the control group. *Bacillus clausii* MTCC5472 exhibited 45.333% at 4 h with a significance value of ($p < 0.0001^{***}$) (Fig. 5E). In comparison, the latter three percentages indicate moderate-to-low hydrophobicity and adhesion capability. The auto-aggregation process is governed by surface and soluble proteins present on bacterial cell surfaces. This is a major step in the communication and colonization of the host gut. An auto-aggregation test of *B. subtilis* JS03 was performed at 1 and 4 h, resulting in values of 57.02% at 1 h ($p < 0.0004^{***}$) and 89.20% ($p < 0.0482^*$) at 4 h after incubation at 15 °C. The isolate showed 89.20% aggregation after 4 h, indicating a strong colonization potential (Fig. 5F). The slightly higher value at 4 h suggests improved biofilm formation. Overall, the consistently high values demonstrated that *B. subtilis* JS03 has a significant capacity for auto-aggregation. In contrast, *Bacillus clausii* MTCC5472 exhibited auto-aggregation rates of 61.52% and 90.86% at 1 h and 4 h, respectively.

Gastrointestinal tolerance test

The survival ability of *B. subtilis* JS03 in simulated gastric fluid was studied at 0, 1, 2, and 3 h. *B. subtilis* JS03 was able to withstand the simulated conditions at pH 3.0 and exhibited a survival rate of 46.16% (T0) ns, 40.39% (T1, $p < 0.0040$), 35.04% (T2, $p < 0.0007$), and 30.10% (T3, 0.0002). Throughout the test, the strain *B. subtilis* JS03 maintained a survival rate of 30–40%.

Growth Curve Analysis and Temperature Optimization for *B. subtilis* JS03

Growth curve analysis was performed using three different media: NB, LB, and BHIB. *B. subtilis* JS03 exhibited slow, steady growth in NB and LB by 48 h, whereas BHIB showed rapid growth between 48 and 72 h; the growth in BHIB and NB reached the logarithmic phase. At 96 and 120 h, NB and LB had attained their stationary phase, as BHIB continued to support the growth of *B. subtilis* JS03, and the growth curve reached its stationary phase (Fig. 7A). The incubation temperature was optimized at 10, 20, 30, and 40 °C for 3, 6, 12, and 24 h. Relative growth was low at 10 °C (0.067), and the highest growth was recorded at 40 °C (0.552) (Fig. 7B). All values were significant ($p < 0.0001^{***}$).

Biofilm Formation

The isolate *B. subtilis* JS03 showed biofilm formation ability at 6 h (84.49%), 12 h (46.046%), 24 h (34.036%), 36 h (27.786%) and 48 h (16.686%) against the positive control *P. aeruginosa* which exhibited gradual increase in the biofilm forming ability 6 h (59.309%), 12 h (74.323%), 24 h (86.074%), 36 h (92.351%) and 48 h (98.333%) (Figure.8A). And the results were found to be significant with respect to the control at $p < 0.0001$.

Biofilm Degradation and Inhibition

The biofilm degradation ability of *B. subtilis* JS03 was tested against clinical pathogens like *E. coli*, *L. monocytogenes*, MSSA, *S. aureus*, *B. cereus*, and *K. pneumoniae*. It was found to be effective against *L. monocytogenes* (77.11%), *S. aureus* (74.88%), *E. coli* (74.57%), MSSA (70.97%), *B. cereus* (60.70%), and *K. pneumoniae* (43.64%). Among the six pathogens its was found to be more effective against *L. monocytogenes* and least effective against *K. pneumoniae* (Figure. 8B). Similarly, biofilm inhibition property of *B. subtilis* JS03 was also carried out and found to be effective against MSSA (59.37%), *K. pneumoniae* (46.08%), *S. aureus* (40.19%), *E. coli* (39.59%), *L. monocytogenes* (32.55%) and *B. cereus* (25.81%) (Figure.8C).

Pre-coated Experiment

The pre-coated experiment is carried out to assess the percentage of pathogenic biofilm formation by clinical pathogens in the presence of preformed *B. subtilis* JS03 biofilm. The highest percentage of pre-coated inhibition was seen in *E. coli* ($23.63\% \pm 4.47\%$), *B. cereus* ($40.95\% \pm 4.68\%$), *Coagulase negative Staphylococcus* species ($42.61\% \pm 11.39\%$), *E. faecalis* ($58.99\% \pm 7.81\%$), MRSA ($59.54\% \pm 4.40\%$), *S. aureus* ($63.62\% \pm 8.60\%$), *L. monocytogenes* ($84.52\% \pm 2.86\%$) and *K. pneumoniae* ($92.90\% \pm 1.54\%$) (Figure.8D). The precoated analysis demonstrated higher anti-adhesive effect against *E. coli*, *B. cereus* and MRSA. It exhibited a very minimal effect on *K. pneumoniae*.

Antimicrobial Activity.

The supernatant of *B. subtilis* JS03 was tested against *S. aureus* MTCC 737, *K. pneumoniae* MTCC 3384, *E. coli* MTCC 728, and *P. aeruginosa* MTCC 2295. *B. subtilis* JS03 showed strong antibacterial activity

against the gram-positive strain *S. aureus* MTCC 737 (20 mm), and the strain *B. subtilis* JS03 was unable to inhibit gram-negative bacteria (**Supplementary Figure S1A, S1B**). When tested against *S. aureus* MTCC 737, *B. subtilis* JS03 produced a clear inhibition zone of 20 mm at a cell density of 1×10^8 CFU/mL. Cross-streaks of *B. subtilis* JS03 against clinical pathogens exhibited good activity against coagulase-negative *Staphylococcus* spp., Methicillin-Sensitive *S. aureus*, and *E. faecalis*. No significant activity was observed against *K. pneumoniae*, *E. coli*, Methicillin-Resistant *S. aureus*, or *P. aeruginosa* (**Supplementary Figure S1C**).

Antibiotic Susceptibility Testing

Antibiotic susceptibility testing of *B. subtilis* JS03 exhibited sensitivity to antibiotics such as norfloxacin, co-trimoxazole, chloramphenicol, meropenem, ciprofloxacin, and imipenem. Intermediate results were observed for trimethoprim, and resistance was observed against nalidixic acid, aztreonam, fosfomycin, colistin, penicillin, streptomycin, tetracycline, ampicillin, and cefotaxime (**Supplementary Figure S4**).

Discussion

The current study was conducted to analyze the probiotic characteristics of the *Bacillus* strain *B. subtilis* JS03 isolated from freshwater sponge *E. fluviatilis*. As the probiotic industry constantly seeks strains with superior probiotic potential [79], our findings for *B. subtilis* JS03 support this search by identifying a strain with a unique combination of stress tolerance and antimicrobial properties. Primarily, the *Bacillus* genus is known to exhibit antibacterial activity against gram-positive bacteria, but *Bacillus*-derived compounds possess a broad spectrum against gram-negative pathogens and fungi [80, 81]. Probiotic strains need to possess antagonistic activity against invasive pathogens to prevent disease and maintain host health [82]. Our findings on *B. subtilis* JS03 exhibited enhanced survival and antimicrobial activity against gram-positive pathogens, which is consistent with the antibacterial properties of the *Bacillus* genus. This is one of the crucial characteristics of a probiotic strain. Antimicrobial agents synthesized by *Bacillus* are peptide and lipopeptide antibiotics [69]. They can also stimulate the immune system and govern the inflammatory response in the mucosal lining of the colon region of the host [69]. The extensive use of antibiotics has adverse effects on the host and can lead to the development of antibiotic-resistant pathogens. Probiotic bacteria serve as the best alternative [83]. Probiotics obtained from food sources have greater benefits over non-food sources, as they adapt to the gastrointestinal tract and exhibit positive outcomes more effectively than probiotic bacteria isolated from other sources [84]. *Bacillaceae* family species, such as *B. subtilis*, *B. licheniformis*, and *B. coagulans*, have been used as probiotic supplements for humans and as animal feed [85].

Sponge-associated bacterial symbionts are the major producers of these antagonistic metabolites [83]. Given the limited studies on the potential of freshwater sponge-associated bacteria, our isolation and probiotic characterization of *B. subtilis* JS03 from *E. fluviatilis* represents a significant contribution. *B. subtilis* JS03 demonstrated promising results towards a broad range of stress tolerance to *in vitro*

gastrointestinal stresses (high bile concentration and pH) and good antibacterial activity against coagulase-negative *Staphylococcus* spp., Methicillin-Sensitive *S. aureus*, and *E. faecalis*, suggesting its potential value from an unexplored niche. Biochemically, the strain was positive for catalase, citrate, Voges–Proskauer, amylase, cellulase, gelatinase, protease, and lipase tests and negative for the methyl red test. It also formed non-haemolytic colonies on blood agar (**Table 1 and Fig. 4**). Adhesion property helps the probiotic to adhere firmly to the intestinal lining of the host. The most common way of calculating adhesion property is through hydrophobicity and auto-aggregation activity [86, 87]. One of the major properties of probiotic bacteria is auto-aggregation and adhesion[88]. In our study, the strain *B. subtilis* JS03 showed good auto-adhesion (66.45%) (4 h) (xylene), demonstrating better adhesion properties compared to the moderate hydrophobicity of *Lactobacilli* (40.0%) in hexadecane [89]. Most probiotic isolates show a greater affinity for xylene solvents owing to their weak electron donation and electron acceptance properties, which indicate a lower hydrophilic surface [64]. The adhesive nature of xylene is a primary indication of hydrophobic surface properties. Vegetative cells contain a peptidoglycan layer on the outer surface, which has lower hydrophobic properties than spores, which contain a protein coat or exosporium [90]. The hydrophobic nature changes with the type of solvent and the cells used (vegetative or spores). The observed adhesion capabilities against xylene are (66.45% at 4 h), suggesting a significant hydrophobic nature, a property that is linked with effective gut colonization [91]. Spores possess higher hydrophobic properties than vegetative cells, allowing them to adhere to gut epithelial cells [92]. *B. tequilensis* isolated from chickens exhibited 70% hydrophobicity with xylene [93]. *Bacillus* strains DS-1, DS-2, DS-3, and DS-4 demonstrated 76.20%, 70.80%, 89.30%, and 84.60% cell-surface hydrophobicity towards toluene, respectively [94]. In addition, *Bacillus* spp. in Korean soy sauce exhibited 25–89% hydrophobicity against xylene [95]. The aggregation properties after 4 h of incubation were 89.20%. Similar results on the auto-aggregation of *Bacillus* strains have been reported previously [96, 97]. A study conducted by Jeon et al. [96] reported that *S. clausii* ATCC 700,160 showed auto-aggregation of 90.86% after 4 h of incubation. Auto-aggregation of *B. subtilis* P223 was 86.03% after 24 h of incubation, and *B. subtilis* MKHJ 1–1 was reported to have an auto-aggregation ability of approximately 91.32% after 24 h [97, 98]. A study conducted by Nithya et al. [99] observed that the probiotic *Bacillus* strain isolated from food sources reported an adhesion of 30–80% and an auto-aggregation value of 70–100%. Strains exhibiting stronger auto-aggregation are correlated with better adhesion properties, which play a key role in GI colonization, whereas co-aggregation ability helps in close interaction with pathogens [100]. Better adhesion properties help probiotic bacteria adhere strongly to the inner gut lining, which aids in major physiological functions [101]. Growth-aggregation helps probiotic bacteria coexist, which, in turn, helps in the formation of biofilms. The same probiotic strain exhibits different degrees of auto-aggregation with various solvents [100]. Hence, the strain *B. subtilis* JS03 is expected to have the highest degree of cellular adhesion compared to previous results.

Guidelines proposed by the FAO/WHO group show that better probiotic selection should be based on gastric acidity resistance and survival based on bile concentration [102]. A better probiotic must transit to the lower GI tract in a more viable state. In order to assess the viability of *B. subtilis* JS03, we subjected it to a simulated gastric fluid (pH 3.0, pepsin, and PBS) assessment test. The strain *B. subtilis*

JS03 exhibited a survival rate of 40.38% at T1, and at T3 it gradually declined to 30.10% by T3. Similar results can be observed in a probiotic strain of *B. subtilis* isolated from a goat farm in Tongcheng, Hubei, China, which showed a 49% survival rate at 1 h and gradually declined to 35% by 3 h [103]. A study conducted by [94] on probiotic *Bacillus* spp isolated from dairy sludge located in Khorasan-e-Razavi, Iran, showed the highest survival rate of up to 90% in a simulated gastric juice tolerance test. The above findings highlight the tolerance of the isolate under simulated gastric conditions. Once they survive the harsh conditions of the stomach, they have to bypass the bile in the small intestine. The bile tolerance test was carried out using a 0.3% ox bile solution, which is similar to human bile juice; moreover, 0.3% serves as a crucial concentration in the bile tolerance evaluation of a probiotic strain [99]. Strain *B. subtilis* JS03 exhibited increased tolerance to various concentrations of bile salt, which is consistent with the *Bacillus* isolates, such as *B. subtilis* and *Bacillus toyoi* [104], and other strains of *B. coagulans* [105]. Strain *B. subtilis* JS03 enhanced survival at 3.0% bile concentration (96.55% at 3 h and 41.57% at 24 h), a result that is comparable to or higher than the survival rate of the probiotic strain *B. subtilis* CE330 [106], which thrives well at higher concentrations of bile. A good probiotic strain must survive the low pH environment of the gastrointestinal tract to reach the desired site in an intact physiological state [99]. The survival of *Bacillus* spp. in acidic environments has been previously reported [107]. Probiotic strains such as *Lactobacillus sporogenes*, *Bacillus laevolacticus*, and *Bacillus racemilacticus* are known to thrive under acidic pH. Among them, *B. laevolacticus* has a good survival rate at pH 2.5 [105]. *B. subtilis* JS03 exhibited 21.24% (3 h) and 72.74% (24 h) survival rates at pH 3.0, whereas at pH 2.0 (26.53% at 24 h) and 1.0 (12.24% at 24 h), a minimal survival rate was registered. [106]. A study conducted by Behbahani et al. [82] reported that *Bacillus* strains DS-1, DS-2, DS-3, and DS-4 isolated from dairy sludge showed 98.80%, 98.54%, 98.16%, and 97.24% viability, respectively, after 3 h at 0.3% bile salt [94]. The *B. subtilis* MKHJ 1–1 strain isolated from fermented soybean exhibited a 42.39% survival rate in the presence of 0.3% bile salt [97]. Zhao et al. demonstrated that *B. subtilis* SOM8 exhibited better survival at pH 3–4 and 1.5% bile concentration, where the final colony-forming units (CFU) remained stable above 6 (Log₁₀ CFU/mL)[108]. The consumption of probiotics along with food as a carrier reduces the chances of exposure to stomach acid [109]. *B. subtilis* JS03 exhibited better relative growth at a 5% NaCl concentration, but the relative growth gradually decreased. The soil-isolated *B. subtilis* HG-15 is a halotolerant bacterium that can thrive at 30% (w/v salinity and assists in wheat growth [110]. *B. subtilis* T9-05 isolated from fish sauce showed better survival at 0–1% salt concentration [111]. *B. subtilis* SJ4 isolated from fermented seafood (Shrimp jeotgal, Korea) exhibited a better survival rate at 1–10%NaCl concentrations [112]. *B. subtilis* FP133 isolated from fermented fish paste can thrive at 5% NaCl [113] and *Bacillus* spp. KYJ 963, a Korean fish sauce, can tolerate salt concentrations ranging from 0 to 15% [114]. Probiotic strains *Lactobacillus acidophilus* CM1 and *Lactobacillus delbrueckii* OS1 exhibited better survival at 4% and 6% NaCl concentrations [115].

Resistance to varied temperatures (higher and lower) helps in transportation and storage. Temperature-tolerant *Bacillus* spp. along with a proper probiotic formulation, assist in efficacy, increase gastrointestinal support, strengthen the immune system, and regulate cholesterol levels in the host. *B. subtilis* JS03 indicated significant viability at 40 °C (98.2%) and 60 °C (88.4%), and a sharp decline at 80

°C. The thermal tolerance is comparable to or even broader than *B. coagulans*, a known probiotic that shows optimal growth between 45 °C-55 °C, whereas some strains can tolerate growth between 30 °C-60 °C [116]. *B. coagulans* X26 isolated from soil exhibited better thermal stress tolerance at 50 °C and pH 2.0, along with bile salts, showing robust survival capabilities [116]. Thermal stability under harsh temperature conditions gives *Bacillus* probiotic products a longer shelf life, making them a better option as probiotic supplements for human and animal applications [117]. The thermal tolerance, enzyme production, and antimicrobial activity of *B. subtilis* JS03 show it as a promising and robust probiotic candidate which has the potential for pharmaceutical applications, which is in alignment with other potential probiotic strains [116]. The *Bacillus* strain *B. subtilis* JS03 was tested for relative growth at 10 °C, 20 °C, 30 °C, and 40 °C. At 10 °C, the strain exhibited reduced growth, whereas at 40 °C, significant growth was observed. The optimal growth of a probiotic strain varies depending on the strain, such as *Lactobacillus salivarius*. Growth occurs between 25°C and 45°C, whereas efficient growth occurs at 37 °C -45 °C, depending on the specific fermentation conditions [118]. The optimal temperature for *Lactobacillus acidophilus* and *Streptococcus thermophilus* is 40–45 °C [119–121]. In contrast, other *Lactobacillus* species grow at 30–40°C [122]. *Bacillus* isolates, such as *B. subtilis*, *B. pumilus*, and *B. licheniformis*, isolated from larvae of *Chitala chitala*, exhibited optimal growth at 30–40 °C [123].

A good probiotic strain must have a better adhesion properties that help in better colonization of the mucosal layer, which acts as a protective barrier towards the invading pathogens. The antagonistic effect of *B. subtilis* JS03 extract upon six clinical pathogens showed better activity against *E. coli*, *L. monocytogenes*, MSSA, and *S. aureus*, and the least activity was recorded for *B. cereus* and *K. pneumoniae*. The antagonistic activity of *B. subtilis* JS03 towards gram negative and gram positive pathogens was due to the secretion of lipopeptides that act as antibiotics [124]. *B. subtilis* is known for its biosynthetic gene clusters (BGCs) encoding antimicrobial compound synthesis. Among the other noticeable species, along with *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, and *B. velezensis* produce a wider class of lantibiotics. These lantibiotics target pathogens' cell membranes and hinder the energy metabolism. Some of the commonly known molecules are subtilin, subtilosin A, sublincen, sublancin 168, lichenicidin, and cerecidins [125]. Bacaucin, a novel lipopeptide from *B. subtilis*, showed wider antibacterial activity against multi-drug resistance gram positive pathogens without inducing bacterial resistance within the pathogens [126]. A study conducted by [94] against *L. monocytogenes* ATCC 19115 exhibited biofilm inhibition of 7.50% when treated with CFS of *B. subtilis* GS3 (DS-1); it also reduced the mature biofilm formation of *L. monocytogenes* ATCC 19115 by 7.50%–51.20%.

Biomass and antimicrobial production by *B. subtilis* JS03 were tested using Brain Heart Infusion broth, NB, LB, and R2 broth. A good probiotic bacterium not only possesses better adhesion to the mucosal layer of the gastrointestinal tract but also has antagonistic activity against gut-invasive pathogens. The antagonistic activity of *B. subtilis* JS03 was tested against *S. aureus* MTCC 737, *K. pneumoniae* MTCC 3384, *E. coli* MTCC 728, and *P. aeruginosa* MTCC 2295. Strain *B. subtilis* JS03 exhibited strong antagonistic activity against gram-positive bacteria *S. aureus* (20 mm zone of inhibition), which is consistent with many *Bacillus*-derived antimicrobial peptides. However, no activity was seen against gram-negative bacteria *K. pneumoniae*, *E. coli*, and *P. aeruginosa*. Suggesting a more selective

antimicrobial profile that aligns with previous observations of *Bacillus* species, often demonstrating strong antagonistic activity against gram-positive pathogens [127]. Previous reports state that NB provides better antimicrobial activity against *P. aeruginosa*, which is higher than that of other bacteriological broths [128]. This is due to the presence of nitrogen sources, such as yeast and beef extracts, which assist in the synthesis of antimicrobial peptides [128]. The specific activity of *B. subtilis* JS03 on *S. aureus* may be due to antimicrobial peptides produced (fengycins and surfactin isoforms) that directly act on Agr quorum sensing (QS) of *S. aureus*, which plays a greater role in virulence and biofilm formation [127]. Administration of *B. subtilis* spores greatly reduced *S. aureus* colonization in the gut and nasal area without disrupting the overall microbiome. This demonstrates specific antagonistic activity against *S. aureus*, rather than a broad spectrum [129].

One of the major properties of a probiotic is to inhibit pathogen adhesion [130, 131]. The anti-adherence effect of *B. subtilis* JS03 precoated onto polystyrene wells on a microtiter plate was carried out to assess the anti-adhesive effect against biofilm-forming clinical pathogens. The study shows that the precoated treatment with *B. subtilis* JS03 has inhibited pathogenic biofilm formation by $23.63\% \pm 4.47\%$ in *E. coli*, $40.95\% \pm 4.68\%$ in *B. cereus*, $42.61\% \pm 11.39\%$ in CONS, and the least inhibition was seen in *K. pneumoniae*, $92.90\% \pm 1.54\%$. Similar results were observed in a study conducted by [132] on the antagonistic activity of *B. subtilis* over Enterotoxigenic *Escherichia coli* (ETEC) 58–72% when tested on Caco-2 cells. Pre-treatment by *B. subtilis* on biofilm-forming *E. coli* showed an inhibition range of 1.42% over IPEC-J2 cells [133]. Apart from *K. pneumoniae*, *B. subtilis* JS03 exhibited better inhibitory properties against *E. coli*, *B. cereus*, and CONS.

Antibiotic susceptibility testing covers aspects such as antibiotic resistance, clinical guidance, surveillance, and improving patient safety. An antibiotic susceptibility test demonstrated that *B. subtilis* JS03 was sensitivity towards norfloxacin (22 mm), co-trimoxazole (25 mm), chloramphenicol (25 mm), meropenem (30 mm), ciprofloxacin (38 mm), and imipenem (42 mm) while exhibiting resistance towards tetracycline (11 mm), ampicillin (9 mm), nalidixic acid (0 mm), aztreonam (0 mm), fosfomycin (0 mm), colistin (0 mm), penicillin (10 mm), streptomycin (0 mm), cefotaxime (11 mm). Certain strains of *Bacillus* are resistant to penicillin but remain susceptible to other classes of antibiotics. [134]. The observed pattern of resistance to tetracycline and ampicillin was also observed in previous studies. [135]. Resistance to streptomycin is common among *Bacillus* spp. [136] and colistin and cefotaxime [137]. Studies have shown that antibiotic resistance gene exchange takes place between pathogenic bacteria and probiotics. [138]. Horizontal gene transfer was not generally involved in *Bacillus* spp. along with gram-negative pathogens such as *Vibrio* and *Aeromonas* [139].

Probiotics find their major applications in the food and medical industry, so ensuring the safety of a probiotic strain is necessary [140]. *In vitro* tests were usually performed to assess the safety of the probiotics, such as hemolytic activity, synthesis of certain enzymes, enterotoxin genes, biogenic amines, and antibiotic resistance genes [141]. Hemolytic activity and lecithinase enzyme screening are associated with virulence characteristics of a bacterial strain [142]. The isolate *B. subtilis* JS03 lacked detectable hemolytic and lecithinase activity under the tested conditions; however, additional genomic

and toxicological analyses are required to fully establish safety [143]. Similar results were reported previously and in correlation with [144–146].

The results of the present study show that the strain *B. subtilis* JS03 isolated from freshwater sponge *E. fluviatilis* can be a potential candidate for a probiotic bacterium based on the tolerance studies conducted in vitro, such as acid, bile, salt, and temperature, absence of hemolytic activity, and antagonistic activity against clinical pathogens. Furthermore, while in vitro findings are promising, they do not fully replicate the complex physiological conditions of in vivo environments. Therefore, follow-up studies, including in vivo trials in relevant host models (eg, livestock for gut health or specific aquaculture species), are important to validate the probiotic efficacy and also to determine the most suitable application fields for *B. subtilis* JS03, such as a plant growth promoter or as a feed supplement in aquaculture. Despite the results, there is still much work that needs to be done to rule out the specific benefits of *B. subtilis* JS03 as a probiotic. The probiotic effect of *Lactobacillus* species was extensively studied when compared to that of *Bacillus* species. Studies focusing on the beneficial effects of *Lactobacillus* species and *Bacillus* species must be conducted to rule out their role in maintaining a healthy gut. Application of *Bacillus* spp. Aquaculture has been growing rapidly in recent years. They act as a better biocontrol agent. *Bacillus*-based probiotics can replicate faster under various environmental conditions, which can be a huge benefit in the aquaculture system. Due to their sporulation property, they are highly stable. A spore-based study must be taken into consideration for evaluating the complete probiotic effect of these isolates. Also, in conclusion, this study demonstrates the potential of *B. subtilis* JS03 and its importance as a probiotic source.

Conclusion

The probiotic potential of *B. subtilis* JS03, which was isolated from the freshwater sponge *E. fluviatilis*, is highlighted in this study. Strong resistance to severe gastrointestinal conditions is demonstrated by the strain, which survived at high bile salt concentrations (96.55% at 3% bile), low pH (80.76% at pH 3–4), and high temperatures (88.4% viability at 60°C). Strong auto-aggregation, hydrophobicity, and the production of many enzymes, including lipase, cellulase, protease, and gelatinase, indicate successful colonization and biofilm development in the host gut. Crucially, JS03 confirmed its safety profile by demonstrating good antibacterial action against clinically important pathogens, including coagulase-negative *Staphylococcus* spp., methicillin-sensitive *Staphylococcus aureus*, and *Enterococcus faecalis*, while remaining non-hemolytic and negative for lecithinase synthesis. Antibiotic susceptibility testing revealed sensitivity to several important drugs. All of these results point to *B. subtilis* JS03 as a viable probiotic candidate with possible uses in the functional food, aquaculture, and human health sectors. The significance of freshwater sponges as repositories of new probiotic bacteria is highlighted by the isolation of this strain from an understudied ecological niche. To confirm its effectiveness and safety and open the door to its development as a next-generation probiotic, more *in vivo* research and genetic analysis are required.

Limitations

Whole-genome screening and *in silico* analysis is been carried out in order to rule out any antibiotic resistance genes that are present in the chromosomal DNA to make sure that it is not transferable and is only intrinsic in nature. Despite stronger auto-aggregation results, the cytotoxic nature of the *B. subtilis* JS03 must be confirmed using a Caco-2 cell line study to confirm the cytotoxic nature of the cell-free filtrate and also the freeze-dried cell-free filtrate.

Abbreviations

E. coli *Escherichia coli*

S. aureus *Staphylococcus aureus*

K. pneumoniae *Klebsiella pneumoniae*

E. faecalis *Enterococcus faecalis*

MSSA Methicillin-sensitive *Staphylococcus aureus*

MRSA Methicillin-Resistant *Staphylococcus aureus*

CONS coagulase-negative *Staphylococcus spp.*

MTCC Microbial Type Culture Collection and Gene Bank

BLAST Basic Local Alignment Search Tool

CFU Colony-forming unit

EPS Exopolysaccharide

B. subtilis *Bacillus subtilis*

NB Nutrient Broth

LB Luria-Bertani medium

E. fluviatilis *Ephydatia fluviatilis*

YEME Yeast Extract and Malt Extract

PBS Phosphate Buffered Saline

R2 Agar Reasoners 2A agar

PCR Polymerase Chain Reaction

NaCl Sodium Chloride

BHIB Brain Heart Infusion Broth

UPGMA Unweighted Pair Group Method with Arithmetic Mean

ANOVA Analysis of variance

KCl Potassium Chloride

Na₂HPO₄ Disodium Hydrogen Orthophosphate

KH₂PO₄ Dihydrogen potassium Orthophosphate

Declarations

Data Availability Statement

No data are available except as included in this article

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Author Contributions

Yaser Arafath: Writing Original Draft, Methodology, Conceptualization, Formal analysis; **Mubashira Mannil:** Formal analysis, Data curation; **Aifa Fathima:** Visualization, Writing Original draft, Software; **Aswathi Bharathan:** Writing review and Editing; **Gokila S:** Formal analysis, Data curation, **George Seghal Kiran:** Supervision, Resources; **Saqib Hassan:** Writing review and Editing; **Joseph Selvin:** Supervision, Conceptualization, Validation, Resources.

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Data Availability

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

Code Availability

Not applicable

Declaration of Competing Interest

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

Ethical Approval

This article does not contain any studies involving animals or human participants.

Consent of participation

is not applicable, as no human participants were involved in this study.

Consent of Publication

Not applicable.

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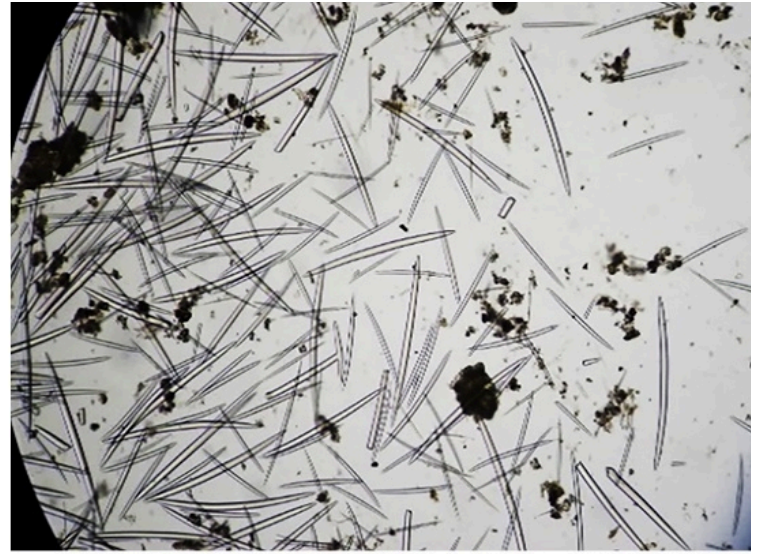
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Figures



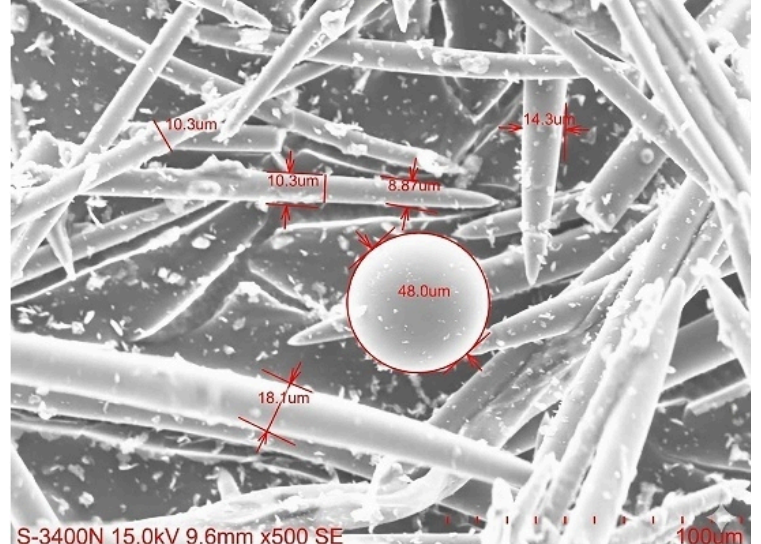
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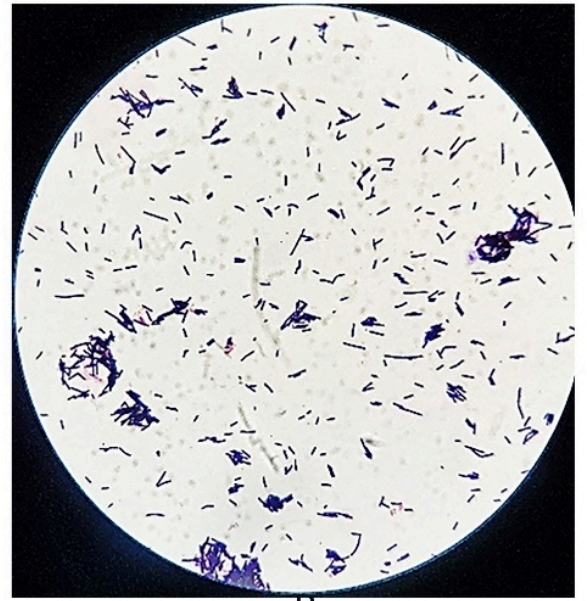
D

Figure 1

Micrographs of isolated *E. fluviatilis*: [A] Gross morphology of sponges, [B] Wet mount preparation showing spicules (40X), [C] *E. fluviatilis* megasclere (50µm scale), [D] *E. fluviatilis* gemmule (48 µm diameter (100 µm scale)).



A



B



C

Figure 2

(A) Pure culture of *B. subtilis* JS03, (B) Gram stain of *B. subtilis* JS03, (C) Antibacterial effect of *B. subtilis* JS03

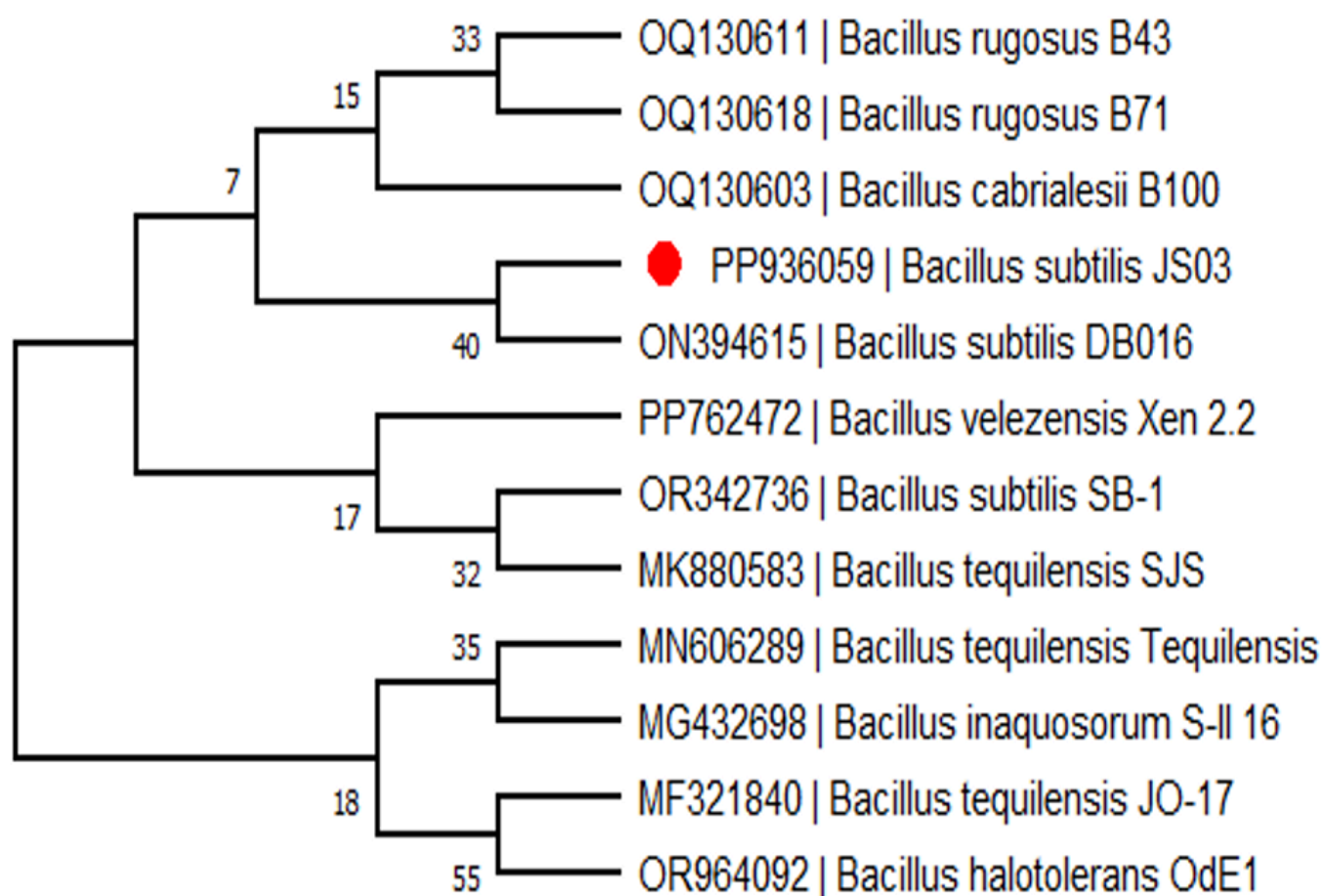


Figure 3

A phylogenetic tree was generated by aligning the 16S rRNA gene sequence (Sanger Sequencing) with the available sequence in the database, and it was found to be *B. subtilis* JS03.

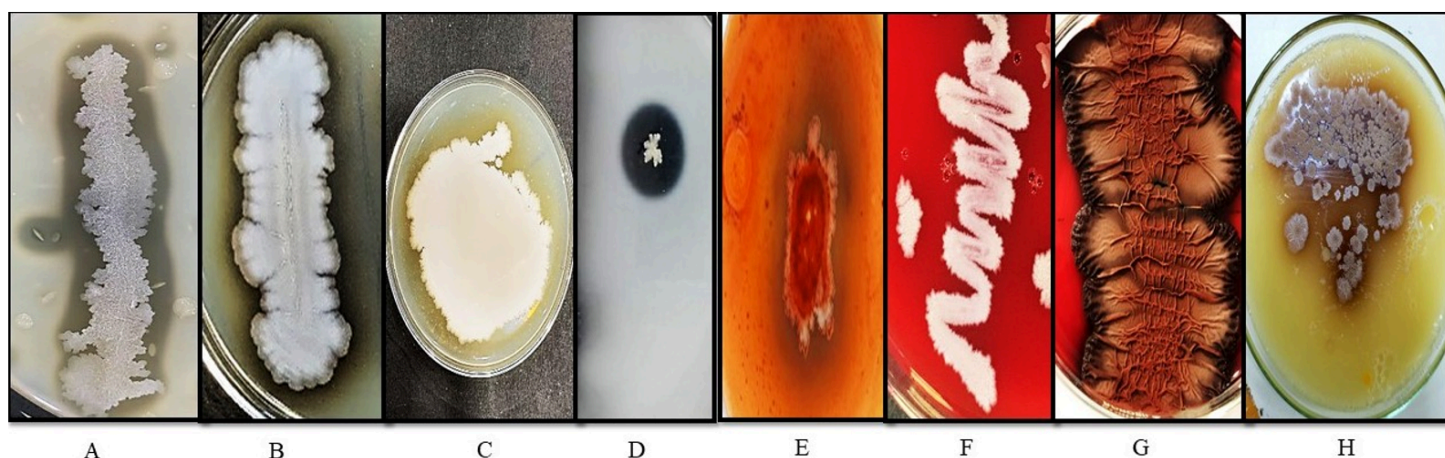


Figure 4

Enzyme screening for *B. subtilis* JS03: A) Protease; B) Lipase; C) Casein hydrolysis; D) Gelatinase; E) Cellulase; F) Blood agar; G) Congo red agar; H) Lecithinase egg yolk agar.

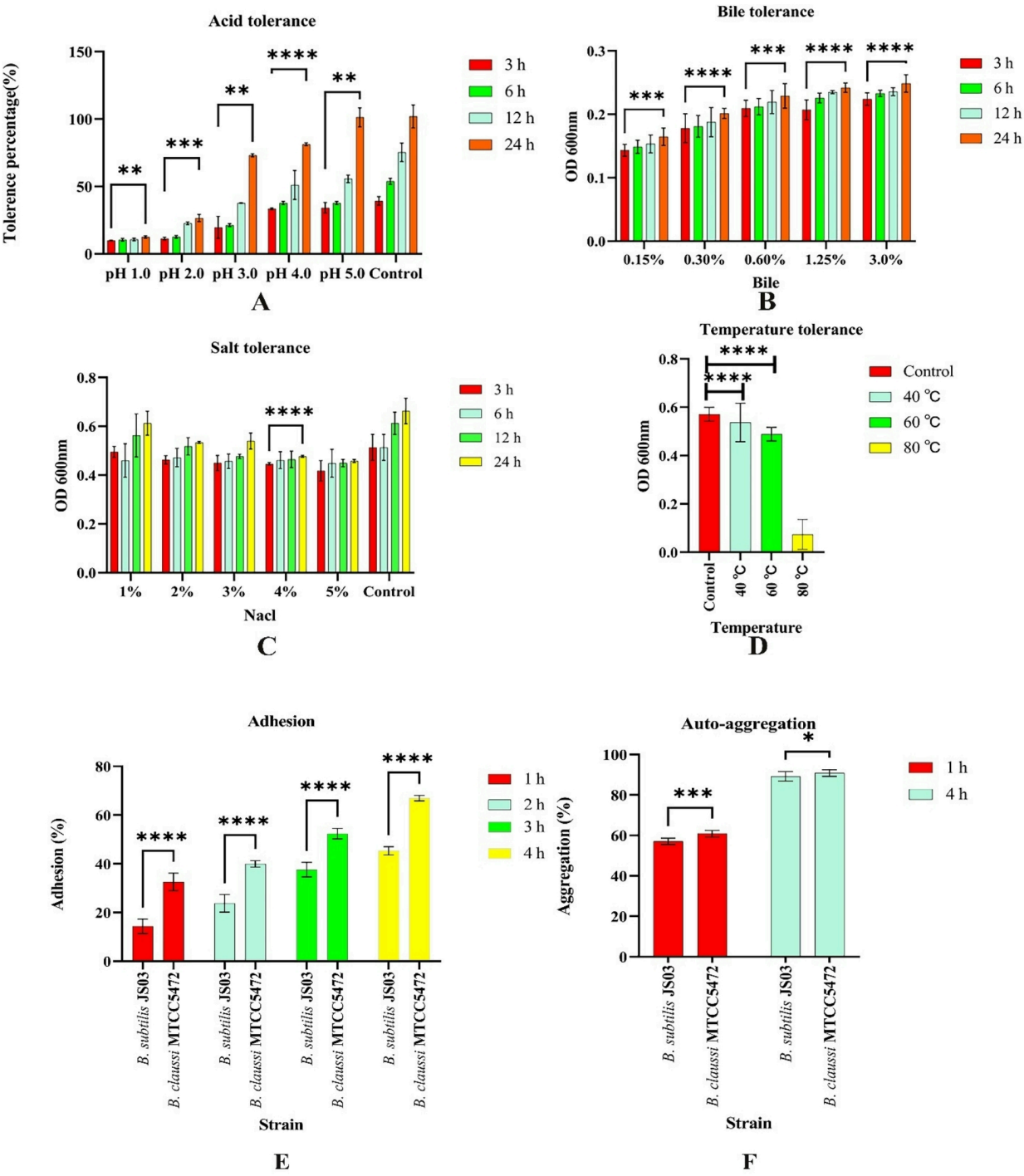


Figure 5

Schematic representation of probiotic characterization of *B. subtilis* JS03 (A) Acid tolerance test (two-way ANOVA, $p < 0.0001$ ***), (B) Bile tolerance test (two-way ANOVA, $p < 0.0001$ ***), (C) Salt tolerance test

(two-way ANOVA, $p<0.0001^{***}$), (D) Temperature tolerance test (Brown-Forsythe ANOVA test, $p<0.0001^{***}$), (E) Adhesion test (two-way ANOVA, $p<0.0001^{***}$), (F) Auto-aggregation test (two-way ANOVA, $p<0.0001^{***}$). All values are expressed as the mean with 95% CI.

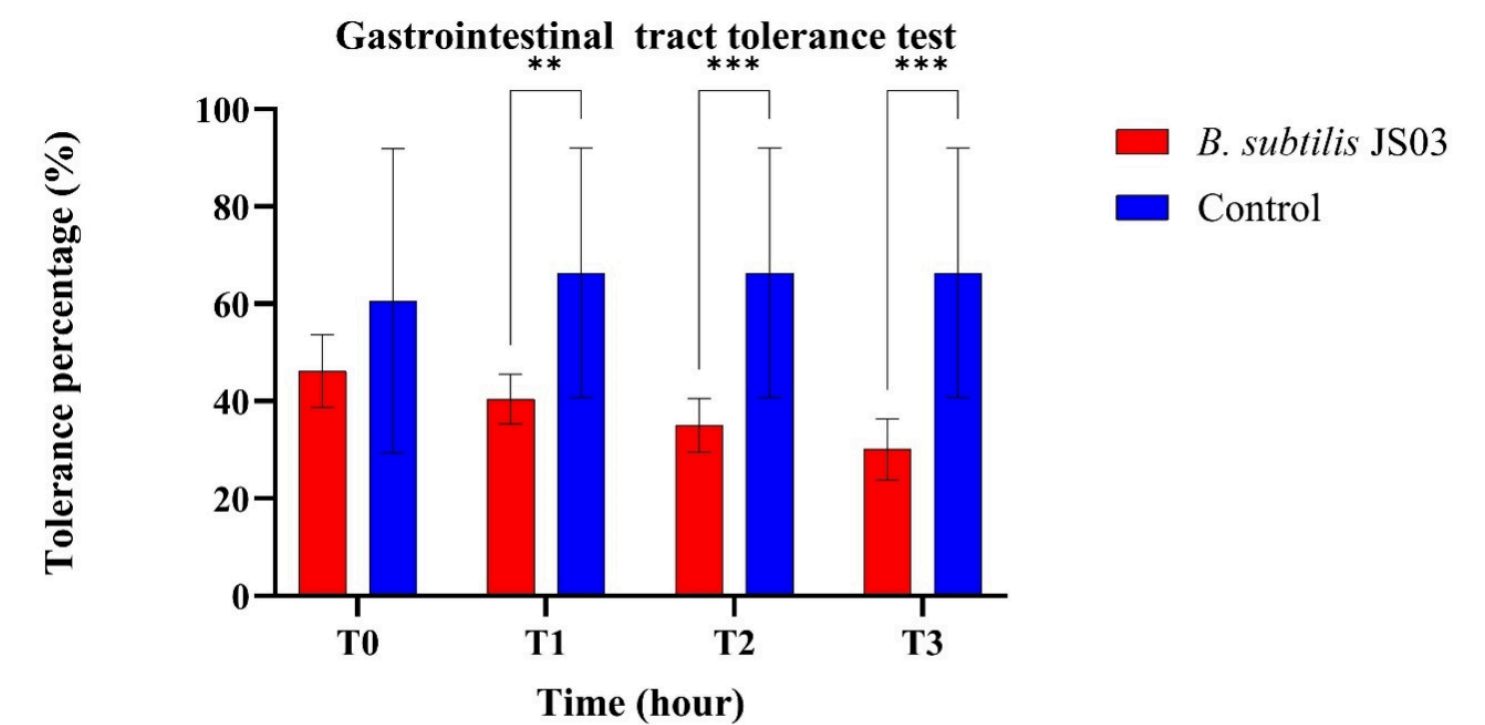


Figure 6

Graphical representation of *B. subtilis* JS03 tolerance in stimulated gastric fluid at different times: T0, T1 ($p<0,0040$), T2 ($p<0.0007$), and T3 ($p<0.0002$) (Two-way ANOVA).

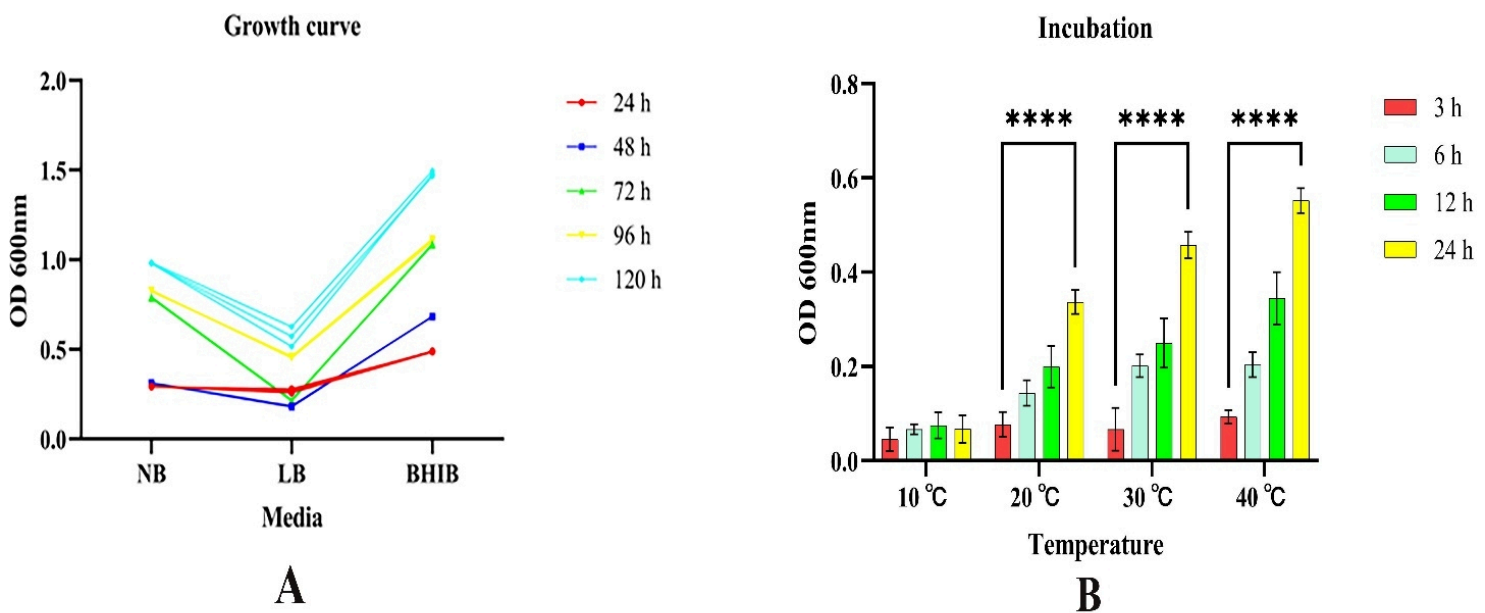


Figure 7

The above-mentioned schematic representation shows (7A) growth curve analysis in various media, such as NB, LB, and BHIB (7B). Relative growth at various incubation temperatures was assessed at 10, 20, 30, and 40 °C. All values were expressed as the mean with 95% CI using two-way ANOVA, with statistical significance at $p < 0.0001$ ***.

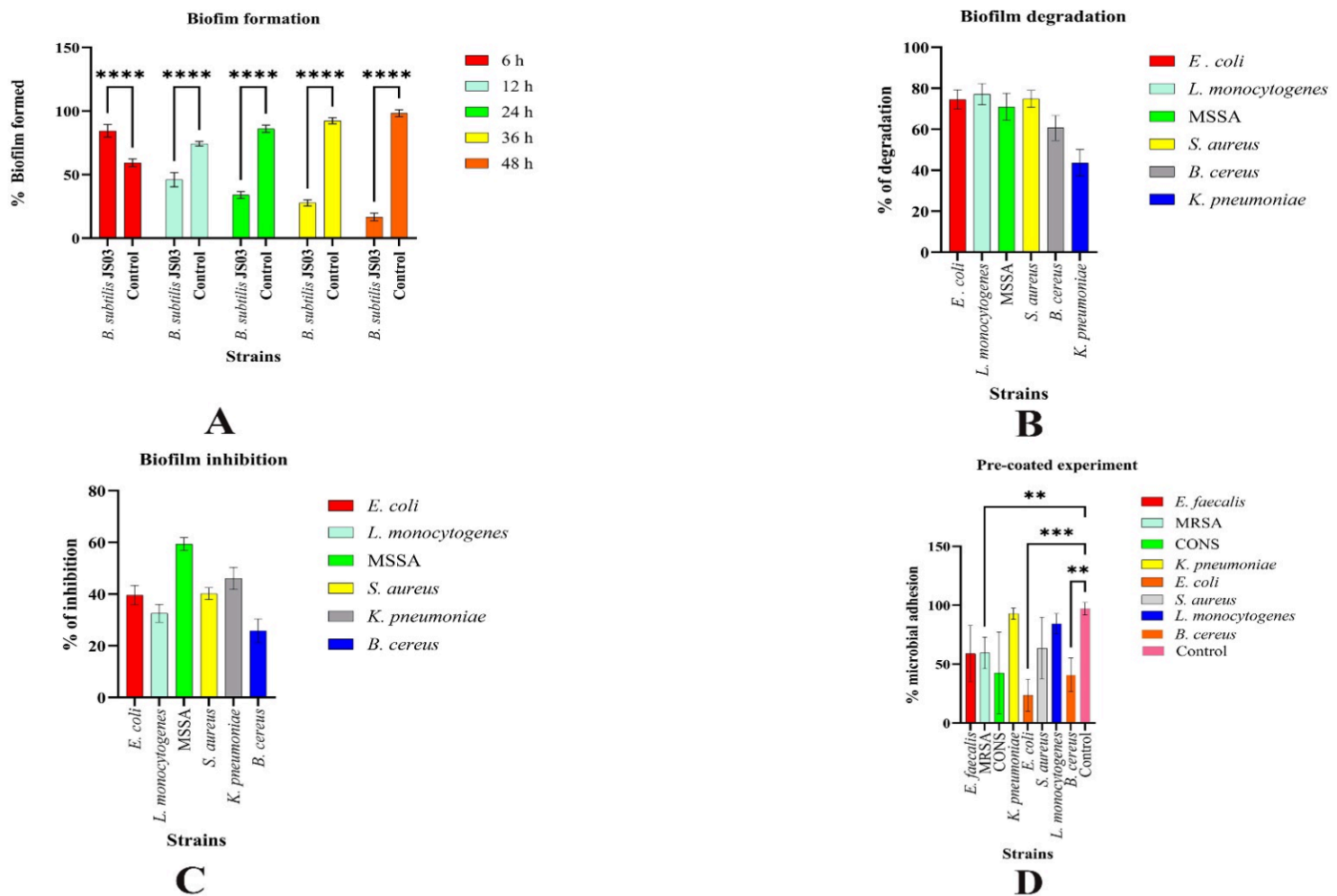


Figure 8

The schematic representation of (8A) Biofilm formation at 6,12,24,36 and 48 h (2-way ANOVA, $p < 0.0001$), (8B) Biofilm degradation (Brown-forsythe and welch ANOVA test, $p < 0.0001$), (8C) Biofilm inhibition (Brown-forsythe and welch ANOVA test, $p < 0.0001$), (8D) pre-coated experiment ((Brown-forsythe and welch ANOVA test).

Supplementary Files

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