Research Square

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Isolation and Probiotic Characterization of Bacillus stercoris VR19 from the polyherbal Ayurvedic fermented formulation Madhookasavam

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Abstract

Probiotics are live microorganisms that provide health benefits to the host when administered in adequate amounts. In this study, bacterial isolates from Madhookasavam, a polyherbal Ayurvedic fermented formulation, were screened to identify potential probiotic candidates. Preliminary safety assessment included Gram staining, hemolysis testing, and antibiotic susceptibility profiling. Among the isolates, VR19 was the only strain to exhibit γ-hemolysis, indicating a non-hemolytic phenotype, and was susceptible to several clinically relevant antibiotics, including Chloramphenicol and azithromycin. The isolate VR19 demonstrated tolerance to acidic conditions and simulated gastrointestinal stresses, including pepsin (0.1–1%), bile salts (0.1–3%), and NaCl (1–10%). Functional characterization revealed cellulase activity of up to 1.95 U/mL, corresponding to the release of 0.959 mg/mL reducing sugars. The isolate also exhibited concentration-dependent antioxidant activity, reaching a maximum DPPH radical-scavenging activity of 72.9%. Probiotic performance was further evaluated using *Caenorhabditis elegans* as an in vivo model. VR19 showed effective intestinal colonization and persistence, as demonstrated by bacterial enumeration and Gram staining of colonies recovered from the nematode intestine. Molecular identification based on, 16S rRNA gene sequencing, and phylogenetic analysis identified VR19 as *Bacillus stercoris*. Collectively, these findings show that *Bacillus stercoris* VR19 possesses promising probiotic properties, including a non-hemolytic phenotype, gastrointestinal stress tolerance, cellulolytic and antioxidant activities, and effective intestinal colonization. The strain therefore warrants further safety and efficacy evaluation for potential therapeutic and functional-food applications.

Introduction

Probiotics are living microorganisms that enhance nutrient bioavailability, maintain intestinal microbial balance, antagonize pathogenic colonization, and reduce serum cholesterol levels [1, 2]. They are being used in a wide range of applications, including dietary supplements in humans, growth promoters in feed supplements, and aquaculture prophylactics [3, 4]. Although researchers have isolated and characterized several potential probiotic strains from dairy-fermented foods and regionally specific traditional fermented foods, the search for other, more effective strains and sources continues [5].

In support of this, recent studies have shown diverse microbial communities, especially probiotic bacteria, in polyherbal fermented traditional medicines, namely Arishta (fermented decoctions) and Asava (fermented infusions), which show potential as valuable sources for isolating and characterizing probiotics [6]. The composition of fermented plant products- carbohydrates, dietary fibers, phenolics, and flavonoids- creates niches that influence microbial metabolism and adaptation [7]. Stress in these environments favors robust strains with probiotic features such as antioxidant activity [8]. Hence, plant-derived probiotics are increasingly viewed as valuable alternatives to dairy-associated strains because of their unique functional characteristics [9]. In the current study, we evaluated the Madhookasavam, a highly recommended polyherbal Ayurvedic fermented medicine (PAFM) for gut-associated dysfunction, for potential probiotic isolates. The ingredients used in Madhookasavam preparations were specific Madhuka (*Madhuka longifera*) flowers, together with honey and auxiliary ingredients including cardamom

(Elettaria cardamomum), lotus stem (*Nelumbo nucifera*), and *sandalwood (Santalum album*), and were fermented by the natural, intrinsic microflora carried by the herbs used in the formulation for a specified period. These Ayurvedic formulations have self-preserving properties due to their high alcohol content (6%-10 %) and low H (~ 5), which improve phytochemical availability and support microorganisms' ability to tolerate harsh environments [10]. Additionally, to exert their beneficial effects, probiotics must resist bile salts to survive in the upper digestive tract and subsequently colonize by adhering to intestinal epithelial cells. Other crucial characteristics to consider for probiotic strain selection include antibiotic susceptibility and consumer safety [11].

Therefore, this study focused on isolation and characterization of potential probiotics from Madhookasavam and assessing their in vivo colonization in the *Caenorhabditis elegans (C. elegans)* model. Ultimately, molecular techniques such as 16S rRNA sequencing were used to validate the isolated probiotic bacteria, as phenotypic and biochemical methods are insufficient to provide clear classification [12].

Material & Methods

1.1. Sampling and Screening of Bacteria

Madhookasavam, a commercially available PAFM, was procured from an Ayurvedic pharmacy in Dehradun, manufactured by Kottakkal Arya Vaidya Sala, Kottakkal, Kerala, India. According to the manufacturer, the formulation was prepared using multiple specific herbs, such as flowers *of Madhuca longifolia* J.F. Macbr., along with honey, *Elettaria cardamomum*, *Nelumbo nucifera*, and *Santalum album*, which are also prescribed in the Ayurvedic Pharmacopeia, through a traditional natural fermentation process. After procurement, the formulation was used under sterile conditions to isolate the harboring bacteria using serial dilution (0.85% NaCl) in Nutrient agar medium supplemented with the antifungal drug fluconazole (15mg/ml). Bacterial colonies on nutrient agar plates were selected based on colony morphology and random distribution for further characterization. We picked 20 colonies onto nutrient agar plates for pure-culture isolation and stored them as glycerol stocks at -80°C [13].

2.2. Hemolysis activity

Before probiotic characterization, we assessed the isolates' safety using blood hemolysis and antibiotic susceptibility assays. We studied the hemolysis pattern following a previously published protocol with some modifications [14]. Briefly, we streaked the isolate on a blood agar plate and incubated it for 24–48 h at 37°C to detect hemolysis patterns. Positive activity was indicated by the formation of a yellowish clear halo around the colonies (β-hemolysis) or a change in the color of the hemolysis halo to green (α-hemolysis). No activity was considered as no hemolysis and was safe to use for further probiotic characterization [15].

2.3. Antibiotic susceptibility

Antibiotic susceptibility profiles of the bacterial isolates were determined using the Kirby–Bauer disk diffusion method in accordance with Clinical and Laboratory Standards Institute guidelines. Fresh bacterial suspensions, adjusted to a 0.5 McFarland standard, were spread uniformly onto Mueller–Hinton agar plates[16]. Commercial antibiotic discs by HiMedia containing Azithromycin, Erythromycin, Gentamicin, Chloramphenicol, Amikacin, Ofloxacin, Tetracycline, Cephalexin, and Clarithromycin were placed on the agar. The plates were incubated at 37°C for 24 h, after which the inhibition zone diameters were measured in millimeters [17]. The results were classified as resistant, moderately sensitive, or sensitive based on CLSI interpretive criteria.

2.4 Low pH Tolerance

To ensure that probiotic isolates survive the harsh acidic environment of the human stomach (pH2.0-6.0), isolates were tested over a range of pH 2.0 to pH 6.0 as described by Hu et al. (2025) [18]. Briefly, isolates were grown in nutrient broth to OD_{600}, harvested by centrifugation, and suspended in nutrient broth previously adjusted to the pH 2.0–6.0 range with HCl, then incubated at 37°C for 3 h. After 3 h of incubation, cells were transferred to conventional, nonacidified nutrient broth medium (pH 6.5), and optical density was recorded at 12 h intervals.

2.5. Pepsin Tolerance Assay

The pepsin tolerance assay is an *in vitro* gastric survival test to assess the isolate's ability to survive acidic, pepsin-containing, stomach-like conditions before reaching the intestine. Briefly, the isolate was incubated with a range of pepsin dissolved in broth (0.1% to 1%) at pH 2.0 for 3 hours and then transferred to nutrient agar medium. Following incubation at 37° C, growth was recorded at 12 h intervals up to 48 h [19].

2.6. Bile Salt Tolerance Assay

Bile salt tolerance assay was used to assess whether the isolate can survive in bile salt (cholate, chenodeoxycholate, and deoxycholate) conditions mimicking the small intestine. The isolate was tested against a range of bile salts (0.1% to 1%), and growth was observed at 12 h intervals up to 48 h [20].

2.7. Osmotic Tolerance

To determine whether the isolate can survive high NaCl concentrations, we performed a salt tolerance assay in the range of 1% to 10% NaCl. Briefly, nutrient broth was prepared with varying salt concentrations and inoculated with a 0.5% seed culture of the isolate for 48 h at 37° C. Isolate growth was observed at 12 h intervals up to 48 h [21].

2.8. Bioactive Cellulase Production and Estimation

Cellulase activity was evaluated to determine the isolate's ability to hydrolyze cellulose into soluble reducing sugars. Activity was determined by quantifying the reducing sugars released from carboxymethyl cellulose using the 3,5-dinitrosalicylic acid method and 0.08g/L Congo red for quantitative analysis. To obtain the crude enzyme extract, the bacterial isolate was inoculated into 100

mL of 1% CMC growth medium and incubated at 37° C with shaking at 200 rpm for 4 days. Following incubation, the 3 mL samples were collected at regular intervals of 24 h; the culture was centrifuged at 8,000 × g for 10 min at 4°C, and the resulting cell-free supernatant was collected as the crude enzyme extract. For the enzyme assay, mix 500 µL of crude enzyme extract with 500 µL of 1% CMC in 50 mM sodium citrate buffer. The reaction mixture was incubated at 50°C for 30 min, then terminated by adding 1 mL of DNS reagent and heating in a boiling water bath for 10 min to develop the characteristic reddish-brown color. After cooling to room temperature, absorbance was measured at 540 nm using a UV–Visible spectrophotometer [22].

2.9. Antioxidant by DPPH Radical Scavenging Assay

The antioxidant potential of the bacterial isolates was evaluated using the 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay. The DPPH assay is a rapid, widely used method for assessing this free-radical scavenging ability. DPPH is a stable free radical that exhibits a deep violet color with a characteristic absorbance at 517 nm. In the presence of antioxidant compounds, DPPH accepts an electron or hydrogen atom and is reduced to a non-radical form, shifting from violet to pale yellow. The decrease in absorbance at 517 nm is directly proportional to the sample's free radical scavenging activity. We quantified antioxidant activity with minor modifications to the method established by Brand-Williams et al. Briefly, the bacterial isolates were initially grown in NAM to prepare the inoculum, which was then transferred into 1% CMC growth medium and incubated for 24 h. Following incubation, the cultures were centrifuged at 10,000 × g for 10 min at 4°C. The resulting cell-free supernatant (CFS) was collected, adjusted to pH 7.0, and filtered through a 0.22 µm syringe filter. The filtered CFS was used as the test sample for the DPPH radical-scavenging assay. Different concentrations of CFS were prepared in methanol, and 100 µL of each preparation was mixed with 100 µL of 0.1 mM DPPH solution, giving a final volume of 200 µL per well. After a 30-minute incubation in the dark at room temperature, we measured absorbance at 517 nm using a microplate reader. Ascorbic acid was utilized as the positive control, while DPPH in methanol served as the negative control [23].

2.10. Bacterial Colonization Assay in *C. elegans*

The N2 Bristol (wild-type) *C. elegans* worms were maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50 strain using standard laboratory procedures. The age-synchronized L4 worms were obtained through bleaching as previously described [24].To inhibit progeny production and prevent interference from newly hatched larvae during the prolonged colonization period, NGM plates used for the assay were supplemented with 120 µM of FuDR. Selected bacterial isolates were cultured at 37°C overnight, harvested by centrifugation at 3000 rpm, washed twice with sterile phosphate-buffered saline, and seeded onto fresh FuDR-supplemented NGM plates. Approximately 5 age-synchronized L4 worms were plated onto the bacterial lawns (OP50, *Lactobacillus sp*. and VR19) and incubated at 20°C for 4 days to allow intestinal colonization. Following the 4-day colonization period, worms were transferred to unseeded NGM plates for 12 h and 24 h of starvation before bacterial enumeration. After starvation, worms were washed five times with sterile M9 buffer containing 100 µg/mL ampicillin and 25 mM sodium azide to remove surface-associated bacteria and immobilize the

worms, respectively. Individual worms were homogenized using sterile disposable micropestles in sterile 1mL M9 buffer. The homogenates were serially diluted, plated onto selective MRS agar medium, and incubated at 37°C for 24 h. Bacterial colonization was determined by counting colony-forming units and expressed as $\log_{10}$ CFU per worm. (Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). [25]

2.11. Molecular Identification of Selected Bacterial Isolate

16S rDNA sequencing of the isolate was performed and outsourced to Biokart, Bangalore. The obtained 16S rRNA gene sequence was edited, assembled, and compared with reference sequences in the National Center for Biotechnology Information database using the Basic Local Alignment Search Tool to determine its closest phylogenetic relatives. Multiple sequence alignment was performed using ClustalW, and a phylogenetic tree was constructed in MEGA version 11 using the Neighbor-Joining method with the Kimura two-parameter model. We evaluated the robustness of the tree topology by bootstrap analysis with 1,000 replications [26].

Results

3.1. Safety analysis of screened isolates

We selected 20 isolates based on distinct colony morphology, using additional parameters such as subculturing colonies from equally distributed quadrants. All 20 isolates were tested in a hemolytic assay: only one isolate showed γ-hemolysis and one isolate showed α-hemolysis. The remaining 18 isolates showed β-hemolysis (Fig. 1A). Because non-hemolytic activity is a key criterion for probiotics, we selected one isolate for further probiotic characterization and named it VR19. Further, VR19 was continued for antibiotic susceptibility assay and showed the zone of inhibitions against multiple antibiotics including, Azithromycin (15 µg, 25.35 ± 2.33 mm), Erythromycin (15µg, 21.65 ± 0.07 mm), Gentamicin (10µg, 19.9 ± 2.55 m), Chloramphenicol (30µg, 31.25 ± 5.0 mm), Amikacin (30µg, 26.10 ± 1.41 mm), Ofloxacin (5µg, 31.25 ± 2.05 mm), Tetracycline (30µg, 36.25 ± 1.63 mm mm), Cephalexin (30µg, 21.15 ± 14.21 mm), Clarithromycin (15µg, 35.90 ± 3.96 mm). The resistance profile was determined based on CLSI guidelines (Fig. 1B). VR19 was susceptible to all antibiotics tested.

3.2. Low acid and pepsin tolerance

The acid tolerance of VR19 was first evaluated by monitoring bacterial growth after 3 h incubation of bacterial cells at pH 2, 3, 4, 5, and 6 and transferring them to normal nutrient broth medium for growth over a 48 h incubation period. The isolate remained viable under all tested pH conditions, though growth varied by pH. Growth was lowest at pH 2.0 and increased progressively with increasing pH. At pH 5 and 6, VR19 showed the highest optical density values, indicating favorable growth under mildly acidic conditions (Fig. 2A). In continuation, VR19 was also tested for pepsin tolerance in the range of 0.1% to 1.0% at pH2.0. VR19 showed reduced growth at 0.1% to 1% compared to the no-pepsin control within the range of 30% to 40% only (Fig. 2B).

3.3. Bile Salt and Osmotic Tolerance Assay

We also evaluated VR19 survival against bile salts, as probiotics encounter bile acids in the small intestine. VR19 survived bile salt concentrations ranging from 0.1% to 3%. At the standard concentration of 0.3% bile salts, VR19 showed survival of more than 70% after 24 h of incubation (Fig. 3A). We evaluated VR19's osmotic stress response using a salt tolerance assay. VR19 showed no effect of high salt concentration on its growth. After 24 h of incubation, VR19 showed significant growth compared with all tested salt concentrations ranging from 1% to 10% (Fig. 3B).

3.4. Bioactive Cellulase Production and Antioxidant Activity

Cellulase production by VR19 was evaluated using the 3,5-dinitrosalicylic acid assay. The isolate exhibited cellulolytic activity by releasing reducing sugars from carboxymethyl cellulose. We quantified these sugars using a glucose standard curve that showed good linearity. VR19 released 0.959 mg/mL of reducing sugars, corresponding to a cellulase activity of 1.95 U/mL, confirming the isolate's cellulolytic potential under the experimental conditions (Fig. 4A).

The antioxidant potential of VR19 was assessed using the DPPH radical scavenging assay at concentrations ranging from 50 to 500 µg/mL. VR19 demonstrated concentration-dependent antioxidant activity, with scavenging capacity increasing progressively as concentrations rose. VR19 showed maximum DPPH radical scavenging activity of 72.9% at 500 µg/mL; however, the ascorbic acid standard consistently showed superior activity across the tested concentrations. The IC_{50} value of VR19 was determined to be 276.0 µg/mL, reflecting a moderate free radical scavenging capacity. Two-way ANOVA revealed significant effects of concentration, sample type, and their interaction on DPPH radical scavenging activity ($P < 0.0001$) (Fig. 4B).

3.5. Bacterial Colonization Assay in *C. elegans*

We evaluated VR19 intestinal colonization potential using the *C. elegans* model, with *Escherichia coli* OP50 and *Lactobacillus sp*. as controls. After exposure, we quantified bacterial populations recovered from worm homogenates as $\log_{10}$ CFU per worm at 12 h and 24 h starvation (Fig. 5A), enumerated on nutrient agar plates (Fig. 5B). VR19 colonized and persisted similarly to *Lactobacillus sp.* Higher than *E. coli* OP50 throughout the experimental period. Although bacterial counts for both strains declined with increasing starvation time, VR19 consistently maintained higher intestinal populations than the control under all tested conditions. A two-way repeated-measures ANOVA confirmed significant effects of bacterial strain, starvation duration, and their interaction on intestinal colonization, indicating the enhanced persistence of VR19 within the nematode intestine.

3.6. Molecular Identification of VR19

We established the taxonomic identity of VR19 through 16S rRNA gene sequencing, followed by BLAST analysis and phylogenetic reconstruction using the Neighbor-Joining method. Sequence analysis identified the isolate as *Bacillus stercoris*, which exhibited high sequence similarity to reference strains in the NCBI database. The phylogenetic tree placed VR19 within the *Bacillus stercoris* clade with strong bootstrap support, confirming the isolate's identity (Fig. 6).

Discussion

Traditional polyherbal fermented ayurvedic formulations are complex microbial ecosystems and promising sources of novel probiotic microorganisms [27]. Previous studies have demonstrated the presence and probiotic potential of bacterial communities associated with polyherbal fermented ayurvedic medicines. Annadurai and Sekar (2019)[28] isolated 35 intrinsic bacterial strains from the fermented polyherbal preparations Ashokarishta, Dasamoolaristha, and Aswagandharistha, several of which exhibited important probiotic characteristics, including tolerance to gastrointestinal conditions, bile salt hydrolysis, hydrophobicity, auto-aggregation, antimicrobial activity, and functional enzymatic activities. Similarly, Chopade et al. (2019) [29] isolated 11 bacterial strains from Takrarishta, among which several isolates displayed probiotic properties, with *Lactobacillus plantarum* TP5 identified as a potent probiotic candidate. More recently, Saha et al. (2024) [12] isolated lactic acid bacteria from different Ayurvedic herbal preparations, including Asava and Aristha, and identified isolates with promising probiotic and antidiabetic properties. In addition, probiotic *Bacillus licheniforms* has been isolated from Ashwagandharistha, further highlighting the microbial diversity and functional potential of fermented Ayurvedic medicines. Collectively, these findings suggest that polyherbal fermented formulations may serve as valuable reservoirs of indigenous microorganisms with potential probiotic and therapeutic attributes.

In the present study, systematic screening of bacterial isolates recovered from Madhookasavam identified VR19 as the most promising probiotic candidate based on its safety profile, gastrointestinal stress tolerance, functional properties, and in vivo colonization ability. Molecular characterization further identified the isolates as *Bacillus stercoris*, an emerging bacterial species with limited but increasing scientific interest [30, 31]. The functional potential of *Bacillus stercoris* has been increasingly documented, highlighting its metabolic versatility and possible biotechnological and probiotic significance. Recent studies have shown that *B. stercoris* can exhibit broad-spectrum antimicrobial activity, particularly against Gram-positive pathogens, including methicillin-resistant *Staphylococcus aureus*, and has been associated with biosynthetic pathways for antimicrobial metabolites such as subtilosin A, bacilysin, bacillaene, and bacteriocin-like compounds [30]. In addition, sponge-associated *B. stercoris* has shown considerable enzymatic and biosynthetic potential, including agarose alginate lyase peptidase production and biosurfactant activity [32]. *B. stercoris* probiotic potential is also supported by its gastrointestinal resilience, bile and lysozyme tolerance, pathogen co-aggregation, antibacterial activity, siderophore production, antioxidant potential, and extracellular production of amylase, protease, cellulase, and lipase. Earlier studies have further reported the production of bioactive antibacterial

metabolites by *B. stercoris* against *Vibrio* and *Aeromonas* species [33]. Collectively, these findings indicate that *B. stercoris* possesses diverse functional attributes.

Safety is the primary criterion in probiotic selection; therefore, among the twenty bacterial isolates evaluated, VR19 was the only isolate exhibiting γ-hemolysis, indicating the absence of hemolytic activity and suggesting a non-virulent phenotype. Furthermore, VR19 remained susceptible to generational antibiotics, supporting its preliminary safety for probiotic applications. Similar safety profiles have been reported for probiotic Bacillus species, including *Bacillus clausii*, which are widely recognized for lacking clinically relevant virulence traits and having acceptable antibiotic susceptibility profiles [34]. Although *Bacillus stercoris* has recently been described, available genomic analyses suggest a similar non-pathogenic property, supporting its consideration as a potential probiotic [30, 31].

An essential characteristic of probiotic microorganisms is their ability to survive the harsh physicochemical conditions encountered during gastrointestinal transit. VR19 demonstrated remarkable tolerance to acidic conditions (pH 2–6), simulated gastric juice containing up to 1% pepsin, 0.3% bile salts, and elevated NaCl concentrations. Survival under stress is particularly important because gastric acidity and bile salts represent the first biological barriers encountered following oral administration [35]. Comparable observations have been reported for probiotic *Bacillus subtilis*, *Bacillus coagulans*, and *Bacillus clausii*, which exhibit high resistance to acidic environments because of their stress-response mechanisms and endospore-forming capability[34]. The sustained growth of VR19 under all tested gastrointestinal stress conditions indicates that the isolate has comparable physiological robustness and may remain viable throughout gastrointestinal passage.

Oxidative stress is increasingly recognized as an important contributor to intestinal inflammation and severe chronic disorders. Therefore, microorganisms that scavenge reactive oxygen species may provide additional therapeutic benefits [36]. VR19 showed concentration-dependent DPPH radical-scavenging activity, demonstrating appreciable antioxidant potential. Similar antioxidant activities have been reported for probiotic *Bacillus subtilis* and *Bacillus velezensis*, where extracellular enzymes, antioxidant peptides, and secreted metabolites contribute to free radical neutralization [37–39]. The antioxidant capacity demonstrated by VR19 suggests that the isolate may help reduce oxidative stress within the gastrointestinal tract.

In addition to its probiotic characteristics, VR19 demonstrated significant cellulase activity. Previous studies have reported the coexistence of cellulolytic activity with acid and bile tolerance and antimicrobial properties in *Bacillus* strains [40]. Cellulolytic *Bacillus* strains have also been reported to enhance cellulose utilization and modulate gut-associated microbial communities [41]. In the gut, cellulose degradation may provide substrates for microbial cross feeding, supporting the growth and activity of other carbohydrate utilizing bacteria and contributing to SCFA production and utilization [42, 43]. Thus, the cellulolytic and probiotic attributes of VR19 suggest its potential as a multifunctional candidate for dietary fiber degradation, modulation of gut microbial communities and promotion of beneficial SCFA associated fermentation.

The ability of probiotic microorganisms to colonize the host intestine is considered an important determinant of their biological efficacy [44]. In the *C. elegans* model, VR19 exhibited significantly greater intestinal colonization and persistence than the standard laboratory food bacterium *E. coli* OP50 and *Lactobacillus sp*. throughout the starvation period. Recovery of Gram-positive colonies from worm homogenates further confirmed successful intestinal persistence. The *C. elegans* model has become an established system for preliminary evaluation of probiotic host interactions because its intestinal physiology shares several conserved features with higher organisms while allowing rapid assessment of bacterial colonizations

[45]. Similar intestinal persistence has been reported for probiotic *Lactobacillus* and *Bacillus* strains evaluated in *C. elegans*, where enhanced colonization correlated with improved host protection against enteric pathogens and increased lifespan [46]. VR19's superior persistence therefore strengthens its candidacy as a probiotic microorganism.

Phylogenetic analysis based on 16S rRNA gene sequencing identified VR19 as *Bacillus stercoris.* Although this species was only recently recognized taxonomically, emerging investigations suggest that *Bacillus stercoris* has metabolic versatility and can produce several biologically active metabolites. Recent reports have proposed its potential utility in environmental biotechnology, biomass degradation, and probiotic development[47, 48]. The present study substantially extends these observations by providing experimental evidence that *Bacillus stercoris* possesses multiple probiotic-associated characteristics, including gastrointestinal stress tolerance, antioxidant activity, cellulase production, and efficient intestinal colonization. Despite these encouraging findings, comprehensive whole-genome sequencing and validation in mammalian models are required to confirm the safety and therapeutic efficacy of *Bacillus stercoris* VR19 before its potential clinical or commercial application.

Overall, these findings show that *Bacillus stercoris* VR19 combines safety, physiological resilience, functional probiotic properties, and intestinal colonization ability comparable to well-established probiotic Bacillus species. These observations further highlight traditional Ayurvedic fermented formulations as valuable reservoirs of previously unexplored probiotic microorganisms and support continued investigation of *B. stercoris* for applications in functional foods, nutraceuticals, and microbiome-based therapeutics.

Conclusion

The present study successfully isolated, characterized and reported *Bacillus stercoris* VR19 from the traditional Ayurvedic fermented formulation Madhookasavam for the first time. This suggests that polyherbal fermented Ayurvedic medicines could be a source of novel probiotics for further exploration. VR19 exhibited the most favorable probiotic profile, demonstrating a non-hemolytic phenotype, susceptibility to clinically relevant antibiotics, tolerance to acidic pH, appreciable antioxidant and cellulolytic activities, and effective colonization of the intestinal tract of *C. elegans*, indicating its ability to survive and persist under gastrointestinal conditions.

Collectively, these findings suggest that *Bacillus stercoris* is a promising probiotic candidate with potential applications in functional foods, nutraceuticals, and microbiome-based therapeutic strategies. Nevertheless, comprehensive genomic safety analysis, detailed metabolic characterization, and validation in mammalian models and human clinical studies are required before recommending its use in humans.

Statement and Declarations

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Competing Interests

The authors declare that they have no competing financial or non-financial interests that could have influenced the research, analysis, interpretation of the results or preparation of this manuscript. The authors have no financial, personal or professional relationships with any organization or individual that could be perceived as influencing the work reported in this manuscript.

Authors Contributions

Vipasha Rathi -Investigation, methodology, experimentation, data collection, data analysis, visualization and manuscript preparation.

Dr. Nikhil Chauhan- Conceptualization, supervision, study direction, critical review and editing of the manuscript.

Dr. Kamesh R Babu- Scientific guidance, review and editing of the manuscript.

Ethics Approval

This study did not involve human participants or vertebrate animals. Therefore, formal ethical approval was not required.

Consent to participate

Not applicable. This study did not involve human participants.

Consent to publish

Not applicable. This manuscript does not contain any individual person’s data or identifiable information requiring consent for publication requiring consent for publication**.**

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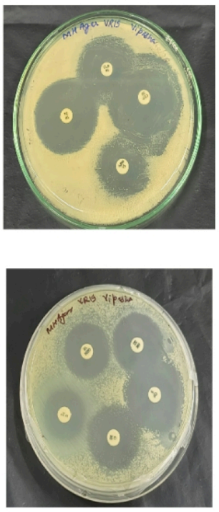
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Figures



(1A)



Antibiotics	Sensitive/Resistance	Mean ± SD
Azithromycin	(27 mm ,23.7 mm)	25.35 ± 2.33 mm
Erythromycin	(21.6 mm,21.7mm)	21.65 ± 0.07 mm
Gentamicin	(21.7 mm,18.1mm)	19.9 ± 2.55 mm
Chloramphenicol	(27.7 mm,34.8mm)	31.25 ± 5.0 mm
Amikacin	(27.1 mm,25.1mm)	26.10 ± 1.41 mm
Ofloxacin	(32.7 mm,29.8mm)	31.25 ± 2.05 mm
Tetracycline	(37.4 mm,35.1mm)	36.25 ± 1.63 mm
Cephalexin	(11.1 mm,31.2mm)	21.15 ± 14.21 mm
Clarithromycin	(38.7 mm,33.1mm)	35.90 ± 3.96 mm

(1B)

Figure 1

Hemolytic assay and antibiotic susceptibility assays of 20 bacterial isolates **(A)**Blood agar assay showed that 18 isolates have β-hemolysis, 1 isolate has α-hemolysis and 1 isolate (VR19) has γ-hemolysis; **(B)**Antibiotic susceptibility assay showed that VR19 did not have resistance to 9 antibiotics: Azithromycin, Erythromycin, Gentamicin, Chloramphenicol, Amikacin, Ofloxacin, Tetracycline, Cephalexin, and Clarithromycin.

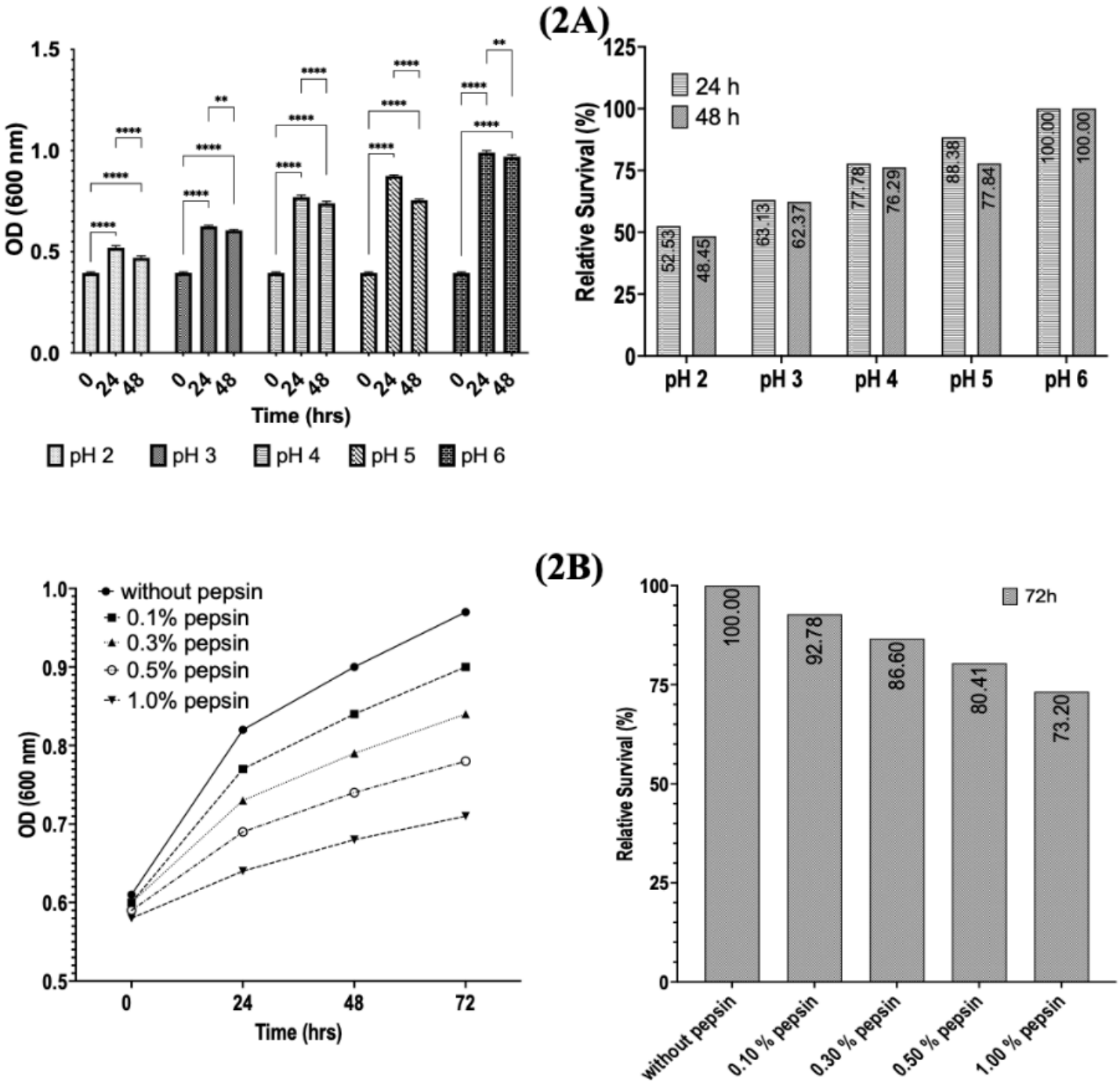


Figure 2

Low acid and pepsin tolerance of VR19 isolate. **(A)** The effect of low acid tolerance (range pH2-pH6) on the growth of VR19 was evaluated at 24 h and 48 h. Significant growth was observed at 24 h and 48 h compared to 0 h. Growth rate data suggested that VR19 optimal pH for growth is pH 6. The relative percentage survival of VR19 was also evaluated after 24 h and 48 h of exposure to different pH conditions; **(B)** VR19 was assessed for pepsin tolerance at concentrations ranging from 0.10% to 1.00%, and growth was observed at 24 h, 48 h, and 72 h. Growth rate data suggested that VR19 optimal growth of more than 50% occurred at 0.3% pepsin. Relative Survival of VR19 in the absence of pepsin is 100% growth.

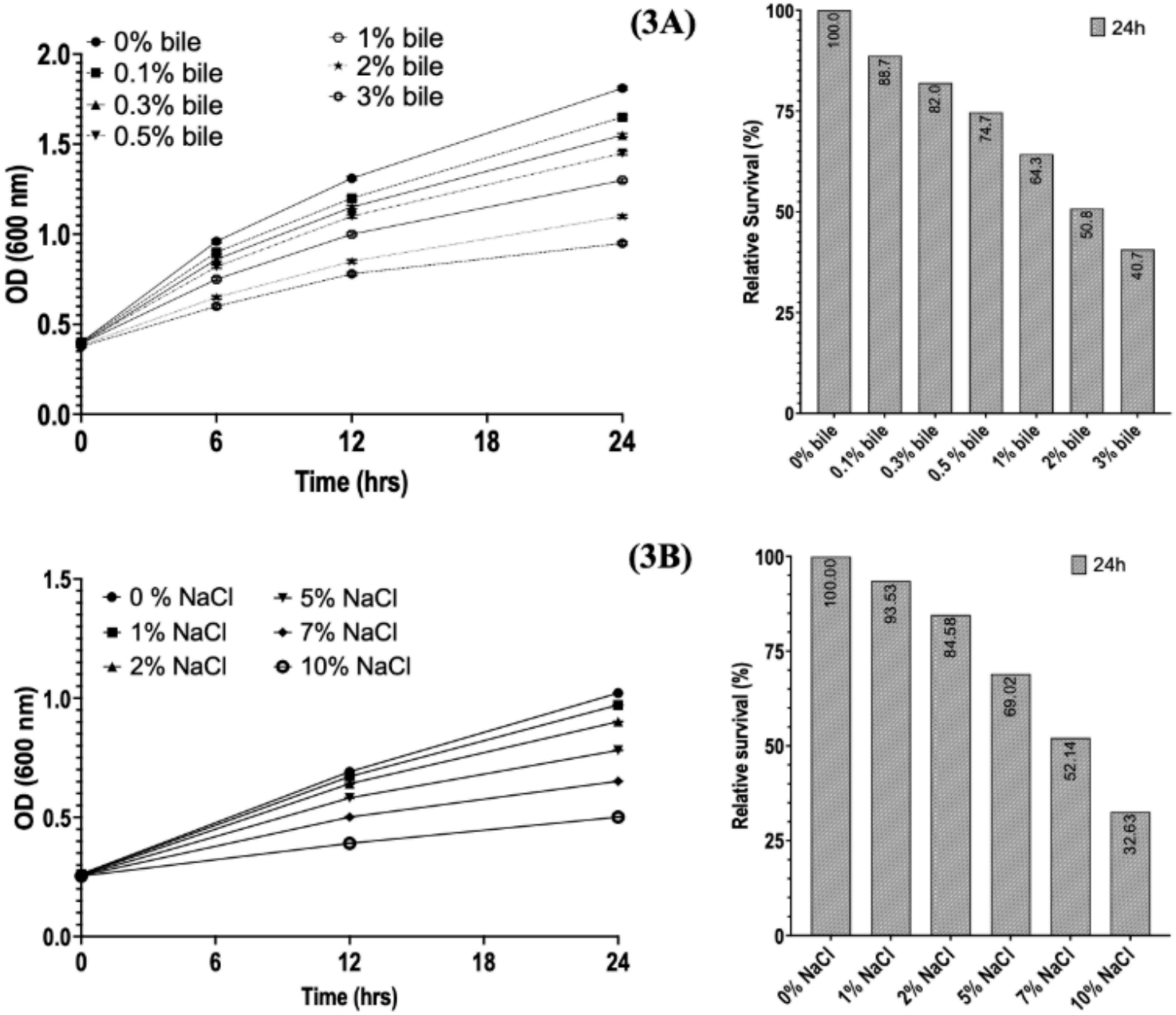


Figure 3

Bile salts and high NaCl tolerance assay. **(A)** Growth was monitored at OD_{600} (ranging 0,12,24 h) in the presence of 0- 3% bile salts; growth decreased with increasing bile concentration but remained detectable at 3% after 24 h. Relative survival of VR19 after 24 h exposure to bile salts: survival declined from 91.2% at 0.1% to 55.7% at 3% bile salt **(B)** Growth was assessed for NaCl tolerance of VR19 at OD_{600} at 0, 12, and 24 h across 0-10% NaCl concentrations, showing concentration-dependent inhibition of growth. Relative survival of VR19 following 24 h NaCl exposure declined from 95.3% at 1% to 50.9% at 10% NaCl.

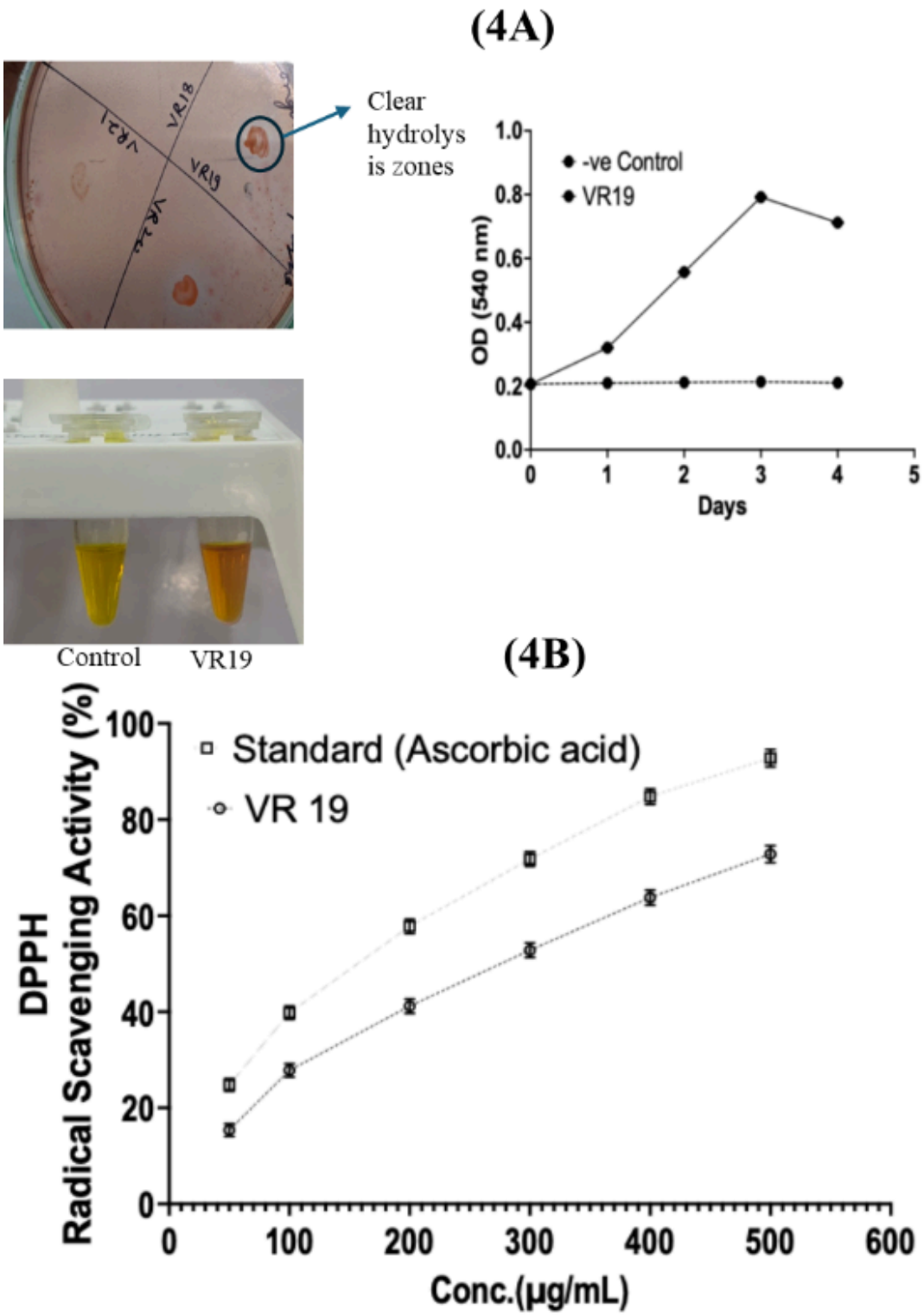


Figure 4

Functional and antioxidant properties of VR19 isolate **(A)** Cellulolytic activity of VR19 determined by Congo red and DNS assays. Congo red assay showing CMC hydrolysis by VR19, evidenced by clear hydrolysis zones. DNS colorimetric assay showing reducing sugar production following CMC hydrolysis. VR19 culture supernatant released 0.959 mg/mL reducing sugars, corresponding to 1.95 U/mL cellulase activity, based on a glucose standard curve (R^2 = 0.9808) **(B)** DPPH radical scavenging activity of VR19

and ascorbic acid. VR19 culture supernatant exhibited concentration-dependent DPPH radical scavenging activity, increasing from 15.2% to 72.9% across 50-500 µg/mL. Ascorbic acid depicted comparatively higher activity, ranging from 24.6% to 92.5% over the same concentration range.

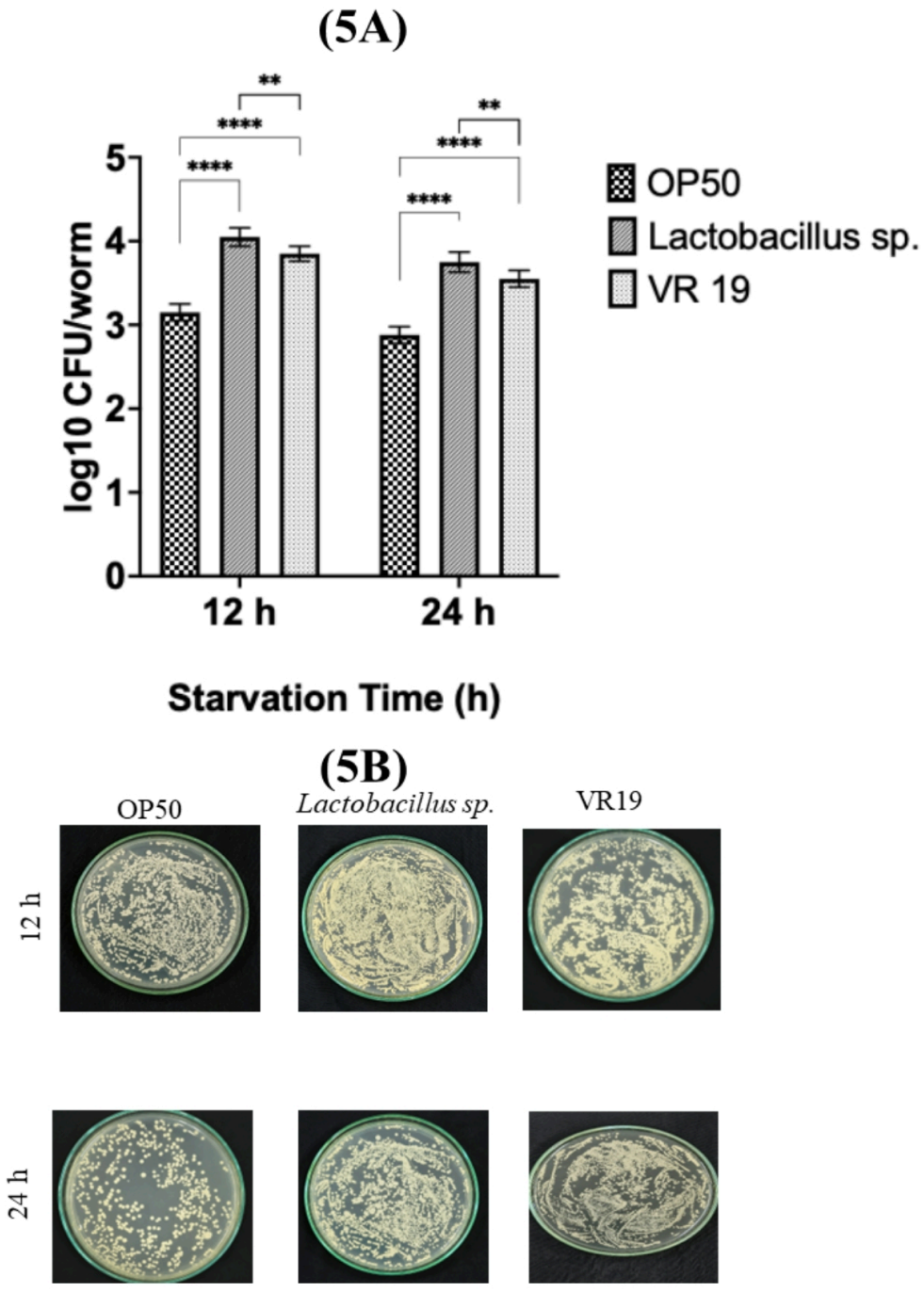


Figure 5

Comparative intestinal colonization and persistence of bacterial strains in *C. elegans* **(A)** Bacterial persistence following 12 h and 24 h starvation, quantified as log10 CFU/worm **(B)** Representative viable count plates for bacterial strains recovered from *C. elegans* following 4 days of colonization with *E. coli OP50*, *Lactobacillus*, VR19.

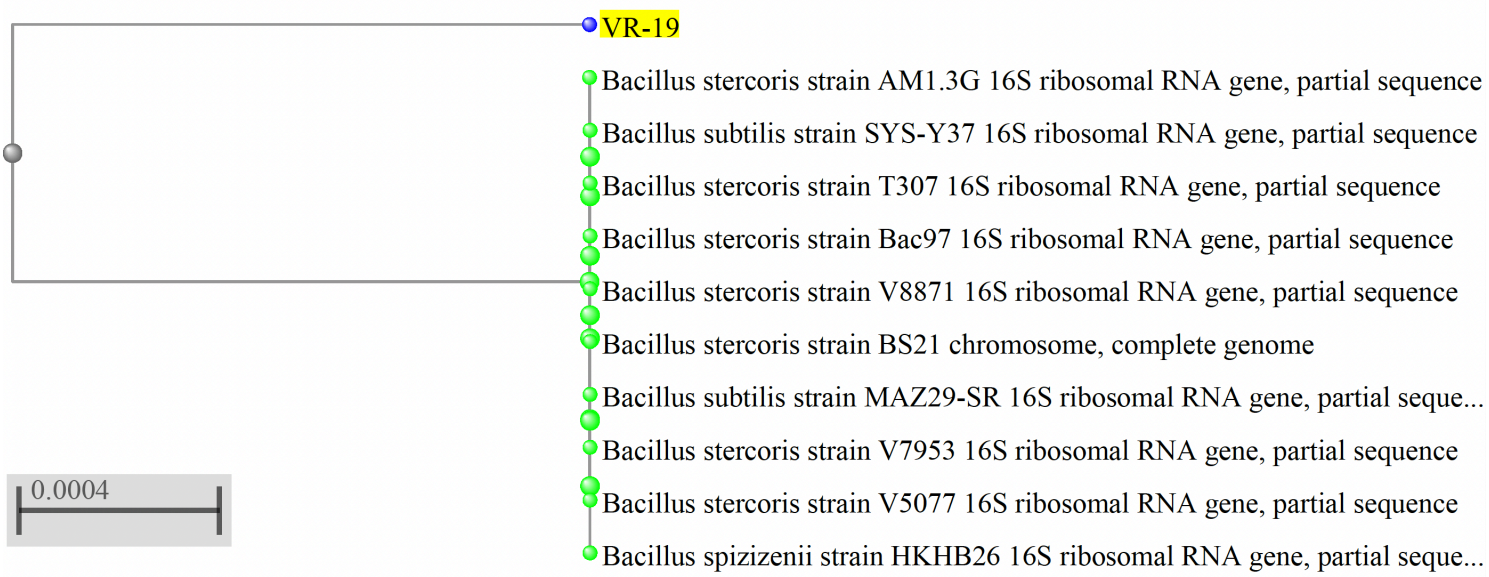


Figure 6

Phylogenetic tree based on the 16S rRNA gene sequence of VR19. The tree shows the phylogenetic relationship of VR19 with closely related *Bacillus* strains. VR19 clustered with *Bacillus stercoris* reference strains. The scale bar represents 0.0004 substitutions per site.