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Characterisation and Evaluation of *Bacillus* Based Probiotics for Environmental Stress Tolerance in Tripura, India

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Abstract

This study evaluated the verification of bacterial claims in commercial probiotic products marketed for biofloc-based fish farming, assessing their performance under conditions characteristic of the North Eastern region of India, where water commonly exhibits high iron content and low pH. Five commercial probiotic products (PR, PB, BG, FB, and LP) labelled as containing multiple *Bacillus* sp. were analysed. Heterotrophic plate count agar was used for bacterial enumeration, followed by isolation, identification, and 16S rRNA sequencing. The findings revealed discrepancies between the label claims and the actual bacterial composition of all five products. The probiotic product PR exhibited lower CFU counts than claimed, while PB closely matched the label claim. Isolated bacteria exhibited low mutual inhibition and low-to-moderate inhibition of pathogenic bacteria. All *Bacillus* isolates produced extracellular enzymes and exhibited varying antibiotic resistance, with *B. pacificus* showing the highest resistance. Probiotic bacteria with antimicrobial resistance are unsafe for aquaculture use because they pose risks of horizontal gene transfer. Significant bacterial growth was observed over a pH range of 4-9 and substantial growth at 15 °C and 20 °C. Iron tolerance assessments indicated that *Brevibacillus borstelensis*, *B. subtilis*, and *B. pacificus* exhibited significant growth at an iron concentration of 5 mg L^{-1}. Additionally, *B. sonorensis*, *B. borstelensis*, and *B. subtilis* demonstrated superior ammonia removal efficiency. These findings provide insights into the performance of probiotic bacteria under region-specific water conditions, highlighting potential candidates for biofloc-based fish farming in Tripura.

Keywords: Probiotics, *Bacillus* sp., antibiotic resistance, ammonia removal, biofloc

Introduction

As the global population continues to rise, food consumption patterns predict a 60% increase in food demand by 2050, putting significant pressure on food production systems, including aquaculture (FAO, 2024). Aquaculture is a vital source of high-quality food and employment; however, it faces increasing criticism for its environmental impact. In intensive aquaculture systems, waste products such as unutilised feed, excrement, and excess nutrients can degrade water quality, leading to issues like eutrophication, oxygen depletion, and disease outbreaks in cultured organisms. These challenges highlight the need for sustainable practices that can reduce aquaculture's environmental footprint.

Biofloc technology has emerged as a promising, environmentally friendly alternative to conventional aquaculture practices for reducing environmental problems (ammonia spikes, high iron content, low temperatures, etc.) in Tripura and other similar climatic conditions. By maintaining an optimal carbon-to-nitrogen (C:N) ratio, biofloc systems

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foster the growth of a symbiotic microbial community including bacteria, algae, and protozoa. This microbial matrix assimilates nitrogenous waste, converting it into high-quality single-cell protein (floc), thereby reducing the need for water exchange and enhancing system sustainability (Ahmad, Rani, Verma, & Maqsood, 2017). Microorganisms, including heterotrophic and chemoautotrophic bacteria, play a key role in these systems by assimilating ammonia and other pollutants, improving water quality, and supporting the decomposition process, oxygenation, and disease prevention (Ebeling, Timmons, & Bisogni, 2006; Kumar, Roy, Behera, Swain, & Das, 2021).

Among the beneficial microorganisms used in biofloc systems, *Bacillus* species are particularly valued for their spore-forming ability, stability under various conditions, and anti-pathogenic properties. *Bacillus* species contribute significantly to the degradation of organic matter and nutrient recycling in aquaculture systems (Hong, Duc, & Cutting, 2005; Lalloo, Ramchuran, Ramduth, Görgens, & Gardiner, 2007; Usta et al., 2019). Despite these advantages, there are concerns about the quality and reliability of commercially available probiotics, with inconsistencies in microbial content and viable cell counts. Some products have been found to contain pathogenic strains, which raises safety and efficacy concerns (Ullah, Raza, Ye, & Yu, 2019; Warzée, Elli, Fall, Cattivelli, & François, 2021).

The performance of commercial probiotics in biofloc systems is particularly important in regions with unique water conditions, such as Northeast India. This region is characterised by high iron content and low pH levels, which can negatively impact aquaculture productivity (Debnath, Saha, Kamilya, Saikia, & Saha, 2012; Anusha, Saha, Saha, & Jagadeesh, 2020). In recent years, several biofloc-based fish farms in North East India have closed or failed; while management practices, infrastructure, and disease outbreaks have been cited as potential causes, there is growing concern that the commercially available probiotics may not be suitable for the region's specific water conditions. The probiotics used in these systems may not function effectively under the region's unique water-quality challenges, including the high iron content and acidic pH.

This study aims to address these concerns by evaluating the bacterial content, claims, and overall performance of commercial probiotics used in biofloc-based aquaculture in North East India. By isolating, identifying, and characterising *Bacillus* sp. from locally available probiotic products, the research will assess their suitability for biofloc systems under region-specific water conditions. Understanding how these probiotics perform under such conditions is essential for optimising biofloc systems and ensuring their long-term sustainability in the region. This investigation seeks to fill the knowledge gap regarding the performance of commercial probiotics and their ability to support successful aquaculture practices in the North East Indian context.

Materials and Methods

In this study, five different probiotic supplements, both liquid (LP) and powder-based (PR, PB, BG, FB), were randomly selected for evaluation and assigned code names. These products were sourced from various agriculture-related retailers and stored at 4 °C to preserve bacterial viability until their expiration date. To ensure anonymity during the analysis, each product was assigned a new code name (mentioned above). However, specific product details and the corresponding codes are provided in Table 1. The viability and detailed microbial content specified on the label are the primary attributes of probiotic preparations. The microbial content's viability was evaluated using growth results on heterotrophic plate count agar, a general-purpose medium for heterotrophic bacterial species.

For powdered probiotic samples, 1 g of the sample was weighed and diluted with 9 mL PBS (Phosphate-Buffer Saline) to prepare the initial suspension. The suspension was vortexed thoroughly to break up the clumps and obtain a homogeneous mixture. For liquid samples, 1 mL of the sample was diluted with 9 mL PBS. From the initial suspension of the respective samples, 1 mL was subsequently mixed with 9 mL of sterile PBS (pH 7.4) to prepare the initial dilution (Khan, Choudhury, Kamilya, Monsang, & Parhi, 2021). This initial dilution was serially diluted before inoculating on agar plates to verify the CFU label claim. Using the pour-plate technique, 1 mL of the final dilution from the respective dilution series was added to three replicate Petri dishes. The plates were gently swirled for dispersion and incubated at 35 °C for 48 hours. Following incubation, colonies were counted, multiplied by the dilution factor, and averaged across replicates to determine the ultimate CFU. Bacterial enumeration

was performed using the standard colony-forming unit (CFU) counting method (Sanders, 2012).

Most of the probiotic products surveyed in this experiment contained a mixture of different bacterial species in a single product. After dissolving each product in PBS, it was serially diluted and plated onto Heterotrophic plate count agar (Hi-Media, India). To rule out the possibility of any gram-negative bacterial contamination, all the products were plated on Luria-Bertani (LB) culture medium (Hi-Media, India) in addition to selective media, as gram-negative bacteria, including opportunistic pathogens such as *Vibrio*, *Aeromonas*, and *Pseudomonas*, are commonly associated with infections in aquatic animals and may pose potential risks to host health if present in probiotic preparations (Austin & Austin, 2012). Bacterial isolates were selected based on morphological differences using the five probiotic products.

The Polymerase chain reaction (PCR) amplification of the 16S rRNA gene using universal bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') was utilised to identify and confirm the 25 bacterial isolates recovered from these five probiotic products, using bacterial genomic DNA as the template (Khan et al., 2021). The reaction mixture of PCR (25 μL) was composed of 1 μL primer (each primer has a concentration of 1 pM), 1 μL template (50 ng), 13 μL of Taq 2X Master Mix (1.5 mM $MgCl_2$) (Hi-Media, India), and 9 μL of nuclease-free water. The PCR (Applied Biosystems) protocol included initial denaturation for 10 min at 94 °C; 35 cycles of denaturation for 1 minute at 94 °C; annealing for 45 sec at 55 °C; extension for 1 min at 72 °C; and a final extension for 7 min at 72 °C. Gel electrophoresis using 1.5% agarose gel was performed for the PCR product, and the amplicons were observed using a gel documentation unit (Gel Doc™, USA). Thereafter, purification was done using a PCR product purification kit (Thermo Fisher Scientific Inc., USA), and the amplified products were sequenced (Bioserve, A REPROCELL COMPANY, Hyderabad). The sequences were blasted against the available sequences in the NCBI database. The sequenced gene was submitted to the NCBI database after identification, and accession numbers were acquired (Table 2). A phylogenetic (neighbour-joining) tree was created by MEGA 6 software using 16S rRNA sequences of the strains and closely related species retrieved from GenBank.

The isolated probiotic bacteria (Table 2) were assayed for antimicrobial activity by agar-well diffusion (Devi, Irungbam, Kumar, Choudhury, & Kamilya, 2023). The pathogenic bacteria (Table 4) were spread on the nutrient agar, and wells were made after complete solidification (Khan et al., 2021). 300 μL of each probiotic bacterial suspension (each with an OD of 0.25) was added to each well, and the inhibition zone was observed after 24 h of incubation at 30 °C. The mutual inhibition capacity between the bacterial isolates was assessed by the parallel streak method, in which the producer strains were streaked across the NA plate in two separate lines (3 cm apart) and incubated at 30 °C for 24 h. After incubation, the indicator strain was streaked in the middle of the producer strain, and the plates were incubated at 30 °C for another 24 h (Khan et al., 2021).

The enzyme-producing capacity of the bacteria for proteinase, amylase, and lipase was determined on gelatin-peptone agar, starch agar, and tributyrin agar, respectively, with slight modifications (Banerjee & Ghosh, 2014). Freshly cultivated bacterial strains were spot inoculated onto the surface of each agar plate, and the plates were then incubated at 30 °C for 24 h. Following incubation, gelatin-peptone agar plates were flooded with 15% $HgCl_2$, and starch agar plates were treated with Lugol's iodine solution. The plates were allowed to stand for 1–2 min before the solutions were decanted. The formation of a clear zone around the colony indicated a positive result. Similarly, a clear zone developed around bacterial growth on tributyrin agar after incubation at 30 °C for 24 h indicated positive lipolytic enzyme production.

A loopful of freshly cultured bacterial strains was transferred into 10 test tubes, each containing nutrient broth, and incubated at 10, 15, and 20 °C to study the thermotolerance of these cultures, as these temperatures represent the average winter climatic conditions in North East India. The bacteria were incubated in a BOD (Biochemical Oxygen Demand) incubator. Growth at various pH levels, *viz*., 4, 7, and 9, was evaluated using 0.5 N HCl and 1 N NaOH in a 50 mL test tube by inoculating a 24 h old bacterial culture in Nutrient broth and incubating for 24 h, and measuring OD at 600nm using Thermo Scientific Multiscan GO Microplate Spectrophotometer (Hoque, 2015; Khan et al., 2021).

Bacterial lawns were prepared using a 24 h old culture of the isolated bacteria. It was tested for

antibiotic sensitivity using Mueller–Hinton agar (Hi-Media, India) against nine antibiotic discs (Hi-Media, India), *viz*., Tetracycline (30 mcg), Azithromycin (15 mcg), Gentamicin (10 mcg), Streptomycin (10 mcg), Penicillin-G (10 units), Vancomycin (30 mcg), Ampicillin (10 mcg), Erythromycin (15 mcg), and Kanamycin (30 mcg). The antibiotic-infused discs were aseptically placed on the agar plate and incubated at 30 °C for 24 h. The interpretation of antibiotic sensitivity was based on the zone of inhibition following the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2008).

Biofilm formation was assessed using Congo Red Agar (CRA) medium (Hi-Media, India) [Brain heart infusion (BHI) broth (Hi-Media, India) - 37 g L^{-1}, sucrose - 50 g L^{-1}, agar -10 g L^{-1} and Congo Red indicator - 8 g L^{-1}] (Freeman, Falkiner, & Keane, 1989). The bacterial strains were inoculated onto CRA plates and incubated at 30 °C for 24 h. Colony colour was then observed to determine biofilm production.

The iron tolerance of the bacterial isolates was assessed following the method of Walujkar et al. (2019) with slight modifications by measuring the optical density (OD) at 600 nm of their culture in nutrient broth against a specific concentration of $FeCl_3$ (5 mg L^{-1}). The broth was inoculated at 30 °C for 24 h.

Stimulated aquaculture water (SAW) was prepared according to Xie et al. (2013) with the following per-litre concentrations: beef extract 0.5 g, sucrose 0.5 g, NaCl 0.25 g, and KH_2PO_4 0.075 g. The SAW was adjusted to an ammonia-N concentration of 10 mg L^{-1}. One mL bacterial suspension with an OD of 0.25 was inoculated into 40 mL SAW in a 45 mL centrifuge tube and incubated at 30 °C for 72 h. Ammonia concentration was recorded at 0 h, 12 h, 24 h, 48 h, and 72 h using the Phenate method (Lipps, Braun-Howland, & Baxter, 2023).

In the haemolytic test (Romanenko et al., 2008), agar plates enriched with 5% chicken blood were used to streak the isolates and then incubated at 30 °C for 24 h. The plates were subsequently observed for zones of haemolysis, which were classified as α (incomplete or partial haemolysis), β (complete haemolysis), or γ (non-haemolytic).

The data were analysed using the IBM SPSS-25 for Windows program (SPSS Inc., Illinois, USA) and Microsoft Excel for illustration of the graph. One-way analysis of variance (ANOVA) was used to compare mean values. The probability threshold was set at 0.05 to evaluate significance.

Results and Discussion

Among the five tested probiotic products, the CFU counts of the PR were found to be lower than the label claim, whereas PB (11.47 ± 0.004 $\log_{10}$ CFU/g) closely matched its label claim (11.602 $\log_{10}$•CFU/g). However, the remaining three products did not specify CFU counts on their labels. Species composition also differed from the information declared on the label. Fourth serial dilution was used for TPC analysis, except for product PB (10^{-8}), which had a higher microbial load. This culture method enabled the determination of microbial viability and enumeration of CFU for the bacteria present in each product (Table 1). These inconsistencies underscore the need for stricter regulatory oversight to ensure quality control in probiotic formulations (Hill et al., 2014; Lewis et al., 2016).

The PR label declared eight *Bacillus* species; however, only two among them were detected. Six labelled species were not detected, while three unlabelled species were identified. With respect to CFU count, PR's label claims 10.69 $\log_{10}$ CFU/g, but the actual count was 7.796 ± 0.006 $\log_{10}$ CFU/g, which is significantly lower. Similarly, PB had an actual count of 11.47 ± 0.004 $\log_{10}$ CFU/g, which was slightly lower than its labelled value of 11.602 $\log_{10}$ CFU/g. However, BG, FB, and LP did not specify CFU counts on their labels. The observed viable counts for these products were 7.74 ± 0.009 $\log_{10}$ CFU/g, 7.91 ± 0.003 $\log_{10}$ CFU/g, and 7.62 ± 0.01 $\log_{10}$ CFU/g, respectively.

Species-level identification of *Bacillus* based solely on 16S rRNA gene sequencing can be challenging because many closely related species share highly similar (> 99%) 16S rRNA sequences, making it difficult to distinguish accurately. Although the detected isolates reliably indicate the presence of *Bacillus* spp., precise species assignment should be interpreted cautiously, as the highlighted taxa may represent closely related or indistinguishable species (Chun & Rainey, 2014). The findings of this study are consistent with those of Weese (2002), who reported the poor composition and improper labelling of commercial probiotic preparations.

The findings revealed that only PB exhibited a viable

Table 1. Contrast in the the product labelling values for viability against against probiotic bacteriological bacteriological culturing

Pro biotic	Label composition[a]	Claimed CFU Counts[a]	Composition found[b]	Labelled Bacteria Not Detected	Unlabelled Bacteria Detected	Actual CFU Count ± SD[b]
PR	*Bacillus coagulans* *B. megaterium,* *Paenibacillus polymyxa* *B. pumilis* *B. cereus* *B. amyloliquefaciens* *B. licheniformis* *B. subtilis*	10.69 $\log_{10}$ CFU/g	*B. licheniformis,* *B. subtilis,* *B. paralicheniformis,* *Brevibacillus borstelensis* *P. thiaminolyticus*	*B. coagulans,* *B. megaterium,* *P. polymyxa,* *B. pumilis,* *B. cereus,* *B. amyloliquefaciens*	*B. paralicheniformis,* *B. borstelensis* *P. thiaminolyticus*	7.796 ± 0.006 $\log_{10}$ CFU/g
PB	*B. subtilis,* *B. licheniformis,* *B. megaterium,* *B. polymixa*	11.602 $\log_{10}$ CFU/g	*B. subtilis,* *B. licheniformis,* *B. sonorensis,* *B. haynesii,* *B. stercoris* *B. pacificus*	*B. megaterium,* *B. polymixa*	*B. sonorensis,* *B. haynesii,* *B. stercoris* *B. pacificus*	11.47 ± 0.004 $\log_{10}$ CFU/g
BG	*Bacillus megaterium* *B. pumilus,* *B. subtilis,*	Not labelled	*B. subtilis*	*B. megaterium,* *B. pumilus,* *B. subterranus,*	NA	7.74 ± 0.009 $\log_{10}$ CFU/g
FB	*B. subtilis,* *B. licheniformis,* *B. polymixa,* *B. megaterium,* *B. pumilis,* *B. coagulans*	Not labelled	*B. subtilis,* *B. licheniformis*	*B. polymixa,* *B. megaterium,* *B. pumilis,* *B. coagulans*	NA	7.91 ± 0.003 $\log_{10}$ CFU/g
LP	*B. subtilis,* *B. amyloliquefaciens* *B. licheniformis*	Not labelled	*B. subtilis,* *B. licheniformis*	*B. amyloliquefaciens*	NA	7.62 ± 0.01 $\log_{10}$ CFU/g

[a]Information labelled on products; [b]Determined in this study; CFU: colony-forming unit

count consistent with its labelled CFU concentration; meanwhile, PR showed a substantial reduction, and BG, FB, and LP lacked labelled CFU information, preventing quality verification. Together, these discrepancies may compromise dosage accuracy and probiotic efficacy, as insufficient or unverified viable cell counts can impair microbial establishment, pathogen suppression, nutrient cycling, and host immunostimulation in aquaculture systems. These inconsistencies underscore the need for stricter regulatory oversight to ensure quality control in probiotic formulations (Hill et al., 2014; Lewis et al., 2016). The present study evaluated the accuracy of bacterial claims in commercial probiotic products used in biofloc-based fish farming. It assessed their performance under the specific environmental conditions of North East India, where water is characterised by high iron content and low pH (Singh & Kumar, 2015), factors that significantly influence fish physiology and disease susceptibility (Anusha et al., 2020).

The detection of unlabelled bacterial species raises concerns about the quality control and transparency of the probiotic formulations, indicating potential issues with the accuracy of the product labelling. Such discrepancies may expose consumers to bacteria not listed on the label, which could have unintended effects. Overall, the findings indicate that the probiotic products did not accurately reflect the bacterial species composition and, where applicable, the CFU counts as claimed on their labels, highlighting the need for better regulatory oversight and quality control in the probiotic industry to ensure product accuracy and consumer confidence.

Among the recovered isolates, nine morphologically distinct colonies were selected using the agar-

overlay method for further characterisation (Table 2).

The dendrogram (Fig. 1) shows the phylogenetic relationships among probiotic strains and the other closely related strains retrieved from the NCBI database. A phylogenetic distance of 0.05 nucleotide substitutions per site is indicated by the scale bar. Species-level identification solely based on 16S rRNA sequencing can misidentify closely related species and high sequence similarity among closely related *Bacillus* species; therefore, additional molecular characterisation is required for definitive species-level identification.

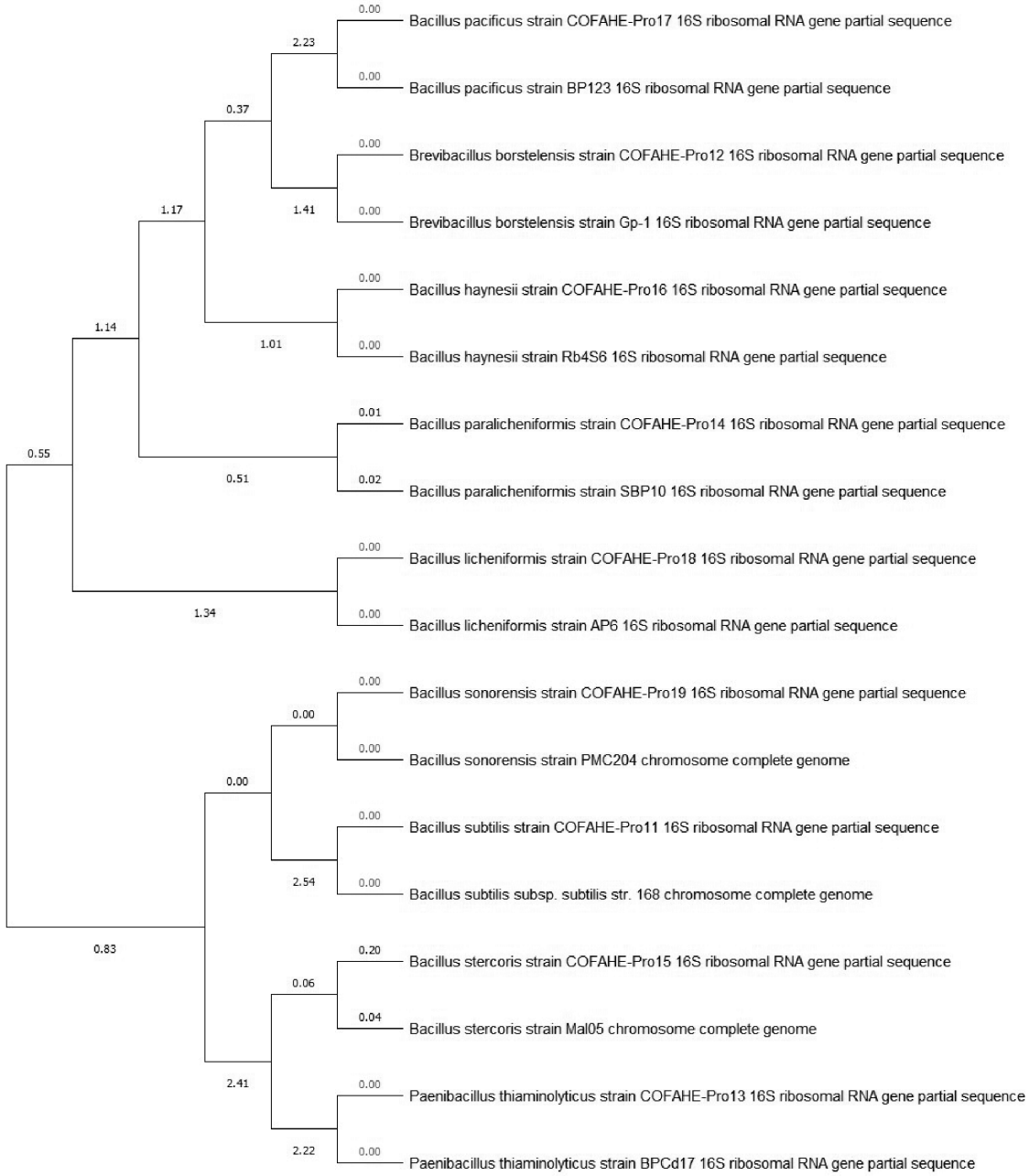


Fig. 1. Dendrogram showing the phylogenetic relationship of the nine selected probiotic strains with other closely associated strains retrieved from the NCBI database

Table 2. Accession no. of the bacterial isolates

Sl. No.	Name of strain	Name of bacteria	Accession no.
1.	COFAHE-Pro11	*Bacillus subtilis*	OR421343
2.	COFAHE-Pro12	*Brevibacillus borstelensis*	OR421344
3.	COFAHE-Pro13	*Paenibacillus thiaminolyticus*	OR421345
4.	COFAHE-Pro14	*Bacillus paralicheniformis*	OR432519
5.	COFAHE-Pro15	*Bacillus stercoris*	OR432520
6.	COFAHE-Pro16	*Bacillus haynesii*	OR740570
7.	COFAHE-Pro17	*Bacillus pacificus*	OR740571
8.	COFAHE-Pro18	*Bacillus licheniformis*	OR740572
9.	COFAHE-Pro19	*Bacillus sonorensis*	OR741757

All the isolated bacteria exhibited weak mutual inhibition (Table 3). However, the isolates exhibited varying degrees of inhibitory activity against different pathogenic bacteria as shown in Table 4. The inhibitory activity likely stems from the production of acids, bacteriocins, or other antimicrobial metabolites (Myllyluoma, Ahonen, Korpela, Vapaatalo, & Kankuri, 2008; Be'er et al., 2009; Grandy, Medina, Soria, Terán, & Araya, 2010). *Bacillus* species, known for their antimicrobial properties, exhibited antagonistic effects against *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, and *Escherichia coli*, supporting their potential use in fish disease management (Verschuere, Rombaut, Sorgeloos, & Verstraete, 2000; Das et al., 2006), consistent with reports on plant-based immunostimulants and alternative therapeutics in aquaculture (Das, Raman, Saha, & Singh, 2015; Devi, Saha, Irungbam, & Saha, 2024).

No inhibition was observed when each bacterial isolate was streaked parallel to itself, as indicated by the absence of an asterisk (*) along the diagonal in Table 3. This was expected, as a species would not inhibit its own growth. All bacterial species exhibited weak mutual inhibition when streaked parallel to other isolates, suggesting that these bacteria produce substances that likely inhibit the growth of other bacterial species, albeit weakly. The inhibitory pattern was generally consistent across all species. For example, *B. subtilis* inhibited the growth of all other isolated species, and similarly, each species inhibited the growth of all other isolates. This indicates a general antagonistic relationship among these bacterial isolates. However, weak inhibition among *Bacillus* species may be attributed to their close phylogenetic relatedness, shared resistance mechanisms, and the production of predominantly narrow-spectrum antimicrobial compounds, which favour coexistence rather than strong antagonistic interactions (Abriouel, Franz, Omar, & Gálvez, 2011).

The antimicrobial activity of the nine bacterial isolates against four indicator species is presented in Table 4, where inhibition levels are denoted as # (low) and ## (medium). *B. subtilis* and *B. licheniformis*

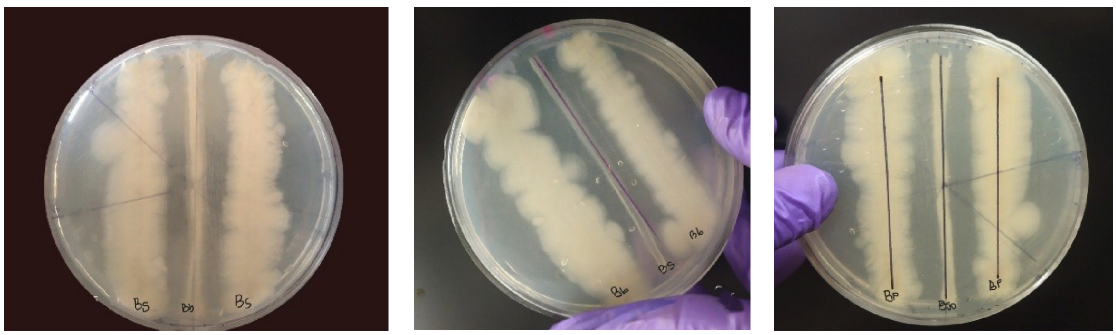


Fig. 2. Mutual inhibition test of probiotic strains by other probiotic strains using parallel streak method

Table 3. Mutual inhibition of the bacterial isolates

Indicator species	Parallel streaking								
	Bacillus subtilis	*Brevibacillus borstelensis*	*Paenibacillus thiamino-lyticus*	*Bacillus paralicheni formis*	*Bacillus stercoris*	*Bacillus haynesii*	*Bacillus pacificus*	*Bacillus licheniformis*	*Bacillus sonorensis*
Bacillus subtilis	-	*	*	*	*	*	*	*	*
Brevibacillus borstelensis	*	-	*	*	*	*	*	*	*
Paenibacillus thiaminolyticus	*	*	-	*	*	*	*	*	*
Bacillus paralicheniformis	*	*	*	-	*	*	*	*	*
Bacillus stercoris	*	*	*	*	-	*	*	*	*
Bacillus haynesii	*	*	*	*	*	-	*	*	*
Bacillus pacificus	*	*	*	*	*	*	-	*	*
Bacillus licheniformis	*	*	*	*	*	*	*	-	*
Bacillus sonorensis	*	*	*	*	*	*	*	*	-

*Weak inhibition; The results of the parallel streaking experiment designed to assess the mutual inhibition among various bacterial isolates. The indicator species listed include *B. subtilis*, *B. borstelensis*, *Paenibacillus thiaminolyticus*, *B. paralicheniformis*, *B. stercoris*, *B. haynesii*, *B. pacificus*, *B. licheniformis*, and *B. sonorensis*. The results indicate the presence of weak inhibition (denoted by *) among these bacterial species.

Table 4. Antimicrobial activity of the bacterial isolates

Indicator species	*A. hydrophila (ATCC 7965)*	*A. hydrophila (ATCC 7966)*	*P. aeruginosa (ATCC27853)*	*E. coli (ATCC 10536)*
Bacillus subtilis	##	#	#	##
Brevibacillus borstelensis	##	#	#	#
Paenibacillus thiaminolyticus	#	#	#	#
Bacillus paralicheniformis	#	#	#	##
Bacillus stercoris	#	#	#	#
Bacillus haynesii	#	#	#	#
Bacillus pacificus	#	#	#	#
Bacillus licheniformis	##	#	##	#
Bacillus sonorensis	#	#	#	#

#, low inhibition; ##, medium inhibition

demonstrated medium inhibition against specific pathogens, with *B. subtilis* showing activity against *A. hydrophila* (ATCC 7965) and *E. coli* (ATCC 10536), and *B. licheniformis* against *A. hydrophila* (ATCC 7965) and *P. aeruginosa* (ATCC 27853). *B. paralicheniformis* exhibited medium inhibition against *E. coli* (ATCC 10536). Other isolates, including *Paenibacillus thiaminolyticus*, *B. stercoris*, *B. haynesii*, *B. pacificus*, and *B. sonorensis*, consistently showed low inhibitory activity against all tested indicator species (Fig. 2). These results highlight the variable antimicrobial potential among the tested isolates, with some demonstrating notable activity against specific pathogens, while others exhibited only limited inhibitory effects.

The formation of halo zones around the colonies qualitatively assessed the extracellular enzyme-producing ability of the bacterial strains. The results showed that all strains produced all three extracellular enzymes: protease, amylase, and lipase (Table 5). The production of these extracellular enzymes by

the isolates suggests their role in enhancing feed utilisation and digestion, an important mechanism for feed additive function, including probiotics (Ghosh, Sen, & Ray, 2002; Balcázar et al., 2006) and phytochemicals (Khan, Saha, & Saha, 2022) in aquaculture.

Based on the zone of inhibition, the isolate susceptibility to nine antibiotics was evaluated. *B. pacificus* showed maximum resistance to three antibiotics (Ampicillin, Penicillin, and Vancomycin), whereas *B. stercoris* and *P. thiaminolyticus* were resistant to penicillin, and *B. licheniformis* was resistant to erythromycin (Table 6). The antibiotic resistance assay revealed that *B. stercoris*, *B. licheniformis*, *B. pacificus*, and *P. thiaminolyticus* exhibited resistance to certain antibiotics, raising concerns about their safety for probiotic applications (FEEDAP, 2012). Identifying antibiotic-resistant strains is essential to prevent the dissemination of antibiotic resistance and to ensure the safety of both humans and aquatic animals.

All the bacterial strains formed black colonies on Congo red agar, indicating positive biofilm-forming ability. In addition, all nine isolates were non-haemolytic (α-haemolysis) as no haemolysis was observed on the blood agar. The biofilm-forming ability of the isolates can enhance bacterial survival and biocontrol activity against pathogens (Davey & O'toole, 2000; Bais, Fall, & Vivanco, 2004).

After subjecting bacterial isolates to varying pH levels, all isolates showed resilience across a broad range, from highly acidic (pH 4) to strongly alkaline (pH 9) conditions (Fig. 3). Interestingly, all strains exhibited their highest rates of proliferation at

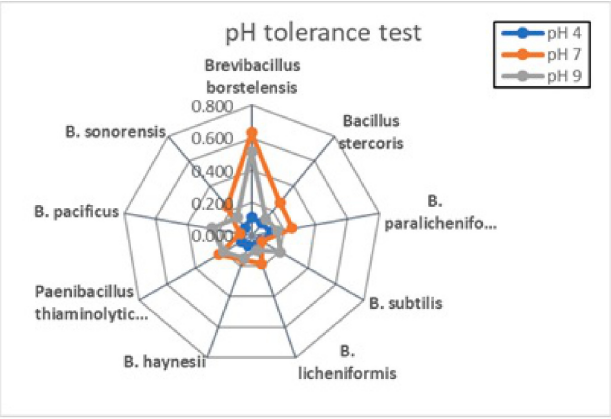


Fig. 3. Radar graph showing growth of bacterial isolates on different pH

neutral pH (pH 7). The ability to tolerate a wide pH range (4–9) suggests that these isolates can survive within the acidic to slightly alkaline conditions of the fish gastrointestinal tract (Montgomery & Pollak, 1988).

None of the bacterial isolates exhibited growth or only minimal growth at 10 °C, indicating limited viability at this temperature. However, all isolates thrived at increasing temperature. Temperature tolerance assessments indicated that while growth was minimal at 10 °C, all isolates showed substantial proliferation at 15 °C and above Fig. 4). Structural modifications at lower temperatures, such as those observed in *B. subtilis*, can influence growth and cell division dynamics (Balassa & Yamamoto, 1970; Neale & Chapman, 1970).

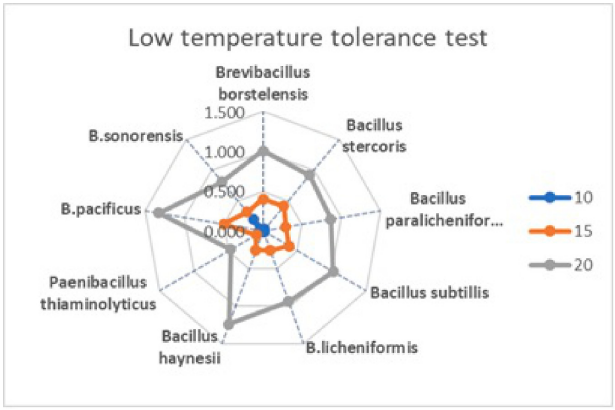


Fig. 4. Radar graph showing growth of bacterial isolates at different temperatures

Except for *B. borstelensis*, *B. subtilis*, and *B. pacificus*, none of the bacterial isolates exhibited significant growth at 5 mg L^{-1} $FeCl_3$ compared with the control, indicating limited iron tolerance at this concentration (Fig. 5). Iron tolerance varied among the isolates, with *B. borstelensis*, *B. subtilis*, and *B. pacificus* demonstrating significant growth at 5 mg L^{-1}, a concentration representative of groundwater iron levels in Tripura (Singh & Kumar, 2015). Given the variability in regional iron content, these strains may be well suited for use in biofloc-based aquaculture systems in North East India. The growth responses of the isolated strains are illustrated in Fig. 4. The evaluation of haemolytic activity, a critical safety criterion, confirmed that all isolates were non-haemolytic, aligning with previous studies that identified *B. licheniformis* and *B. subtilis* as safe probiotic candidates (Ghosh et al., 2002; Dumitru et al., 2018).

Table 5. Enzyme production assay of the bacterial isolates

Sl.no.	Name of bacteria	Result		
		Amylase test	Lipase test	Protease test
1.	*Brevibacillus borstelensis*	+	+	+
2.	*Bacillus stercoris*	++	++	+
3.	*Bacillus sonorensis*	+	++	+
4.	*Bacillus haynesii*	++	++	+
5.	*Bacillus licheniformis*	++	++	+
6.	*Bacillus pacificus*	+	+	+
7.	*Paenibacillus thiaminolyticus*	+	+	+
8.	*Bacillus paralicheniformis*	++	++	+
9.	*Bacillus subtilis*	++	++	+

Table 6. Antibiotic susceptibility of different bacterial isolates

Sl.no.	Name of bacteria	Antibiotics								
		TE	AZM	GEN	AMP	S	P	E	VA	K
1.	*Brevibacillus borstelensis*	++	++	++	++	+	++	+	++	+
2.	*Bacillus stercoris*	+	++	+	+	+	R	+	+	+
3.	*Bacillus sonorensis*	+	++	+	+	+	+	++	++	+
4.	*Bacillus haynesii*	+	++	++	+	+	+	+	++	+
5.	*Bacillus licheniformis*	++	++	+	+	+	+	R	+	+
6.	*Bacillus pacificus*	++	+	+	R	+	R	+	R	+
7.	*Paenibacillus thiaminolyticus*	+	+	+	+	+	R	+	+	+
8.	*Bacillus paralicheniformis*	++	++	++	+	+	+	+	+	+
9.	*Bacillus subtilis*	++	++	++	+	++	+	+	+	++

++, susceptible; +, intermediate; R, resistance

Except for *B. borstelensis*, all bacterial isolates exhibited notable ammonia-N removal after 12 h of incubation (Fig. 6). However, by the 24-h mark, *B. pacificus, B. haynesii, B. stercoris*, and *B. subtilis* displayed an increase in ammonia-N levels. After 48 h, all isolates except *B. paralicheniformis* continued to demonstrate ammonia-N removal. Nonetheless, after 72 h, ammonia-N production was observed in all bacterial isolates, except for *B. subtilis*. Although *B. borstelensis* exhibited a dip in ammonia-N removal at 48 h, its performance improved by 72 h. Additionally, *B. sonorensis*, *B. borstelensis*, and *B. subtilis* exhibited greater ammonia removal efficiency, highlighting their potential role in biofloc technology for maintaining water quality (Gupta & Gupta, 2001). Although *Bacillus* species are generally less efficient at ammonia removal compared to autotrophic bacteria, their heterotrophic nitrification capacity makes them valuable for aquaculture applications (Xie et al., 2013).

In conclusion, the present study provides important insights into the performance of commercial probiotic products under region-specific environmental conditions, particularly low pH, low temperature, high iron content, and elevated ammonia levels typical of North East India. The findings indicate that several probiotic formulations used in biofloc systems do not meet the expected quality standards, with discrepancies in CFU counts, inaccurate labelling, and the presence of antibiotic-resistant bacteria, raising significant safety concerns for aquaculture

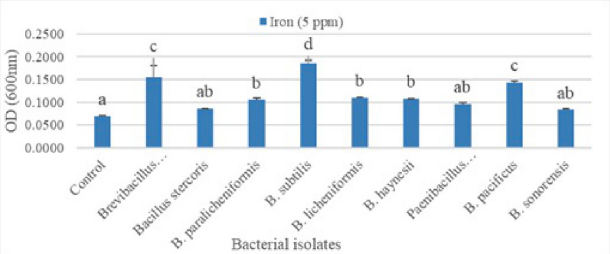


Fig. 5. Iron tolerance of different bacterial isolates at 5 mg L^{-1} $FeCl_3$. The data are presented as mean ± SE. A significant difference is shown by different lowercase superscript letters in the bar chart ($p < 0.05$)

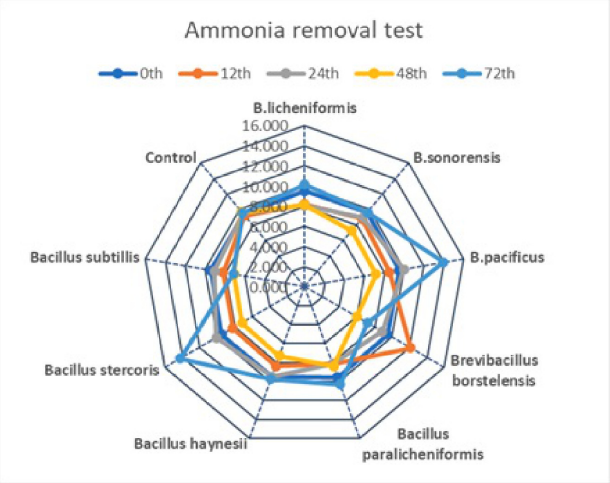


Fig. 6. Radar graph showing ammonia removal capacity of different bacterial isolates.

applications. Among the evaluated isolates, *B. subtilis, B. borstelensis*, and *B. sonorensis* demonstrated robust adaptability under stress conditions, exhibiting tolerance to a wide range of pH, low temperatures, elevated iron concentrations, and efficient ammonia removal. These attributes highlight their potential suitability for biofloc-based aquaculture systems, particularly under challenging environmental conditions. Overall, the study underscores the urgent need for stringent quality control measures, accurate labelling practices, and continuous evaluation of probiotic formulations to ensure their efficacy and safety in aquaculture. Future research should focus on long-term impacts of probiotics on fish health and water quality, deeper mechanistic understanding of their action, and the development of strategies to mitigate risks associated with antibiotic resistance.

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