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Research Article

PROBIOTIC CHARACTERIZATION OF *BACILLUS SUBTILIS* STRAIN ISOLATED FROM INFANT FECAL MATTER REVEALED BY 16S rRNA GENE AND PHYLOGENETIC ANALYSIS

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ABSTRACT

Objective: The rationale of our study was to isolate and identify the putative probiotic strain from infant fecal matter exhibiting a broad range of antimicrobial activity and to analyze the effect of different culturing conditions on its probiotic properties and the production of antimicrobial metabolites.

Methods: In the present study, bacterial strains were screened for probiotic properties and antimicrobial activity from infant fecal matter (6 months–2 years). The effect of varying culture conditions such as tolerance to acid, bile salt, phenol, NaCl, pH, incubation period, and temperature along with autoaggregation assay, hydrophobicity, and hemolysis was studied. The characterization of the potent strain was studied by morphological, biochemical, and 16S rRNA gene sequencing along the phylogenetic affiliation of the strain was studied.

Results: Two putative probiotic bacteria (DAM and IFM) were isolated, identified, characterized, and predicted at pH 2.0, 3.0, and 4.0, the isolate IFM had 50%, 60%, and 70% survivability, while isolate DAM had 55%, 63%, and 75% survivability, respectively. At a bile salt concentration of 0.5%, both isolates had a 75% survival rate. The isolates exhibited a high percentage of hydrophobicity and autoaggregation. The isolates also had non-hemolytic activity and were susceptible to many clinical tested antibiotics (tetracycline, erythromycin, ampicillin, gentamycin, penicillin, etc.). The isolate showed antimicrobial activity against enteric pathogens such as *Staphylococcus aureus*, *Escherichia coli*, and *Shigella dysenteriae*. The accession number of *Bacillus subtilis* MT279753 and MK453362 was submitted to NCBI.

Conclusion: The result revealed that isolates have potent probiotic properties and possess a direct influence on the production of antimicrobial metabolites. These parameters can be modified for the improvement of the potentiality of the isolates.

Keywords: Probiotics, Antimicrobial activity, Phylogenetic analysis, *Bacillus spp.,* 16s rRNA Secondary structure.

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INTRODUCTION

Probiotics are considered to be live microbial feed supplements. It is known to exert beneficial effects on the host by improving its intestinal microbial balance [1,2]. Most of the commercially available probiotic strains are *Lactobacillus* sp. and *Bifidobacterium* sp [3,4]. However, *Bacillus* sp. have been considered as potential probiotic strains in various dietary supplements [5,6]. The advantage of *Bacillus* sp. over other probiotic strains is they can survive in foods that require harsh processing conditions such as high temperature and pressure [7]. *Bacillus* sp. which are commonly used in different feed supplements as probiotics are *Bacillus coagulans*, *Bacillus subtilis*, *Bacillus licheniformics*, *Bacillus cereus*, *Bacillusnatto* (*subtilis*), *Bacillus clause*, *Bacillus pumilus*, *Bacillus amyloliquefaciens,* and *Bacillus polyfermenticus* [8,7]. *Bacillus* sp. are considered to be a highly potential probiotic due to its beneficial effects i.e. secretion of antimicrobials, stimulation of immune system, resistance to high acid, bile salt, non-hemolytic activity, and improvement of the gut microbiome [4,8].

In general, *B. subtilis* as a probiotics strain has shown outstanding health-boosting records [9-12]. Their application with certain immune-deficient populations especially for critically ill, neonates, and elderly groups should be evaluated and regulated carefully since reports related to bacteria in an immune-compromised patient treated with spore former and other probiotics have been recorded, repetitively [13]. Food additives and therapeutic supplements are lately seeing extensive use of probiotics, especially as digestion enhancers. Ubiquitously found endospore-forming Gram-positive bacterium of the *Bacillus* group produces several vitamins and digestive enzymes and *B. subtilis* are used as probiotics.

In the present study, we undertook the task of screening and examining potential probiotic bacteria, particularly those with antimicrobial and bacteriocin-producing properties from the feces of healthy newborn babies collected from Mayurbhanj District Hospital. The probiotic products being marketed as functional food, dietary supplements, or drug are also elucidated by the state of probiotic strains. The quality of commercial probiotic products is an important issue to be considered for regulation. In our research, the findings proved the probiotic potency of known probiotic *B. subtilis* is used as the clear implication for future research in the field and to develop our isolates as probiotics for human and animal nutrition. Studies and screening of lactic acid bacteria (LAB) from fermented vegetables, steamed buns, yogurt, and other materials have previously been pursued by many researchers but a few attempts is initiated to screen antibacterial LAB from the feces of newborn infants.

METHODS

Isolation

Samples were collected from infant fecal matter (6 months–2 year old), from Mayurbhanj district hospital, Mayurbhanj, Odisha, India. They were diluted by serial dilution method and plated using MRS agar media by spread plate and pour plate method. Plates were then incubated at 37°C for 48 h. Colonies were randomly selected based on morphological differences. The isolates were maintained on MRS agar slants at 30°C and preserved as frozen glycerol stocks. From the stock, propagation of isolates was done to MRS broth medium which was considered as working culture.

Identification of source organism

One gram of fecal matter sample was added in 100 mL distilled water and kept in a rotator shaker for about 30 min. After shaking the sample was serially diluted. 100 µL of each from 10^{-4} to 10^{-7} tubes were taken from the diluted sample and were spread over the MRS agar plates. The samples were incubated at 37°C for 12–24 h. The isolates were identified using Bergey's manual of determinative bacteriology approach, which included morphological and biochemical characterization [14]. Further, 16S rRNA sequence analysis of the isolates was performed to ensure that the isolates were correctly identified to species level.

Morphological characterization of isolate

Bacterial isolates were inoculated on MRS media and incubated for 12–24 h at 37°C. The colony morphology was noted with detail to shape, size, color, and texture, and colonies were detected under a high-power magnifying lens.

Screening for probiotic properties

All the isolates were screened for their probiotic properties. The screening was done for acid tolerance, bile salt tolerance, phenol tolerance, NaCl tolerance, antibiotic sensitivity, antimicrobial activity, autoaggregation, and hydrophobicity of all the isolation using standard methods [15].

Tolerance to acid

Acid tolerance of isolates was done following the method of Del Re *et.al*. [16]. In the process, each isolate was grown overnight in MRS broth medium at 37°C. The culture was then centrifuged (8000 rpm for 5 min) and the cell pellets were collected. The collected cell pellets were washed twice in sterile phosphate buffer saline (PBS, pH 7.3) and re-suspended in 1 mL of PBS of different pH (1, 2, 3, 4) sequentially. Bacteria cells from the condition were transferred to BHI agar plates and incubated at 37°C. The viable cells in each plate at the time interval of 0, 60, 120, and 180 min were recorded and CFU/mL was calculated. Based on the values, survival percentage of strains was calculated.

Tolerance to bile salt

Bile salt tolerance was tested according to the method given by Patel *et al*. [17]. 100 µL of overnight grown isolate culture were inoculated into 900 µL MRS broth which was pre supplemented with 0.3, 0.5, 1.0, 1.5, 2.0, 2.5, 3, 3.5, and 4% of bile salt. The inoculation was then incubated for 24 h at 37°C. From each bile salt concentration medium, 100 µL of culture was plated into MRS agar plate medium and incubated again for 24 h at similar conditions. The growth of bacterial strains was presented in $\log_{10}$ CFU/mL and the percentage of survival of bacteria was determined.

Tolerance to phenol

To estimate the phenol tolerance, 100 µL of isolate culture grown overnight were inoculated into 900 µL MRS broth medium supplemented with 0.1–0.5% of phenol and were incubated for 24 h at 37°C [18]. After incubation, the absorbance of each medium at 600 nm was measured taking MRS broth medium without phenol as a reference and the survival percentage of each strain was determined.

Tolerance to NaCl

To estimate the NaCl tolerance, 100 µL of isolate culture grown overnight were inoculated into 900 µL MRS broth medium supplemented with 2, 4, 6, 8, 10, and 12% of NaCl and were incubated 24 h at 37°C [19]. After incubation, the absorbance of each medium at 600 nm was measured taking MRS broth medium without NaCl as a reference and the survival percentage of each strain was determined.

Antibiotic susceptibility test

The antibiotic susceptibility test of isolates was done against some regular antibiotics, including levofloxacin (5 mg), gentamycin (10 mg), tetracycline (10 mg), ampicillin (10 mg), norfloxacin (10 mg), erythromycin (15 mg), chloramphenicol (30 mg), and cefoperazone (75 mg). The isolates were spread on the lawn of MRS agar plates and were incubated overnight at 37°C for 24 h. The antibiotics were then placed at each plate aseptically and incubated. After incubation, a zone of inhibition was observed [20].

Determination of antimicrobial activity

For the antimicrobial test, the test pathogens were collected from MTCC, IMTECH, Chandigarh, India. The pathogens were *Staphylococcus aureus*, *Shigella dynsentriea*, *Candida albicans*, *Escherichia coli*, and *Listeria monocytes*. The antimicrobial activity of isolates was done by a good diffusion method where the overnight culture of pathogens was inoculated in a nutrient agar medium and the isolates were incubated in an MRS broth medium for 24 h at 37°C. After incubation, the pathogens were overlaid on the test bacterial spot. The zone of inhibition was observed and the antimicrobial activity of each isolate against each test pathogen was evaluated [18].

Auto aggregation assay

Auto aggregation assay was performed according to Del Re *et.al*. [16]. In the assay, each isolate was grown overnight in an NA broth medium at 37°C. The culture was then centrifuged (8000 rpm for 5 min) and the cell pellets were collected. The collected cell pellets were washed twice in sterile (PBS, pH 7.3) and re-suspended in 1 ml of PBS to get an OD of 0.5 at A_{600} and considered as $A_{0.}$ After that, 4 ml culture was mixed by gentle vortexing for 10 s and incubated for 1 h at 37°C. After incubation absorbance of the upper suspension was measured as A_t.

Auto aggregation % was expressed as:

$$A_0 - (A_t/A_0) \times 100$$

(Where at represents the absorbance at time t = 1 h and A_0 the absorbance at t = 0 h.)

Hydrophobicity of strains

According to Rosenberg *et al* [21], hydrophobicity of strain was measured. In this method, each isolate was grown overnight at 37°C. The cells were pelleted at 8000 rpm for 5 min and washed twice with PBS pH 7.3, re-suspended in 0.1 mol/L KNO3 (pH 6.2). Absorbance at A_{600} was measured as A_0 using a spectrophotometer (ultraviolet [UV]-visible 1601 Spectrochem, Mumbai). One milliliter of solvent (xylene, acetone, and heptane) was added to 3 mL of cell suspension. After 10 min pre-incubation at room temperature, two phases were mixed by gentle vortexing for 2 min and incubated at room temperature for 20 min.

Hemolysis of red blood cells

Each bacterial isolate was streaked on NA agar supplemented with 5% sheep blood and incubated at 37°C for 24 h. The presence of a clear zone around colonies indicated the lysis ability of those colonies and was considered as a positive result.

Secondary screening by specific assay methods Bacitracin like Inhibition Studies (BLIS)

BLIS was adapted for characterizing the antibiotic-producing strains against human test pathogens. This specific assay was also known as secondary screening [22]. The isolate was cultured in a broth and incubated at 37°C, for 24 h. The test isolate was lawn cultured (1 inch) at the center of the MHA plate with the help of a sterile cotton swab and incubated at 37°C, for 24 h. After the incubation period, the bacterial mass was swept out from the plate aseptically with the help of a sterile slide then the plate was exposed to chloroform vapor for 40 min followed by exposure to UV radiation for 1 h, to ensure the death of the cells. Then freshly grown cultures of the test pathogens were streaked across the plates and incubated at 37°C [23].

Analysis of isolates

Strains were analyzed from infant fecal matter using the 16S rRNA gene and phylogenetic approach [24,25]. The 16S rRNA gene makes it the "Ultimate molecular chronometer" [26] and the most common genetic marker. The 16S rRNA genes are a useful target for phylogeny and clinical identification. This study was done through amplification of the 16S rRNA gene using a set of primer (27F 5'-AGAGTTTGATCTGGCTCAG -3'1492R

5'-CGGTTACCTTGTTACGACTT-3'). The gene is used for phylogenetic studies due to its highly conserved region among bacterial species. For this reason, 16S rRNA is considered as the reliable molecular clock that lineages distantly related bacteria which have similar functionality. The 16S rRNA gene sequences are very often used in the identification, classification, and quantification of microbes [27,28]. The DNA sequence was analyzed using an online (nucleotide Basic Local Alignment Search Tool) (n BLAST) [29]. The phylogenetic tree was prepared by neighbor-joining using pair-wise alignment in MEGA 7 software.

Modeling of RNA secondary strain

For the modeling of 16S rRNA secondary structure, M fold software is used. This algorithm widely uses the minimal free energy state. M fold calculates energy metrics that determine all optimal and suboptimal secondary structures for RNA molecules. Plot fold displays the optimal and suboptimal secondary structure for an RNA molecule. The stem-loop finds all possible stems and stems can be plotted with the Dot plot.

Statistical analysis

There were three replicates of experiments conducted for the independent characterization of two isolates. Hence, the mean values of data were taken for statistical analysis. Pearson-correlation analysis was performed to find out the linear association among the variables. All the analysis was carried out using SPSS statistical software, version 20.

RESULTS AND DISCUSSION

Strain isolation and identification

According to the method of Bergey's manual of determinative bacteriology, the two selected isolates (DAM and IFM) were identified as *B. subtilis*. Allowing to the morphological features the two isolated strains appeared yellowish roughed surfaces with irregular margins. Microscopically, the isolates appeared as Gram positive. The bacterial strains grow at pH 5.5–8.5 and temperature at 25–30°C.

Phylogenetic analysis was done using 16S rRNA gene analysis of isolates with expected base pairs of DAM and IFM, respectively [29]. After performing a BLAST search isolates DAM and IFM exhibited close association with known *B. subtilis* with a GC content of 53% and 55%, respectively. The sequences were aligned by cluster alignment method and a phylogenetic tree was generated using MEGA software. The phylogenetic relationship among different isolates of *B. subtilis* based on nucleotide region of ribosomal 16S rRNA sequence was showing rooted "neighbor-joining" tree. In the phylogenetic tree, DAM and IFM were assigned to the *B. subtilis* group, while the rest of the accessions were assigned to the other group. The phylogenetic tree reveals that the *B. subtilis* isolated from infant fecal matter has a great diversity. The GC content percentage for the two isolated strains was calculated and the GC content found more than 52% (Figs. 1 and 2).

At the center and interspecies levels, the bacteria 16S rRNA sequences are good indicates of phylogenetic relationship. At the genus level, the study of 16S rRNA gene sequence analysis provided the best identification of the isolates. Partial sequencing of the 16S rRNA gene is considered a good alternative for phenotypic identifications and placing isolates in their appropriate taxonomic position. It is confirmed that the two samples from different sources belong to *B. subtilis* isolates. The 16s amplified products of isolate with 100bp ladder shown in Fig. 3.

Probiotic properties

Acid tolerance

The *in vitro* screening for resistance to the acidity of the stomach is one of the characteristics to learn about the probiotic effects of strains in the gut. Therefore, while studying the growth and viability of the isolates at different pH, it was observed that the isolate IFM could show survivability (60–70% viability) at pH 2 and about 80% survivability at pH 3. Similarly, the isolate DAM also demonstrated 80% viability, at pH indicating that it had probiotic characteristics. Due to the high acidic nature of the gastrointestinal tract, the bacterial spores must

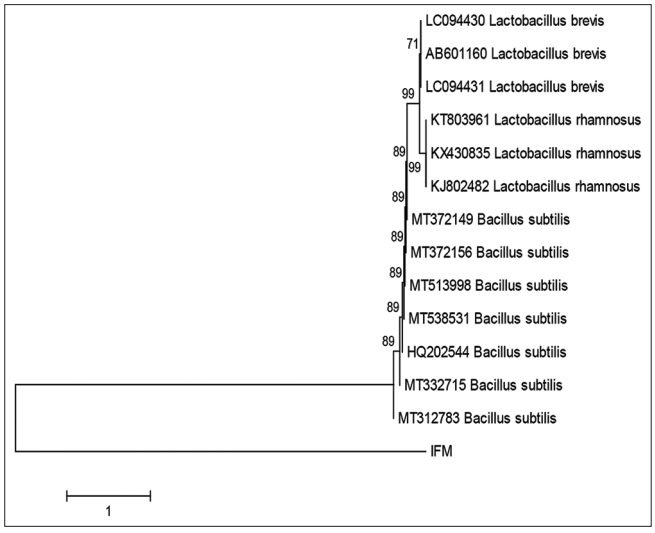


Fig. 1: Evolutionary taxa (phylogenetic tree by NJ Method)

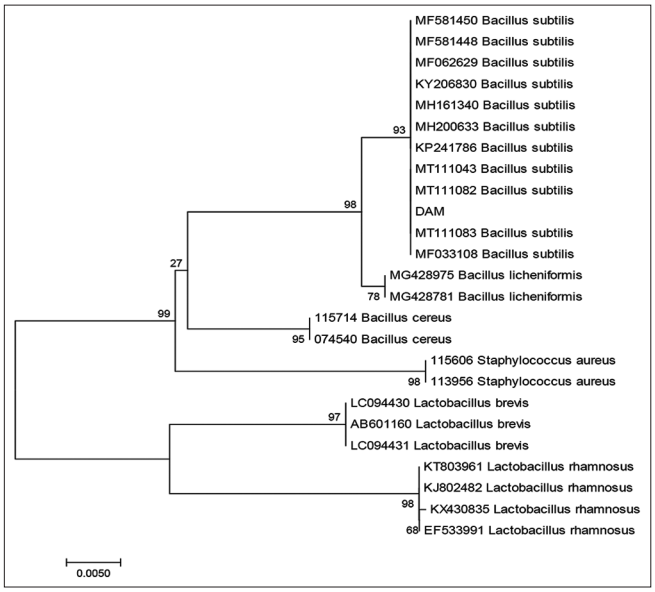


Fig. 2: Evolutionary taxa (phylogenetic tree by NJ Method)

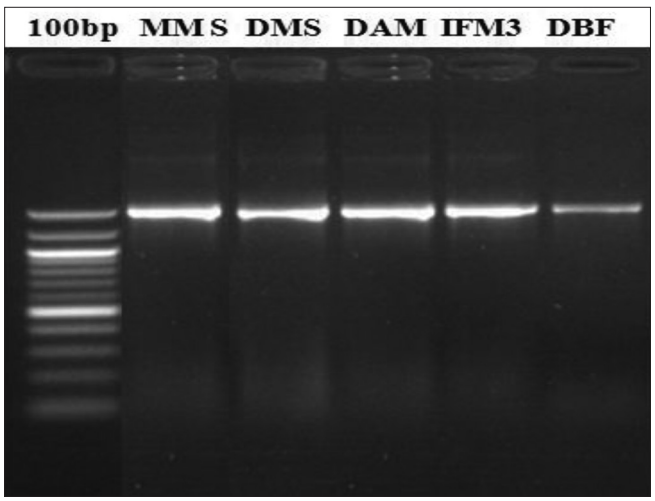


Fig. 3: 16S Amplified products of isolates with 100bp ladder (DAM & IFM)

survive and transit to the intestine to exert their properties [18,30]. Consequently, it is needed for the bacteria to have protection systems and to counter the low pH in the stomach. In this study, the selections of potentially probiotic *B. subtilis* strains were examined for their capability to withstand low pH. According to the activity of the human GI tract varies from 1.5 to 4.5; however, the *in vitro* studies are mostly performed at pH 3 because the viability below is very low. A similar study was reported by Kumar *et al.*, (2009) [31] that *E. coli* showed higher acid tolerance at pH3 in comparison to pH 2. Gangadharan *et al.* (2010) [18] also reported that viability of Lactococcus at pH 3 was 80% and 60% viability at pH 2 which was the same as our study (Fig. 4).

Bile salt tolerance

Bile salt tolerance is one of the important characteristics of probiotic organisms as they need to survive bile salts in the duodenum to exert their beneficial effects in the gut [31]. The tolerance of the two isolates to bile salts (0–4%) was investigated, and it was observed that in the bile salt range of 0.3–2%, the two isolates had a consistent survival rate of 50–80%, and the viability declined as the bile salt concentration increased (Fig. 5). In *Bacillus* strains, the variability to bile salts is important in estimating bile resistance and selecting viable probiotics. For their positive effects in the gut, probiotic strains must be able to withstand bile salts and allow them to survive and colonize the gastrointestinal tract through enterocyte adhesion [17].

Erkkila and Petaja (2000) [32] reported a similar case study where it showed that strains *Pediococcus, acidilactici (P2). Lb. curvatus (RM 10) and Lb. sakei (L2)* was resistant to 3 g/L bile salt at pH 6. Bhakta *et al*. [33] reported that LAB showed the highest tolerance to bile salt at 4 g/L concentration.

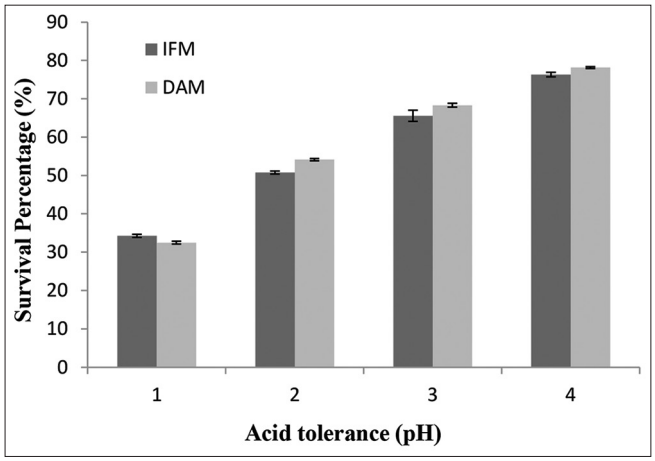


Fig. 4: Acid tolerance of the strain against different pH

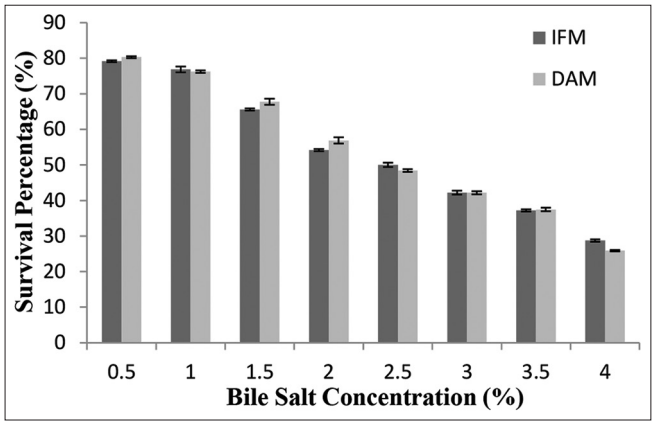


Fig. 5: Survival % of strain against bile salt concentration

Phenol tolerance

Tolerance to phenol is beneficial to probiotic bacteria as the aromatic amino acids are derived from dietary-produced proteins and can be delaminated in the gut [34]. For probiotic strain, it was proved that the phenolic compounds can be used in the bacteriostatic effect testing for the resistance and survival in gastrointestinal conditions [35]. Gangadharan *et al.* [18] reported a relatively high survivability rate (70–80% at 0.2% phenols, which was reduced to 50% at 0.4% phenol and 10% at 0.6% phenol, respectively). In our investigation, the results of phenol tolerance of the two isolates' showed a relatively high survival rate (70–80%) at 0.2% phenol and 60% survival at 0.4% phenol (Fig. 6).

NaCl tolerance

In our study, *B. subtilis* showed 70–90% viability at 2% (w/v) and 4% (w/v) salt concentrations and the percentage of viability reduced to 20% at 8% (w/v) of salt concentration (Fig. 7). As the concentration of NaCl increased, the viability of the cells began to decline. The growth was completely inhibited/stopped at 12% NaCl. Our study was highly similar to the study reported by Panda *et al*. (2014) [36] in which Lactobacillus showed 59–99% viability at 2% and 4% salt concentration. The viability decreased to 5% and 8% at 12% NaCl concentration. At high salt concentrations uncoupling between growth and energy production occurs where some strains cannot remain viable [37]. A decrease in microbial viability at high salt concentration is often osmotic stress and a significant challenge for microorganisms surviving in the fermentation process [37]. In another study by Wang *et al*, (2016) [38] reported that Lb. Plantarum ATCC 14917 was able to survive and show the highest viability up to 6% NaCl concentration.

Antibiotic resistance study

For selecting the potential and highly effective probiotic strain, antibiotic resistance plays an important role [39]. The strains of

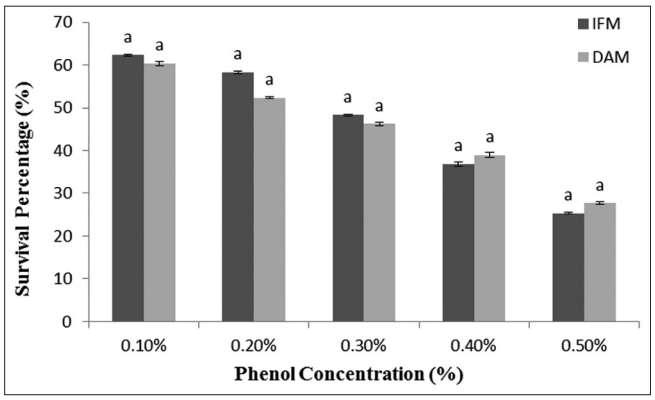


Fig. 6: Survival % of strain against different phenol concentration

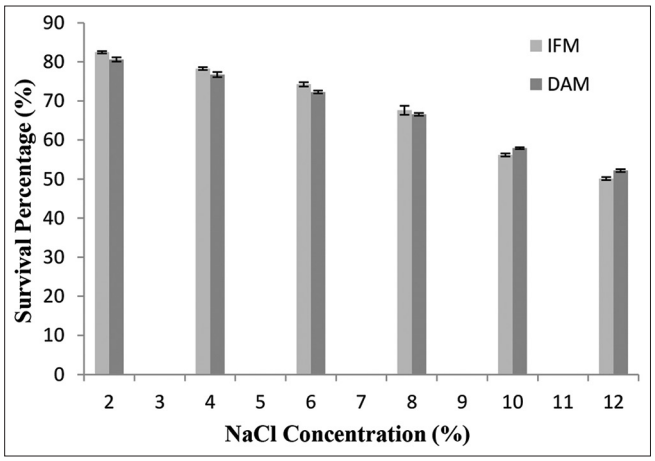


Fig. 7: Survival % of strain against different NaCl concentration

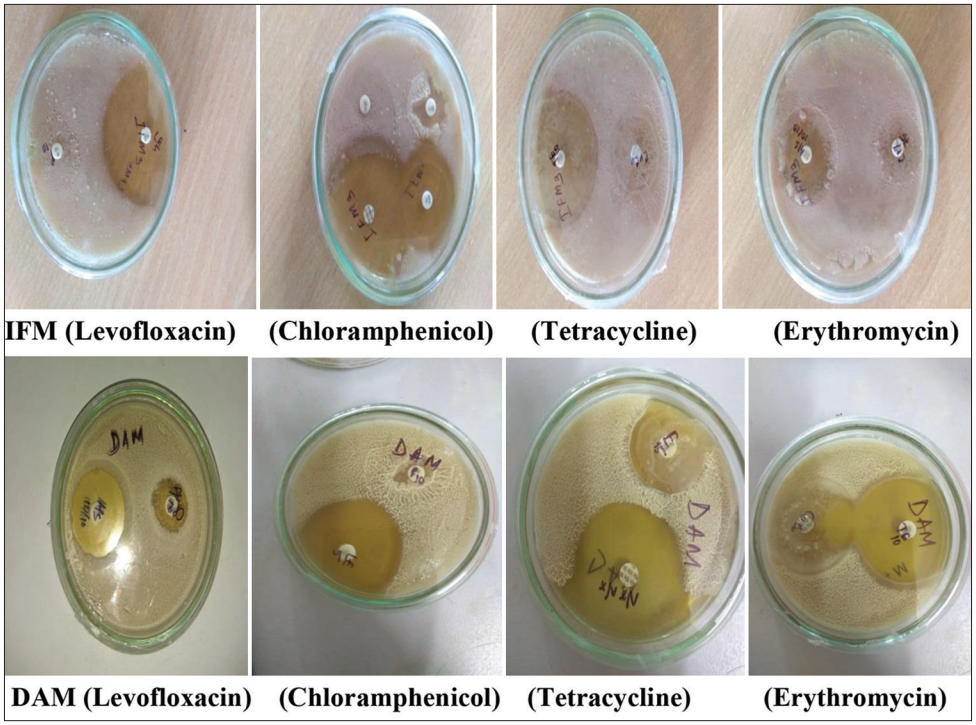


Fig. 8: Antibiotic activity of *Bacillus subtilis* (IFM and DAM)

B. subtilis (DAM and IFM) were highly susceptible to Levofloxacin, chloramphenicol, and tetracycline and moderately susceptible to all other clinically antibiotic tested (Fig. 8). The antibiotic-resistant properties also indicated that the isolate (*B. subtilis*) was able to survive in undesirable conditions caused due to the high concentrations of antibiotics. Panda *et al.* (2017) [40] reported a similar result which was concomitant to our study. They reported that isolate Jc13 was highly susceptible to cefoperazone, tetracycline, and chloramphenicol. Alanber and Khaled (2020) [41] also reported a similar study in which Bacillus Strains were resistant to cefoperagone, ceftazidime, cefepimeetc, and highly sensitive to gentamicin and amikacin. Most of the *Bacillus* sp. are spore produces and possess the ability to transfer antibiotic resistance genes [42], which carry the property of bacillus for antibiotic resistance.

Antimicrobial activity

Antimicrobial activity is the most significant property of a viable and functioning probiotic. The antibacterial efficacy of *B. subtilis* strain against several pathogen microorganisms was tested in this study. The isolates showed moderate antimicrobial activity against *Klebsiella* sp. and *E. coli*, *S. aureus*, and *Listeria monocytogens*. Antimicrobial activity may be a result of organic acids (Fig. 9). The result of antimicrobial activity of two isolates indicates the capacity to inhibit food-borne outbreaks caused by pathogens [18]. The antimicrobial activity possessed by several bacterial strains may be the result of bacteriocins and organic acid production, that is, lactic acid, acetic acid, and formic acid. The effectiveness of antibiotic treatment is however constantly decreasing due to the increasing spread of resistant pathogens [43]. Efremenkova *et al.* reported a similar study in 2019, where *B. subtilis* 534 showed a wide range of antimicrobial activities against clinical tested pathogens and fungi [44].

Autoaggregation assay

Autoaggregation (an important property for probiotics) reflects the adhesion ability to enterocytic cellular lines which provides resistance to peristaltic elimination [42]. Based on sedimentation characteristics of isolates, the autoaggregation was investigated. IFM and DAM showed more than 30% of autoaggregation ability. Similar work was reported by Patel *et al.* [17] on *Bacillus* sp. and showed 32.6% autoaggregation activity. The colonization of bacteria to intestinal cells is dependent on the cell surface of hydrophobicity (Table 1). By the probiotics microbes, the colonization of the tissue can prevent pathogens access by steric interactions.

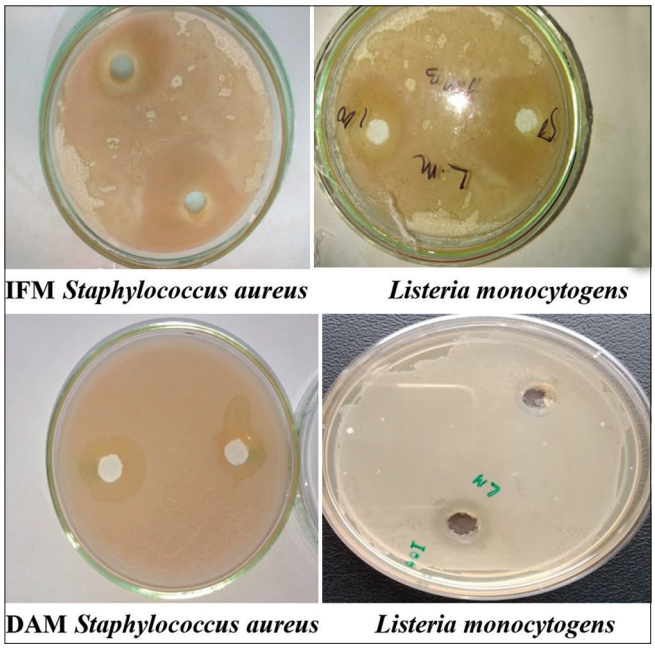


Fig. 9: Antimicrobial activity of *Bacillus subtilis* (IFM and DAM)

Table 1: Percentage of autoaggregation by DAM and IFM

Isolates	Initial OD at 600 nm	Final OD at 600 nm	Percentage of auto aggregation
DAM (*B. subtilis*)	0.378	0.274	34.68%
IFM (*B. subtilis*)	0.376	0.268	33.67%

B. subtilis: *Bacillus subtilis*

Hydrophobicity of strains

The IFM isolate showed high hydrophobicity (45.8%) with acetone whereas the isolate DAM showed 43.7% hydrophobicity with xylene. High hydrophobicity may be due to the presence of hydrophobic molecules (lipids, cytoplasmic, array, and wall intercalated proteins) on the bacterial surface. Kos *et al.* [45] reported the 70.96% and 36.06% hydrophobicity of *Lactobacillus* with xylene and chloroform, respectively. In another study, *B. subtilis* adhered to solvents such as chloroform and hexane with 19 and 11.46% adhesion, respectively, which was concomitant to the result of Hamadi and Latrache [46] (Table 2).

Hemolytic activity

The two isolates of *B. subtilis* were found non-hemolytic (γ- hemolysis) on 5% sheep blood agar. Hemolytic properties contain α-hemolysis, β-hemolysis, and γ-hemolysis. β- Hemolysis is considered harmful and α- and γ-hemolysis are considered as safe. α- Hemolysis causes the incomplete lysine of erythrocytes, resulting in a green – had zone around the bacterial colonies. γ- Hemolysis is without hemolysis. In probiotic strain, the absence of hemolytic nature is considered to be a positive trait for bacteria.

Secondary screening by specific assay methods (BLIS)

The isolates showed positive antibacterial activity (disc and spot methods) which were subjected to secondary screening. Plates were observed for growth on either side of the lawn and no growth at the center represents the antibacterial activity of the isolate against the test pathogens [22]. It was demonstrated that the two putative probiotic isolates can produce bacteriocins (Fig. 10).

Modeling of RNA secondary strain

Different strains of *B. subtilis* were studied for secondary structure modeling. The strategy based on free energy proved useful for the identification and thermodynamic characterization of *B. subtilis* strains.

Table 2: Percentage of hydrophobicity of two isolates against different solvent

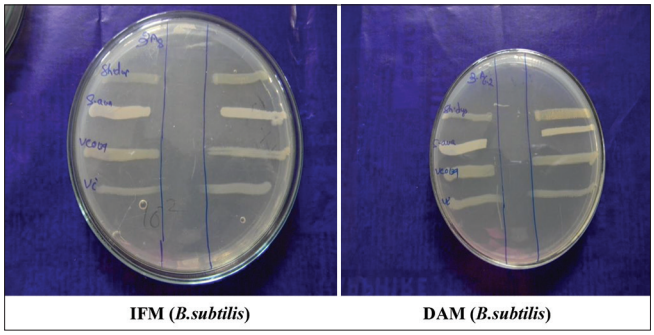
Isolates	Solvents	Initial OD at 600 nm	Final OD at 600 nm	% of Hydrophobicity
DAM (*B. subtilis*)	Heptane	0.292	0.192	36.55%
	Xylene	0.292	0.183	33.47%
	Acetone	0.292	0.219	45.8%
IFM (*B. subtilis*)	Heptane	0.292	0.196	37.92%
	Xylene	0.292	0.213	43.74%
	Acetone	0.292	0.203	40.32%

B. subtilis: *Bacillus subtilis*

Table 3: *B. subtilis* strains with optimal energy of 16S rRNA secondary structure

Accession number	Optimal energy Kcl/mol
MT279753 IFM (*B. subtilis*)	−499.43 Kcl/mol
MK453362 DAM (*B. subtilis*)	−488.17 Kcl/mol

B. subtilis: *Bacillus subtilis*



IFM (*B.subtilis*) **DAM (*B.subtilis*)**

Fig. 10: Bacteriocin like inhibition studies experiment (Secondary screening)

The IFM *B. subtilis* showed −499.43 Kcal/moL of optimal energy while DAM *B. subtilis* showed −488.17 Kcal/moL of optimal energy for 116S rRNA secondary structure (Table 3). The nucleic acid secondary structure dot plot is a triangle plot that represents base pairs as dots. In this dot plot method, the base pairs display in more than one folding at 37°C [47].

Large well-determined structural domains are identified by visual inspection of an "energy dot plot." These plots show suboptimal output from the RNA folding algorithm. These plots often contain a mixture of clear regions and cluttered regions. Clear regions define well-determined structural domains in the optimal folding. Nucleotides within these regions do not interact with other regions of the molecule in suboptimal folding. Cluttered regions indicate portions of the molecule that have the potential to form numerous alternative structures. Predicted structures in the optimal folding that are located in cluttered regions of the plots are considered to be poorly determined.

It can be used to compare a few folders. Using the efficient algorithm, it is too complicated to multi-branch loops. Therefore, slightly simple rules are used for folding. This simple rule gives the initial free energies and the final energies expressed with the best rules [48,49]. This study helped to predict the secondary structure of 16sr RNA and to identify conformational changes (Fig. 11a and 12a). The M fold web server gives suboptimal folding as well as energy dot plots (Fig. 11b and 12b). Energy dot plots are shown the base sets in the anticipated ideal folding (lower left triangle), just as all conceivable base matches on the whole potential foldings inside 12 kcal of the ideal anticipated folding (upper right triangle), and Minimum Free Energy appeared in the figure.

Dynamic writing of certain computer programs is by and large utilized for the optional construction forecast, which likewise helps in the figuring of thermodynamic free energy. To check the proper function of RNA molecule secondary structure, as the optional design is important. We use the M fold web worker since it utilizes the neighbor energy rules to ascertain the secondary construction of the RNA. M fold ascertains energy networks that decide all ideal and imperfect optional secondary

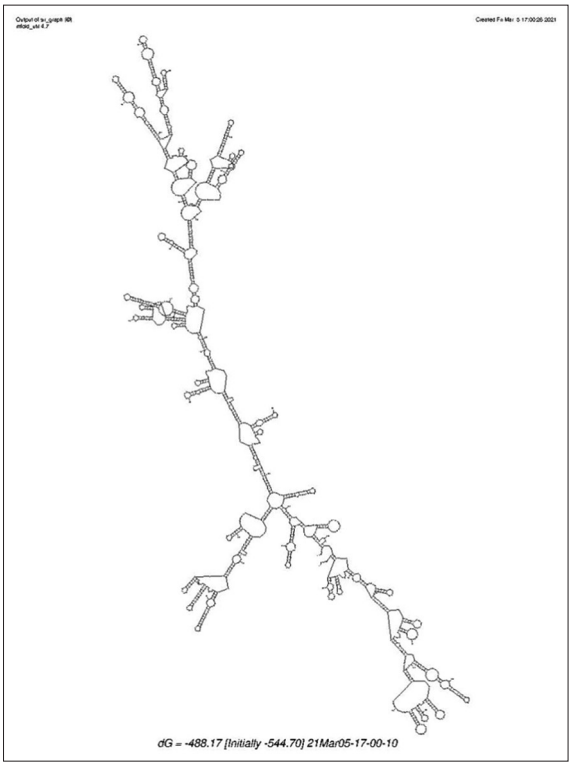


Fig. 11: (a) 16s rRNA of secondary structure, *Bacillus subtilis* (DAM)

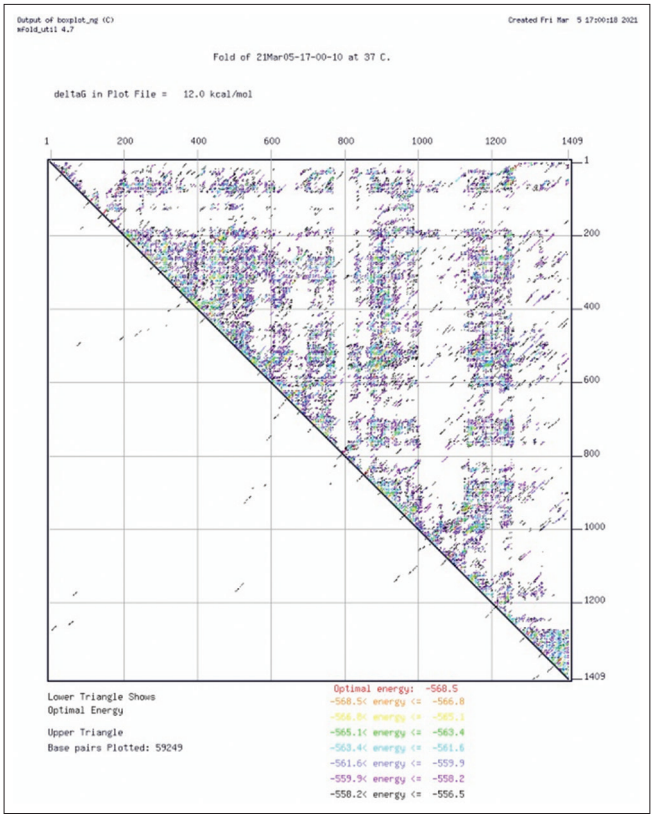


Fig. 11: (b) Energy dot plot for the annotated structure plot of 12-kcal/mol (DAM)

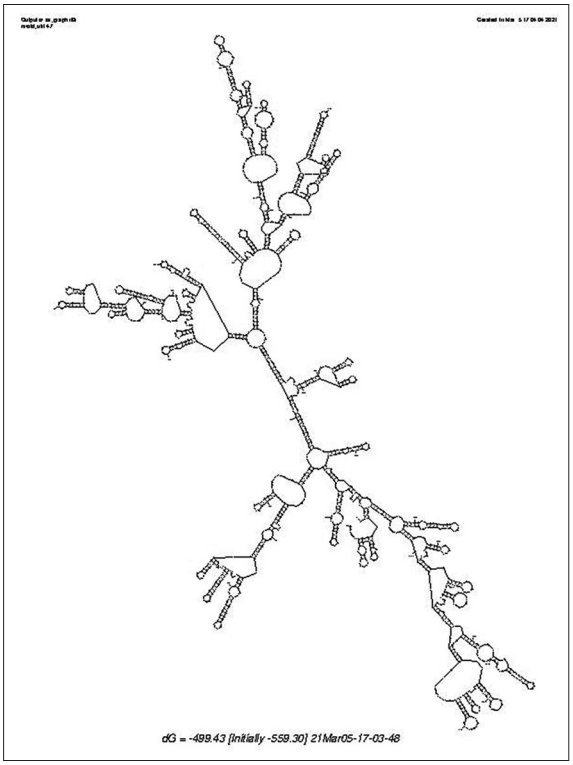


Fig. 12: (a) 16s rRNA of Secondary structure, *Bacillus subtilis* (IFM)

structures for an RNA molecule. Plot Fold shows the ideal and imperfect optional constructions for an RNA particle anticipated by M fold. Stem

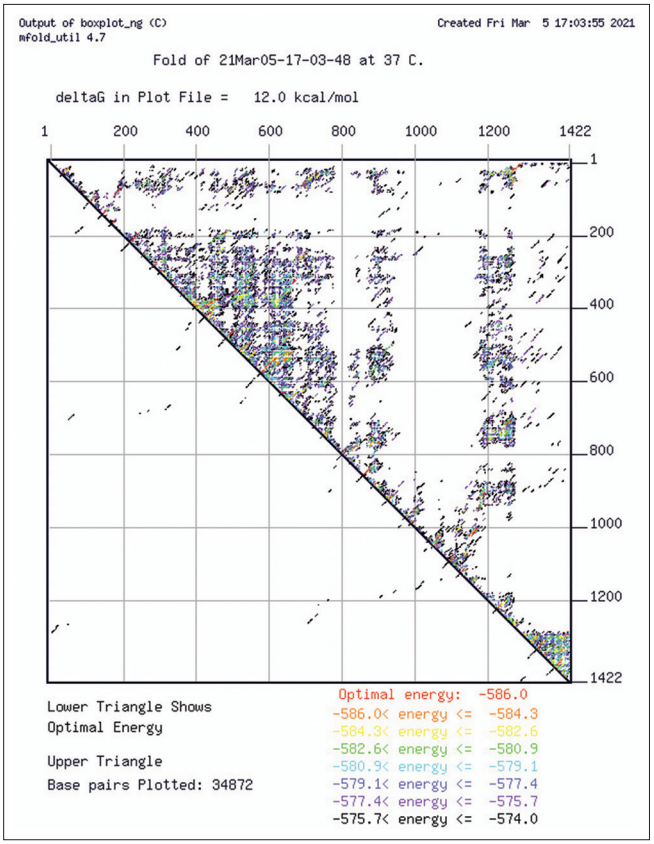


Fig. 12: (b) Energy dot plot for the annotated structure plot of 12-kcal/mol (IFM)

Table 4: Correlation coefficient analysis between bile salt and survivability of two isolates

Bile salt conc. (%)	DAM	IFM
1.000	−0.823**	−0.780**
	1.000	−0.988**
		1.000

**Correlation is significant at the 0.01 level (2- tailed)

Loop discovers every single imaginable stem and stems can be plotted with DotPlot. *B. subtilis* one of the significant microorganisms will create spores because of survivalists under outrageous temperatures. The nucleic acid secondary structure dot plot is triangular a three-sided plot that addresses base sets as dots. The benefits of this speck smudge are that it shows the base sets in more than one folding at 37°C. It can be used to compare few folders. Utilizing the productive calculation, it is too muddled to even consider creating multi-branch circles.

The outcomes introduced here show that the PC program M fold predicts RNA secondary structures. Diagrammatic portrayal of energy dot plot has appeared in the figure individually for each rRNA. The advancement of computational devices gives the interconnection of grouping and underlying data to comment on and find a secondary design of rRNA.

Statistical analysis

Pearson's correlation analysis showed that the entire above variable had a significant negative correlation with two isolates (DAM and IFM). When the concentration of bile salt, NaCl and phenol increased the survivability rate of the isolates decreased. For example, DAM- bile salt tolerance (−0.823, $p<0.01$), DAM- Phenol tolerance (−0.928 $p<0.01$), IFM- bile salt tolerance (−0.988, $p<0.01$), IFM- NaCl tolerance (−0.924, $p<0.01$) (Tables 4-6).

Table 5: Correlation coefficient analysis between phenol and survivability of two isolates

Phenol conc. (%)	DAM	IFM
1.000	−0.928**	−0.856**
	1.000	−0.819**
		1.000

**Correlation is significant at the 0.01 level (two-tailed)

Table 6: Correlation coefficient analysis between NaCl and survivability of two isolates

Nacl conc. (%)	DAM	IFM
1.000	−0.960**	−0.893**
	1.000	−0.924**
		1.000

**Correlation is significant at the 0.01 level (two- tailed)

CONCLUSION

The two strains of *B. subtilis* have been proved to be potential probiotic strains due to their survival under higher tolerance to bile salt, NaCl, phenol, and highly acidic conditions. The colonization efficiency was proved by exhibiting high autoaggregation and hydrophobicity. The absence of hemolytic nature made the isolates to be considered potential probiotic strains. Further, the present study helped to predict the secondary structure of 16s rRNA and to identify conformational changes with the M fold web server giving suboptimal folding as well as the "energy dot plot." Depending on energy level which gives different secondary structures and the most stable structure has been selected for the study. All base pairs participated in the folding of the RNA is represents the dot blot and the folding was carried out with the minimum folding energy. And the predicted models are the suited best models.

The study of probiotics as a new concept receiving rapid scientific interest is a recent phenomenon. In the recent scenario, *B. subtilis* being the potential probiotics is widely used as food bio preservatives. *B. subtilis* is also produces lipopeptides which show antimicrobial activity [50]. The emergence of probiotics as new science and its application in farming and aquaculture has evolved as an alternative to antibiotics and prophylactics in humans.

CONTRIBUTIONS

DD isolated the strains, performed morphological, biochemical, probiotic, bioinformatics analysis (phylogenetic and secondary structure prediction), and wrote the manuscript. SHP designed the study, performed statistical analysis of the study. SHP, CCR, and NM supervised the study and revised the manuscript. All authors read and approved the final version of the manuscript.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest regarding the publication of this paper.

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