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Research Article

In-Depth Functional Characterization of *Bacillus subtilis* PLSSC Revealing its Robust Probiotic Attributes

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Abstract

Functional assessment of a probiotic strain is imperative to substantiate its potential to offer health benefits to the host upon adequate consumption. Present study was designed to study probiotic attributes, safety aspects of *Bacillus subtilis* PLSSC *in vitro* with detailed investigation on antimicrobial activity, aligned to the regulatory guidance. We found that *Bacillus subtilis* PLSSC spore powder preparation is stable at real time ICH stability conditions for 30 months (99.71% viability). It remained viable in acid and bile stress and during *in vitro* simulated gastrointestinal digestion (99.48-100% viability). It tolerated thermal exposure well up to 80°C (86.18 % viability) up to 6 h and survived (100.0% viability) pasteurization. It showed excellent aqueous stability under ICH recommended storage conditions for one year with 96.21-99.9 % survival. Cell surface adhesion properties indicated affinity towards non-polar solvents and ability to aggregate with pathogens. *Bacillus subtilis* PLSSC showed broad spectrum antimicrobial activity including anti-listerial with 125 mg L^{-1} MIC. Its antimicrobial compound identified as subtilosin A (3108 Da by mass spectrometry) showed good stability in broad pH range (1.0-11.0) and temperatures (40-100°C). Peptide was sensitive to pepsin treatment but remained stable when exposed to trypsin. Thus, it showed excellent *in vitro* probiotic potential with well characterized antimicrobial activity as well as suitability for industrial processing implying wide applications.

INTRODUCTION

Probiotics have gained considerable attention in recent years for their potential health benefits and therapeutic applications. These live microorganisms, when administered in adequate amounts, confer a beneficial effect on the host by improving the microbial balance in the gut and promoting various physiological functions [1]. Among the diverse group of probiotic bacteria, *Bacillus subtilis* has emerged as a promising candidate due to its remarkable probiotic attributes and well-established safety profile [2-6].

Bacillus subtilis (*B. subtilis*), a Gram-positive, endospore-forming bacterium, has a long history of safe use in food and feed applications [7]. It possesses several characteristics that make it an attractive probiotic candidate. First, *B. subtilis* exhibits robust survivability under harsh conditions, including the acidic environment of the stomach and the bile-rich environment of the small intestine, enabling it to reach the colon in viable form [8]. Second, it has a wide range of antimicrobial properties, including the production of antimicrobial peptides, enzymes, and secondary metabolites, which contribute to its ability to inhibit the growth of pathogens and modulate the gut microbiota [9]. Third, *B. subtilis* has demonstrated immunomodulatory effects, promoting a balanced immune response and potentially reducing the risk of certain inflammatory conditions [10,11].

Despite the extensive use of *B. subtilis* in various industries, comprehensive characterization and evaluation of specific strains for their probiotic potential are essential for their successful application in human health. The present study on characterization of *B. subtilis* PLSSC strain as a potential probiotic candidate will contribute to the growing body of knowledge on probiotics and provide insights into the selection and development of effective probiotic formulations. *B. subtilis* PLSSC is a safe probiotic strain with GRAS status from USFDA [7]. The strain has been proven safe for human use through a validated phase 1 clinical trial [12].

This study aims to thoroughly characterize a *B. subtilis* PLSSC strain and evaluate its probiotic properties, including its survival and stability in simulated gastrointestinal conditions, suitability for industrial processes, production of antimicrobial compound, and its characterization.

MATERIALS AND METHODS

Bacterial strains, media and chemicals

Bacillus subtilis PLSSC (*B. subtilis* PLSSC) was made available

from the in-house culture collection at Advanced Enzyme Technologies ltd., Maharashtra, India. The culture was identified using 16S rRNA sequencing (Data not shown). Bacterial and fungal strains used as pathogenic strains for assessing antimicrobial activity are given in Table 1 with their growth and assay media. Cultures were maintained in 20% glycerol and stored at -80^{0}C. *B. subtilis* PLSSC was revived in nutrient broth at 37 °C, 160 rpm for experimental purpose wherever applicable. All the chemicals and reagents were purchased from Sigma-Aldrich, India and microbiological media from Hi-Media Labs India Pvt. Ltd.

Spore preparation and viable spore count

Spray dried preparation of spores of *B. subtilis* PLSSC used in the present study was carried out at Advanced Enzyme Technologies Ltd. (India) following a proprietary good manufacturing process. Viable spore count was determined using pour plate method. Approximately, 1.0 g (or 1.0 mL) sample was withdrawn at predetermined time interval from each of the test conditions and serially diluted in tween peptone water [composition, % (w v^{-1}): proteose peptone 1.00, sodium chloride 0.50, disodium phosphate 0.35, monosodium phosphate 0.15, and tween-80 0.20, pH 7.2±0.2]. Spores were then activated via heat shock treatment for 15 min at 70ºC and immediately cooled to about 45-50°C. Activated spore suspension (1.0 mL) was plated and poured with pre-sterilized molten nutrient agar (M001, HiMedia, Mumbai, India). Solidified media plates were incubated at 37°C for 24 h. Viable spore activity was expressed in colony forming units per mL or g (Log_{10}CFU mL^{-1} or g^{-1}) by taking the mean of three independent analyses.

Powder stability

Spray dried powder preparation of *B. subtilis* PLSSC spores was assessed for stability over 30 months under real time stability conditions (25±2°C, 60%±5% RH) in stability chamber [13]. Viable spore count was determined as described earlier.

Acid and bile stability

Ability of *B. subtilis* PLSSC spores to survive in acid and bile was studied as described by Maity C et al. [14]. Briefly, *B. subtilis* PLSSC spore suspension (5×10^8 CFU) was prepared in sterile miliQ water with pH adjusted to 1.5, 2.5, 3.0, 5.0 & 7.0. In another set, spores (2×10^9 CFU) were exposed to different concentrations of bile salts (SRL) as 0.01, 0.1, 0.2, 0.3, 0.5, 0.7, and 1.0%, w v^{-1}. Both the sets were incubated at 37°C for 6 h. Viable spore count was determined every hour as described earlier.

In vitro stability by static gut model

Stability of *B. subtilis* PLSSC spores was assessed under simulated *in vitro* conditions of human gastrointestinal (GI) tract as per standard harmonized procedure based on an international consensus developed by the COST INFOGEST network [15]. Fasting (free spores) and fed conditions along with different diets- pasteurized milk (PM), powdered baby food (PBF), standard American diet (SAD) and standard European diet (SED) were created for the assessment. Briefly, the suspension (1×10^9 CFU mL^{-1}) was sequentially exposed (50 rpm, 37°C) to simulated salivary fluid (oral phase = 2min, pH = 7.0) followed by simulated gastric fluid (gastric phase = 2h, pH = 3.0) and simulated intestinal fluid (intestinal phase = 2h, pH = 7.0). Samples (1 mL) were withdrawn at specific interval (up to 240 min) and analyzed as described earlier. Each experiment was carried out with freshly prepared digestive juices under aseptic conditions.

Thermal and aqueous stability

B. subtilis PLSSC spores (1×10^9 CFU) were tested for stability at various temperatures (0, 40, 60, 80, 90°C) for 6 h. Samples were taken every hour, immediately cooled in ice-cold water and analysed. Additionally, *B. subtilis* PLSSC spores (2×10^9 CFU) were suspended per 100 mL serving of phosphate buffer (pH 7.2 ± 0.05), milk (pH 6.7 ± 0.05), and juice (freshly prepared orange juice, pH 4.5 ± 0.05). The prepared suspensions were exposed to three different pasteurization temperatures such as 63°C, 72°C and 90°C in oil bath. Samples were taken at specific time intervals [16] and immediately cooled in ice-cold water. Viable spore count was determined as described earlier. An aqueous spore suspension of *B. subtilis* PLSSC (1.5×10^8 CFU) was prepared in sterile DW (pH 7.0) and stored in accordance with ICH guidelines [13] for refrigerated (5 ±3°C), intermediate (25 ±2°C, 60% ±5% RH) and accelerated (at 40 ±2°C, NMT 75% RH) stability. Viable spore count was determined at specific intervals and as described earlier.

Bacterial adhesion to hydrophobic solvents (BATH)

Cell surface hydrophobicity was assessed as described by Lim H et al. [17], with slight modifications. Briefly, *B. subtilis* PLSSC was grown in nutrient broth overnight at 37°C, centrifuged at 3500 rpm for 15 min. Cell pellet was washed twice and resuspended with phosphate buffered saline (PBS; pH 7.4) and OD_{600} was recorded (A_0). Culture suspension and hydrophobic solvents (xylene, toluene, and ethyl acetate) were mixed in 1:1 proportion and vortexed for 5 min (Labquest Borosil, MTV012). The mixtures were then allowed to rest for 30 min at ambient temperature. From the two separated phases aqueous layer was removed and OD_{600} (A_1) was recorded. The cell surface hydrophobicity percentage was calculated according to the formula:

$$\text{Hydrophobicity } (\%) = \frac{A_0 - A_1}{A_0} \times 100$$

A_0 = Optical density before mixing with solvents; A_1 = Optical density after mixing with solvents

Aggregation and co-aggregation ability

Autoaggregation and co-aggregation was assessed as described by Lim et al. [17] with slight modifications. Briefly, *B. subtilis* PLSSC was grown in nutrient broth overnight at 37 °C, centrifuged at 3500 rpm for 15 min. Cell pellet was washed twice and resuspended with phosphate buffered saline (PBS; pH 7.4) and OD_{600} was recorded (A_0). The suspension was allowed to

stand at ambient temperature and OD_{600} was recorded at 6 h (A_t). The autoaggregation percentage was calculated according to the formula:

$$\text{Autoaggregation (\%)} = \frac{A_0 - A_t}{A_0} \times 100$$

At = Optical density at 6 h at 600 nm; A0 = Optical density of 0 h at 600 nm.

To assess co-aggregation ability with pathogens, initially OD_{600} of suspension of *B. subtilis* PLSSC and pathogens under study was recorded individually. Then, equal proportion of the two was mixed. The mixtures were incubated at ambient temperature under static conditions. OD_{600} of the mixtures was recorded at 0 and 6 h. The co-aggregation was calculated according to the formula:

$$\text{Co} - \text{aggregation (\%)} = \left(\frac{(A_{Path} + A_{Pro})}{2} - A_{Mix}\right) \div \left(\frac{A_{Path} + A_{Pro}}{2}\right) \times 100$$

A_{path}, A_{pro} = Optical density of the pathogen and the probiotic strain suspension at t, respectively;

A_{mix} = Optical density of the mixed suspension at t.

Antimicrobial activity and characterization

Screening and production of antimicrobial compound (AMC): The production of AMC by *B. subtilis* PLSSC was studied as described by Ahire JJ, et al. [18]. Briefly, a single axenic colony of *B. subtilis* PLSSC was inoculated in 10 mL of clarified Mueller Hinton (MH) Broth. After incubation at 37°C, 120 rpm for 24 h, inoculum (1%) was transferred to 100 mL clarified MH Broth and incubated under same conditions. The culture was then mixed with 4 % activated sterile XAD16N beads and evenly poured onto sterile MH agar plates. The plates were incubated at 37°C for 5 days. The beads were then collected, washed several times with MilliQ water, followed by 30 % ethanol and again with MilliQ water. The AMC was eluted with 80 % isopropanol (IPA) containing 0.1 % trifluoroacetic acid (TFA). The eluted sample was filtered (0.2 µ cellulose acetate, Axiva) and concentrated using Rotavapor (Rotavapor® R-300, Buchi, Switzerland). The antimicrobial activity of the concentrate was determined against *M. luteus* by spot-on-the-lawn assay [19]. Briefly, 50 µL of *M. luteus* culture grown overnight from single colony was mixed with 25 mL molten MH agar and poured in petri dish. After drying, 25 µL of sample was spotted onto the agar surface and the plates were incubated overnight at 37^{0}C. Antimicrobial activity was measured as zone of inhibition in mm.

Purification of AMC: The concentrated sample showing antimicrobial activity was purified using reverse phase C_{18} cartridge (Sep-Pak, 35 cc Vac Cartridge, 10 g Sorbent, Waters, United States). The C_{18} column was preconditioned as per manufacturer's protocol. The sample was loaded, washed with water and eluted with 10 % to 90 % gradient of IPA containing 0.1 % TFA with 10 % increment at a flow rate of 1 mL min^{-1}. The antimicrobial activity of all the eluted fractions was determined against *M. luteus* by spot-on-the-lawn assay as described earlier. Eluted fractions showing antimicrobial activity were pooled together and concentrated using Rotavapor and lyophilized (Lyophilizer, Lyo Lab, United States). Lyophilized sample was stored in amber color bottle at -20^{0}C until further use [18].

Characterization of AMC: ***Molecular characterization*:** Molecular mass of purified AMC (1 mg mL^{-1} in acetonitrile) was determined using triple quadrupole mass spectrometry (Shimadzu LCMS 8040). Approximately 1 mg mL^{-1} of lyophilized AMC was prepared in filter sterilized DW. Absorption spectrum (190 – 800 nm) was recorded at data interval of 0.2 nm and scan speed 100 nm min^{-1} (UV spectrophotometer JASCO V-730).

Antimicrobial spectrum and MIC: Antimicrobial activity of lyophilized AMC (5 mg mL^{-1}) was determined against a set of 17 pathogens (Table 1) by spot-on-the-lawn assay as described earlier. Minimum Inhibitory Concentration (MIC) was determined against *L. monocytogenes* by agar dilution method according to Wiegand I et al. [20]. *L. monocytogenes* was grown overnight in Brain Heart Infusion Broth (BHIB) at 37^{0}C under shaking conditions (120 rpm). Different concentrations of lyophilized AMC (1 – 1000 mg L^{-1}) were prepared in 15 mL sterile molten MH-agar; five µL of overnight grown culture of *L. monocytogenes*

Table 1: Bacterial and fungal pathogenic strains used in the study

Pathogen	Cultivation conditions	Spot assay medium
Bacillus cereus ATCC 33019	Nutrient broth, 37 °C, aerobic	Mueller Hinton agar
Bacillus circulans ATCC 4516	Nutrient broth, 37 °C, aerobic	Mueller Hinton agar
Bacillus subtilis subsp. Spizizenii ATCC 6633	Brain heart infusion broth, 37 °C, aerobic	Brain heart infusion agar
Candida albicans ATCC 90028	Potato dextrose broth, 25 °C, aerobic	Mueller Hinton agar
Clostridium sporogenes NCIM-5125(Equivalent to ATCC 19404)	Reinforced clostridium medium, 37 °C, aerobic	Mueller Hinton agar
Enterobacter cloacae ATCC 13047	Nutrient broth, 37 °C, aerobic	Mueller Hinton agar
Escherichia coli ATCC 700728	Nutrient broth, 37 °C, aerobic	Mueller Hinton agar fortified with yeast extract (5 g L^{-1})
Escherichia coli ATCC 9002 NCTC	Nutrient broth, 37 °C, aerobic	Mueller Hinton agar
Klebsiella pneumoniae ATCC BAA-1144	Soyabean casein digest broth	Mueller Hinton agar
Listeria monocytogenes ATCC 19115	Brain heart infusion broth, 37 °C, aerobic	Brain Heart Infusion agar
Micrococcus luteus MTCC 106^T	Mueller Hinton Broth, 37 °C, aerobic (MH Broth)	Mueller Hinton agar
Pasteurella multocida ATCC 12945	Brain heart infusion broth, 37 °C, aerobic	Brain heart infusion agar
Pseudomonas aeruginosa ATCC 9027	Nutrient broth, 37 °C, aerobic	Mueller Hinton agar
Salmonella abony NCIM-2257(Equivalent to ATCC 6017 NCTC)	Nutrient broth, 37 °C, aerobic	Mueller Hinton agar
Salmonella enterica ATCC 14028	Nutrient broth, 37 °C, aerobic	Mueller Hinton agar
Staphylococcus aureus ATCC 6538P	Nutrient broth, 37 °C, aerobic	Mueller Hinton agar
Vibrio parahaemolyticus ATCC 17802	Nutrient broth, 37 °C, aerobic	Mueller Hinton agar fortified with yeast extract (5 g L^{-1})

was spotted on the agar surface. The plates were incubated at 37^{0}C for 48 h. The lowest concentration of AMC in the agar medium that inhibited visible growth was considered MIC.

Stability of AMC: Stability of AMC was determined under various pH, temperature and enzyme conditions [18]. For pH stability, pH of DW was adjusted to different pH viz. 1,3,5,7,9,11 and 14 using 0.1 N NaOH/HCl, and mixed with 5 mg mL^{-1} AMC. After incubation at 37^{0}C for 30 min, the pH was readjusted to 7.0 ± 0.2. To determine temperature stability, 1 mg mL^{-1} of AMC was prepared in 0.1% TFA water and kept at 40^{0}C, 60^{0}C, 80^{0}C and 100^{0}C. Samples were collected at 10, 30 and 60 min incubation. In addition, a set of samples was autoclaved at 121^{0}C for 15 min. Stability of AMC in presence of proteolytic Enzymes namely trypsin and pepsin was determined. One mg mL^{-1} AMC was mixed with 1 mg mL^{-1} pepsin and trypsin prepared in 0.1% TFA water and Tris HCl (pH 8.0) respectively. After 4 h of incubation at 37^{0}C, the enzyme was inactivated by heating at 95^{0}C for 5 min, and cooled. In all the 3 sets of experiments an untreated AMC (1 mg mL^{-1}) was used as control. Antimicrobial activity of filter sterilized (0.2µ) samples against *monocytogenes* was determined by spot-on-the-lawn assay as described earlier.

Statistical analysis

Viable activity of *B. subtilis* PLSSC spores was expressed as mean Log_{10} CFU mL^{-1} or g^{-1} of three repeated measurements. Statistical analysis was carried out using GraphPad Prism version 9.5.1 (GraphPad Software Inc., USA, https:// www.graph pad. com/ scien tific- softw are/ prism/). Significant differences between the means were calculated at $p<0.05$ using Two-way analysis of variance (ANOVA) followed by Tukey's HSD or Dunnett multiple comparison test.

The whole genome sequencing data for strain B. subtilis PLSSC is submitted to GenBank with accession no. CP031129.1.

RESULTS

Powder stability

During long term storage of spray dried powder of *B. subtilis* PLSSC, initial viable spore count (10.41±0.78 Log_{10}CFU g^{-1}) reduced by 0.03 Log_{10}CFU over 30 months (10.38±0.78 Log_{10}CFU g^{-1}) indicating excellent stability (Table 2). Stability data for three independent batches of *B. subtilis* PLSSC spore powder preparation is provided in supplementary table (Supplementary Table S1).

Acid and bile stability

B. subtilis PLSSC spores remained viable when exposed to acidic environment up to 5 h (Figure 1a). Viability up to 5 h was least affected at pH 7.0, 5.0 and 3.5 when compared with initial count. At pH 1.5 and 2.5, significant difference in viability was seen from 1 h (P =0.0003; P=0.0001). Five h exposure led to 1.15 and 0.47 Log_{10}CFU reduction at pH 1.5 and 2.5 respectively, whereas 0.15-0.30 Log_{10}CFU reduction was observed in case of pH 3.5, 5.0 and 7.0. Viability at different bile salt concentrations was maintained for *B. subtilis* PLSSC spores for up to 5 h (Figure 1b). Among the different concentrations studied maximum reduction in viability of 0.07 Log_{10}CFU was seen at 1.0 % bile after 5 h; albeit negligible as compared with the initial count.

Table 2: Real time long term stability of *B. subtilis* PLSSC spore powder preparation

Time (months)	Viability (Log_{10}CFU g^{-1})
0 day	10.41±0.78
3	10.41±0.79
6	10.40±0.79
9	10.40±0.79
12	10.40±0.79
18	10.40±0.79
24	10.39±0.79
30	10.38±0.78

Table S1: Real time long term stability of B. subtilis PLSSC spore powder preparation carried out for three independent batches (Log_{10} CFU g-1)

Batch I	Batch II	Batch III
9.94	9.98	11.32
9.94	9.97	11.31
9.93	9.97	11.31
9.92	9.97	11.31
9.92	9.97	11.31
9.92	9.96	11.31
9.91	9.96	11.30

In vitro stability by static gut model

Stability under *in vitro* static gut model showed ability of *B. subtilis* PLSSC spores to survive adverse conditions in human gastrointestinal tract. Free spores remained viable under all simulated phases of GI digestion (Figure 1c). During gastric phase of digestion viability of free spores showed reduction of 0.5 Log_{10}CFU which was statistically non-significant (P=0.5250). Presence of food matrices did not alter the viable activity; initial viability of approximately 9.00 Log_{10}CFU was maintained until the end of the intestinal phase in presence of powdered baby food (P>0.9999), milk (P=0.9973), standard American diet (P>0.9999) and standard European diet (P=0.9973).

Thermal and aqueous stability

B. subtilis PLSSC spores exhibited thermal stability from 0 to 80°C up to 6 h (P=0.0609). The viability at 90°C significantly reduced over 6 h duration. Statistically significant reduction in viability (2.60 Log_{10}CFU) was seen after 3 h exposure (P<0.0001). As depicted in Figure 2a viability was completely lost after 6 h (9.00 Log_{10}CFU reduction). At pasteurization temperatures *B. subtilis* PLSSC spores remained viable in all the 3 matrices. Viability of 9.38±0.02 Log_{10}CFU in PBS, 9.43±0.01 Log_{10}CFU in milk and 9.43±0.02 Log_{10}CFU in orange juice was observed after 30 min at 63°C (Figure 2b). Viable count remained 2×10^9 per serving for all the 3 matrices at 72°C and 90°C till 30 sec (99.8-100.0% viability) (Figure 2c). Viability of *B. subtilis* PLSSC spores was maintained as aqueous suspension throughout the stability duration of 1 year under ICH conditions (Figure 2d).

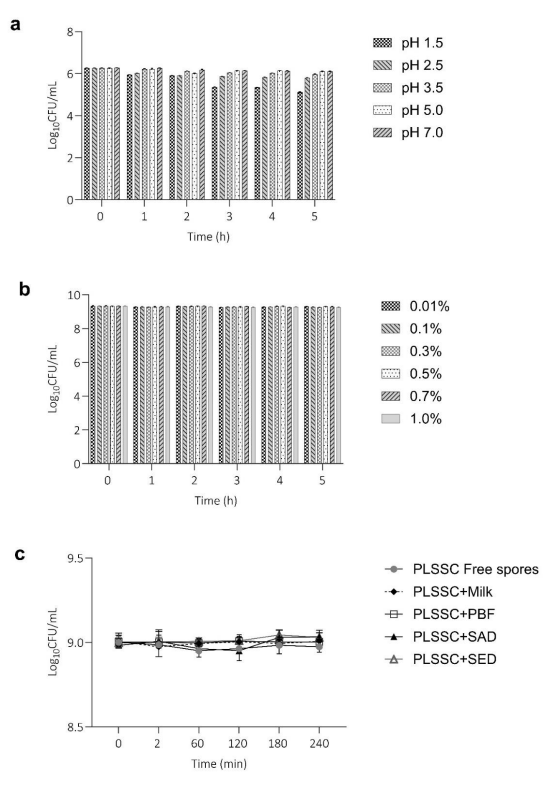

Figure 1 Stability of *B. subtilis* PLSSC spores at **(a)** different pH, **(b)** bile(%) and **(c)** in simulated gastrointestinal tract as free spores; in Milk, PBF-Powdered baby food, SAD-Standard American diet, SED-Standard European Diet.

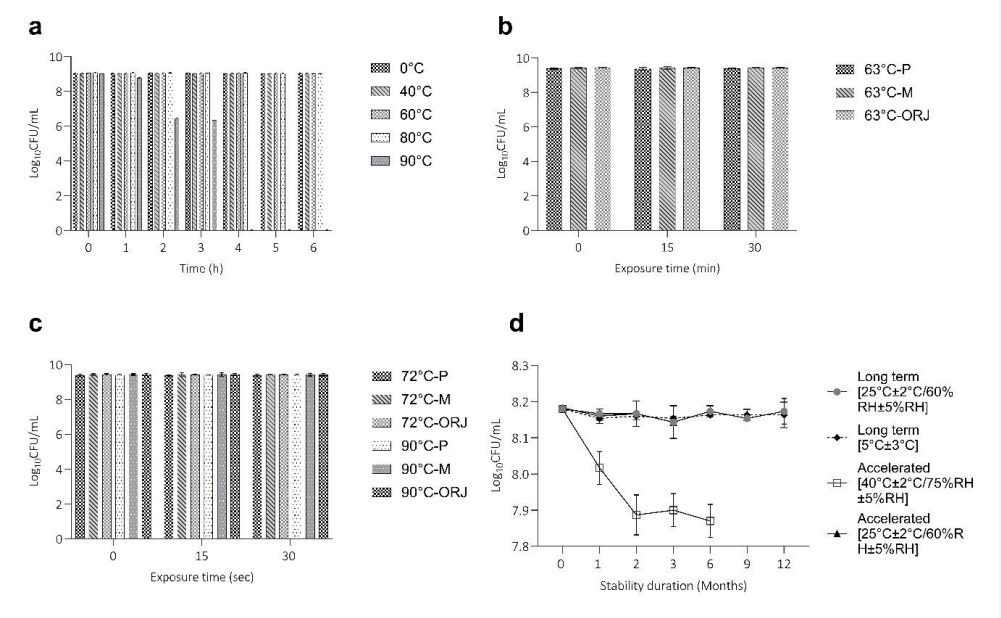

Figure 2 Stability of *B. subtilis* PLSSC spores after exposure to **(a)** different temperatures, **(b)** pasteurization at 63°C in PBS(63-P), Milk(63-M), and orange juice(63-ORJ), **(c)** pasteurization at 72°C in PBS(72-P), Milk(72-M), and orange juice(72-ORJ), at 90°C in PBS(90-P), Milk(90-M), and orange juice(90-ORJ), **(d)** aqueous stability of *B. subtilis* PLSSC under ICH recommended conditions.

Long term stability under shelf and refrigerated storage showed negligible Log_{10}CFU reduction in 1 year.. No significant change in viability (0.04 Log_{10}CFU reduction; P=0.4486) was seen during accelerated stability under refrigerated conditions [25°C ± 2°C/60% RH ± 5% RH]. Viability was affected under accelerated conditions on the shelf [40°C ± 2°C/75% RH ± 5% RH] with 0.31 Log_{10}CFU reduction (P=0.0234) at the end of 6 months.

Aggregation ability and BATH

We observed bacterial adhesion to non-polar solvents in the range of 21-25 % with maximum adhesion to xylene and least to ethyl acetate in 6 h (Figure 3a). We observed 23.81% autoaggregation for *B. subtilis* PLSSC in 6 h (Figure 3b). As depicted in Figure 3b,c co-aggregation of *B. subtilis* PLSSC was highest (23.11%) with *S. aureus* ATCC 6538P, 20.09% with *C. albicans* ATCC 90028 and lowest (4.48%) with *B. cereus* ATCC 33019.

Antimicrobial activity and characterization

Antimicrobial activity of probiotic *B. subtilis* PLSSC was examined in the present study. XAD16N beads acted as an adsorbent for the AMC produced by *B. subtilis* PLSSC. The AMC was further eluted with 80% IPA – 0.1% TFA and its rotavap concentrate showed 11 mm zone of inhibition against indicator organism *M. luteus*. The Sep-Pak C18 column eluted fractions with 50%- 90% IPA-TFA were found to be active fractions showing effective zones of inhibition against *M. luteus* (Figure 4). The active fractions were pooled, concentrated and freeze-dried to obtain 150 mg of AMC powder.

The UV-VIS spectrum analysis of the freeze-dried AMC powder revealed presence of absorption peaks in region 200 – 230 nm with intense peak at 221 (shoulder peak), 225 and 228 nm. Mass spectrometric analysis (LCMS) showed presence of precursor ion corresponding to 1037 m/z in positive ionization mode (Figure 5).

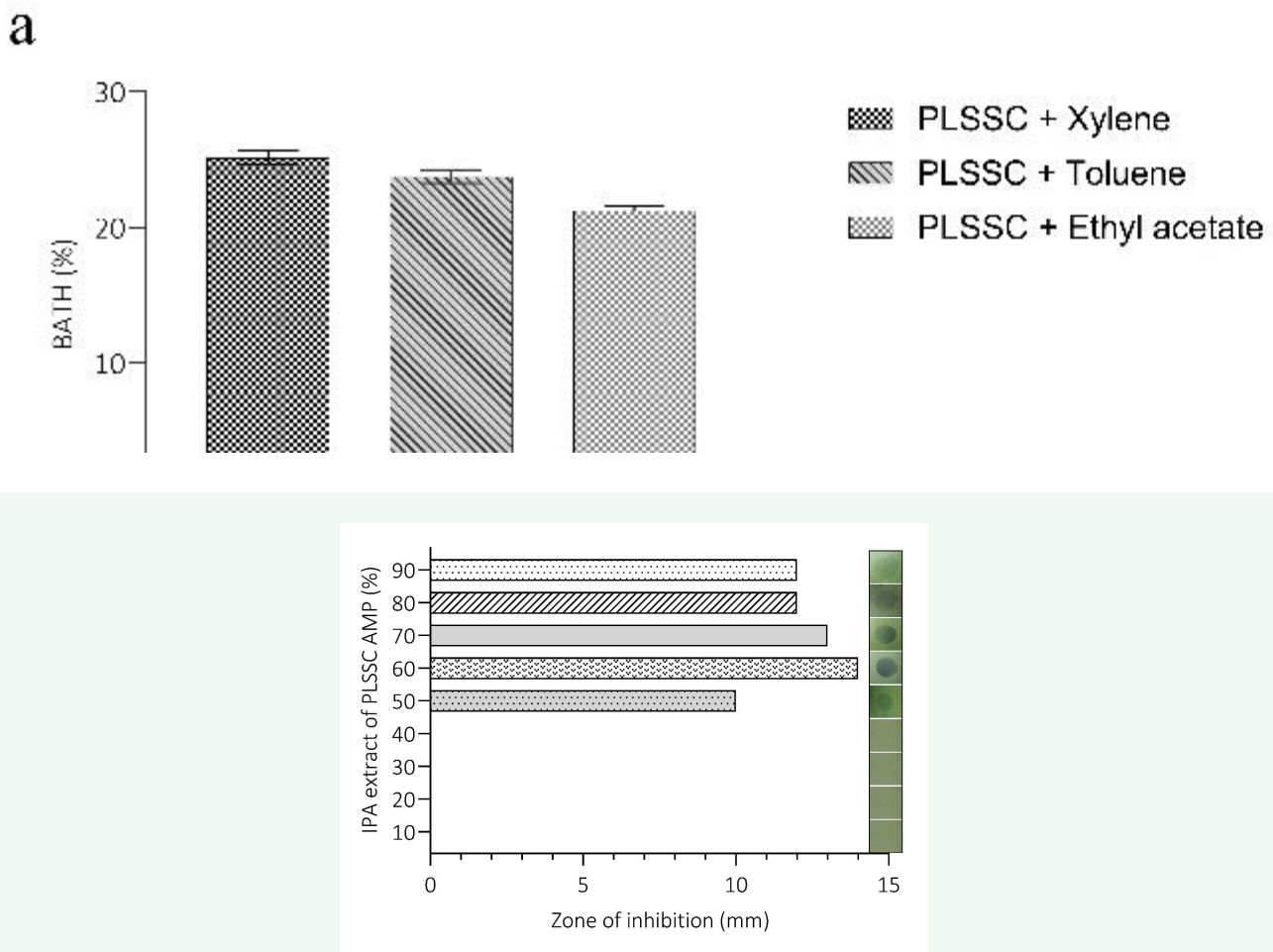

Figure 4 Antimicrobial activity of IPA extracts of antimicrobial compound produced by *B. subtilis* PLSSC

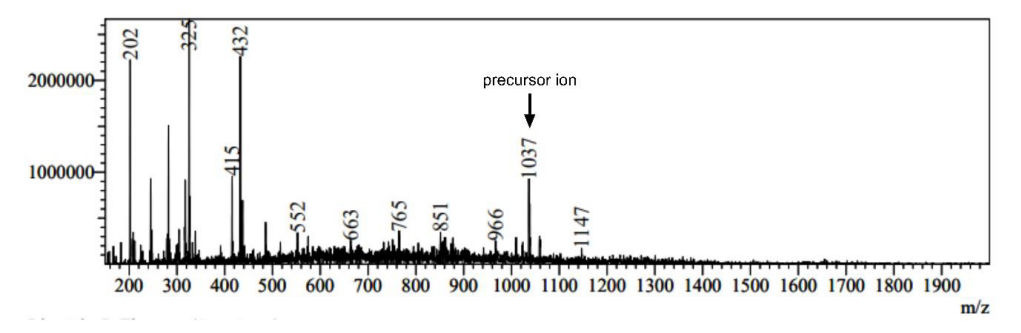

Figure 5 Mass spectrum (Triple quadruple mass spectrometry) for antimicrobial compound produced by *B. subtilis* PLSSC

Antimicrobial spectrum against 17 pathogens revealed broad spectrum antimicrobial activity of AMC produced by *B. subtilis* PLSSC (Table 3). Antimicrobial activity was not observed against *C. albicans*, *P. aeruginosa, E. coli* 700728, *B. cereus* while rest all pathogens were inhibited by AMC. Maximum inhibition at 5 mg mL^{-1} concentration was seen for *E. cloacae* (15 mm), *L. monocytogenes* (15 mm), *B. circulans* (15 mm), *S. aureus* (14 mm), *and B. subtilis subsp. Spizizenii 6633* (13 mm). The MIC of AMC produced by *B. subtilis* PLSSC determined against *L monocytogenes* was found to be 125 mg L^{-1}.

Effect of pH, proteolytic enzymes and temperature on AMC was studied and is presented in Table 4. PLSSC AMC was found to be tolerant to proteolytic treatment; when treated with trypsin 100 % activity was retained and with pepsin 78.57%. Antimicrobial activity remained unaltered in the pH range of 1 to 11. Complete inactivation of AMC occurred at pH 14.0. Thermal tolerance of AMC was also observed where antimicrobial activity was retained at temperatures 40, 60, 80 and 100^{0}C for 60 min. Antimicrobial activity disappeared when AMC was autoclaved for 15 min at 121^{0}C.

DISCUSSION

Probiotics are gaining a lot of attention owing to a plethora of health benefits they offer to the host upon consumption. Extensive screening is required to obtain a good probiotic candidate that can reach the target site in adequate numbers. In the present study, we substantiated probiotic characteristics of *B. subtilis* PLSSC *in vitro* and assessed its antimicrobial potential followed by characterizing the AMC.

Spray dried powder of *B. subtilis* PLSSC spores showed excellent stability and 100.00 % viability (Log_{10}CFU) was maintained for ICH stability conditions for 30 months. Stability of *B. subtilis* PLSSC spores is similar to the previously reported stability for other probiotic strains of *B. subtilis*- DE111 [4], SG 188 [5], MB40 [6]. Good stability of a probiotic strain eliminates the need to add overages into formulations for probiotics to reach in adequate numbers at the target site *in vivo*. Oral probiotics encounter exposure to gastric acid and bile during

Table 3: Inhibition zones (mm) exhibited by AMC produced by B. subtilis PLSSC

Sr. No.	Pathogen	PLSSC AMC (5mg mL^{-1})	Positive control (10 µg mL^{-1})
1	*Escherichia coli* ATCC 9002 NCTC	10	15^C
2	*Salmonella abony* NCIM-2257 (Equivalent to ATCC 6017 NCTC)	7	15^C
3	*Clostridium sporogenes* NCIM-5125 (Equivalent to ATCC 19404)	9	16^C
4	*Pseudomonas aeruginosa* ATCC 9027	0	4^N
5	*Salmonella enterica* ATCC 14028	9	17^C
6	*Enterobacter cloacae* ATCC 13047	15	7^N
7	*Klebsiella pneumoniae* ATCC BAA-1144	10	20^C
8	*Escherichia coli* ATCC 700728	0	18^C
9	*Vibrio parahaemolyticus* ATCC 17802	25	12^N
10	*Pasteurella multocida* ATCC 12945	24	33^C
11	*Listeria monocytogenes* ATCC 19115	15	15^C
112	*Bacillus cereus* ATCC 33019	0	12^N
13	*Staphylococcus aureus* ATCC 6538P	14	20^C
14	*Candida albicans* ATCC 90028	0	nd
15	*Micrococcus Luteus MTCC 106^T*	8	19^N
16	*Bacillus subtilis subsp. Spizizenii ATCC 6633*	13	13^C
17	*Bacillus circulans ATCC 4516*	15	11^C

C: Cefixime; N: Neomycin; nd: not done; Due to the partially purified nature of AMC slightly high concentration (5 mg.ml^{-1}) was used for determining antimicrobial spectrum.

Table 4: Stability of AMC produced by *B. subtilis* PLSSC under conditions of extremes of pH, temperature and proteolysis

Treatment	Zone of inhibition (mm)	Activity(%)
Control (without any treatment)	14	100
Enzymes		
Trypsin (1 mg mL^{-1})	14	100
Pepsin (1mg mL^{-1})	11	78.57
pH		
1	14	100
3	14	100
5	15	100
7	15	100
9	14	100
11	13	92.85
14	0	0
Temperature		
40^{0}C, 60 min	12	85.71
60^{0}C, 60 min	11	78.57
80^{0}C, 60 min	11	78.57
100^{0}C, 60 min	11	78.57
121^{0}C, 15 min	0	0

their passage from oral cavity to colon. Hence, it is desirable for a good probiotic strain to be able to withstand the stress. Acid and bile stress can adversely affect bacterial survival via proton accumulation and disruption of pH gradient, disruption of essential enzyme functions or macromolecules such as DNA [21]. In the present study, acid and bile exposure did not affect the viability of *B. subtilis* PLSSC from pH 3.5 to 7.0; we observed minor reduction similar to the literature reports at pH 1.5 and 2.5 [17-23]. Other *B. subtilis* strains have shown roughly 0.10 to 0.60 Log_{10}CFU reduction [22]. About 90 and 40% survival has been reported for *B. subtilis* MKHJ 1-1 when exposed to pH 2.5 and 0.3% bile salt [17]. *B. subtilis* FTC01 survived to 80 and 96% at pH 2 and 3 for 2 h [23].

B. subtilis PLSSC spores in presence of various food matrices (mimicking fed conditions) as well as free spores (fasting condition) remained viable throughout all the phases of simulated GI digestion. *B. subtilis* MA139 [24] and few other strains of *B. subtilis* have been studied for their tolerance and stability in simulated gastrointestinal conditions and were found to remain unaffected by gastric or intestinal conditions [25,26].

Temperature tolerance of *B. subtilis* PLSSC was found to be reasonably good as it maintained viability (99.33%) up to high temperatures of 80°C for 6 h. Guo X, et al. [22] have also reported similar tolerance for four *B. subtilis* strains but for a shorter exposure of 5 to 10 min. Viability at pasteurization temperatures (63,72,90°C) in 3 matrices (PBS, milk, orange juice) was 100.0%. Our results are in agreement with a recent report by Mazhar S et al. [27] on *Bacillus subtilis* DE111® spores' viability across three pasteurization processing temperatures (45, 75, and 90°C) in PBS, Oat milk and Apple juice. Our results indicate that B. subtilis PLSSC can be considered to be the ideal probiotic candidate for incorporation in foods and nutraceuticals undergoing high temperature processing.

Our results indicate ability of *B. subtilis* PLSSC spores to remain viable in aqueous environment under humid conditions for one year. It implies potential to incorporate the strain in aqueous suspensions. The primary reason for a good stability under adverse environmental or processing conditions is the ability of *B. subtilis* PLSSC to form endospores. The combination of protective layers, reduced metabolic activity, and molecular mechanisms for DNA protection and repair enables spores to withstand the harshest environments until conditions become favorable for germination and growth [28].

BATH, autoaggregation and co-aggregation ability of *B. subtilis* PLSSC strongly indicate its adherence and survival potential in the gut. BATH as reported by other researchers is comparable to our results; it varies from 5 to 57% with other strains of *B. subtilis* and non-polar solvents [17,23]. Autoaggregation ability enables a probiotic strain to establish itself in the intestinal lumen. *B. subtilis* PLSSC showed comparable autoaggregation and co-aggregation ability. The variation in aggregation abilities arises as this property is strain dependent; it ranges from 63% in 5 h to 88% in 4 h for various other strains of *B. subtilis* [17,29]. Co-aggregation of *B. subtilis* P223 with *S. aureus* ATCC 6538P (57.09%) and *B. subtilis* MKHJ 1-1 with *S. aureus* KCTC 3881 (24.75%) is reported in the literature [17,21].

Ability to antagonize pathogens is a well established mechanism through which probiotics offer protection to the host from various infections. *B. subtilis* is known to produce plethora of AMCs of various types including ribosomal peptides (RPs), volatile compounds, polyketides (PKs), non-ribosomal peptides (NRPs), and hybrids between PKs and NRPs [9]. We studied the ability of *B. subtilis* PLSSC to produce AMC on solid media. Adsorptive polymeric resin Amberlite® XAD16N was used for their property to retain hydrophobic compounds. It is a nonionic, hydrophobic, crosslinked polymer with macroporous structure, high surface area, and the aromatic nature. With its characteristic pore size distribution, it is known to adsorb hydrophobic molecules from polar solvents such as recovery and purification of antimicrobial substances from fermentation broth [30,31]. The compounds adsorbed onto XAD16N were eluted with 80% IPA-0.1% TFA which showed zone of inhibition confirming the antimicrobial nature of the extracted compounds. AMC was purified by column chromatography and lyophilized for further analysis. Khochamit N et al. [30], and Epparti P et al. [32], have reported triply charged precursor ions of 1134.86^{3+} m/z and 1140.19^{3+} m/z respectively, corresponding to subtilosin A by quadrupole-time-of-flight (QTOF) analysis. We obtained precursor ion with 1037 m/z in LC-MS using triple quadruple as a detector. This precursor ion could be triply charged computing to molecular mass of 3111 corresponding to subtilosin A. However, multiple charges cannot be confirmed using triple quadruple detector as it is limited by unit mass resolution. For accurate mass determination and compound identification High Resolution Mass Spectrometry (HRMS) technology (Q-TOF, MALDI-TOF) would be required. Additionally, our results are supported by whole genome sequencing data for *B. subtilis* PLSSC (GenBank: CP031129.1) where genes for subitlosin A family bacteriocin and subtilosin A maturase are annotated. Subtilosin A is a sactipeptide produced by *B. subtilis* known to possess antibacterial activity against both Gram-positive and Gram-negative pathogens. Subtilosin A attaches to a membrane receptor, and binds plasma membrane via electrostatic interaction. Transmembrane pH gradient gets disrupted as a result of electrostatic binding and effluxes intracellular ATP. Eventually cell dies due to starvation. Subtilosin A can block quorum sensing and inhibit biofilm formation [33].

Subtilosin A produced by *B. subtilis* PLSSC showed broad spectrum antimicrobial activity. Notably, it was able to inhibit Gram positive food borne pathogen *Listeria monocytogenes* and is a prominent characteristic of our strain *B. subtilis* PLSSC. Additionally, subtilosin A showed clear inhibition of two poultry pathogens *E. coli* ATCC 9002 NCTC and *S. enterica* ATCC 14028 indicating its potential application in poultry feed. Bacteriocins bind to specific receptors in the target cells prior to initiating antimicrobial effect [34]. Precisely for this reason, though broad spectrum, strain specificity has been noted for each independent bacteriocin. Partly, this could be the reason

in differences in antimicrobial effect seen for different strains of the same species of a target pathogen e.g. *E. coli* ATCC 9002 NCTC (Poultry pathogen) and *Escherichia coli* ATCC 700728 (Human pathogen) used in the present study. Broad-spectrum antimicrobial activity of *B. subtilis* and anti-listerial potential of subtilosin A is well established [23, 27, reviewed in 35, 36]. MIC of subtilosin A against *L. monocytogenes* was found to be 125 µg mL^{-1} which is higher than the MIC reported in previous literature (19-26 µg mL^{-1}) and could be due to its partially purified nature [32,37]. Subtilosin A exhibited good antimicrobial activity across a broad pH range including pH as low as 1.0 and up to pH 11.0. This trend is similar to the one reported by Karagiota A et al. [38] where AMC fractions from *B. subtilis* subsp. *subtilis* NCIB 3610 showed stable antimicrobial activity on acidic range of pH but was adversely affected at extreme alkaline pH such as pH 10.0. Similar observation is showed in other reports [39,40]. Thermal stability for subtilosin A coincides with earlier reports [38, 39]. We observed no activity for subtilosin A after autoclaving. Kim SY et al. [41], has also reported complete loss of antimicrobial activity for subtilin KU43 when autoclaved. On the contrary there are few reports of *B. subtilis* AMCs namely subtilin JS-4 [42], Subpeptin JM-4A & B [43] to have retained their activity after autoclaving. The AMC produced by *B. subtilis* PLSSC was resistant to proteolytic enzymes trypsin and pepsin; it was more sensitive to pepsin than trypsin indicating its peptide nature. Ample of evidence on adverse effect of pepsin on AMC produced by *B. subtilis* is available [30,38,39,42].

CONCLUSION

The present study comprised of probiotic characterization of *B. subtilis* PLSSC strain in terms of its ability to survive adverse gastrointestinal conditions to reach colon in adequate amounts. Its stability during thermal processing and ICH recommended storage was established. The strain was also studied for production of AMC which was identified as subtilosin A. Antimicrobial spectrum and stability of subtilosin A was also assessed. The findings of this study will provide valuable information for further research and contribute to the development of evidence-based probiotic interventions for promoting human health and well-being.

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AUTHOR CONTRIBUTION

Project was conceived and manuscript was reviewed by Dina Saroj. Manuscript was reviewed and revised by Aruna Inamdar. Experiments and data collection was performed by Yogini Dixit, Namrata Bhingardeve. Data was analysed and first draft of the manuscript was written by Yogini Dixit. All authors have read and agreed to the manuscript.

DATA AVAILABILITY

All data supporting the findings of this study are available within the paper and its Supplementary Information.

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