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Isolation and Characterization of Potential Probiotic Lactic Acid Bacteria with Antimicrobial Properties from Fermented Bamboo Shoots of Arunachal Pradesh, India

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Keywords: Probiotics; Lactobacillus plantarum MBS17; Antimicrobial activity; Proteinase K; GC-MS Analysis.

Abstract

The study aimed to isolate potential probiotic strains from fermented bamboo shoots of Arunachal Pradesh, India based on functional traits such as acid tolerance, simulated conditions of the gastrointestinal tract, antimicrobial activity, antibiotic susceptibility, bacterial cell surface hydrophobicity, auto-aggregation assay and co-aggregation ability. Proximate nutrient composition was evaluated. Crude protein was found to be 3.74%, crude fiber was 1.92% and fat was found to be negligible. Antioxidant activity, Total Phenolic Content (TPC) and Total Flavanoid Content (TFC) of fermented bamboo shoots was also determined. It was found that fermented bamboo shoot have great antioxidant property almost more than ascorbic acid which suggests as a great nutraceutical food source. 36 different bacteria were isolated from 06 fermented bamboo shoots grown on differential growth media. After screening out, isolate MBS17 was found to be a potential probiotic 16SrRNA gene sequencing revealed the isolate to be *Lactobacillus plantarum* MBS17and its gene sequence was submitted to gene bank with accession no. MW043712. The survivality count in simulated gastric juices was found greater than 6.1 log CFU/ml after 4 hrs of treatment. Hydrophobicity ranged from 55.61 to 77.37 ± 1.74 % with various hydrocarbons. The strain MBS17 showed co-aggregation ability with *Klebsiella pneumonia*, *Pseudomonas aeruginosa Salmonella enterica typhimurium* and *Listeria monocytogenes*, it showed variable susceptibility towards commercial antibiotics but resistant for Oxacillin, Vancomycin and Methicillin. Filtered cell free supernatant of *L.plantarum* MBS17 was found to be antagonistic towards 6 major pathogenic strains. This lactic acid bacterial strain was first to be have antibiofilm activity against Salmonella typhimurium. GC-MS analysis of the ether extract of sterilized cell free supernatant was performed and its major peaks were mostly short to medium chain fatty acids. These results establish the probiotic potentiality of MBS17 and also as alternative food biocontrol agent.

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Introduction

Probiotics are defined as "Live microorganisms administered in adequate amounts conferring health benefits on the host belonging to the GRAS (Generally Recognized as Safe) category". In the recent years, many probiotics were identified for their beneficiary effects on the human such as immune modulation, anticancer, antioxidant, and antimicrobial effect [1]. Nowadays, probiotics are used as an alternative therapy antagonistic to drug-resistant pathogens, however, they are also known to maintain healthy gut microflora and reduce gut-related disorders and heart diseases. An increase in the population of these species can lead to severe gastrointestinal tract infections. It was found that some *Lactobacillus* sp. strains can produce antimicrobial substance or metabolite like bacteriocin, organic acid, catalase enzyme, and peptide responsible for the antagonistic activity. Studies showed that the direct application of LAB, or even only their Cell-Free Supernatant (CFS), inhibits the growth of spoilage causing bacteria in chicken breast, beef, bread, and cheese [2]. LAB can metabolically produce various antibacterial compounds including organic acids, fatty acids, bacteriocins, cyclic dipeptides, phenolic compounds, and hydrogen peroxide. However, studies have also shown that most of the aforementioned metabolites exist in lower concentration than their Minimum Inhibitory Concentration (MIC).Consequently, the antibacterial effects of LAB may not only be due to one compound, but can be attributed to the joint action of several compounds, and often involves synergistic effects among organic acids, Roche-mycin, fatty acids, cyclic dipeptides, and bacteriocins [3]. Biotechnological potentials, such as antitumor activity, antibiofilm formation, antimicrobial activity, and antioxidant activity of EPSs from Lactobacilli are still not fully understood. They also compete with the pathogens for nutrients and attachment to the intestinal surface, thus blocking the sites for the pathogens [4].

Traditionally fermented bamboo shoots have been prepared and consumed by the local communities of North East India since ages, yet their probiotic potential is not entirely known. They are consumed in different forms raw and cooking and their local names are Khorisa, Ekung, Mesu, Soibum, Soidon, Soijim, Heccha, Eup, Hirring, Lung-saij, Tuaithur, soidonmahi, Tabah bam shoot pickle, Naw-maidong and Jiang-sun. The nature of products, and their mode of preparation are community specific. Fermentation of bamboo shoots not only makes it palatable in terms of flavor, aroma, texture and appearance but also makes it nutritious with extended shelf life because of the action of lactic acid bacteria making the product acidic and good for digestion. Nutritional analysis reveals that fermented bamboo shoots are rich in fiber and protein low in carbohydrates and fat. It has been reported that they consist of many nutritional elements like antioxidants and medicinal components which play a key role in the prevention of metabolic disorders by [5]. The states of Assam and Arunachal Pradesh located in the Northeast region of India is famous not only for its traditionally fermented food and beverages but also blessed with biodiversity hotspots. Ekung and Khorisa are ethnic fermented food product prepared from edible tender bamboo shoots found in Assam and mostly used in a variety of food preparations including fish and meat. Nevertheless, the study of molecular dynamics and nutritional value of Khorisa is still limited.

Fermented bamboo shoots are nutritional and medicinally important food. Fermented bamboo shoots are consumed by many local tribes of North East India such as Tai-Ahom, Bodo, Thadau, Mosang, Tiwa, Karbi Anglong Tribes as they are rich in dietary fibres, macronutrients and low fat. Bamboo shoots contain phytosterols and high fibre help in reducing fat and cholesterol levels in blood. The dietary fibre possesses number of health benefits as it controls blood pressure, hypertension, and obesity and also protects our body from coronary diseases and potential carcinogens [6]. There was increase in the frequency of bowel movement and faecal volume indicating its role in cholesterol lowering and diabetes prevention in individuals provided with bamboo diets. Bamboo shoots are used by local tribes for treatment of irregular menstrual cycle, heavy bleeding after delivery, infertility problems, reducing labour pain, and also for inducing puberty in young female [7]. Therefore, the present study aims on the isolation of potential bacterial isolates from traditionally fermented bamboo shoots collected from different districts of Arunachal Pradesh and to characterize specific probiotic properties in addition to antibacterial property, stability, and composition of LAB-derived antibacterial substances and their effects on principal gut pathogens.

Materials and Methods

Chemicals and reagents

The media used for culturing bacteria were purchased from HiMedia India Pvt. Ltd. and the other necessary chemicals and consumables were procured from Sigma India Pvt. Ltd.

Bacterial strains and growth conditions

Salmonella typhimurium ATCC 14038, *Listeria monoytogenes* AMDK2, *Klebsiella pneumonia* MTCC 618, *Pseudomonas aeruginosa* MTCC 2247, *Staphylococcus aureus* MTCC 3160, *Candida albicans* MTCC 3017 indicator strains and *Lactobacillus plantarum* MBS17 a potential probiotic strain were used in the study. Luria Broth (LB) and Tryptone Soya broth (TSB) was used to revive the pathogenic strains at 37°C and 28°C, respectively and *L.plantarum* MBS17 was cultured in De Man Rogosa (MRS) broth. The strains were sub-cultured till they achieve 0.4 OD at 600 nm and further used for the experimental analysis.

Sampling and Isolation of bacteria

Ekung, an indigenous fermented bamboo shoot product, was collected from West Kameng district, Arunachal Pradesh, India located at Latitude 27.01 N and Longitude 92.63 E to screen the potential probiotic strains. Samples were collected in sterile containers and stored at 4°C until the study was performed. Serial dilution upto10^{-9} was performed using sterile phosphate buffer solution at pH 7.4 and 100µL of each dilution was plated on de Man, Rogosa and Sharpe (MRS) agar. The plates were incubated at 37°C for 24 h. After incubation, different types of colonies were selected on the basis of colony morphology and transferred to sterile broth culture. Only gram positive and catalase negative isolates were considered for in-vitro probiotic properties like low pH tolerance, bile salt tolerance, cell survivaibilty under simulated conditions of intestinal and gastric juices, Cell surface hydrophobicity assay, Cell auto-aggregation and co-aggregation assay, antimicrobial activity and adhesion to intestinal epithelium cell line.

Nutritional analysis

The sample was further processed to detect nutrient composition, particularly carbohydrate, protein, fat, fiber and moisture. Total carbohydrate was estimated by using the Anthrone method. The main reagents are 2.5 HCL, Anthrone reagent: 200 mg of anthrone in100 mL of ice-cold 95% H2SO4 and standard

glucose at 1mg/ml concentration. The protein content (N x 6.25) was evaluated by determining the nitrogen (N) content by using the micro Kjeldahl method and multiplying it by 6.25 *(Codex Guidelines on Nutrition Labelling).* Fiber present in the sample is estimated by the Fibre Extractor. Fat content was determined by using the Soxhlet Extraction method [8] by using petroleum ether.

Antioxidant activity

1,1-Diphenyl-2-Picryl-Hydrazyl (DPPH) assay was used to check the antioxidant capacity of the sample as described by [9] with slight modification. Briefly, 10 µl of sample solution (1.0 mg/ml) concentration in methanol was added to 190µl of 60 µM DPPH solution, mixed and kept in dark for 30 min at room temperature. Ascorbic acid was used as postive control while only methanol and DPPH as negative control. The absorbance was measured at 517 nm and DPPH scavenging capacity was calculated by this following formula.% DPPH Scavenging activity = $(A_{control}-A_{sample}) / A_{control} \times 100$

Total phenolic content

Folin–Ciocalteu reagent was used to determine the total phenolic content of extract [10] with some slight modifications. Briefly, 25µl of extract (1mg/ml) was mixed with 100 µl of 0.075 g/ml Sodium Carbonate, and 125 µl of 10% v/v Folin–Ciocalteu reagent.The reaction mixture was incubated for 30 min at room temperture and absorbance was measured at 765 nm. The calibration curve of gallic acid was used to calculate the total phenolic content and was expressed as mg gallic acid equivalent per g dry weight.

Total flavanoid content

The total flavonoid content of the extract was evaluated using Aluminium chloride colorimetric method [11] with some slight modifications. 25 µl of extract (1mg/ml) was mixed with 75 µl of 95% ethanol, 10 µl of 10% w/v AlCl3, 10 µl of 10% w/v Potassium acetate, and 140 µl deionized water. The reaction mixture was incubated for 40 min at room temperature and absorbance was measured at 415 nm. The calibration curve of quercetin (Sigma) was used to calculate the total flavonoid content and was expressed as mg quercetin equivalent per g dry weight.

Biochemical and molecular characterization

The following standard guidelines of Bergey's Manual of Systematic Bacteriology [12] was used characterization for bacterial isolates. The Molecular identification of the isolates was done by 16S rRNA sequence analysis followed by phylogenetic studies. The screened isolate was grown in MRS broth for overnight at 37°C and genomic DNA was isolated using the isolation kit (Hi Media, India). Universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used to amplify the conserved 16SrRNA gene sequence [13]. The PCR amplification was conducted in Eppendorf thermocycler on initial denaturation at 95°C for 5 min, then 30 cycles of 94°C for 1 min, Primer annealing at 54°C for 40 sec, Elongation at 72°C for 1 min, and final Extension at 72°C for 10 min. The PCR product was electrophoresised in 1.5% agarose gel and the amplicon so obtained was sent to Molbiogen, (1st Base, Apical Scientific, Singapore) for sequencing. The sequence obtained was submitted to NCBI (National Center for Biotechnology information) Genbank for its accession number. The BLAST result showed that the strain was *Lactobacillus plantarum* MBS17. The phylogenetic tree was generated by Neighbor-Joining (NJ) method using MEGA 5.05 [14].

Screening of probiotic bacterial strain

Tolerance to low pH

Resistance to low pH was assessed according to the method described by [15] with slight modifications. The freshly grown bacterial strain MBS17 was harvested by centrifugation at 5000 rpm for 10 min at 4°C followed by washing with phosphate buffered saline (PBS, pH 7.4) and then re-suspended (10 cfu/mL) in PBS solutions of different pH values such as 1.5, 2.5, 3.5 and 5. The bacteria were incubated at 37°C for 60 mins. The resistance of *L.plantarum* in every condition was assessed in terms of viable colony count on MRS agar after 1 hr respectively and pH 7.2 was considered as control.

Tolerance to bile salts

The resistance to bile salts was assessed according to [16] MRS broth culture of the isolated strain was centrifuged at 5000 rpm for 10 min at 4°C and bacterial cells were resuspended in PBS (pH 7.4) supplemented with 0.2, 0.3, 0.4 and 0.5 % (w/v) bile (Oxgall) and incubated for 0, 1, 2, and 3 h at 37°C. The PBS suspension without bile was considered as the control. Resistance in every condition was assessed in terms of viable colony count on MRS agar plate after the treatment. The tolerance to various concentrations of bile salts was evaluated through the plate assay. Plates were incubated at 37°C in anaerobic condition and growth was recorded after 24-48 h. A bile-free plate was employed as the positive control. The experiment was repeated thrice.

Survibility of bacteria under simulated conditions of intestinal and gastric juice

In order to test simulated gastrointestinal transit tolerance, bacterial cells were suspended in simulated gastric fluid (SGF) and intestinal fluid (SIF) adjusted in the different PBS with various pH values inorder to mimic the environment of human gastrointestinal tract [17]. SGF was prepared by supplementing sterilized PBS (pH 2, 3 and 4) (adjusted with 1 N HCl) with pepsin (HiMedia) to a final concentration of 0.3 mg/ml. To prepare SIF, pancreatin (1g/L,Sigma) was supplemented to the sterilized PBS of pH 6.8 and pH 8 (adjusted with 1N NaOH/ 1N HCl). To perform the test, bacterial cells from18-24 h culture were harvested by centrifugation at 3000 x g for 5 mins at 4°C, PBS washed twice (pH7.4) and resuspended in the concentration of 10^8 CFU mL^{-1}) in SGF and SIF treatment for 0 to 4 h respectively. The resistance of the isolate MBS17 was evaluated in terms of viable colony count on MRS agar after the treatment.

Cell surface hydrophobicity assay

The ability of bacterial cells to adhere to the epithelium of the Gastroinestinal tract of the human host can be determined by its capacity to adhere to the hydrocarbons. The hydrophobicity of the bacterial isolate was examined by the adhesion to the non-polar solvents as described by [18]. The overnight grown culture at 37°C in MRS broth was pelleted (6000 × *g*, 5 min) and washed twice with cold PBS. The absorbance of the cell suspension was adjusted to 0.6 at 600 nm. The cell suspension 2ml was added to the different tubes containing equal volume of chloroform, ethyl acetate, n-hexane and xylene. The two phase system was mixed well by vortexing for 2 min and incubated for 1 h at room temperature. After the incubation period, the aqueous phase was gently taken to measure its absorbance at

600 nm. The surface hydrophobicity was evaluated as change in the O.D of the aquoes phase after mixing and phase separation compare to that of original suspension (A_0) as: Hydrophobicity [%] = 100 × At – A0/A0

Where 'At' represents the absorbance at time t = 1 h and 'A0' the absorbance at t = 0.

Auto-aggregation of cells

Autoaggregation assay was performed according to [19] with slight modifications. The auto-aggregation ability is the precondition necessity for the bacterial cells for colonization on the epithelial cells of the GIT tract so the bacterial cells were tested for auto-aggregation assay. The bacterial cell suspension 2ml was vortexed for 10 sec and incubated at 37 C. An aliquot of 0.1 ml collected from the upper surface at regular interval and mixed with 0.9 ml PBS and A_{600} was measured. Autoaggregation percentage was expressed as:

Autoaggregation [%] = 1- At/Ao x 100

Where A_t -absorbance at different time intervals (2h, 4h, 24h) and A_0 -absorbance at 0h.

Co-aggregation assay

The co-aggregation assay can determine interbacterial adherence capability of probiotic strain, protecting the host by inhibiting the colonization of pathogens. The bacterial cell suspension was prepared similarly as 2.8.5. Equal volume (2mL) of each cell and pathogen suspension were mixed together in pairs by vortexing for 10 sec. The control tubes were set up at the same time, containing 4 mL of each bacterial suspension. The absorbance (A_{600}) of the suspensions was measured followed by mixing and 5h of incubation at room temperature.An aliquot of 0.1 mL collected from the upper surface was mixed with 0.9 mL and measured its absorbance at $A_{600.}$

The percentage of coaggregation was calculated using the equation of [20].

$$\text{Autoaggregation [\%]} = \frac{\left[\frac{(Ax + Ay)}{2}\right] - A(x + y)}{Ax + A\,y/2}$$

Where x and y represent each of the two strains in the control tubes, and (x+y) represents the mixture.

Safety Assessment Assay

Hemolytic Assay

The bacterial isolate was evaluated for haemolytic activity using Sheep Blood Agar plates containing 5% (*v/v*) commercially available mammalian blood and incubated at 37°C for 24h [21]. *Staphylococcus aureus* was taken as positive control for the test. Clear zone around the tested organism indicated positive test for haemolysis.

Antibiotic susceptibility

The antibiotic susceptibility of *L.plantarum* was evaluated against the erythromycin (15µg), Clindamycin (30µg), co-trimoxazole (25µg), levofloxacin (5g), oxacillin (1g) ampicillin(10 µg),ciprofloxacin(30µg), CoTrimoxazole (Sulpha/Trimethoprim) (3µg), vancomycin (30µg), tetracycline (30 µg), chloramphenicol (30 µg), kanamycin (30 µg) and gentamicin (10µg) Piperacillin (30g) antibiotic discs (Himedia Ltd., India) following the method suggested by Shao et al. (2015). The freshly grown bacterial suspension 50 µl (after dilution to about 10^5-10^6cfu/ml) was spread evenly on the surface of the MRS agar plate. Antibiotic discs were placed on the plates followed by incubation at 37°C for 24–48 h. Results were expressed by measuring the diameter of the zone of inhibition and interpreted as sensitive (S), resistant (R) and intermediate (I) as per the manufacturer's protocol.

Assessment of antimicrobial activity

The antimicrobial potentiality of the isolated bacterial strain was assessed by Agar well diffusion method. Firstly, the isolate was cultured for 18-24 hr at 37°C and centrifuged at 10,000 rpm for 10 mins at 4°C and the supernatant was collected, filtered through a 0.22 mm syringe filter and used for the antimicrobial test. The log phase 100 µL cultures of *Candida albicans, Psuedomonas aeruginosa Salmonella typhimurium, Listeria monocytogenes*, *Klebsiella pneumonia* were seeded on Luria Bertani (Miller) media. Approximately, 7.5 mm-diameter wells were made into the surface and loaded with 100µL cell free supernatant, Gentamicin was taken as positive control, sterilized broth culture as negative control and incubated for 24h.The clear zones around the wells indicated the antimicrobial activity [22]. The nature of antimicrobial substance was checked by neutralizing the CFS with 0.5 M NaOH/ 1N HCL, further treating with Proteinase K, Pepsin and Trypsin at a final concentration of 1.5 mg/ml. The antimicrobial test was performed similarly like the above.

Anti Biofilm Assay (Tissue Culture Plate method)

CFSM was prepared from probiotics which was used to assess the antibiofilm formation activity as described by [23]. Five different groups labeled as test agents probiotic CFSM in various concentrations (10%, 20%, 30%, 40% and 50%) vol/vol along with their experimental control (broth medium) were prepared. In each group, 100 µl of the test antibiofilm CFSM I the above concentrations prepared was added in triplicate to the corresponding wells of sterile 96-well microtitre plate (Sigma Aldrich, USA) except for the negative controls. Briefly, 10 µl of 0.4 OD600 of *Salmonella* culture was poured in wells of 96-well-flat bottom tissue culture plate. CFSM was added in different concentrations followed by addition of TSB to make the final volume of 200µl. The plates were wrapped in aluminium foil and incubated at 37°C for 24 h in a static condition. To wash off the planktonic bacteria and the media the wells were washed twice with Phosphate-buffered saline (PBS, pH 7.4) after incubation, followed by staining of adhered cells with 0.1% Crystal Violet. After 30 min of incubation the wells were washed with PBS to remove excess stain and the plate was air dried. The biofilm was quantified by checking optical densities at 570 nm (Thermo Scientific Multiscan GO) after solubilizing the bound crystal violet in methanol by Gupta et al. (2019). Sterilized MRS Broth and furanone (1mM) were used as negative and positive control respectively. The mean absorbance ($OD_{595\ nm}$) of test organisms was determined and the percentage inhibition was calculated using the following formula (Eq. 1):

% Biofilm inhibition = ODcontrol-ODtest / ODcontrol × 100

Where ODcontrol and ODtest is the absorbance of untreated control and treated culture respectively.

GC-MS analysis of the ether extract.

Antimicrobial substance from the broth culture of the isolates are extracted using solvent extraction method described by [24]. Bacterial culture of 48h is centrifuged and the superna-

tant separated. The supernatant is saturated by NaCl. Now, for 100 ml of supernatant 20 ml of 50% HCl is added followed by extraction with diethyl ether and concentration under vacuum. The antimicrobial substance in the broth culture of bacteria is identified by GC-MS (Agilent 7890A GC and 240 - MS Ion Trap). The conditions for GC-MS are as follows : GC column with c l 30 m, d 0.320 mm, f 0.25 µm and temperature range 60 - 325°) The GC oven temperature is programmed and maintained at 60°C for 2 min, followed by 115°C with a rate of 15°C per min and held for 15 min by [25]. All the major metabolites were identified by comparing with mass spectra obtained from commercial library.

Statistical analysis.

The results are subjected to ANOVA using GraphPad Prism software (Graph Pad Software, version 5.0 Inc.CA, USA).

Results

Isolation of the bacterial strain and biochemical characterization

Forty-five bacterial strains were isolated from 06 Ekung fermented bamboo shoot samples on different growth media basically MRS (de Mann Rogosa Sharpe) media with different types colony morphology. The strains were further screened for probiotic characteristics and only one *Lactobacillus* sp. strain was found to be potential probiotic. This strain is gram-positive, rod-shaped and non-spore forming bacterium. It exhibited negative tests for catalase and carbohydrate utilization revealing it to be able to ferment lactose, maltose, dextrose, mannose, sucrose, l-arabinose and galactose (Table 4)

Nutritional analysis

The nutrient composition in the fermented bamboo shoot sample is evaluated and presented in Table 5. Fat content is 0.03 ± 0.0172 g/100g, protein content 3.74 ± 0.18393 g/100g and fibre in the sample 1.92 ± 0.353 1g /100g. This has signified the importance of the Ekung, fermented bamboo shoot as a perfect functional food with high protein and low fat content.

Antioxidant activity

The methanolic extract (1.0 mg/ml) of fermented bamboo shoot samples exhibited significant free radical scavenging activity ranging between 39.43 and 48.60 % and it was found that the positive control Ascorbic acid (1.0 mg/ml) exhibited almost 85.82% of DPPH scavenging activity.

Total flavanoid and phenolic content

The gallic acid calibration curve (R^2 = 0.9485) was used to calculate total phenolic content and it was found to be 41.07 ± 0.93 mg gallic acid equivalents/g. Similarly, to estimate total flavonoid content quercetin calibration curve (R^2 = 0.9993) was used and was found to be 31.33 ± 1.50 mg quercetin equivalent/g.

Molecular characterization of isolate

The derived 16S rRNA gene sequence obtained is submitted to NCBI Genbank for its accession number.The BLAST result as proved the strain to be *Lactobacillus plantarum* MBS17.The maximum sequence similarity is observed in *Lactobacillus plantarum* CAU-226 approximately with 99% similarity. The phylogenetic tree was generated by Neighbour–Joining (NJ) method in Figure 1 using MEGA 5.05 (Tamura et al. 2011).

Screening of probiotic characterization

Tolerance to low pH

In this study, the viability of *L.plantarum* is investigated at the different pH values. Figure 2 shows the log of viability of the target strain after 1h of exposure to the desired pH. As the pH of stomach increases, the bacteria survive more indicating possible effect of the stomach environment.

Tolerance to bile salts

The strain is treated with different concentrations (0.2%, 0.3%, 0.4%, 0.5%) of bile salts. The survivability of the isolate is higher till 2hr of treatment but gradually decreased after exposure upto 3h (Figure 2). Significantly the viability was more than 6.5 log cfu/ml in 0.2 and 0.3% bile salt concentration but the populations declined to 5.1 log cfu/ml in 0.5% bile salt after 3 hr of treatment.

Survibility under simulated conditions of intestinal and gastric juices

The tolerance of the isolate to simulated gastrointestinal juice is investigated in–vitro by treating the cells with pepsin and pancreatin juice at different pH value ranging frompH2-8 which mimic the different environment of the stomach and small intestine. Treatment with SGF (Simulated gastric fluid) has showed its tolerance to the acidic conditions, the population is 6.62 log cfu/ml and finally after 3h the population is 5.94 log cfu/ml and it the survibility at pH4 is near 7cfu /ml (Figure 2). The population of the cell is greater than 7.5 log cfu/ml in SIF (Simulated Intestinal Fluid) after 4h treatment.

Cell surface hydrophobicity assay

Hydrophobicity is demonstrated by using hydrocarbons like chloroform,ethyl acetate, n-hexane and xylene. The isolate has showed the highest hydrophobicity of 77.37% with chloroform and 72.63% with ethyl acetate and least 55.61% with xylene (Table 1).

Auto-aggregation assay

The auto-aggregation of the strain was found to be 13.73 % after 2 h of incubation at 37°C (Table 2) at static condition and has significantly inclined to 79.23% after 24 h of incubation.

Co-aggregation assay

Co-aggregation assay is performed to check the co-aggregation ability of strain MBS17 with food pathogenic bacteria *Klebsiella pneumonia*, *Psuedomonas aeruginosa*, *Salmonella typhimurium* and *Listeria monocytogenes.* It is observed that the isolate has co-aggregated with most of the pathogens and mostly the co-aggregation ability is more than 40.5% the highest with *Salmonella typhimurim* (53.37%) followed by *Psuedomonas aeruginosa* (45.55%) (Table 2).

Safety Assesment Test

Hemolytic assay

No hemolysis of *L.plantarum* was observed in sheep blood agar plate (Figure 3) which showed safety toward use of the isolate as a potential probiotics. The non-haemolytic nature of *L.plantarum* on mammalian blood agar plates also advocates its non-cytoxic nature.

Antibiotic susceptibility test

L.plantarum is susceptible to antibiotics Clindamycin, Erythromycin, Gentamicin, Levofloxacin, Fusidic Acid, Chloramphenicol, Piperacillin, Co-Trimoxazole, Piperacillin, Tetracycline and Rifampicin except Oxacillin, Vancomycin and Methicillin (Table 3)

Antifungal and antibacterial activity

Antagonistic potentiality of cell free supernantant of *L.plantarum* in (Figure 4 and Table 6) is assessed against the pathogenic bacteria and fungal indicator strains. The test indicator strains are *Pseudomonas aeruginosa* MTCC 2247, *Listeria monocytogenes* (AMDK2 KT894986), *Salmonella typhimurium* ATCC14038, *Staphylococcus aureus* MTCC 3160, *Klebsiella pneumonia* MTCC 618, *Yersinia pestis* and the fungal strain *Candida albicans* 3017.The inhibitory effect is the highest on *Pseudomonas aeruginosa* MTCC2247(16±0.7), *Listeria monocytogenes* AMDK2 (KF894986) (16 ± 1.0) and lowest on *Salmonella* sp. ATCC 14038. It is observed that the isolate inhibited the growth of *Candida albicans* MTCC 3017 incubated at 28°C to a greater extent and the zone of inhibition is more than 24 mm ± 1.5

Anti-biofilm assay of CFS against *Salmonella typhimurium*

The SCFS of the selected LAB exhibited dose dependent biofilm inhibition as shown in the Figure 5. This method depicted a dose dependent and significant biofilm inhibition. The biofilm formation was reduced to 75.88 ± 2.57 at a concentration of 50 (v/v %) of SCFS.

Characterization of antimicrobial substance

Antimicrobial substances present in the CFS is determined by neutralizing the pH of the supernatant to 7.0 with 1M NaOH treating with the different enzymes such as Proteinase K, Pepsin and Trypsin respectively. Clear zones of inhibition around the wells are seen even after treatment with Proteinase K, Pepsin and Trypsin but the neutralized CFS has fail to show any zone of inhibition which predicts that pH of CFS of *L.plantarum* is slightly acidic and the activity may be due to some organic acids, hydrogen peroxide, low molecular weight, antimicrobial substances. The pH of CFS is checked and found to be ranging between 3.8-4.0, as the clear zones disappeared after protease hydrolysis of the CFS, so it can be determined that the metabolites present in CFS which are responsible for the antimicrobial effects may not be proteinaceous in nature. Therefore it is necessary to further analyse the CFS to evaluate its major constituents.

GC-MS analysis of CFS

The antimicrobial substance is extracted from the broth culture of isolate using solvent extraction method. The Ether extract of *L.plantarum* MBS17 is analyzed by GC-MS (Figure 6 and Table 7) and the compounds analysed by the major peaks are Propanoic acid hexyl ester, Acetic acid 2-hydroxy-1-methylethyl ester, Propyl acetate, Butanoic acid 3-hydroxy, 3-Nonynoic acid and Benzoic acid 2-hydroxy-phenylmethyl ester.

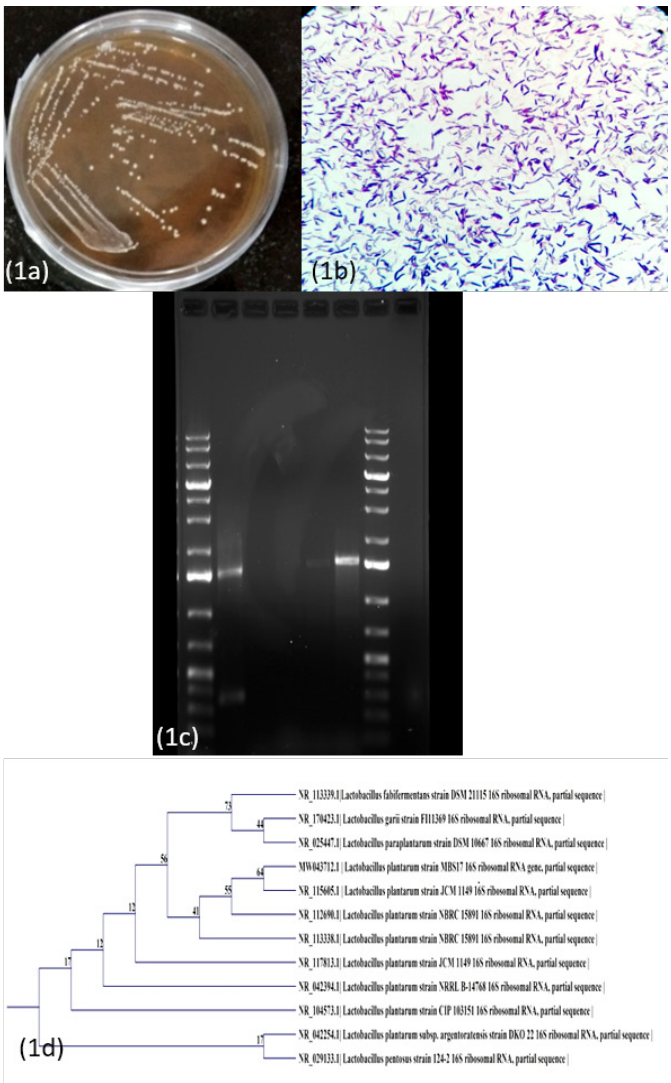

Figure 1: Isolation and identification of L.plantarum MBS17. **(a)** Colonial morphology; **(b)** Cells form of LP- MBS17; **(c)** Agarose gel electrophoresis of LP-MBS17 16s rDNA PCR amplification product; **(d)** Phylogenetic analysis of LP-MBS17.

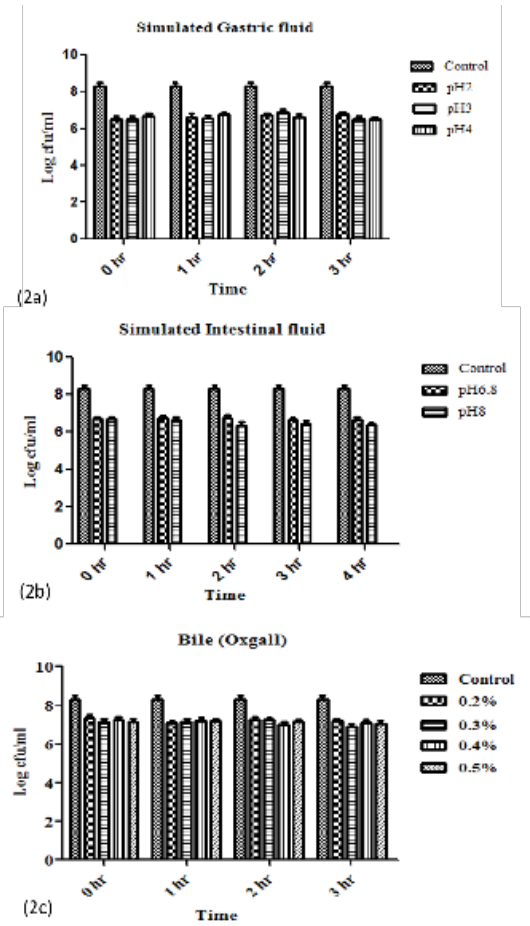

Figure 2: Viability of LP - MBS17 in (A) Simulated gastric fluid at pH 2.0, pH 3.0 and pH 4.0 and (B) Simulated intestinal fluid at pH 6.8 and pH 8.0 in Time intervals of 0,1,2 3 & 4 hr c) different concentration of bile (Oxgall).

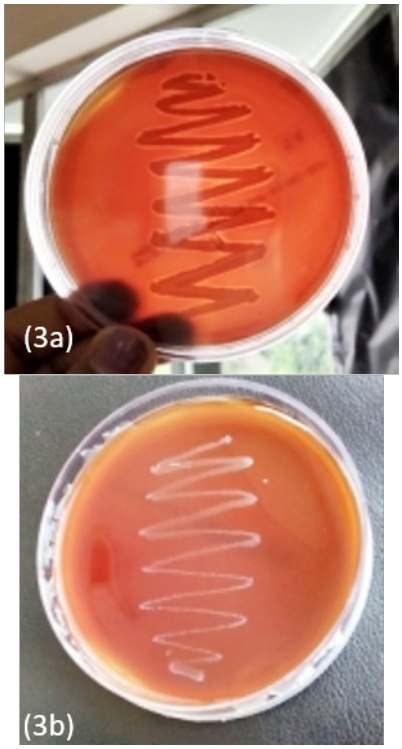

Figure 3: Hemolysis Assay on mammalian Blood Agar **(a)** Clear zones around the colonies of S. aureus (control) indicate hemolysis **(b)** Absence of clear zones around the colonies of L.plantarum MBS17 indicates no hemolysis.

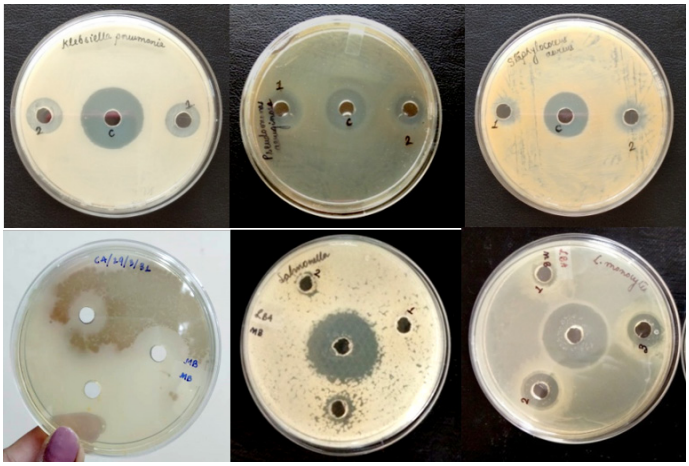

Figure 4: Agar well diffusion assay for assessment of Antimicrobial activity A. Salmonella ATCC 14038 B. Listeria monocytogenes AMDK2 (KF894986) C. Klebsiella pneumoniae MTCC 618 D. Pseudoonas aeruginosa MTCC 2247 E. Staphylococcus aureus MTCC 3160 F. Candida albicans MTCC 3017.

Table 1: Bacterial Cell Surface Hydrophobicity Assay of LP - MBS17.

	Hydrocarbons	Hydrophobicity percentage
	Chloroform	77.37 ± 0.74
	Ethyl acetate	72.63 ± 1.12
	n hexane	62.89 ± 0.49
	Xylene	55.61 ± 1.70

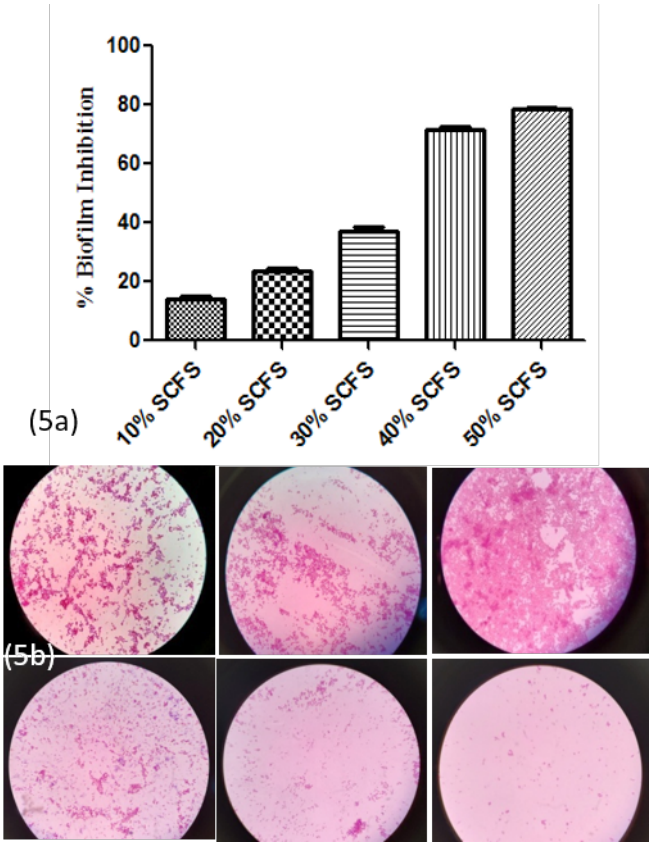

Figure 5: (A). Effect of filtered cell free supernatant on biofilm inhibition by Tissue culture method and error bars represent the standard deviations of 3 measurements. **(B)**. Light microscopic visualization of antibiofilm activity at 100 X magnification.

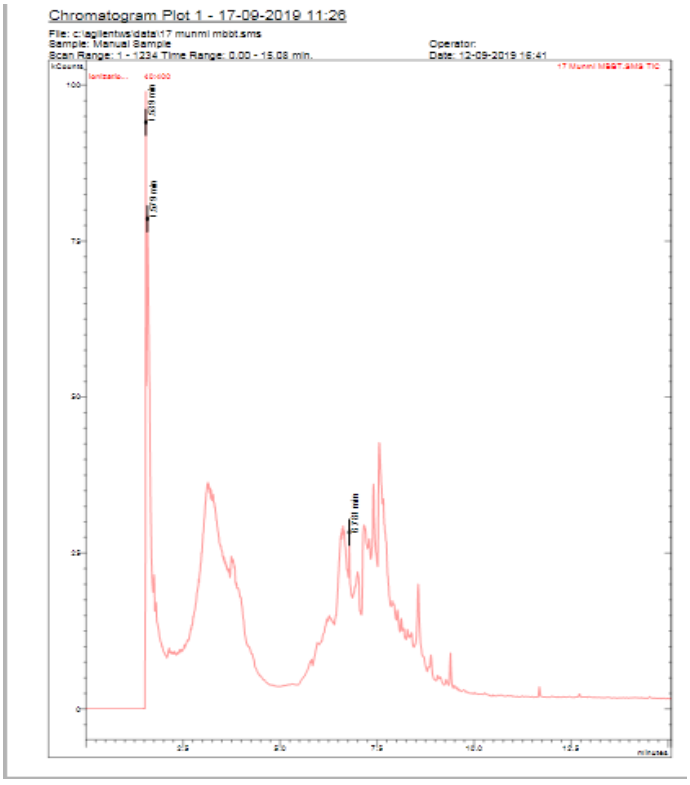

Figure 6: GC-MS Chromatogram of ether extract of LP-MBS17 culture.

Table 2: Auto-aggregation and Co-aggregation ability of LP - MBS17.

Auto-aggregation (%)		Co-aggregation(%)			
		Klebsiella pneumonia MTCC 618	*Pseudomonas aeruginosa* MTCC 2297	*Salmonella enterica typhimurium* ATCC 14038	*Listeria monocytogenes* AMDK2 (KF894986)
2hr	13.73 ± 0.18	18.73 ± 0.11	17.56 ± 0.44	18.31 ± 0.67	16. 54 ± 0.91
4hr	25.83 ± 0.21	23.47 ± 0.55	32.15 ± 0.17	24.77 ± 0.22	21.30 ± 0.52
24hr	79.23 ± 0.91	40.95 ± 0.90	45.55 ± 0.33	53.37 ± 0.46	42.85 ± 0.155

Table 3: Antibiotic Susceptibility of LP - MBS17 (CLSI guidelines).

Antibiotics	Concentration	Susceptibility
Clindamycin	30 mcg	S
Erythromycin	15 mcg	S
Gentamicin	10 mcg	S
Levofloxacin	05 mcg	S
Oxacillin	01 mcg	R
Vancomycin	30 mcg	R
Fusidic Acid	30 mcg	S
Chloramphenicol	30 mcg	S
Methicillin	05 mcg	R
Cefepime	30 mcg	S
Rifampicin	05 mcg	S
Tetracycline	30 mcg	S
Co- Trimoxazole (Sulpha/ Trimethoprim)	25 mcg	R
Doxycycline hydrochloride	30 mcg	R
Linezolid	30 mcg	R
Penicillin	25 mcg	S
Piperacillin	30 mcg	S

Table 4: Biochemical test of LP- MBS17.

Sl. No.	Carbohydrate fermentation test Carbohydrate used	Results
1	Dextrose	+ve
2	Fructose	+ve
3	Galactose	+ve
4	Mannose	+ve
5	Melibiose	-ve
6	L-Arabinose	+ve
7	Lactose	+ve
8	Maltose	+ve
9	Xylose	-ve
10	Raffinose	-ve
11	Trehalose	-ve
12	Sucrose	+ve

Table 5: Proximate Nutrient Composition in Ekung, fermented bamboo shoot.

Proximate composition	(g/100 g fresh weight basis)
Protein	3.74 ± 0.18393
Fat	0.03 ± 0.0172
Crude Fiber	1.92 ± 0.3531
Carbohydrate	1.2 ± 0.40
Moisture	92.43 ± 0.134
Ash	0.77 ± 0.11

Table 6: Antimicrobial Activity by filtered CFS of LP - MBS17.

Sl No.	Pathogenic strain	Zone of inhibition (mm)
1	Salmonella ATCC 14038	**06 ± 0.2**
2	Listeria monocytogenes AMDK2 (KF894986)	16 ± 0.8
3	Klebsiella pneumoniae MTCC 618	15 ± 0.5
4	Pseudoonas aeruginosa MTCC 2247	16 ± 0.7
5	Staphylococcus aureus MTCC 3160	13 ± 1.0
6	Candida albicans MTCC 3017	24 ± 2.1

Table 7: Metabolites identified in the cell free supernatants of LP - MBS17 against pathogenic indicator strains.

Compound name	Molecular Formula	Molecular Weight	Retention time (min)	% of Peak Area
1. Propanoic acid, hexyl ester	C9H18O	158.23	1.576	28.713
2. Acetic acid, 2-hydroxy-1-methyl ethyl ester	C5H10O3	118.13	3.123	4.736
3. Propyl acetate	C5H10O	102.13	3.150	1.437
4. Butanoic acid, 3-hydroxy-	C5H10O3	118.13	3.782	1.437
5. 3-Nonynoic acid	C9H16O2	156.22	5.970	3.616
6. Benzoic acid, 2-hydroxy-, phenylmethyl ester	C14H12O	228.2	7.179	6.177

Discussion

In the present study, substantial effort has been made to designate the probiotic bacterial strains originating from the indigenous fermented product 'Ekung' of Arunachal Pradesh on the basis of technological criteria. As fermented products are generally safe to use and have various health benefits so it is justifiable to isolate probiotic bacteria from them. A key property for probiotic microorganisms is that it must tolerate and grow in the stressful and inhostible gastrointestinal conditions and colonize the GIT. The pH of the gut varies in different region and found to be lowest in the stomach region so it is crucial for the microorganism to be resistant to acid and bile and other digestive enzymes in order to be viable enough by [26]. Acid tolerant property in bacteria is necessary not only for withstanding gastric stresses, but it also enables the strains to survive for longer periods in high acid carrier foods, such as yogurt, other without reduction in their number [27]. The resistance of the identified bacterial strain *L.plantarum* MBS17 to lower pH value was checked by mimicing the gastrointestinal conditions and

treating the bacterial cells at final concentration of 10^8cfu/ml in simulated pepsin 3g/L and pancreatin enzymes 1g/L at different pH 2–8 [28]. reported that the resistance to low pH by *Lactobacillus* is due to the presence of F0F1-ATPase activity. The incubation of *L. plantarum* MBS17 in pH2 resulted in the decline of the bacterial population, however the viability was more than 6 log cfu/ml in all the treatments which suggests that it can be a potential probiotic that can successfully colonise the human GIT. However it should be capable of tolerating higher pH upto8.5 and also the emulsification of the bile salts in the large intestine reported [29].

Bile salts are released in the small intestine which are biosurfactant in nature and have vital role in digestion of fats and lipids. Bile salt tolerance is considered as important criterion for lactic acid bacteria to survive. The surviviblity of *L.plantarum* MBS17 is tracked in 0.5% bile salt concentration however it was stated by [16] that the normal human stomach contains 0.3% of bile salt and it is concluded that it was tolerant to bile salts. The bacteria could not to survive in high concentration of bile which implies that the cell membrane of the bacteria is effected by high concentration of bile salts. Bacterial cell surface hydrophobicity and autoaggregation assays are important for the selection of potential probiotic strains. Hydrophobicity determines the ability of a bacterial strain to adhere hydrocarbons which establishes a strong relationship with colonization on the epithelium of the digestive tract [30]. Hydrophobicity of the selected strain is done against chloroform, ethyl acetate, n-hexane, xylene and it is found to be more than 55.5% with all the hydrocarbons. Hydrophobicity capacity reflects its adhesion ability to epithelial surfaces. Autoaggregation or capacity to form cellular aggregates is a desirable character of potential probiotic to get attached and grow on gut epithelial cells and it can inhibit adherence of pathogenic bacteria to intestinal mucosa either by forming a barrier via self-aggregation or co-aggregation with commensal organisms on the intestinal mucosa or by direct co-aggregation with the pathogens to facilitate clearance [31].

L.plantarum MBS17 is susceptible to almost all tested antibiotics but resistant to vancomycin, oxacillin, methicillin, linezolid, and co-trimoxazole (Sulpha/Trimethoprim). These antibiotics are generally used for the prevention of bacterial infections such as pneumonia (a lung infection), bronchitis (infection of the tubes leading to the lung, 'travelers' diarrhea, urinary infection, intestine infection and the underlying mechanism of the antibiotics is the disruption of bacterial growth with the inhibition of the protein synthesis [32,33].

Reported that LAB strains (*L.plantarum* K132, *L.paracasei* K114 and *L.lactis* E124) isolated from Ethiopian fermented foods were remarkedly antagonistic against *Salmonella Typhimurium* DT104 in the mouse model. The mortality rate of the challenged mice with bacterial strain was prevented by pretreatment with mixed probiotic LAB strains (*L.lactis,L.plantarum* and *L.paracasei*). Consequently,the population of the infected mice with *Salmonella Typhimurium* DT104 without probiotic treatment after 15 days was 33.33% less than those treated with probiotic bacteria [34]. Characterized the antimicrobial activity of 3 *L.plantarum* strains respectively M7, S11 and P1 against *E.coli* ATCC 35128 *S.typhimurium* ASI1174 and *S.aureus* ATCC12600. They found that 3 *L.plantarum* strains could inhibit the growth of *S.aureus* and *L.plantarum* strains M7 and S11 exhibited prominent antimicrobial activity against *E. coli* with zone of inhibition 11.4 and 11.2 mm but they did not show any ability to inhibit the proliferation of *Salmonella*.*L. plantarum* S11 had the highest antimicrobial activity against *Salmonella.* They had isolated the strains from Chinese traditional dairy foods. The secreted antimicrobial substances were found to be organic acids mainly lactic and Acetic acids [35] with small amount of tartaric, malic and citric acids.In the investigation, effort is also made to determine the microbial and nutritional value of 'Ekung' derived from fermented bamboo shoot of Arunachal Pradesh. The isolate is identified as *Lactobacillus plantarum* MBS17 and assigned with the accession no. MW043712**.** The strain has exhibited excellent antimicrobial and antifungal activity against the pathogenic indicator strains of *Yersinia pestis, Salmonella* ATCC 14038, *Listeria monocytogenes* AMDK2, *Staphylococcus aureus* MTCC 3160, *Klebsiella pneumonia* MTCC 618, *Pseudomonas aeruginosa* MTCC 2247 and *Candida albicans* MTCC 3017 with zone of inhibition exceeding 15 ± 0.2 mm separately except *Salmonella* with 8 mm.

In the present study, the GC-MS analysis of the ether extract of bacterial CFS revealed the presence of few esters and organic acids. Propanoic acid hexyl ester, Aceticacid 2-hydroxy-1-methyl ethyl ester, Propyl acetate, Butanoic acid 3-hydroxy-, 3-Nonynoic acid and Benzoic acid 2-hydroxy-, phenylmethylester are detected. The peak area is the highest in Propanoic acid hexyl ester followed by Benzoic acid 2-hydroxy-, phenylmethyl ester so it can be stated that the antimicrobial activity is cumulative in nature [36]. assessed the antimicrobial activity of Butanoic acid against *Escherichia coli ATCC 25922, Escherichia coli F18, Salmonella enterica Typhimurium* ATCC 14028 *Salmonella enterica Typhimurium*, *Campylobacter jejuni* ATCC 33560, *Campylobacter jejuni, Enterococcus faecalis* ATCC 29212 *Clostridium perfringens* ATCC 12915 *Streptococcus pneumonia* ATCC 49619 and *Streptococcus suis* ATCC 43765, the MIC values for the Gram negative bacteria was found to be 2300 mg/L.

The MIC values of butyric acid and valeric acid were both 2000 mg/L for *Enterococcus faecalis* (*E.faecalis*),1200 and 1300 mg/L for *Clostridium perfringens* (*C. perfringens*), 700 and 1000 mg/L for *Streptococcus pneumoniae* (*S. pneumoniae*), and 700 and 1000 mg/L for *Streptococcussuis.* Medium chain fatty acids are also reported for their potential antimicrobial activities and their potential ability to suppress the development of antibiotic-resistant genes in bacteria [37]**.** Tested the ability of acetic acid to inhibit the growth of pathogens, obstructing the formation of biofilms and destroy the pre-formed biofilms.LAB bacteria have antimicrobial activities against pathogenic strains due to the antimicrobial substances produced including organic acids, proteinaceous substances, bacteriocin, plantaricin, hydrogen peroxide and biosurfactants [37].

Conclusion

Fermented bamboo shoots were used to isolate probiotic microbial strain which fulfill all the basic probiotic criteria. The isolate MBS17 was found to be the most potential probiotic strain and identified as *Lactobacillus plantarum* MBS17 on the basis of 16S rRNA gene sequencing with the accession number MW043712. The safety assessment was evaluated against antibiotics and it was found to be safe. *L.plantarum* MBS17 exhibited remarkable antimicrobial activity against respiratory and food pathogens indicating its application in food preservative. Synthetic chemical additives are widely used as preservatives which reduces the nutritional property of any food and in longer period they could be a source of carcinogens so *L.plantarum* MBS17 can be a suitable candidate for natural antimicrobial agents as it produce natural organic compounds.

Conflict of interest statement

The authors declare that they do not have any conflict of interest.

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