Acta Scientific Microbiology (ISSN: 2581-3226)

Volume 7 Issue 11 November 2024

Research Article

Exploring the Probiotic Capabilities of *Lactiplantibacillus plantarum* 022AE: *In Vitro* Characterization and Functional Insights

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DOI: 10.31080/ASMI.2024.07.1450

Received: August 12, 2024

Published: October 30, 2024

Abstract

Probiotic strains are emerging as promising organic means to improve overall well-being and are becoming integral to modern-day lifestyles. Their *in vitro* characterization to substantiate functional properties is fundamental to their successful development. In the present study, *Lactiplantibacillus plantarum* 022AE was thoroughly investigated for its *in vitro* probiotic characteristics. This strain demonstrated stability in both acidic and bile environments and exhibited excellent survival under gastrointestinal simulation conditions. The cell surface properties of *Lactiplantibacillus plantarum* 022AE showed a strong ability to adhere to mucin, suggesting its potential to persist in the gastrointestinal tract through mechanisms such as autoaggregation and affinity towards non-polar solvents. Additionally, the antimicrobial compounds produced by *Lactiplantibacillus plantarum* 022AE showed antagonistic effects against several pathogens, including *Clostridium perfringens* ATCC® 13124™ (a poultry pathogen), enterotoxin-producing *Bacillus cereus* ATCC 33019, and *Listeria monocytogenes* ATCC 19115. The strain also exhibited DPPH free radical scavenging ability, indicating significant antioxidant potential. Furthermore, its enzymatic capabilities, including the production of β-galactosidase and bile salt hydrolase, suggest its potential to alleviate lactose intolerance and reduce serum cholesterol levels. *Lactiplantibacillus plantarum* 022AE also demonstrated compatibility with industrial processing, showing stability under thermal stress and in liquid storage conditions. Overall, *Lactiplantibacillus plantarum* 022AE presents itself as a robust and potential probiotic strain with multiple beneficial properties.

Keywords: *Lactiplantibacillus plantarum*; Probiotic; Antimicrobial; Antioxidant; Cholesterol Reduction; Lactose Intolerance

Abbreviations

AMC: Antimicrobial Compound; ATCC: American Type Culture Collection; BAs: Bile Acids; BATH: Bacterial Adhesion to Hydrocarbons; BSH: Bile Salt hydrolase; CE: Cefixime; CFS: Cell Free Supernatant; CFU: Colony Forming Units; CP: Ciprofloxacin; DPPH: 2,2-diphenyl-1-picrylhydrazyl; EFSA: European Food Safety Authority; FDA: Food and Drug Administration; GI: Gastrointestinal; GRAS: Generally Regarded as Safe; ICH: International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; IPA: Isopropyl Alcohol; MRS: De Man–Rogosa–Sharpe; MTCC: Microbial Type Culture Collection; OD: Optical Density; ONPG: Ortho-nitrophenyl beta-D-galactopyranoside; PBS: Phosphate Buffered Saline; QPS: Qualified Presumption of Safety; ROS: Reactive Oxygen Species; SAD: Standard American Diet; SD: Standard deviation; SED: Standard European Diet; SGF: Simulated Gastric Fluid; SIF: Simulated Intestinal Fluid; SSF: Simulated salivary Fluid; TFA: TrifluoroAcetic Acid; β-gal: β-galactosidase

Introduction

Probiotics have gained significant attention in recent years due to a combination of scientific, medical, and consumer-driven factors. They are live microorganisms which when administered in suf-

ficient quantities have beneficial effects on the host [1]. Probiosis is a multifaceted process involving modulation of gut microbiota and immunity, production of bioactive metabolites, and elimination of pathogens, etc. [2]. Emerging research suggests a link between gut health and mental health, often referred to as the "gut-brain axis." Probiotics may help alleviate symptoms of anxiety, depression, and stress [3]. Increasing consumer interest in natural and preventive health measures has driven the demand for probiotics as part of a healthy lifestyle [4,5]. Probiotics are now widely available in the market as food products or dietary supplements. Regulatory bodies are working incessantly towards establishing quality standards and regulatory requirements for probiotic products. Probiotic activity is considered to be strain specific and every new emerging strain has to exhibit certain *in-vitro* properties to qualify as probiotic such as ability to establish and persist in gastro-intestinal tract, inhibit pathogens, produce beneficial metabolites, etc. Thus, *in vitro* functional characterization forms the primary basis for animal and clinical studies and overall product development of a probiotic strain. They provide a cost-effective way to gather preliminary data on the potential benefits and mechanisms of action of probiotics.

Lactiplantibacillus plantarum (*L. plantarum*) is a Gram-positive, facultative anaerobic bacterium recognized for its robustness and diverse metabolic capabilities [6]. Its long history of safe use in fermented foods supports its suitability for dietary supplementation [7]. Various strains of *L. plantarum* namely NCIMB 30562 [8], DSM 33452 [9], Lp-115 [10], 299v [11] have obtained GRAS (Generally Recognized as Safe) status by USFDA when consumed within recommended doses. Consequently, in recent years, extensive *in vitro* studies have highlighted its potential as a beneficial probiotic, particularly when used as a dietary supplement [12]. *In vitro* characterization has elucidated its ability to survive and thrive in the harsh conditions of the gastrointestinal tract [13], including tolerance to gastric acidity and bile salts [14]. This resilience underscores its potential to colonize the gut and exert beneficial effects on host health. Furthermore, research has demonstrated that *L. plantarum* strains exhibit strain-specific probiotic attributes, including the modulation of gut microbiota composition [15] and enhancement of mucosal barrier function [16]. These mechanisms contribute to improved digestion, nutrient absorption, and immune system modulation, which are crucial for maintaining overall health. *L. plantarum* strains are capable of producing different antimicrobial compounds, such as hydrogen peroxide, organic acids (primarily lactic and acetic acid), anti-aflatoxin and bacteriocins. The latter act against a wide range of bacterial pathogens, in the broad and narrow spectra. The plantaricins (or two-peptide bacteriocins) are usually produced by *L. plantarum*, e.g. E/F and J/K [17].

In this regard, a commercial probiotic strain *Lactiplantibacillus plantarum* 022AE (*L. plantarum* 022AE) was assessed for its *in vitro* probiotic potential. The strain's safety has been thoroughly evaluated earlier and meets the safety criteria for its intended use as a food ingredient or supplement. It has been given a GRAS status by USFDA (GRN 1108) [18] and is included in the QPS list [19]. In the present study, *L. plantarum* 022AE was extensively investigated for *in vitro* probiotic characteristics such as stability and survival in gastrointestinal simulations, adhesion properties, functional aspects such as antimicrobial and antioxidant activity, production of enzymes such as Bile Salt Hydrolase (BSH) and Beta- galactosidase.

Materials and Methods

Bacterial strains, media and chemicals

L. plantarum 022AE preparation (400×10^9 CFU/g) was manufactured by an in-house proprietary process at Advanced Enzyme Technologies Ltd. Pathogenic bacterial and yeast strains are mentioned in Table 1 along with their cultivation conditions and growth media. All the chemicals, reagents were procured from SigmaAldrich, India while microbiological media from HiMedia Labs Pvt. Ltd. India.

Acid and bile stability

Stability of *L. plantarum* 022AE cells when exposed to different pH and bile concentrations was analysed as described by Dixit., *et al*. [20]. Briefly, *L. plantarum* 022AE cells (2×10^9CFU/mL) were exposed to pH 1.5, 2.5, 3.0, 5.0 & 7.0 and bile salt solutions of 0.01, 0.1, 0.2, 0.3, 0.5, 0.7, and 1.0%, w/v at 37°C. Stability was evaluated in terms of viable activity analysed by a standard viable count method using pour plate technique. One mL of sample was withdrawn every hour from each set up to 5 h. Withdrawn samples were 10-fold serially diluted in tween peptone water [compositions, (g/L): proteose peptone 10.0, sodium chloride 5.0, disodium hydrogen phosphate 3.5, monosodium dihydrogen phosphate 1.5, tween-80 2.0] and pour plated in pre-sterilized molten MRS agar (M963). Solidified agar plates were incubated at 37°C for 48 h. Viable activity was expressed in colony forming units per mL (CFU/mL) by taking the mean of three independent analyses.

Sr. no.	Pathogens	Growth medium	Assay medium
1	*Bacillus cereus* ATCC 33019	Nutrient broth	Mueller Hinton agar
2	*Bacillus circulans* ATCC 4516	Nutrient broth	Mueller Hinton agar
3	*Bacillus subtilis subsp. spizizenii* ATCC 6633	Brain heart infusion broth	Mueller Hinton agar
4	*Candida albicans* ATCC 90028	Potato dextrose broth	Mueller Hinton agar
5	*Clostridium difficile* ATCC 9689	Reinforced Clostridial medium broth #	Reinforced Clostridial medium agar #
6	*Clostridium perfringens* ATCC 13124	Reinforced Clostridial medium broth #	Reinforced Clostridial medium agar #
7	*Clostridium sporogenes* NCIM-5125(Equivalent to ATCC 19404)	Reinforced Clostridial medium broth #	Mueller Hinton agar
8	*Enterobacter cloacae* ATCC 13047	Nutrient broth	Mueller Hinton agar
9	*Escherichia coli* ATCC 700728	Nutrient broth	Mueller Hinton agar##
10	*Escherichia coli* ATCC 9002 NCTC	Nutrient broth	Mueller Hinton agar
11	*Klebsiella pneumoniae* ATCC BAA-1144	Soybean casein digest broth	Mueller Hinton agar
12	*Listeria monocytogenes* ATCC 19115	Brain heart infusion broth	Brain heart infusion agar
13	*Micrococcus luteus* MTCC 106T	Brain heart infusion broth	Mueller Hinton agar
14	*Pasteurella multocida* ATCC 12945	Brain heart infusion broth	Tryptone soy agar
15	*Pseudomonas aeruginosa* ATCC 9027	Nutrient broth	Mueller Hinton agar
16	*Salmonella abony* NCIM-2257(Equivalent to ATCC 6017 NCTC)	Nutrient broth	Mueller Hinton agar
17	*Salmonella enterica* ATCC 14028	Nutrient broth	Mueller Hinton agar
18	*Staphylococcus aureus* ATCC 6538P	Nutrient broth	Mueller Hinton agar

Table 1: Bacterial and yeast pathogenic strains used in the present study.

#: Supplemented with 1g/L L-Cysteine; ##:supplemented with 5 g/L yeast extract.

In vitro stability of *L. plantarum* 022AE in static gut model conditions

The *in vitro* stability of *L. plantarum* 022AE was evaluated using a static gut model and various dietary substrates. Specifically, *L. plantarum* 022AE preparation was aseptically added to 100 mL of distilled water, pasteurized milk, powdered baby food, the standard American diet (SAD), and the standard European diet (SED) to a final viable count of 1 ×10^9 CFU/mL. For the static gut model simulation, 5 mL of each *L. plantarum* 022AE-enriched food sample was used. The simulation of gastrointestinal digestion followed the standard harmonized method established by the COST INFOGEST network [21]. Electrolyte solutions were prepared for the oral (SSF), gastric (SGF), and intestinal (SIF) master mixes. Each experiment was carried out under aseptic conditions with freshly prepared digestive fluids, including enzyme solutions, bile, and pH adjustments. The *L. plantarum* 022AE-supplemented diets underwent sequential exposure to simulated salivary fluid (2 minutes at pH 7.0), simulated stomach fluid (2 hours at pH 3.0), and simulated intestinal fluid (2 hours at pH 7.0) while being agitated at 50 rpm and 37°C. After the designated gastrointestinal transit times, 1.0 mL samples were extracted from the reaction flasks, and the viable activity was determined using the pour plate method as described previously.

Cell surface hydrophobicity

The bacterial adhesion to hydrocarbons (BATH) was used to measure the hydrophobicity of the cell surface of *L. plantarum* 022AE [22]. Briefly, overnight grown culture of *L. plantarum* 022AE cells was centrifuged and washed pellet was resuspended in PBS at a pH of 7.4. Optical density at 600 nm was adjusted to 1.0 (A_0) was recorded. Liquid-liquid extraction was carried out with equal volumes of organic solvents of different polarities namely xylene, ethyl acetate, toluene by vortexing for 5 min at 1800 rpm (Labquest, Borosil, MTV012). Aqueous and organic phases were allowed to separate during the incubation for 30 min at 37 °C. Optical density at

600 nm (A_1) was recorded for the aqueous layer of the two layers. Percentage cell surface hydrophobicity of the bacterial cells adhering to solvents was calculated using the following equation.

$$\text{Cell Surface Hydrophobicity} = \frac{A_0 - A1}{A0} \times 100$$

Auto-aggregation

For the auto-aggregation test, *L. plantarum* 022AE cells were harvested by centrifugation from an overnight culture grown in MRS broth at 37 °C at 120 rpm. The resulting pellet was washed and re-suspended in a PBS to OD_{600} at 0.70 ± 0.05. Suspension was incubated at 37 °C for 6 h, mixed for 10 seconds, and the OD600 of the samples was measured [23]. The auto-aggregation percentage was calculated using the following equation:

$$\text{Auto} - \text{aggregation}\ (\%) = \frac{A_0 - A_6}{A_0} \times 100$$

Where (A_6) represents the absorbance at 6 h, and (A_0) represents the initial absorbance.

Co- aggregation with pathogens

Similar to, autoaggregation, overnight grown cultures of *L. plantarum* 022AE and pathogenic bacteria (Table 1) were centrifuged at 3500 rpm for 15 min to obtain the pellet. After subsequent washing of pellet with PBS twice, OD_{600} was adjusted to 0.7 ± 0.05 for *L. plantarum* 022AE and each pathogen. Equal volumes of *L. plantarum* 022AE and pathogen cell suspensions were mixed and incubated at 37°C under static condition. OD_{600} was determined at 0 and 6 h [24]. Co-aggregation (%) was determined using the equation below:

$$Co - aggregation\ (\%) = \frac{\left(\frac{Apat + Aprobio}{2} - Amix\right)}{\left(\frac{Apat + Aprobio}{2}\right)} \times 100$$

Apat, Aprobio = the absorbance of the pathogen and the probiotic strain at time t, respectively;
Amix = the absorbance of the mixed culture at time t.

Mucin adhesion

Adhesion to mucins was studied as per the protocol given by Mazzantini., *et al*. [25] with slight modifications. Briefly, 1 mL glycerol stock of *L. plantarum* 022AE was inoculated in MRS broth and grown overnight at 37°C, 120 rpm. This inoculum was transferred aseptically to fresh MRS broth (100 mL) under same cultivation conditions and grown to an OD_{600} of ~ 1.5. Cultures were centrifuged at 4500 rpm for 10 min at 4 °C, pellets washed twice and reconstituted with sterile PBS. The suspensions (OD_{600} ~1.5) 100 µL were added to 96 well plates (Nunc® Edge 2.0, Sigma) containing 120 µL of mucin agar (pH 6.8) (Test). Suspension incubated with only 1% (w/v) bacteriological agar served as agar control. Plates were incubated at 37 °C at 50 rpm. After 90 min incubation, the liquid phase was discarded and wells washed two times with 100 µL of PBS to dislodge loosely adhered cells. Solidified mucin and bacteriological agar were removed mechanically using sterile spatula and homogenized in 1.5 ml of peptone saline. This sample was serially diluted 10-fold and pour plated in MRS agar. Viable activity was determined in terms of CFU/well for both agar control and mucin test well.

B-galactosidase activity (β-gal)

β-galactosidase activity of *L. plantarum* 022AE was carried out as described by Harley and Prescott [26]. Briefly, overnight grown *L. plantarum* 022AE cells were inoculated in sterile ONPG broth and incubated at 37°C for 24 h under aerobic condition. Yellow coloration compared with uninoculated ONPG broth indicated β-galactosidase activity.

Bile salt hydrolase activity (BSH)

BSH activity of *L. plantarum* 022AE was assessed by growing in Soft MRS agar containing 0.3% Ox bile salt and 0.37 g/L $CaCl_2$ for 48h under an atmosphere of 5% v/v CO_2 environment [27]. Precipitation around the colony indicated BSH activity.

Antioxidant activity via DPPH radical scavenging

Antioxidant activity by scavenging of DPPH radical was measured by 96-well microtitre plate assay as described by Cai., *et al*. [28] with modifications. Briefly, 150 µL of 0.20 mM DPPH reagent was mixed separately with equal volumes of PBS (Blank), 100 µg/mL ascorbic acid, uninoculated MRS broth (medium control), washed cell pellet re-suspended in PBS (OD_{600} 1.0) and CFS of *L. plantarum* 022AE grown in MRS broth (Test). In addition, alcohol blanks were included for test and medium control. Post incubation at 37°C in the dark absorbance was read at 517 nm. The DPPH-free

radical scavenging activity for *L. plantarum* 022AE CFS was calculated by following equation:

$$Radical\ scavenging\ activity\ (\%RSA) = \left\{\left(1-\frac{A_t - A_{tb}}{A_d}\right)\times 100\right\} - \left\{\left(1-\frac{A_m - A_{mb}}{A_d}\right)\times 100\right\}$$

Where A_d represents absorbance of DPPH; A_t represents absorbance of test; A_{tb} represents absorbance of alcohol blank of test; A_m represents absorbance of medium control; A_{mb} represents absorbance of alcohol blank of medium control. A_d represents absorbance of 0.20 mM DPPH. This ensured elimination of error arising due to uninoculated medium. The DPPH-free radical scavenging activity for *L. plantarum* 022AE cell pellet was calculated by following equation:

$$Radical\ scavenging\ activity\ (\%RSA) = \left(1-\frac{A_t}{A_d}\right)\times 100$$

Antimicrobial activity

Production and extraction of antimicrobial compound (AMC) from *L. plantarum* 022AE was carried out as described by Dixit., *et al*. [20] with modifications based on the specific growth requirements. Overnight grown *L. plantarum* 022AE was mixed with XAD16N beads and allowed to grow on clarified MRS agar at 37°C for 5 days. AMC adsorbed onto XAD16N beads was eluted using 80% isopropanol (IPA) containing 0.1% trifluoroacetic acid (TFA), concentrated by Rotavapor (Rotavapor® R-300, Buchi, Switzerland) and analyzed. Ability of *L. plantarum* 022AE to antagonize 18 pathogens was studied by spot-on-the-lawn assay [29]. Zone of inhibition (mm) obtained against each pathogen was recorded.

Thermal and liquid stability of *L. plantarum* 022AE

L. plantarum 022AE cell suspension (2 ×10^9 CFU/mL) was prepared in different liquid matrices namely, aqueous (distilled water), aqueous-glycerol (20% glycerol), buffer (Mcilvaine buffer pH 6.5), buffer-glycerol (20% glycerol in Mcilvaine buffer pH 6.5), oil (sunflower oil) and emulsion (sunflower oil). Stability was evaluated as per ICH-guidelines (Q1A(R2) for long-term (Real time) stability at 5 ± 3 °C and accelerated stability at 25 ± 2 °C, 60% ± 5% RH [30]. Sampling and viable activity analyses were done at day 0, 1, 2, 3, 6, 9, and 12th month as per the procedure as described earlier. Thermal stability was evaluated by analysing viable activity of *L. plantarum* 022AE cell suspension (2 ×10^9 CFU/mL) exposed to temperatures- 4, 25, 30, 40, 50°C for 6 h.

Statistical analysis

All the experiments were performed in independent triplicates and data were expressed as the mean ± standard deviation (SD) of Log_{10}CFU/g or mL. Both statistical analyses and graphs were prepared using GraphPad Prism version 8.0.2 (GraphPad Sofware Inc., USA, https://www. graphpad.com/scientifc-sofware/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. For mucin adhesion assay, the two-tailed Student's t-test was used to compare the CFU/well obtained for agar control and mucin test wells.

Results

Acid and bile stability

L. plantarum 022AE cells remained viable at pH 3.5 to 7.0 up to 5 h with no significant difference in the viable activity 9.137 Log_{10}CFU.mL^{-1} compared with initial count 9.300 Log_{10}CFU.mL^{-1} (P-value = 0.1668). At pH 2.5, viable activity showed no significant reduction up to 3 h (P-value = 0.0998); but reduced significantly to 8.693 Log_{10}CFU.mL^{-1} after 4 h (P-value = 0.0088) and 5 h 8.683 Log_{10}CFU.mL^{-1} (P-value = 0.0103). Viability of *L. plantarum* 022AE cells was significantly affected at pH 1.5 within one hour of exposure (P-value = 0.0063). After 5 h, 6.480 Log_{10}CFU.mL^{-1} remained viable from initial count of 9.300 Log_{10}CFU.mL^{-1} (P-value = 0.0001) (Figure 1a).

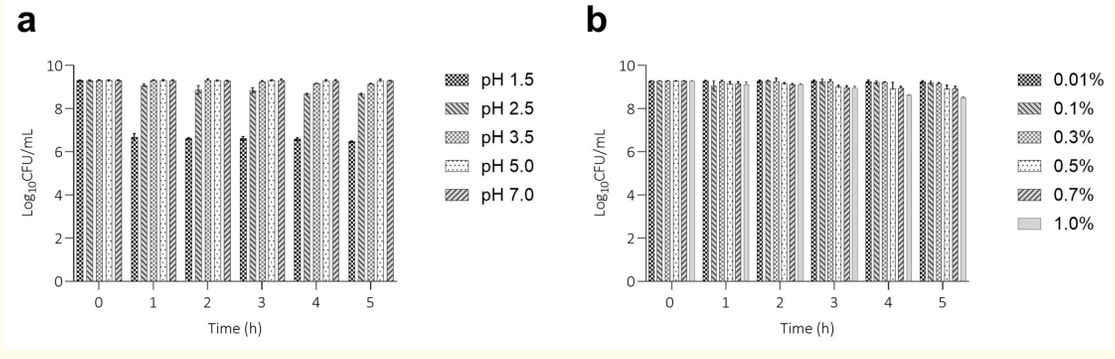

Figure 1: Viable activity of *L. plantarum* 022AE cells at (a)different pH values and (b)concentrations of bile, up to 5 h, expressed as mean ± SD.

Viable activity of *L. plantarum* 022AE cells remained unaffected (Initial viability 9.293 $Log_{10}CFU.mL^{-1}$) at bile concentration of 0.01 to 0.7 % up to 5 h (8.927 $Log_{10}CFU.mL^{-1}$); no statistical significance was noted (P-value = 0.0997). At bile concentration as high as 1%, viable activity showed no significant difference up to 3 h (8.950 $Log_{10}CFU.mL^{-1}$, P-value = 0.0856). After 4 and 5 h, 8.620 and 8.500 $Log_{10}CFU.mL^{-1}$ remained viable with P-value = 0.0008 and 0.0021, respectively (Figure 1b).

In vitro stability of *L. plantarum* 022AE in static gut model

L. plantarum 022AE cells in free form (without any food matrix mimicking fasting conditions) survived all the three phases of static gut model i.e. salivary, gastric and intestinal (Figure 2). Exposure of 2h in gastric compartment showed no significant reduction in viability (P-value = 0.7929). Viability of *L. plantarum* 022AE free cells was 9.010 $Log_{10}CFU.mL^{-1}$ at the end of the intestinal phase (240 min, P-value = 0.7748). In presence of various food matrices viable activity of *L. plantarum* 022AE cells remained unaffected (milk and powdered baby food) or improved as in case of SAD and SED. Viable activity of *L. plantarum* 022AE cells was 9.253 and 9.117 $Log_{10}CFU.mL^{-1}$ in presence of milk and powdered baby food respectively. In case of SAD food matrix, in gastric phase *L. plantarum* 022AE cells showed no significant difference in viability (9.313 $Log_{10}CFU.mL^{-1}$, P-value = 0.2542) from initial (9.040 $Log_{10}CFU.mL^{-1}$); in intestinal phase its viability improved (9.470 $Log_{10}CFU.mL^{-1,}$ P-value = 0.0205). Similarly, presence of SED improved viability of *L. plantarum* 022AE cells after entering intestinal phase (9.407 $Log_{10}CFU.mL^{-1,}$ P-value = 0.0052).

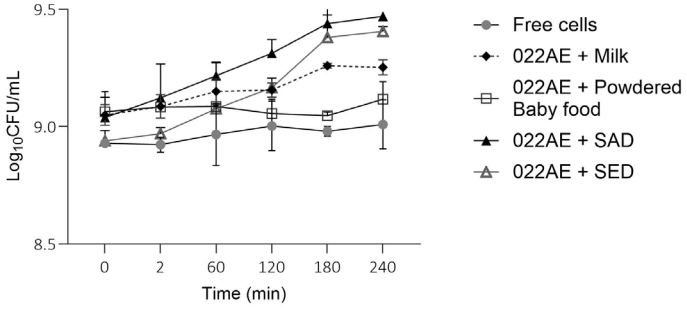

Figure 2: Viable activity of *L. plantarum* 022AE cells under *in vitro* static gut model under fasting (free cells) as well as fed (Food Matrices-Milk, Powdered baby food, Standard American diet-SAD, Standard European diet-SED), expressed as mean ± SD.

Cell surface properties and mucoadhesion

Bacterial adhesion of *L. plantarum* 022AE to non-polar solvents showed maximum adhesion to ethyl acetate (25.2 ± 0.08%) followed by xylene (21.23 ± 0.15%) and least to toluene (17.55 ± 0.08%) in 6 h. Autoaggregation for *L. plantarum* 022AE was 16.13% in 6 h. Co-aggregation of *L. plantarum* 022AE was seen highest with *C. albicans* ATCC 90028 (20.45%), *S. aureus* ATCC 6538P (14.75%) and lowest with *P. aeruginosa* ATCC 9027 (6.70%). *L. plantarum* 022AE showed adherence to mucin as indicated by significant difference between mean CFU/well for mucin test ($3.06 \pm 1.37 \times 10^8$) and agar control ($1.10 \pm 0.45 \times 10^8$) with P-value 0.034.

ß-galactosidase activity

β-galactosidase activity was determined by using ONPG method, development of yellow coloration was observed after 24 h of inoculation of *L. plantarum* 022AE in sterile ONPG broth indicating production of β galactosidase.

Bile salt hydrolase activity

Visible halos around the spot growth further surrounded by precipitation were observed for *L. plantarum* 022AE indicating production of bile salt hydrolase.

Antioxidant activity

Antioxidant potential of *L. plantarum* 022AE was evaluated in terms of its ability to scavenge DPPH free radicals. Cell free supernatant showed 24.61 ± 3.03% scavenging while that for the intact cells was 18.17 ± 4.41%. Activity seen for standard ascorbic acid was 39.45 ± 1.01%.

Antimicrobial activity

Antimicrobial activity was checked against 18 pathogens by spot-on-the-lawn assay method (Table 2). *L. plantarum* 022AE AMC showed zone of inhibition against 15 tested pathogens out of 18 pathogens, no activity was seen against *Pasteurella multocida* ATCC 12945, *Candida albicans* ATCC 90028, *M. luteus* MTCC 106^T (Figure 3).

Thermal stability of *L. plantarum* 022AE

Survival of *L. plantarum* 022AE cells was studied at various temperatures (4-50^0C) up to 6h wherein considerably good stabil-

ity at high temperatures (50^{0}C) was recorded (Figure 4). Viability of *L. plantarum* 022AE cells remained unchanged at 4-25^{0}C up to 6h (9.037 Log_{10}CFU.mL^{-1}, P-value = 0.0578). *L. plantarum* 022AE cells were stable at 40^{0}C till 5h (9.110 Log_{10}CFU.mL^{-1}, P-value = 0.1903) and 50 ^{0}C till 2h (8.877 Log_{10}CFU.mL^{-1}, P-value = 0.1011) post which viability reduced significantly. Viability at 6h was 8.967 Log_{10}CFU.mL^{-1} (P-value = 0.0408) and 5.517 Log_{10}CFU.mL^{-1} (P-value = 0.0053) at 40 and 50°C, respectively.

Liquid Stability of *L. plantarum* 022AE

In presence of various liquid matrices, the viability of *L. plantarum* 022AE cells was checked for 1, 2, 3, 6 and 9 months under 4°C and 25°C. At 4°C, *L. plantarum* 022AE preparations showed 8.973 Log_{10}CFU/mL (P-value = 0.0527, 97.8%) in Mcilvaine buffer and 6.217 Log_{10}CFU/mL (P-value = 0.0005, 66.8%) in DW during 6 months of storage. Matrices such as oil (3.043 Log_{10}CFU/mL, P-value<0.0001) and buffer-glycerol (1.583 Log_{10}CFU/mL, P-value = 0.0001) showed the significant reduction in viability after 6 months. Viability was completely lost in aqueous glycerol after 3 months (Figure 5-a).

Sr. no.	Pathogen	022AE AMC	Positive control (10 µg/mL)
1	*Bacillus cereus* ATCC 33019	7.6	9 ^{CP}
2	*Bacillus circulans* ATCC 4516	12.83	20 ^{CP}
3	*Bacillus subtilis subsp. spizizenii* ATCC 6633	17.33	24 ^{CP}
4	*Candida albicans* ATCC 90028	0	0
5	*Clostridium difficile* ATCC 9689	12.83	14 ^{CP}
6	*Clostridium perfringens* ATCC® 13124™	13.8	15 ^{CP}
7	*Clostridium sporogenes* NCIM-5125*(*Equivalent to ATCC 19404)	10	29 ^{CP}
8	*Enterobacter cloacae* ATCC 13047	12	12.5 ^{CE}
9	*Escherichia coli* ATCC 700728	11	22.5 ^{CE}
10	*Escherichia coli* ATCC 9002 NCTC	11	21 ^{CE}
11	*Klebsiella pneumoniae* ATCC BAA-1144	9	26 ^{CE}
12	*Listeria monocytogenes* ATCC 19115	9.33	15 ^{CE}
13	*Micrococcus luteus* MTCC 106^T	0	10.5 ^{CP}
14	*Pasteurella multocida* ATCC 12945	0	33.5 ^{CE}
15	*Pseudomonas aeruginosa* ATCC 9027	11.35	25.5 ^{CP}
16	*Salmonella abony* NCIM-2257	9.1	26 ^{CP}
17	*Salmonella enterica* ATCC 14028	12.33	25 ^{CE}
18	*Staphylococcus aureus* ATCC 6538P	10.6	0

Table 2: Zone of inhibition (mm) shown by *L. plantarum* 022AE.

CE: Cefixime; CP:Ciprofloxacin.

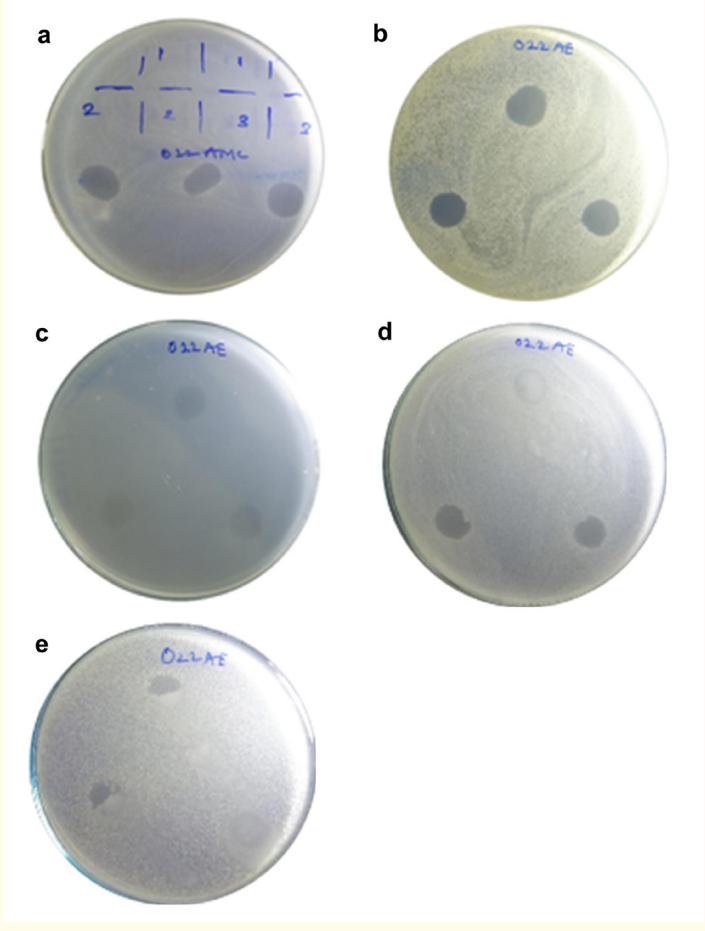

Figure 3: Zone of inhibition exhibited by AMC produced by *L. plantarum* 022AE against (a) *S. enterica* ATCC 14028 (b) *B. subtilis* ATCC 6633 (c) *B. circulans* ATCC 4516 (d) *Cl. difficile* ATCC 9689 and (e) *Cl. perfringens* ATCC 13124.

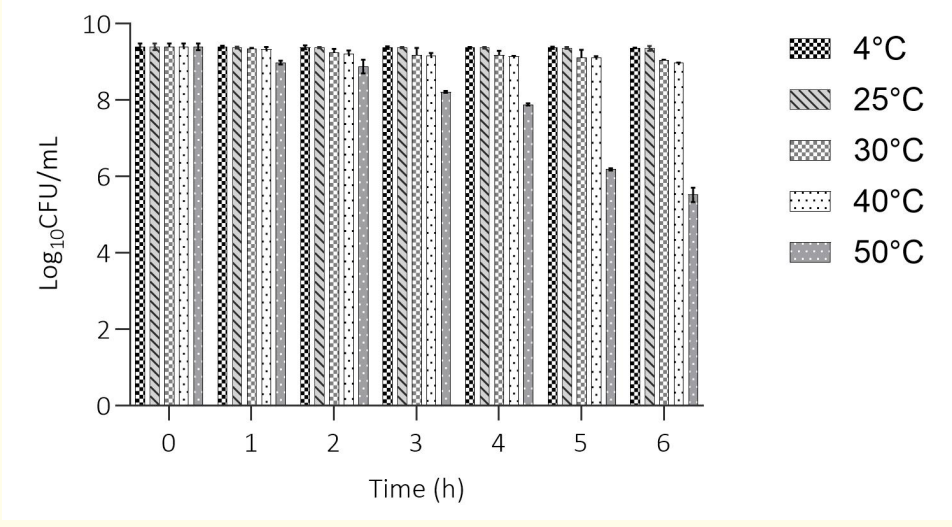

Figure 4: Viable activity of *L. plantarum* 022AE cells at different temperatures up to 6h.

After six months of storage at 25°C, the preparation of *L. plantarum 022AE* exhibited the highest viability 5.367Log_{10}CFU (89.5%, P-value = 0.0012) in buffer matrix, followed by aqueous 5.367 Log_{10}CFU (57.6%, P-value<0.0001). Lowest viability was observed in matrices such as oil emulsion 1.967 Log_{10}CFU (21.5%) and buffer-glycerol 0.4433 Log_{10}CFU (4.9%). No viable activity was found in aqueous glycerol and oil after 3 months (Figure 5-b).

Discussion

Probiotic bacteria show humungous potential in benefitting the host, when consumed in adequate amounts. Each probiotic is considered unique as the properties exhibited are strain specific. This necessitates evaluation of each strain in vitro, at pre-clinical and clinical level. *In vitro* evaluation thus forms the basis for the probiotic product development. In the present study, we evaluated

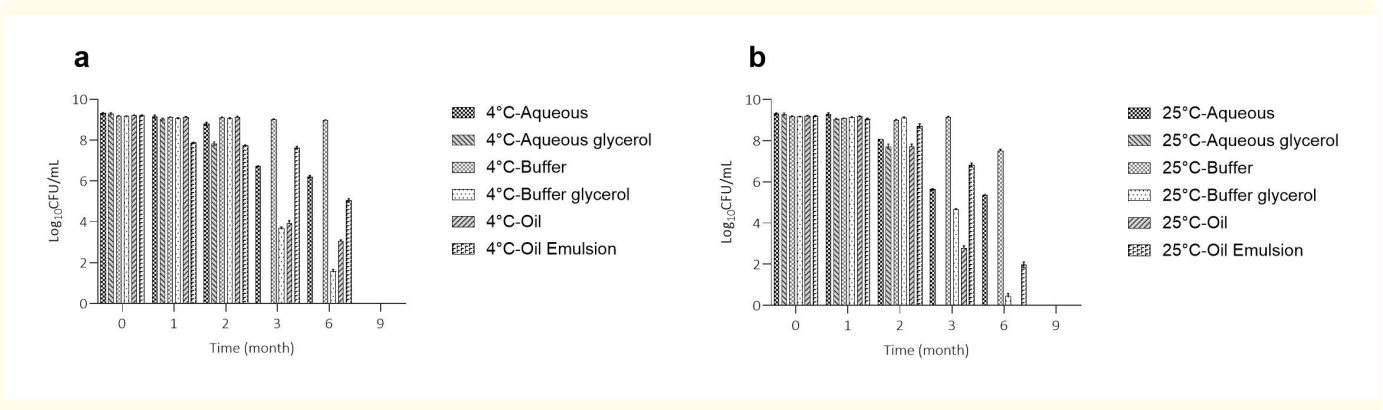

Figure 5: Viability of *L. plantarum* 022AE cells in different matrices for 1, 2, 3, 6 and 9 months at 4°C (a) and 25°C (b).

suitability of a commercial probiotic strain *L. plantarum* 022AE as a probiotic by thorough investigation of its *in vitro* probiotic functional characteristics.

Following oral ingestion, probiotic bacteria encounter a number of human defence systems that are associated with secretions, among them are stomach acid and intestinal bile [31]. For them to reach colon in adequate numbers, tolerance to acid, bile and digestive enzymes is very essential. In the present study, *L. plantarum* 022AE cells showed good stability in acidic environment at pH 2.5 for 5 h (93.36% viability) and viability was adversely affected only at extreme pH of 1.5 (69.67% viability). It showed very good tolerance to various bile concentrations up to as high as 1.0% for 5 h (91.46 % viability). Both these outcomes correlated well when *L. plantarum* 022AE was studied in static *in vitro* gut model. Different phases of gastrointestinal digestion namely- oral, gastric and intestinal phase were simulated *in vitro* and probiotic strain was exposed to each one after the other for a predetermined duration. Additionally, in this unique study, fasting conditions were mimicked by exposing the strain in absence of any food matrices and fed conditions in presence of them. Viability of *L. plantarum* 022AE was maintained under all tested conditions throughout the simulated GI conditions. Specifically designed diets such as SAD, SED improved viability of the strain which was evidently noted in the intestinal phase. Thus, *L. plantarum* 022AE showed ability to survive *in vitro* GI conditions which may help establish itself in human gut. *L. plantarum* GXL94 showed similar acid and bile stability where it maintained 96% viability at pH 2.5, 95% at 1.0% bile, 99% in simulated gastric juice and 95% in simulated intestinal juice for 3 h [32]. *L. plantarum E680* showed good tolerance to acid (pH 2.0, 85%) and bile (0.3%, 80%) for 3 h [33]. Zhong., *et al*. [34] reported survival of various strains of *L. plantarum* -B2 (98%), YJ24 (93%), YJ14 (90%), and HN9 (92%) in simulated gastric juice for 3 h. In general, tolerance to acid and bile salts varies from strain to strain due to differences in the origin of the strain and experi-

mental conditions. Mechanisms of acid and bile tolerance are very well studied in *L. plantarum*. Acid stress affects bacterial cell viability by interfering in intracellular pH homeostasis. According to the Hamon., *et al*. [35] cell protecting proteins GrpE, MetE and RpsB are induced in response to acid stress and are abundantly seen in the constitutive proteome of acid resistant phenotypes of *L. plantarum*. On the other hand, bile stress is a multifaceted impact including detergent action, low-pH, oxidative and osmotic stresses. Therefore, the ability of microbes to tolerate bile and bile acids is recognised as important for their survival and persistence in the GI tract. General stress response proteins ClpP, Dps, GroEL, Hsp1, and Hsp3 were shown to play role in bile stress. Proteins GshR1, GshR4 were found to be protective against oxidative stress while OpuA (a representative ABC transporter), was abundantly found in resistant strains of *L. plantarum* in response to osmotic stress [36].

Successful colonization of GI tract by a probiotic strain further depends on its cell surface properties. Intestinal mucosa is considered as excellent niche for probiotics. Interaction of probiotics with mucus layer is primarily via its most abundant glycoprotein-mucin [37]. Thus, ability to adhere to mucin *in vitro*, forms an integral part of the probiotic characterization. *L. plantarum* 022AE was able to adhere to mucin, in vitro, as assessed by statistical significance. Various other strains of *L. plantarum* have shown mucoadhesion ability to varying degree. *L. plantarum* UBLP40 showed similar mucoadhesion [38]. Out of 31 strains of *L. plantarum* studied by Tallon., *et al*. [39] 299V (human intestine), CBE and FV (corn silage) showed very good mucoadhesion where as MRS22 (Chikwangue), 415RW (cow milk) showed poor adhesion. Ability to form autoaggregates further facilitates colonization of GI tract in higher numbers. *L. plantarum* 022AE showed 16.13% autoaggregation. Coaggregation of probiotic with pathogens may exclude pathogens from attaching to intestinal mucosa thus facilitating their elimination. *L. plantarum* 022AE could coaggregate with *C. albicans* ATCC 90028 and *S. aureus* ATCC 6538P. If a probiotic strain has affinity towards non-polar solvents *in vitro*, it may adhere to non-polar GI surfaces better, *in-vivo*. *L. plantarum* 022AE showed affinity towards xylene (21.23 ± 0.15%). In comparison, *L. plantarum* BBC33 showed 24.8% affinity to xylene and 37.2% autoaggregation [40]. Four strains of *L. plantarum* namely LpE, LpF, LpG, LpH showed autoaggregation in the range of 8 to 20% [41]. *L. plantarum* strains isolated from infant faeces (68-72%), showed better mucoadhesion compared with the isolates from shrimp intestines (47-53%) and fermented foods (32-55%) [42]. Variation in the cell surface properties among different strains, ascribed to strain property and origin of isolation is well reported. Overall, good cell surface properties and excellent adhesion to mucin indicate that *L. plantarum* 022AE may be able to adhere to intestinal epithelium. Further *in vitro* investigation on adhesion to intestinal cell lines would be required to substantiate the same.

β-galactosidase is responsible for hydrolysis of lactose into subunits galactose and glucose. Probiotic bacteria having ability to produce β-galactosidase are presumed to have role in alleviating symptoms of lactose intolerance [43]. In the present study, ONPG broth was used to detect β-galactosidase production. β-galactosidase acts on ONPG substrate (similar to lactose) and cleaves it into galactose and o-nitrophenol. The release of o-nitrophenol turns the broth yellow indicating production of enzyme β-galactosidase while uninoculated ONPG broth remains colourless [44]. Similar to *L. plantarum* 022AE, several other strains namely *L. plantarum* Ln4 and G72 have been reported to produce β-galactosidase [45]. Bile salt hydrolase plays a specific role in reduction in serum cholesterol levels. Primary bile acids produced at the expense of cholesterol in liver are conjugated to form bile salts. These are then excreted in small intestine and utilised for solubilization of dietary lipids. Large proportion of these salts are brought back to liver via enterohepatic circulation. The remaining portion reaches colon where gut bacteria produce bile salt hydrolase to bring about deconjugation of bile salts. These further undergo transformation into secondary bile acids and are excreted through faeces. Resultant decrease in proportion of conjugated bile salts available for lipid solubilisation redirects liver to produce more of primary bile acids using cholesterol, thus leading to a serum cholesterol-lowering effect [46]. Further, secondary BAs produced as a result of BSH activity modulate lipid and glucose metabolism via the interaction with several receptors involved in mechanisms regulating host energy harvest [47]. The bile salt hydrolase assay using ox bile and calcium chloride works on the principle of detecting the enzymatic hydrolysis of conjugated bile salts into deconjugated bile acids and amino acids. Bile salts, such as taurocholate or glycocholate present in ox bile are hydrolysed by bile salt hydrolase releasing free bile acids and taurine or glycine. The deconjugated bile acids released during the hydrolysis react with calcium chloride and precipitate as insoluble calcium salts. Visual detection of precipitate indicated production of bile salt hydrolase. *L. plantarum* 022AE showed ability to produce bile salt hydrolase *in vitro*, indicating its potential to reduce serum cholesterol. *L. plantarum*

is widely known for its hypocholesterolemic action both *in vitro* and *in vivo* [46].

Reactive oxygen species (ROS) are produced as a byproduct of numerous metabolic reactions brought about in living cells. Certain level of these ROS aids in immune response against pathogens; their exceeding levels are harmful due to the exerted oxidative stress. Probiotic bacteria are known to reduce oxidative stress via various mechanisms including free radical-scavenging [48]. In the present study we studied ability of *L. plantarum* 022AE to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical. Cell free supernatant of *L. plantarum* 022AE showed 24.60 ± 3.03% (18 µg ascorbic acid equivalents) while that for cell pellet was 5.28 ± 0.42% (~4 µg ascorbic acid equivalents). These results indicated that antioxidant activity was associated with extracellular metabolites of *L. plantarum* 022AE showing postbiotic potential. Similarly, that *L. plantarum* DMDL 9010 showed higher DPPH free radical scavenging activity in cell free supernatant compared with cell precipitates [49]. In contrast, Ahire., *et al*. have shown antioxidant activity of *L. plantarum* UBLP40 to be associated with cell wall components [38].

Antimicrobial activity of probiotics is integral to its functional characterization. Ability to antagonize pathogens gives protection to host from infectious diseases. *L. plantarum* demonstrates antimicrobial effect through production of bacteriocins. We observed production of antimicrobial compound (AMC) by probiotic strain *L. plantarum* 022AE on solid medium adsorbed onto Amberlite® XAD16N and extracted using 80% IPA-0.1% TFA. The AMC showed noticeable inhibition of few key pathogens namely *Bacillus subtilis subsp. spizizenii* ATCC 6633 and *Clostridium perfringens* ATCC® 13124™ (Poultry pathogen), enterotoxin producing *Bacillus cereus* ATCC 33019 and *Listeria monocytogenes* ATCC 19115. In comparison, *L. plantarum* Q7 strain also showed anti-listerial activity inhibiting formation of biofilm *Listeria monocytogenes* ATCC 19115 [50]. Production of plantaricins and other bacteriocins by numerous strains of *L. plantarum* isolated from fermented foods is reviewed extensively by Rocchetti., *et al*. [51]. Similar to these strains, whole genome sequencing annotation for *L. plantarum* 022AE revealed presence of two-peptide plantaricin gene (Data not shown) confirming ability to antagonize pathogens, *in silico*. Further purification and characterization studies would be needed to identify the antimicrobial compound produced by *L. plantarum* 022AE.

Probiotic bacteria get exposed to harsh manufacturing and processing conditions before reaching the consumer. Their viability may get compromised during this stress affecting overall quality of a probiotic product. A strain showing good tolerance to thermal stress or good stability under ambient storage conditions gets better industrial acceptance. *L. plantarum* 022AE cells showed reasonably good thermal resistance at 50°C (1.54 h D value); at temperatures lower than (4-40°C) that it showed 95% viability up to 6 h with D values ranging from 195 to 16 h. Thermal resistance of various other *L. plantarum* strains has been studied and the outcomes are in line with ours. As an example, the mean D value of 20 *L. plantarum* strains ranged from 0.8 to 19 min at 55°C [52]. *L. plantarum* strains heat shock responses studied by Angelis., *et al*. [53] revealed induction of DnaK and GroEL in *L. plantarum* DPC2739 cells adapted to heat stress when in mid-exponential phase. These proteins may play a role in heat resistance through temperature sensing, chaperone activity, function control and stability of ribosome.

Another interesting aspect of probiotics of industrial utility is their stability in liquids. We studied viability of *L. plantarum* 022AE in different liquid matrices which mimic the key ingredients in liquid products available in the market as foods or dietary supplements. Following ICH guidance, under refrigerated (97.8% viability) as well as ambient temperature (89.5% viability) storage, *L. plantarum* 022AE remained stable only in buffer for 6 months. Matrices such as aqueous glycerol and buffer glycerol did not support viability of *L. plantarum* 022AE. Incorporation of *L. plantarum* 022AE in liquid dietary supplements seems promising with stability up to 6 months whereas oil based products may not suitable for storage and viability.

Probiotics being live microorganisms coming from natural habitat or fermented foods are being considered as an organic alternative to modern medicine by consumers globally. While the real worth of probiotic consumption can be substantiated only through clinical evidence, the basis for its functionality is formed *in vitro*. In the present work, *L. plantarum* 022AE showed excellent probiotic functionalities when studied *in vitro*. It can survive GI conditions and has ability to persists in GI tract via adhesion and other cell surface properties. It has broad spectrum antagonistic activity and antioxidant potential. Its enzyme functionalities can be extrapolated to its probable role in alleviation of lactose intolerance and hypocholesterolemic action. Additionally, it also showed promis-

ing industrial compatibility. In conclusion, *L. plantarum* 022AE is a highly potential probiotic strain for human and animal nutrition.

Acknowledgement

Authors gratefully acknowledge the support of Mr. V.L. Rathi and Mr. M. Kabra at Advanced Enzyme Technologies Ltd., India.

Conflict of Interest

The authors declare that there exist no conflicts of interest, whether internal, financial or of any other kinds.

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