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RESEARCH ARTICLE

Isolation and identification of lactic acid bacteria with probiotic potential from fermented flour of selected banana varieties grown in Sri Lanka

D.M.W.D. Divisekera[1], J.K.R.R. Samarasekera[1*], C. Hettiarachchi[2], J. Gooneratne[1], M. Iqbal Choudhary[3,4] and S. Gopalakrishnan[5]

[1] *Industrial Technology Institute, 363, Bauddhaloka Mawatha, Colombo 07.*
[2] *University of Colombo, 94, Kumaratunga Munidasa Mawatha, Colombo 07.*
[3] *H.E.J. Research Institute of Chemistry, International Centre for Chemical and Biological Sciences, University of Karachi, Karachi, Sindh 75270, Pakistan.*
[4] *Dr Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi, Sindh 75270, Pakistan.*
[5] *International Crops Research Institute for the Semi-Arid Tropics, Patancheru, Hyderabad, Telangana 502324, India.*

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Abstract: This study aimed at isolating and identifying lactic acid bacteria (LAB) with probiotic potential from fermented flour of selected banana varieties grown in Sri Lanka and evaluating their probiotic and safety attributes *in vitro*. Ten lactic acid bacteria were isolated from six varieties of fermented banana flour, *kolikuttu*, *seeni parakum*, *ambul nadee*, *ambum*, *seeni* and *anamalu*. The isolates were screened for phenotypical and biochemical characteristics. The selected isolates were identified by 16 S rRNA sequencing as *Enterococcus durans* (two strains), *E. gallinarum*, *E. hirae*, *E. faecium* (two strains), *Lactobacillus plantarum*, *L. curieae*, *Weissella cibaria* and *Pediococcus acidilactici* and their partial sequences were deposited in NCBI. Among them, six isolates were selected based on the results of *in vitro* safety attributes and evaluated for their probiotic attributes. Three isolates, namely, *E. durans* MF405179.1, *E. faecium* MF574466.1 and *L. curieae* MF405178.1 isolated from *kolikuttu*, *seeni parakum* and *ambul nadee*, respectively demonstrated tolerance to acid, gastric juice, bile, salt, phenol and temperature under gastric conditions, and also showed susceptibility to tested antibiotics. Among the selected isolates, *E. durans* MF405179.1 demonstrated the highest hydrophobicity and auto-aggregation of 69.91 % and 76.53 %, respectively. Further, it exhibited highest adhesion to both HCT116 and HT29 cell lines demonstrating 72.5 % and 74.16 % adhesion, respectively. This is the first report of isolation and characterisation of LAB strains with probiotic potential from flour of banana varieties *kolikuttu*, *seeni parakum*, *ambul nadee*, *ambum*, *seeni* and *anamalu* grown in Sri Lanka.

Keywords: Fermented banana flour, lactic acid bacteria, probiotics.

INTRODUCTION

Probiotics are defined as 'live microorganisms, which when administered in adequate amounts confer health benefit to the host' (FAO/WHO, 2002). Prebiotics are non-digestible food components that are utilised by probiotics, which ultimately provide health benefits to the host (FAO, 2007). A positive relationship has been established between probiotic food and health benefits that include reduction of non-communicable diseases (such as hyperlipidemia, hypertension, colorectal cancers, kidney diseases and hepatic diseases), reduction of gastro intestinal diseases (such as irritable bowel syndrome, lactose intolerance, constipation, *Helicobacter pylori* infection and gastric ulcers), improved immunity, uro-genital health, oral health, metabolism and reduction of upper respiratory tract infections, sexually

* Corresponding author (radhika@iti.lk; https://orcid.org/0000-0002-0480-5725)

transmitted diseases, allergies, inflammation reactions, neurological disorders and pregnancy and childhood associated disorders (Felley *et al*., 2001; Chmielewska & Szajewska, 2010; Guglielmetti *et al*., 2011; Homayouni *et al*., 2012; Kechagia *et al*., 2013; Sharma & Shukla, 2016). In order to exert beneficial effects to the host, potential probiotic candidates should possess a number of desirable characteristics, such as tolerance to gastric concentrations of acid, bile, salt, phenol, gastric juice and gastric temperature, ability to aggregate and adhere to epithelial cells and be free from virulence causing factors. These characteristics facilitate smooth transition of probiotics through the gut and enable colonisation (Gibson & Fuller, 2000). Due to proven health benefits, probiotic foods have gained a high market revenue (Sharma *et al*., 2013) and is expected to reach 64.02 billion USD by 2022 (*rnrmarketresearch.com*). Majority of probiotic foods are of dairy origin, considering several known health risks associated with the consumption of dairy based probiotic foods, i.e. intolerance to milk sugar lactose, allergy to milk proteins, high fat and cholesterol content in the milk has led scientists to pursue alternative substrates to produce non-dairy probiotics (Kumar *et al*., 2015).

Banana (*Musa* species) is cultivated abundantly in tropical and subtropical regions and contributes to the economy of developing countries. Surprisingly, about one-fifth of the banana harvest is wasted and redundant (Rayo *et al*., 2015). In Sri Lanka, banana cultivation covers about 69–70 % of the land area grown under fruits. At the Plant Genetic Resource Centre in Gannoruwa, Sri Lanka, 29 banana cultivars and 2 wild species are preserved (Ekanayaka *et al*., 2011) including popular varieties such as *seeni parakum, kandula, ambul nadee, nethrappalam, ambum, kolikuttu, seeni, anamalu* and *rathkesel*. Besides being an abundant low-cost ingredient, banana flour is rich in prebiotics including dietary fibre components such as resistant starch, arabinoxylans and beta-glucans (Topping & Clifton, 2001) and therefore is an ideal substrate for the non-dairy probiotic food industry. Several authors have successfully proved the compatibility of banana flour in food product development (Alves *et al*., 2016; Gomes *et al*., 2016; Segundo *et al*., 2017) including probiotic foods such as banana flour based probiotic drinks (Batista *et al*., 2017).

No studies have been reported to evaluate the flour of banana varieties grown in Sri Lanka as a potential probiotic and prebiotic source. With this background, the objective of this study was to isolate and identify lactic acid bacteria (LAB) with probiotic potential from fermented flour of selected banana varieties grown in Sri Lanka and evaluate the *in vitro* probiotic and safety attributes.

METHODOLOGY

Sample collection, preparation and isolation of lactic acid bacteria

Nine banana varieties were selected for the study (Figure 1). Five varieties, *kolikuttu, ambul nadee*,

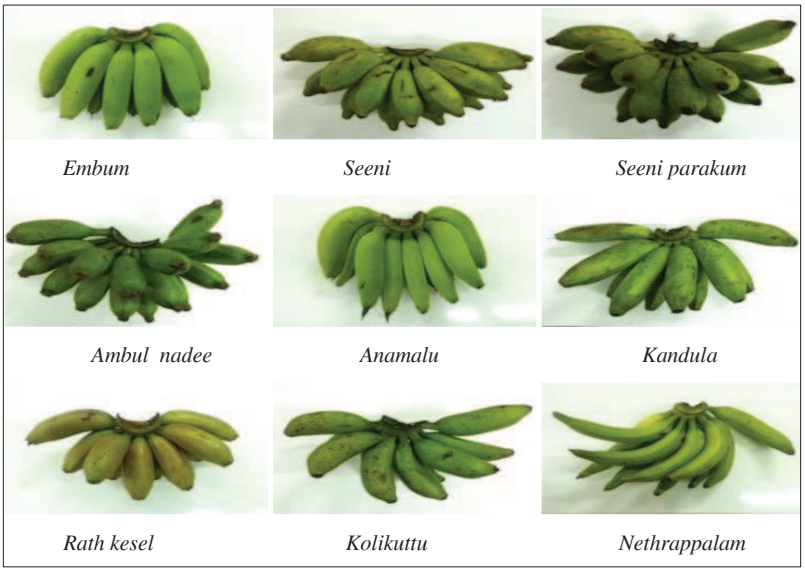

Figure 1: Banana varieties used in the study

kandula, rath kesel and *anamalu* were collected from the Agriculture Research Centre, Girandurukotte, Sri Lanka. Three varieties; *seeni*, *seeni parakum* and *nethrappalam* were collected from the Agriculture Research Station, Thellijjawila, Sri Lanka. *Ambum* was collected from the Regional Agriculture Research and Development Centre, Angunakolapelessa, Sri Lanka. All banana varieties were grown in the first quarter of the year 2014 and the fruits were completely matured and green in colour at the time of harvesting. The fruits were transported to the Microbiology Laboratory, Industrial Technology Institute, Sri Lanka within 6 h at < 20 ± 2 °C. Peels of the fruits were removed, flesh was cut into 4 mm thick slices and soaked in 1 % (w/v) solution of sodium metabisulphite for 10 min to avoid enzymatic browning. Soaked banana pieces were dried in a tray dryer (JKO3RD KINKAI, China) at 35 ± 2 °C till the moisture content reduced to < 10 %. The dried banana slices were ground in a variable speed rotor mill (Fritsch Pulverisette 14, Germany) and passed through a sieve (0.5 mm), packed in commercially sterile pouches and stored at 4 °C. Twenty-five grams of flour of each variety of banana (n = 6) was transferred into pre-sterilised glass beakers covered with aluminum foil.

The flour samples were mixed with 100 mL of sterilised tap water and left to ferment in a biological safety cabinet at 26–28 °C for 18 h. Each fermented sample was serially diluted up to 10^6 in sterilised saline (0.85% NaCl, w/v), spread on culture plates containing solidified De Man, Rogosa and Sharpe (MRS) agar (Oxoid, UK) (Reis *et al*., 2016) and incubated at 37 °C for 48 h.

Phenotypical and biochemical characterisation of lactic acid bacteria

Culture plates were observed for colonies with typical LAB morphology and the isolated colonies were further streaked on fresh MRS agar at 37 °C to determine the purity. Colony morphology (form, size, shape, surface, texture, colour, elevation and margin) of the purified LAB isolates were evaluated as per the standard protocols (Kunchala *et al*., 2016). Phenotypical characterisation was performed using Gram staining, endospore staining (Collee *et al*., 2006) and motility evaluation (Pyar & Peh, 2014). Biochemical tests including indole, methyl red, Voges Proskauer, citrate utilisation, catalase, oxidase, starch hydrolysis, urease, and amino acid hydrolysis (arginine, orinithine and lysine) were performed according to the methods given in Collee *et al*. (2006). Sugar fermentation pattern of LAB isolates were investigated according to Nazari *et al*. (2012) for sugars including glucose, fructose, maltose, lactose, galactose, melezitose, melibiose, arabinose, ribose, sucrose, salicin, sorbitol, mannitol, cellulose, cellobiose and dextrose (Sigma Aldrich, UK).

Molecular identification of lactic acid bacteria

Ten isolates that exhibited phenotypical and biochemical characteristics similar to LAB were selected for molecular identification. The selected LAB isolates were inoculated into MRS broth and incubated at 37 °C for 18 h. From each bacterium, 2 mL of centrifuged (14000 × g at 4 °C for 2 min) pellet was mixed with Tris EDTA buffer (200 µL) and re-centrifuged (14000 × g at 4 °C for 2 min). This procedure was repeated once more. To each pellet, 10 µL of proteinase K (100 µgµL^{-1}, w/v) was added and mixed well. Subsequently, sodium dodecyl sulfate (10 %, 10 µL) was added and incubated at 50 °C for 1 h. After incubation, equal volumes of phenol and chloroform (110 µL) were added and centrifuged (14000 × g at 4 °C for 2 min). Ethanol (30 µL, ≥ 99.8, v/v) and sodium acetate (15 µL, 3 M) were added to the aqueous layer, mixed in a vortex mixture and incubated in an ice bath for 1 h. After incubation, the tubes were centrifuged (14000 × g at 4 °C for 5 min). Subsequently, the pellets were mixed with ethanol (1 mL, 70 %, v/v) and centrifuged (14000 × g at 4 °C for 5 min). Supernatants were discarded and ethanol was evaporated. Finally, each pellet was dissolved in ultra pure PCR grade water (40 µL) and stored at -20 °C (Shahriar *et al*., 2011 modified). The extracted DNA was analysed for its quantity and purity using a gel documentation system (BIO RAD, UK) by mixing 5 µL of DNA with 2 µL of gel loading dye, and the gel was run at 60 V for 15 min. PCR was performed using Dr. Max DNA Polymerase in a DNA Engine Tetrad 2 Peltier Thermal Cycler (BIO-RAD, UK) under the following conditions: initial denaturation (95 °C, 5 min) followed by 35 cycles of denaturation (95 °C, 30 s), annealing (55 °C, 30 s), elongation (72 °C, 1 min), and final elongation (72 °C, 10 min). The universal primers 27F (5′AGAGTTTGATCCTGGCTCAG3′) and 1492R (5′GGTTACCTTGTTACGACTT3′) were used in PCR (Doi *et al*., 2013) and PCR product purification was carried out using the multiscreen-PCR filter plate (Millipore, USA). The 16S ribosomal RNA gene of the purified DNA products was sequenced at Macrogen, South Korea. Sequence analysis was done using Bioedit sequence alignment editor 7.0.2 software (Ibis therapeutics, Carlsbad, CA). The database search for homologous sequences was performed by basic local alignment tool of the National Center for Biotechnology Information (NCBI, USA). Sequences with an identity of 99 % or higher to those in the databases were allocated

to the same species (Altschul *et al*., 1990). The partial sequences of 16S rRNA gene of LAB isolates were deposited at NCBI, USA. Phylogenetic analyses were conducted according to neighbour-joining method (Saitou & Nei, 1987) in MEGA7.

In vitro safety attributes of lactic acid bacteria

The LAB isolates were investigated for safety attributes including hemolysis, DNase and gelatin hydrolysis (*in vitro*). For the hemolysis test, blood agar was prepared by supplementing blood agar base (Hi media, India) with sheep blood (5 %, v/v). For the gelatin hydrolysis test, tryptone neopeptone dextrose agar (TND) was prepared (w/v) using tryptone (1.7 %), peptone (0.3 %), dextrose (0.25 %), NaCl (0.5 %), K_2HPO_4 (0.25 %), agar (1.5 %) and gelatin-bacteriological (0.4 %). For the DNase test, DNase agar (Oxoid, UK) was prepared according to manufacturer's instructions. The enzyme activities were performed by inoculating the LAB isolates (10^5 cfumL^{-1}) into the respective agar as described above and incubated at 37 °C for 48 h (Gupta & Malik, 2007). Blood agar plates were observed for the presence of hemolysis zones β, α and γ. TND agar plates were saturated with ammonium sulfate and observed for clear zones around the inoculated area. DNase agar plates were observed for thick plaque of growth around the colonies. The controls used were *Streptococcus pyogenes* ATCC 19615 and *Serratia marcescens* ATCC 13880 for hemolysis and DNase/gelatin liquefaction assays, respectively.

Probiotic attributes of lactic acid bacteria exposed to *in vitro* gastric conditions (acid, bile, salt, phenol, gastric juice and temperature)

The isolated LAB strains that confirmed safety attributes were inoculated into tubes containing sterile MRS broth and incubated at 37 ± 1 °C for 18 h. After incubation, the tubes were centrifuged at 10,000 × g at 4 °C for 15 min. Subsequently, the pellets were washed with sterile saline (0.89 NaCl, w/v) and centrifuged at 10,000 × g at 4 °C for 15 min. Each pellet was individually suspended in MRS broth and adjusted to 0.5 MacFarland turbidity standards (1.5×10^8 cfumL^{-1}). From each bacterial suspension 100 µL was inoculated into MRS broth adjusted as per the gut conditions (*in vitro*) including pH (1.5, 3 and 4, v/v HCl and pH 7 as control), bile concentrations (0.2, 0.5, 1.0, 1.5, and 2.0 %, w/v, porcine bile), salt concentrations (4, 5, 8, and 12 %, w/v NaCl), phenol concentrations (0.1, 0.2, 0.3, 0.4, and 0.5 % v/v phenol) and simulated gastric juice at pH 2 (0.05 gL^{-1} porcine bile, 0.10 gL^{-1} lysozyme, and 0.10 gL^{-1} pepsin) according to the method given by Aswathy *et al*. (2008). All the attribute tests were performed in triplicate and repeated twice.

Antibiotic susceptibility of lactic acid bacteria

The LAB isolates that survive in gastric conditions were selected for investigating the susceptibility/ resistance to antibiotics. Agar disc diffusion method (CLSI, 2012) was performed to evaluate the antibiotic susceptibilities of LAB using commercially available antibiotic discs (Oxoid) cefotaxime (15 µg), gentamycin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), sulphamethoxazole (25 µg), ciprofloxacin (5 µg), amikacin (30 µg), bacitracin (52 µg), ampicillin (10 µg), amoxicillin (30 µg), cephalothin (30 µg), vancomycin (30 µg) and erythromycin (30 µg). Cells were prepared as mentioned in the attribute tests. LAB cell pellets were adjusted to 0.5 MacFarland turbidity standards (1.5×10^8 cfumL^{-1}). From each bacterial suspension, 100 µL was tested against respective antibiotics. The plates were incubated at 37 ± 1 °C for 24 h. The experiment was performed in triplicate and repeated twice.

Auto-aggregation ability of lactic acid bacteria

The LAB isolates that survive in gastric conditions were selected for investigating the auto-aggregation ability. Auto-aggregation of LAB strains was investigated *in vitro* according to the method of Kos *et al*. (2003) with some modifications.

Test isolates were inoculated into sterile MRS broth and incubated at 37 ± 1 °C for 18 h. After incubation, the tubes were centrifuged at 10,000 × g at 4 °C, 15 min. Subsequently, the pellets were washed with sterile saline (0.89 NaCl, w/v) and re-centrifuged at 10,000 × g at 4 °C for 15 min. Each pellet was individually suspended in MRS broth and adjusted to 0.5 MacFarland turbidity standards (1.5×10^8 cfumL^{-1}). The cell suspensions (3 mL^{-1}) were mixed for 10 s and incubated at 37 ± 1 °C for 5 h. At intervals of 0, 1, 2, 3, 4 and 5 h, 200 µL of the upper suspension of each reaction mixture was transferred to 96 well plates. Absorbance was measured at λ= 620 nm. The auto-aggregation percentage was expressed as a function of time using the following formula;

$$\text{Auto-aggregation (\%)} = 1 - \frac{A_t}{A_0} \times 100$$

A_t (Absorbance at time t =1, 2, 3, 4 h); A_0 (absorbance at t = 0)

Cell surface hydrophobicity of lactic acid bacteria

Cell surface hydrophobicity was measured according to the method given by Kos *et al*. (2003). LAB isolates were inoculated into sterile MRS broth and incubated at 37 ± 1 °C for 18 h. The cells were harvested by centrifugation (Centurion Scientific k3 series, UK) at 5000 × g at 4 ± 1 °C for 15 min. The pellets were washed twice with phosphate buffered saline (PBS). The cells were adjusted to 10^8 cfumL^{-1} and re-suspended in KNO_3 solution (0.1 Moldm^{-3}, pH 6.2). The initial absorbance of cell suspensions was measured at ƛ= 620 nm (A_0). Each LAB strain was investigated for adhesion with 3 solvents; xylene (non-polar solvent), ethyl acetate (non-polar and basic solvent) and chloroform (non-polar and acidic solvent). One milliliter of each solvent was added separately to 3 mL of each cell suspension and incubated for 10 min at 37 ± 1 °C. The two phase system was mixed for 2 min. After mixing, the reaction mixture was incubated at 37 ± 1 °C, 20 min. The aqueous phase was removed and absorbance was measured at 620 nm. Controls (LAB cells 10^8 cfumL^{-1} suspended in KNO_3 solution) were prepared without adding the solvents and were measured parallel to tests. The percentage of LAB cell adhesion to solvents was calculated using following formula;

$$\text{MATS (\%)} = 1 - \frac{A_1}{A_0} \times 100$$

A_1 = Absorbance of aqueous layer; A_0 = Absorbance of control

Cell adhesion of lactic acid bacteria

The ability of the selected LAB strains to adhere to two epithelial cell lines, namely, *Homo sapiens* colon colorectal adenocarcinoma ATCC HTB-38 (HT-29) and *Homo sapiens* colon colorectal carcinoma ATCC CCL-247 (HCT-116) *in vitro* was performed according to Duary *et al*. (2011) with modifications. The cell lines were obtained from the bio bank of the ICCBS, University of Karachi, Pakistan and maintained in Dulbecco's modified Eagles medium **(**DMEM) supplemented with 10 % fetal bovine serum (v/v), 1 % l-glutamine (v/v), 1 % penicillin-streptomycin solution (v/v) and 7.5 % $NaHCO_3$. The medium was sterilised by filtration and stored at 4 ± 1 °C until use. Monolayer of cell lines with 80 % confluence was used in the study. LAB strains were grown in MRS broth and adjusted to 0.5 MacFarland turbidity standards (1.5 × 10^8 cfumL^{-1}) as described earlier. LAB cells were further serially diluted in tubes containing sterilised PBS and the cell concentration was adjusted to 1.5 × 10^2 cfumL^{-1}. From each cell line, 10^6 cellsmL^{-1} in 0.5 mL of DMEM was placed in 6 well tissue culture plates and incubated at 37 ± 1 °C for 24 h in 5 % CO_2. The cell culture media flasks containing HT-29 and HCT-116 were discarded and new media were added every other day. After 14 days of incubation (14 days post confluence phase) mono-layers were washed twice with sterile PBS. One milliliter of LAB strain and 1 mL of DMEM media were added into the wells containing monolayers (n = 9). The plates were further incubated (Nuaire NU-8700E, USA) at 37 ± 1 °C for 1 h in 5 % CO_2. After 1 h, the monolayers were washed five times with sterile PBS, fixed with methanol and observed under the microscope (Optica B 500 i, Italy) at a magnification of 100×. Subsequently, the attached LAB cells/well were counted and the adhesion ability was expressed as the percentage ratio of the LAB cells initially inoculated and the LAB cells remained attached after washing with PBS.

Statistical analysis

All experiments were conducted in triplicate and repeated twice. The mean and standard error of the data obtained from parallel experiments were calculated using Minitab 14. One-way ANOVA (unstacked) followed by multiple comparisons using tukey's family error rate was performed to analyse the data. Values $p < 0.05$ were considered as significant.

RESULTS AND DISCUSSION

Phenotypical characteristics of lactic acid bacteria

A total of 150 bacteria were isolated from seven varieties of fermented banana flour, namely, *ambul nadee* (20), *ambum* (29), *seeni parakum* (26)*, kolikuttu* (16)*, kandula* (10), *seeni* (27) and *anamalu* (22). No bacteria were isolated from *rathkesel* and *nethrappalam* varieties. Among them, 99 bacterial colonies demonstrated to have typical LAB colony morphology on MRS agar as described by De Man *et al.* (1960) of which, 65 were Gram positive cocci/bacilli that are non-spore forming and non-motile organisms isolated from *ambul nadee* (7), *ambum* (8), *kandula* (13), *seeni parakum* (8), *kolikuttu* (7), *seeni* (9) and *anamalu* (13). LAB are usually Gram positive cocci or bacilli, non-spore forming and non-motile organisms, hence these new isolates were tentatively identified as LAB, based on the phenotypic characters exhibited.

Biochemical characteristics of lactic acid bacteria

Biochemical characterisation of the 65 tentatively identified LAB revealed that 10 isolates, namely, K08, K14, K16, SP13, SP25, SP26, AN18, AM22, S27 and

A29 were negative for catalase, oxidase, indole, Voges Proskauer, methyl red, citrate utilisation and starch hydrolysis, and LAB isolates SP25 and K14, and AN18 and SP13 were positive for H_2S and urease, respectively. K16 isolate showed starch hydrolysis, while isolates K08, SP13, SP26 and AM22 hydrolysed arginine and orinithine. None of the LAB isolates hydrolysed lysine. LAB usually show negative reactions to some biochemical tests including indole, methyl red, Voges Proskauer, citrate utilisation, catalase and oxidase (Chowdhary *et al*., 2012). H_2S production protects the bacterial cells from oxidase stress and antibiotics (Kimura, 2014), while urease activity helps probiotic LAB strains to counteract acid stress during fermentation processes (Mora & Arioli, 2014). Amino acid hydrolysis during growth provides energy to LAB for their metabolic activities during fermentation (Pessione & Cirrincione 2016). Hence, these 10 LAB isolates were selected for further studies.

Sugar fermentation pattern of lactic acid bacteria

LAB represents a group of microorganisms that are functionally related by their ability to produce lactic acid during fermentation. The ability to ferment different carbohydrates is one of the important characteristics that makes LAB ideal for fermentation. All 10 selected LAB isolates could ferment sugars including glucose, fructose, maltose, lactose, galactose, sucrose and dextrose. LAB strains AM22 and K14 could also ferment cellulose, while LAB isolates such as AN18, A29, SP13, SP25, SP26, K14 and S27 were found to ferment sorbitol. Except isolate S27, all the other isolates fermented mannitol. None of the isolates could ferment melezitose, melibiose, arabinose, cellobiose and ribose (Table 1). Similar observations on sugar fermentation have been reported by Langston and Bouma (1960) on LAB, which was isolated from grass silage. All the LAB isolates were observed to be hetero-fermentative producing lactic acid and CO_2.

Table 1: Summary of the carbohydrate fermentation pattern of isolated lactic acid bacteria

LAB isolates	G	F	M	L	G	MZ	MB	A	R	SU	SA	SO	Mn	C	CB	D
K08	+	+	+	+	+	-	-	-	-	+	-	-	+	-	-	+
K14	+	+	+	+	+	-	-	-	-	+	-	+	+	+	-	+
K16	+	+	+	+	+	-	-	-	-	+	-	-	+	-	-	+
SP13	+	+	+	+	+	-	-	-	-	+	-	+	+	-	-	+
SP25	+	+	+	+	+	-	-	-	-	+	-	+	+	-	-	+
SP26	+	+	+	+	+	-	-	-	-	+	-	+	+	-	-	+
AN18	+	+	+	+	+	-	-	-	-	+	-	+	+	-	-	+
AM22	+	+	+	+	+	-	-	-	-	+	-	-	+	+	-	+
S27	+	+	+	+	+	-	-	-	-	+	-	+	+	-	-	+
A29	+	+	+	+	+	-	-	-	-	+	-	-	+	-	-	+

The letters K, SP, AN, AM, S and A display lactic acid bacteria isolated from banana varieties *kolikuttu*, *seeni parakum*, *ambul nadee*, *ambum*, *seeni* and *anamalu*, respectively. (G) glucose; (F) fructose; (M) maltose; (L) lactose; (G) galactose; (MZ) melezitose; (MB) melibiose; (A) arabinose; (R) ribose; (SU) sucrose; (SA) salicin; (SO) sorbitol; (Mn) mannitol; (C) cellulose; (CB) cellobiose; (D) dextrose; (+) ferment; (-) do not ferment.

Table 2: Molecular identification of newly isolated lactic acid bacteria

LAB isolates	Genus /species identification	NCBI gene bank accession
K08	*Enterococcus durans*	MF405179.1
K14	*Enterococcus gallinarum*	MF480436.1
K16	*Lactobacillus plantarum*	MF405177.1
SP13	*Weissella cibaria*	MF480445.1
SP25	*Enterococcus hirae*	MF480429.1
SP26	*Enterococcus faecium*	MF574466.1
AN18	*Lactobacillus curieae*	MF405178.1
AM22	*Enterococcus durans*	MF480435.1
S27	*Pediococcus acidilactici*	MF480433.1
A29	*Enterococcus faecium*	MF480430.1

The letters K, SP, AN, AM, S and A display lactic acid bacteria isolated from banana varieties *kolikuttu*, *seeni parakum*, *ambul nadee*, *ambum*, *seeni* and *anamalu*, respectively.

Molecular identification of newly isolated lactic acid bacteria

The LAB were identified by comparing 16S rRNA partial sequences with those present in NCBI gene bank.

Sequences showed 98 – 99 % identity to the existing sequences, which was assigned to the same genus and species. The accession numbers obtained after depositing partial sequences in NCBI gene bank are presented in Table 2.

Safety attributes of isolated lactic acid bacteria

Out of the 10 LAB strains isolated from fermented banana flour, six isolates, namely, K08, K16, SP26, AN18, AM22 and S27 were free from hemolysis, DNase and gelatin hydrolysis activity and possess safety attributes (Table 3). Hemolysis, DNase and gelatin hydrolysis activity contribute to the incidence of virulence in microorganisms; therefore ideal probiotic candidates should be free from these virulence factors (Halder *et al*., 2017).

Table 3: Safety attributes of isolated lactic acid bacteria (*in vitro*)

LAB isolates	Hemolytic activity	DNase activity	Gelatin hydrolysis
K08	γ	negative	negative
K14	γ	negative	positive
K16	γ	negative	negative
SP13	γ	negative	positive
SP25	α	negative	negative
SP26	γ	negative	negative
AN18	γ	negative	negative
AM22	γ	negative	positive
S27	γ	negative	negative
A29	γ	negative	positive
Streptococcus pyogene[a]	β	negative	negative
Serratia marcescens[b]	γ	positive	positive

The letters K, SP, AN, AM, S and A display lactic acid bacteria isolated from banana varieties; *kolikuttu*, *seeni parakum*, *ambul nadee*, *ambum*, *seeni* and *anamalu*, respectively. α - partial lysis of the red cells in the media; β - complete lysis of the red blood cells in the media; γ - non hemolytic. n = 9. [a] Positive control of hemolysis; [b] positive control of DNase and gelatin hydrolysis

Probiotic attributes of lactic acid bacteria (*in vitro*)

Six LAB isolates K08, K16, SP26, AN18, AM22 and S27 demonstrated significant differences ($p < 0.05$) in their probiotic attributes. Except isolate AM22, others tolerate acid up to pH 1.5 (Table 4). Probiotic bacteria need to survive passage through the stomach, where the pH can lie between 1.5–2.0 and further are required to stay viable for 4 hours or more before they move to the gastro-intestinal tract. Hence, the primary host factors that may affect commercial probiotics are the elevated levels of acidity in the proventriculus and ventriculus. Therefore, being tolerant to acidic conditions is an important criterion to be considered throughout the selection of potential probiotic isolates to assure their viability and functionality (Dunne *et al*., 2001; Bakari *et al*., 2011). The average concentration of bile salts in the small intestine is around 0.2–0.3 %, and may increase up to 2 % (w/v) depending upon the host physiology as well as the type and amount of food ingested (Bakari *et al*., 2011; Menconi *et al*., 2013). Healthy humans commonly have about 0.3 % bile (Vicente *et al*., 2008) and hence commercial probiotic bacteria need to tolerate at least up to 0.3 % bile (Dunne *et al*., 2001). Except isolate S27, all the isolates tolerated up to 1.5 % bile. Isolates K08, SP27 and AN18 tolerated up to 2 % bile (Table 5). All isolates grew at temperature between 30–42 ^{o}C (Table 6).

Table 4: Acid tolerance of isolated lactic acid bacteria

LAB isolates	Survival of LAB in pH after 6 h (OD at 620 nm)			
	control	pH 4	pH 3	pH 1.5
K08	1.94 ± 0.01 [a]	1.53 ± 0.03[b]	0.88 ± 0.06[c]	0.35 ± 0.02[d]
K16	1.87 ± 0.03 [a]	1.60 ± 0.04[b]	0.87 ± 0.02[c]	0.37 ± 0.03[d]
SP26	1.99 ± 0.00 [a]	1.67 ± 0.04[b]	0.84 ± 0.03[c]	0.38 ± 0.03[d]
AN18	1.88 ± 0.00 [a]	1.92 ± 0.01[a]	1.61 ± 0.05[b]	0.59 ± 0.03[c]
AM22	1.70 ± 0.04 [a]	1.35 ± 0.01[b]	0.84 ± 0.00[c]	0.01 ± 0.00[d]
S27	1.80 ± 0.03 [a]	1.41 ± 0.02[b]	0.42 ± 0.03[c]	0.24 ± 0.02[d]

Data is expressed as mean ± SEM, n = 9. Within a row mean values superscripted with different letters are significantly different ($p < 0.05$). The letters K, SP, AN, AM and S display lactic acid bacteria isolated from banana varieties *kolikuttu*, *seeni parakum*, *ambul nadee*, *ambum* and *seeni*, respectively. Control - MRS broth of pH 6.

Table 5: Bile tolerance of isolated lactic acid bacteria

LAB isolates	Survival of LAB in bile after 6 hours (OD at 620 nm)					
	Control	0.2 %	0.5 %	1.0 %	1.5 %	2.0 %
K08	1.94 ± 0.01^{a}	1.65 ± 0.01^{b}	1.45 ± 0.00^{c}	1.15 ± 0.02^{d}	0.74 ± 0.01^{e}	0.48 ± 0.00^{f}
K16	1.87 ± 0.03^{a}	1.46 ± 0.01^{b}	1.27 ± 0.00^{c}	0.96 ± 0.02^{d}	0.26 ± 0.02^{e}	0.09 ± 0.00^{f}
SP26	1.99 ± 0.00^{a}	1.85 ± 0.01^{b}	1.43 ± 0.00^{c}	1.18 ± 0.00^{d}	0.54 ± 0.00^{e}	0.23 ± 0.01^{f}
AN18	1.88 ± 0.00^{a}	1.52 ± 0.01^{b}	1.17 ± 0.01^{c}	0.65 ± 0.01^{d}	0.34 ± 0.01^{e}	0.12 ± 0.00^{f}
AM22	1.80 ± 0.03^{a}	1.44 ± 0.01^{b}	1.19 ± 0.00^{c}	0.98 ± 0.00^{d}	0.28 ± 0.00^{e}	0.01 ± 0.00^{f}
S27	1.70 ± 0.04^{a}	1.34 ± 0.01^{b}	1.18 ± 0.00^{c}	0.88 ± 0.03^{d}	0.09 ± 0.00^{e}	0.03 ± 0.00^{f}

Data is expressed as mean ± SEM, n = 9. Within a row mean values superscripted with different letters are significantly different ($p < 0.05$). The letters K, SP, AN, AM and S display lactic acid bacteria isolated from banana varieties *kolikuttu*, *seeni parakum*, *ambul nadee*, *ambum*, and, *seeni* respectively. Control-isolates in MRS broth without bile salts.

Table 6: Temperature tolerance of isolated lactic acid bacteria

LAB isolates	Survival of LAB at different temperatures after 6 hours (OD at 620 nm)		
	30 °C	37 °C	42 °C
K08	1.46 ± 0.01^{b}	1.74 ± 0.03^{a}	1.25 ± 0.00^{c}
K16	1.54 ± 0.01^{b}	1.79 ± 0.01^{a}	1.22 ± 0.01^{c}
SP26	1.46 ± 0.01^{b}	1.88 ± 0.00^{a}	1.33 ± 0.01^{c}
AN18	1.38 ± 0.01^{c}	1.52 ± 0.01^{b}	1.83 ± 0.04^{a}
AM22	1.26 ± 0.02^{c}	1.57 ± 0.01^{a}	1.45 ± 0.01^{b}
S27	1.35 ± 0.01^{c}	1.95 ± 0.03^{a}	1.55 ± 0.01^{b}

Data is expressed as mean ± SEM, n = 9. Within a row mean values superscripted with different letters are significantly different ($p < 0.05$). The letters K, SP, AN, AM and S display lactic acid bacteria isolated from banana varieties *kolikuttu*, *seeni parakum*, *ambul nadee*, *ambum* and *seeni*, respectively.

The ability to tolerate normal body temperature enables the probiotics to have an active metabolism in the gut. On the other hand, the ability to tolerate high temperature enables a better rate of growth as well as a high yield of lactic acid production during fermentation and reduces the contaminations in fermentation processes (Ibourahema *et al*., 2008). All isolates K08, K16, SP26, AN18, AM22 and S27 tolerated up to 12% salt, except isolate AM22, which could tolerate salt up to 8 % (Table7). When LAB survive in 6.5 % NaCl, they are considered as osmo-tolerant. This osmo-tolerant characteristic of LAB enables them to carry out metabolism and lactic acid production even in the presence of high concentrations of salts in the gut (Menconi *et al*., 2014).

Except isolate AM22, all the other LAB tolerated simulated gastric juice of pH 1.5 (Table 8). The ability of

Table 7: Salt tolerance of isolated lactic acid bacteria

LAB isolates	Survival of LAB in NaCl after 6 hours (OD at 620 nm)				
	Control	4 %	5 %	8 %	12 %
K08	1.96 ± 0.01^{a}	1.57 ± 0.01^{b}	1.36 ± 0.03^{c}	1.17 ± 0.01^{d}	0.37 ± 0.01^{e}
K16	1.99 ± 0.00^{a}	1.80 ± 0.04^{b}	1.65 ± 0.01^{c}	1.49 ± 0.01^{d}	1.28 ± 0.01^{e}
SP26	1.94 ± 0.01^{a}	1.65 ± 0.01^{b}	1.47 ± 0.00^{c}	1.27 ± 0.00^{d}	0.95 ± 0.01^{e}
AN18	1.88 ± 0.01^{a}	1.47 ± 0.01^{b}	1.27 ± 0.01^{c}	0.88 ± 0.00^{d}	0.65 ± 0.01^{e}
AM22	1.75 ± 0.01^{a}	1.36 ± 0.00^{b}	0.96 ± 0.00^{c}	0.36 ± 0.01^{d}	0.07 ± 0.00^{e}
S27	1.87 ± 0.02^{a}	1.67 ± 0.00^{b}	1.15 ± 0.01^{c}	0.55 ± 0.01^{d}	0.24 ± 0.00^{e}

Data is expressed as mean ± SEM, n = 9. Within a row mean values superscripted with different letters are significantly different ($p < 0.05$). The letters K, SP, AN, AM and S display lactic acid bacteria isolated from banana varieties *kolikuttu, seeni parakum, ambul nadee, ambum* and *seeni*, respectively.

Table 8: Simulated gastric juice tolerance of isolated lactic acid bacteria

LAB isolates	Survival of LAB in simulated gastric juice after 6 hours (OD at 620 nm)	
	Control	Simulated gastric juice of pH 1.5
K08	1.96 ± 0.01	0.31 ± 0.00^{c}
K16	1.99 ± 0.00	0.38 ± 0.00^{b}
SP26	1.94 ± 0.01	0.13 ± 0.00^{d}
AN18	1.88 ± 0.01	0.87 ± 0.00^{a}
AM22	1.75 ± 0.01	0.02 ± 0.00^{e}
S27	1.87 ± 0.02	0.37 ± 0.00^{b}

Data is expressed as mean ± SEM, n = 6. Within column mean values superscripted with different letters are significantly different ($p < 0.05$). The letters K, SP, AN, AM and S display lactic acid bacteria isolated from banana varieties *kolikuttu*, *seeni parakum*, *ambul nadee*, *ambum* and *seeni*, respectively. Control - LAB in MRS broth.

potential probiotic strains to survive in the human gastric juice is the key indication that displays the ability of the strains to survive passage through the stomach (Shewale *et al*., 2014). Except isolates K16 and S27, others tolerated 0.4 % phenol (Table 9). Phenols are formed in the intestines by gut bacteria that deaminate various aromatic amino acids delivered by the diet or produced by endogenous proteins. These phenol compounds can inhibit the growth of probiotic LAB. Therefore, phenol tolerance is essential for their survival in the gastrointestinal tract (Yadav *et al*., 2016). The results of this study indicated that not all desirable probiotic characteristics are present within a single isolate, where many isolates displayed varying but promising capabilities. LAB isolates *E. durans* (K08), *E. faecium* (SP26) and *L. curieae* (AN18) demonstrated superior probiotic attributes; hence were selected for further studies.

Table 9: Phenol tolerance of isolated lactic acid bacteria

LAB isolates	Survival of LAB in phenol after 6 hours (OD at 620 nm)					
	Control	0.1 %	0.2 %	0.3 %	0.4 %	0.5 %
K08	1.96 ± 0.01^{a}	1.67 ± 0.01^{b}	1.24 ± 0.00^{c}	0.86 ± 0.01^{d}	0.66 ± 0.01^{e}	0.55 ± 0.00^{f}
K16	2.00 ± 0.00^{a}	1.35 ± 0.01^{b}	0.86 ± 0.00^{c}	0.58 ± 0.00^{d}	0.02 ± 0.00^{e}	0.01 ± 0.00^{e,f}
SP26	1.94 ± 0.01^{a}	1.76 ± 0.01^{b}	1.52 ± 0.03^{c}	1.13 ± 0.01^{d}	0.75 ± 0.01^{e}	0.56 ± 0.01^{f}
AN18	1.87 ± 0.01^{a}	1.53 ± 0.01^{b}	1.37 ± 0.00^{c}	0.97 ± 0.01^{d}	0.56 ± 0.01^{e}	0.33 ± 0.01^{f}
AM22	1.75 ± 0.01^{a}	1.66 ± 0.01^{b}	0.64 ± 0.02^{c}	0.23 ± 0.01^{d}	0.38 ± 0.00^{e}	0.01 ± 0.00^{f}
S27	1.86 ± 0.02^{a}	1.25 ± 0.01^{b}	0.86 ± 0.01^{c}	0.56 ± 0.01^{d}	0.04 ± 0.00^{e}	0.01 ± 0.00^{e,f}

Data is expressed as mean ± SEM, n = 9. Within a row mean values superscripted with different letters are significantly different ($p < 0.05$). The letters K, SP, AN, AM and S display lactic acid bacteria isolated from banana varieties *kolikuttu*, *seeni parakum*, *ambul nadee*, *ambun* and *seeni*, respectively.

Antibiotic susceptibility of lactic acid bacteria

LAB isolates demonstrated significant differences ($p < 0.05$) in susceptibility/resistance at tested antibiotic concentrations. Isolates K08 and SP26 were susceptible to all the antibiotics at tested concentrations (Table 10). Isolate AN18 was susceptible to all the antibiotics at tested concentrations except chloramphenicol at 30 μgmL^{-1} concentration. Resistance or susceptibility to antibiotics alone will not cause risk in probiotic LAB candidates. Their ability to transfer the respective antibiotic resistance encoding genes is the real cause of risk (Gueimonde *et al*., 2013) and therefore there is a need to investigate prior to commercial applications.

Auto aggregation ability of lactic acid bacteria

Probiotics that are able to auto-aggregate have the potential to adhere into the gut epithelium. Therefore, aggregation is considered as a prerequisite of an ideal probiotic candidate (Kos *et al*., 2003). In this study, isolate K08 (*E. durans*) demonstrated highest auto-aggregation of 76.5 %, which is higher than that reported by previous authors. Jeevaratnam and Nallala (2017) reported 53 % auto-aggregation in *E. durans* at the end of 5 hour incubation. Significant differences ($p < 0.05$) in auto-aggregation were observed among the LAB strains (Table 11). Auto-aggregating ability of the LAB strains was observed to be time dependent and therefore,

Table 10: Antibiotic susceptibility/resistance pattern of isolated lactic acid bacteria

LAB isolates	Diameter (in mm) of the inhibition zone of the LAB strains		
	K08	SP26	AN18
Cefotaxime	19.00 ± 0.0^{b}	18.75 ± 0.25^{b}	20.00 ± 0.0^{b}
Gentamycin	15.25 ± 0.25^{c}	19.00 ± 0.71^{a}	$16.00 \pm 0.0^{b,c}$
Tetracycline	26.75 ± 0.25^{a}	11.00 ± 0.41^{c}	11.00 ± 0.0^{c}
Chloramphenicol	17.75 ± 0.25^{b}	21.00 ± 0.71^{a}	0.0 ± 0.0^{c}
Sulphamethoxazole	12.50 ± 0.96^{c}	14.00 ± 0.0^{c}	27.00 ± 0.71^{a}
Ciprofloxacin	18.50 ± 0.29^{b}	14.75 ± 0.63^{c}	21.50 ± 0.50^{b}
Amikacin	19.00 ± 0.0^{d}	$19.25 \pm 0.25^{c,d}$	21.00 ± 0.71^{c}
Bacitracin	10.00 ± 0.91^{f}	22.75 ± 0.25^{c}	31.25 ± 0.95^{g}
Ampicillin	27.00 ± 0.0^{a}	11.25 ± 0.75^{c}	17.25 ± 0.48^{b}
Amoxicillin	27.50 ± 0.50^{a}	$12.00 \pm 0.0^{c,d}$	11.00 ± 0.0^{d}
Cephalothin	$21.50 \pm 0.29^{b,c}$	22.00 ± 0.8^{c}	$20.25 \pm 0.75^{b,c}$
Vancomycin	24.50 ± 0.50^{a}	23.00 ± 0.0^{a}	12.00 ± 0.0^{b}
Erythromycin	18.75 ± 0.25^{c}	16.75 ± 0.75^{d}	11.50 ± 0.29^{e}
Control	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}

Data is expressed as mean ± SEM, n = 9. Within a row mean values superscripted with different letters are significantly different ($p < 0.05$). The letters K, SP and AN display lactic acid bacteria isolated from banana varieties *kolikuttu, seeni parakum* and *ambul nadee*, respectively. Control - no antibiotic. Cefotaxime-15 µg; gentamycin-10 µg; tetracycline-30 µg; chloramphenicol-30 µg; sulphamethoxazole-25 µg; ciprofloxacin-5 µg; amikacin-30 µg; bacitracin-10 units; ampicillin-10 µg; amoxicillin-30 µg; cephalothin-30 µg; vancomycin-30 µg; erythromycin-30 µg.

Table 11: Auto-aggregation percentage of isolated lactic acid bacteria with time

LAB isolates	Auto-aggregation percentage of LAB with time (hours)				
	1 h	2 h	3 h	4 h	5 h
K08	21.33 ± 0.65^{a}	31.66 ± 0.74^{a}	37.12 ± 1.28^{a}	42.01 ± 0.87^{b}	76.53 ± 0.59^{a}
SP26	$2.63 \pm 0.17^{d,e}$	7.04 ± 0.30^{d}	$10.45 \pm 0.32^{d,e}$	45.53 ± 0.55^{a}	61.10 ± 1.19^{c}
AN18	4.45 ± 0.39^{c}	7.00 ± 0.54^{d}	11.97 ± 0.49^{d}	22.32 ± 0.70^{f}	38.51 ± 1.02^{e}

Data is expressed as mean ± SEM, n = 9. Within a column mean values superscripted with different letters are significantly different ($p < 0.05$). The letters K, SP and AN display lactic acid bacteria isolated from banana varieties *kolikuttu, seeni parakum* and *ambul nadee*, respectively.

increment of auto-aggregation was observed with the increase of incubation period.

Hydrophobicity of lactic acid bacteria

Probiotics should exhibit hydrophobic surfaces for better adherence to cells and solid materials in the gut (Del-Re *et al*., 2000). Results revealed that isolates K08 (*E. durans*) and SP26 (*E. faecium*) demonstrated 69.9 % and 60.2 % affinity to xylene respectively. Therefore these strains have elevated hydrophobic properties (Giarous *et al.,* 2009). Further, it was observed that these strains demonstrated the highest auto-aggregation ability. Del-Re *et al*. (2000) suggested that hydrophobicity improves the auto-aggregation and observations of this study completely agree with the statement. Isolate AN18 (*L. curieae)* demonstrated 67.4 ± 0.2 % affinity to ethyl acetate, which is a basic solvent and electron donor (Figure 2).

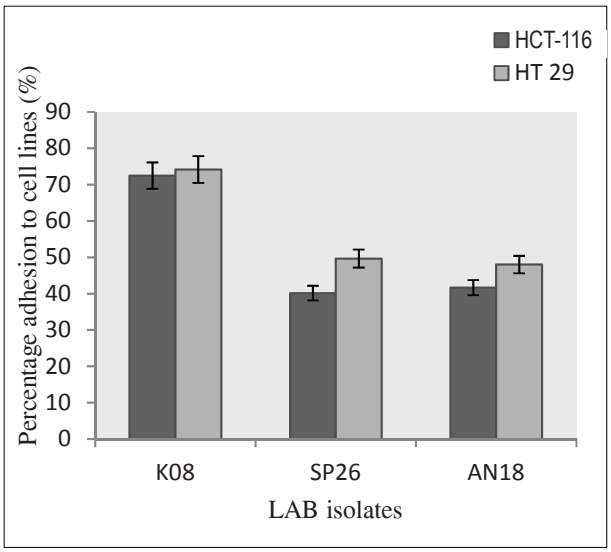

Figure 2: Hydrophobicity of LAB isolates
Data is expressed as mean ± SEM, n = 9. The letters K, SP and AN display lactic acid bacteria isolated from banana varieties *kolikuttu, seeni parakum* and *ambul nadee*.

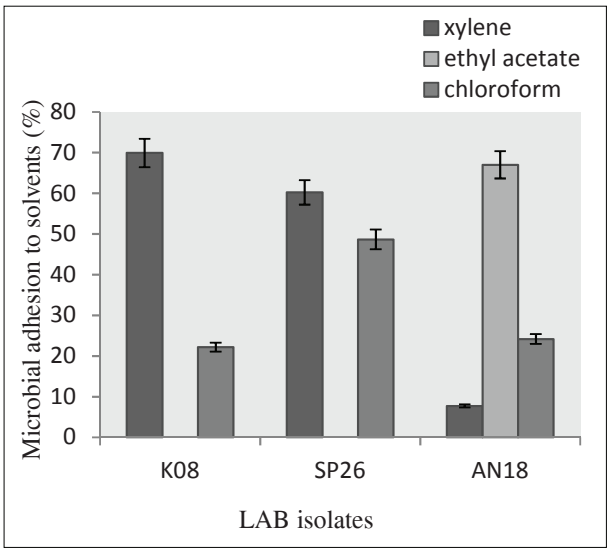

Figure 3: Cell adhesions of LAB isolates
Data is expressed as mean ± SEM, n = 9. The letters K, SP and AN display lactic acid bacteria isolated from banana varieties *kolikuttu, seeni parakum* and *ambul nadee*.

Adhesion to epithelial cell lines by lactic acid bacteria

Significant differences ($p < 0.05$) in adhesion of LAB strains to HCT-116 and HT-29 cell lines were observed. The results revealed that isolate KO8 (*E. durans*) exhibited the highest cell adhesion, with HCT-116 as well as HT-29 demonstrating 72.5 ± 5.9 % and 74.2 ± 4.9 %, respectively. The lowest cell adhesion was observed in isolate AN18 (*L. curieae)* that demonstrated 11.7 ± 2.2 % and 11.5 ± 1.7 % with HCT-116 and HT-29, respectively. Results of this study completely agree with Reid *et al*. (1988) on the relationship of auto-aggregation and cell adhesion (Figure 3).

CONCLUSION

In this study, three LAB strains *E. durans* (MF405179.1), *E. faecium* (MF574466.1) and *L. curieae* (MF405178.1) isolated from banana var. *kolikuttu*, *seeni parakum* and *ambul nadee* respectively, exhibited superior probiotic characteristics. Isolates K08 (*E. durans*) and SP26 (*E. faecium*) demonstrated superior hydrophobic properties and auto aggregative properties, which enable them to exhibit cell adhesion. Therefore, the present study provides evidence on the potential application of banana flour as an ideal substrate to produce non-dairy probiotic food. It is recommended to investigate the compatibility of banana flour in novel non-dairy food formulations including banana flour incorporated supplements/preparations, non-dairy yoghurts, drinks including frizzy drinks and confectionaries that can be developed into commercial products.

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