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ORIGINAL ARTICLE

Lactic acid bacteria isolated from fermented flour of finger millet, its probiotic attributes and bioactive properties

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Abstract

This study aims to isolate and identify lactic acid bacteria from fermented flour of selected finger millet varieties grown in Sri Lanka and to evaluate their probiotic attributes and bioactive properties in vitro. Fifteen lactic acid bacteria were isolated from three varieties of fermented finger millet flour namely *ravi*, *raavana* and *oshadha*. These isolates were screened for phenotypical and biochemical characteristics. The selected isolates were identified by 16 S rRNA sequencing as *Bacillus cereus* (five strains), *Streptococcus lutetiensis*, *Lactobacillus plantarum*, *Lactobacillus fermentum* (two strains), *Brevibacillus borstelensis*, *Paenibacillus* species, *Lactococcus lactis* subspecies *lactis*, *Enterococcus faecium*, *Pediococcus acidilactici*, and *Enterococcus lactis*, and their partial sequences were deposited in GenBank. Among them, five isolates including two isolates, *L. plantarum* MF405176.1 and *L. fermentum* MF033346.1 isolated from *ravi*; two isolates, *L. lactis* MF480428.1 and *E. faecium* MF480431.1 isolated from *raavana*; and *P. acidilactici* MF480434.1 isolated from *oshadha* varieties respectively, exhibited in vitro safety attributes and could tolerate acid, gastric juice, bile, salt, phenol, and temperature under simulated gastric conditions, and also were susceptible to antibiotics tested. Further, they demonstrated bactericidal activity against both drug-sensitive and multidrug-resistant pathogens. Among the selected isolates, *L. plantarum* MF405176.1 demonstrated highest hydrophobicity and adhesion to both colon colorectal adenocarcinoma and colon colorectal carcinoma cell lines. *L. lactis* subspecies *lactis* MF480428.1 exhibited the highest auto-aggregation and 2, 2, diphenyl-1-picrylhydrazyl free radical scavenging activity. *P. acidilactici* MF480434.1 demonstrated the lowest IC_{50} values against HCT-116 and HT-29 cells. None of the LAB isolates could assimilate > 10% cholesterol in vitro.

Keywords Fermented finger millet flour · Lactic acid bacteria · Probiotics · Bioactivity

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 utilized by probiotics, which ultimately provide health benefits to the host (FAO/WHO 2007). 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 gastrointestinal diseases (such as irritable bowel syndrome, lactose intolerance, constipation, *Helicobacter pylori* infection, and gastric ulcers), improvement of immunity, upper respiratory tract infections, uro-genital health, oral health, sexually

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transmitted diseases, allergies, inflammation reactions, metabolism, neurological disorders, and pregnancy- and childhood-associated disorders (Felley et al. 2001; Chmielewska and Szajewska 2010; Guglielmetti et al. 2011; Homayouni et al. 2012; Kechagia et al. 2013; Sharma and Shukla 2016). In order to exert beneficial effects to the host, potential probiotic candidate should possess a number of desirable characteristics, such as tolerance to gastric concentrations of acid, bile, salt, phenol, gastric juice, and temperature; ability to aggregate and adhere in to epithelial cells; and free from virulence causing factors. These characteristics facilitate smooth transition of probiotics through gut and enable colonization (Gibson and Fuller 2000). Due to proven health benefits, probiotic foods have gained a high market revenue (Sharma et al. 2013) and expected to reach US$64.02 billion by 2022 (rnrmarketresearch.com) (RnR Market Research 2017). Majority of probiotic food are of dairy origin, considering several known health risks associated with 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 have led scientists to pursuit alternative substrates to produce non-dairy probiotic food of non-dairy origin (Kumar et al. 2015).

Finger millet (*Elucine coracana*) is the most commonly consumed cereal after rice in Africa and South Asia and has been denoted as the crop for future use due to its high yield, resistance to pests and diseases, and superior adaption to diverse environmental conditions (Chandra et al. 2016). Besides being an abundant, low-cost, highly nutritive ingredient, finger millet flour is rich in prebiotics including resistant starch and dietary fiber (Saleh et al. 2013); therefore, it is an ideal substrate for non-dairy probiotic food. In Sri Lanka, over 200 germplasm accessions of finger millet varieties are preserved at the Plant Genetic Resource Centre, Gannoruwa, Sri Lanka (Dasanayaka 2016). Among them, three varieties, *Ravi*, *Ravana*, and *Oshadha*, are recommended as early maturity and high yielding varieties by the Department of Agriculture, Sri Lanka. Although the compatibility of finger millet flour in development of non-dairy probiotic food is extensively reported in African continent (Adebiyi et al. 2017), to the best of our knowledge, no studies have been reported to evaluate flour of finger millet varieties grown in Sri Lanka as a potential probiotic and prebiotic source. Among the probiotic potential different microorganisms, lactic acid bacteria (LAB) are extensively reported as commercial probiotics (Menconi et al. 2014). With this background, the objective of this study was to isolate and identify lactic acid bacteria from fermented flour of selected finger millet varieties grown in Sri Lanka and to evaluate their probiotic attributes and bioactive properties in vitro.

Materials and methods

Sample collection and isolation of lactic acid bacteria

Three finger millet varieties, *Ravi*, *Ravana*, and *Oshadha*, were selected for the study. Seeds were collected from the germplasm of the Field Crop Research and Development Institute, Mahailluppallama, Sri Lanka. The seeds were transported to the microbiology laboratory, Industrial Technology Institute within 6 h at $< 20 \pm 2$ °C. Samples of each variety were washed with sterilized water under aseptic conditions and oven dried at 35 °C (Memmert, Germany) till the moisture content reduced to < 10%. Dried samples were ground in a rotor mill (Fritsch, Germany), passed through sieve (0.5 mm), and were packed in commercially sterile pouches and stored at 4 °C. Twenty-five grams of each variety ($n = 6$) was transferred into pre-sterilized glass beakers covered with aluminum foil. The flour samples were mixed with 100 ml of sterilized 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 sterilized saline (0.85% NaCl, *w*/*v*), spread on 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 characterization of lactic acid bacteria

Plates were observed for the colonies with typical LAB morphology. These were isolated and further streaked again on fresh MRS agar at 37 °C to check the purity. Colony morphology (form, size, shape, surface, texture, color, elevation, and margin) of the purified LAB isolates was evaluated as per the standard protocols (Kunchala et al. 2016). Phenotypical characterization was performed using Gram staining, endospore staining (Collee et al. 2006), and motility evaluation (Pyar and Peh. 2014). Biochemical tests including indole, methyl red, voges proskauer, citrate utilization, catalase, oxidase, starch hydrolysis, urease, and amino acid hydrolysis (arginine, orinithine and lysine) were performed according to the methods given in Mackie and MaCartney, 14th edition. Sugar fermentation pattern of LAB isolates were investigated according to Nazari et al. 2012 for sugars including glucose, fructose, maltose, lactose, galactose, melezitose, melibiose, arbinose, ribose, sucrose, salicin, sorbitole, mannitole, cellulose, cellobiose, and dextrose (Sigma Aldrich, UK).

Molecular identification of lactic acid bacteria

Among the newly isolated bacteria, ten isolates that exhibited phenotypical and biochemical characteristics similar to LAB were selected for molecular identification. The selected isolates were inoculated in to MRS broth and incubated at 37 °C

for 18 h. From each bacterium, 2 ml was centrifuged (14,000×*g* at 4 °C for 2 min). Pellets were mixed with Tris EDTA buffer (200 μl) and re-centrifuged (14,000×*g* at 4 °C for 2 min). This procedure was repeated once more. To each pellet, 10 μl of proteinase K (100 μg/μl, *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 (14,000×*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 ice bath for 1 h. After incubation, the tubes were centrifuged (14,000×*g* at 4 °C for 5 min). Subsequently, the pellets were mixed in ethanol (1 ml, 70%, *v*/*v*) and centrifuged (14,000×*g* at 4 °C for 5 min). Supernatants were discarded and ethanol was evaporated. Finally, each pellet was dissolved in ultra pure water (40 μl) and stored at − 20 °C (Shahriar et al. 2011 modified). The extracted DNA was analyzed for its quantity and purity using a gel documentation system (BIO-RAD, UK) by mixing 5 μl of DNA with 2 μl gel loading dye, and gel was run at 60 V for 15 min. Polymerase chain reaction (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), initial elongation (72 °C, 1 min), and final elongation (72 °C, 10 min). The universal primers 27F (5′AGAGTTTGATCCTGGCTCAG 3′) and 1492R (5′GGTTACCTTGTTACGACTT3′) were used in PCR (Doi et al. 2013), and the PCR product purification was carried out using 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). Sequences with an identity of 98–99% or higher than those in databases were allocated to the same species (Alschul et al. 1990). The partial sequences of 16S rRNA gene of LAB isolates were deposited at GenBank NCBI, USA. Phylogenetic analysis was conducted according to neighbor-joining method (Saitou and Nei. 1987) in MEGA7.

In vitro safety attributes of lactic acid bacteria

Identified LAB isolates were investigated for safety attributes including hemolysis, DNAse, and gelatin hydrolysis (in vitro). For the hemolysis test, blood agar 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 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%) (*w*/*v*). For the DNAse test, DNAse agar (Oxoid, UK) was prepared according to the manufacturer's instructions. The enzyme activities were performed by inoculating the LAB isolates (105 cfu/ml) in to the respective agar described above and incubated (37 °C) for 48 h (Gupta and 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 the clear zones, around the inoculated area. DNAse agar plates were observed for thick plaque of growth around the colonies. The reference strains used were *Streptococcus pyogene* 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)

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, 15 min. Subsequently the pellets were washed with sterile saline (0.89 NaCl, *w*/*v*) and centrifuged at 10,000×*g* at 4 °C, 15 min. Each pellet was individually dissolved in MRS broth and adjusted to 0.5 Macfarland turbidity standards (1.5×10^8 CFU/ml). From each bacteria, 100 μl was inoculated in MRS broth adjusted as per the gut conditions (in vitro) including pH (1.5, 3, and 4 *v*/*v* HCl and 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*), and simulated gastric juice of pH 1.5 containing 0.05 g/l porcine bile, 0.10 g/l lysozyme, and 0.10 g/l pepsin according to the method given by Aswathy et al. 2008.

Antibiotic susceptibility of lactic acid bacteria in vitro

The LAB isolates that survive in gastric conditions were investigated for the susceptibility/resistance to antibiotics. Agar disc diffusion method (CLSI 2012) was performed to evaluate the antibiotic susceptibilities of newly isolated LAB against cefotaxime (15 μg), gentamycin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg), sulfamethoxazole (25 μg), ciprofloxacin (5 μg), amikacin (30 μg), bacitracin (10 units), ampicillin (10 μg), amoxicillin (30 μg), cephalothin (30 μg), vancomycin (30 μg), and erythromycin (30 μg). The LAB isolates were grown on MRS agar with respective antibiotics.

Auto-aggregation ability of lactic acid bacteria in vitro

The LAB isolates that survived in gastric conditions were further investigated for their auto-aggregation ability in vitro according to the method of Kos et al. (2003) with some modifications. LAB isolates were grown in MRS broth and adjusted to 0.5 Macfarland turbidity standards (1.5×10^8 CFU/ml) as described earlier. The cell suspensions 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 suspensions of each reaction mixer was transferred in to the 96-well plates. Absorbance was measured at 620 nm. The auto-aggregation percentage was expressed as a function of time using the following formula.

$$\text{Auto-aggregation}(\%) = 1 - \frac{At}{A0} \times 100$$

At (absorbance at time·$t = 1, 2, 3, 4\,\text{h}$), $A0$ (absorbance at $t = 0$)

Cell surface hydrophobicity of lactic acid bacteria in vitro

Cell surface hydrophobicity was measured according to the method given by Kos et al. 2003. The LAB isolates were inoculated in 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, 15 min. The pellets were washed twice with PBS. The cells were adjusted to 10^8 CFU/ml and re-suspended in KNO_3 solution (0.1 Mol/dm^3, pH 6.2). Initial absorbance of cell suspensions were measured at 620 nm (A_0). Each LAB isolate was investigated for their ability to adhere into three solvents: xylene (non-polar solvent), ethyl acetate (non-polar and basic solvent), and chloroform (non-polar and acidic solvent). One milliliter of solvent was added to 3 ml of 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. The percentage microbial adhesion to solvents (MATS) was calculated using following formula:

$$\text{MATS}\ (\%) = 1 - \frac{\text{A1}}{\text{A0}} \times 100$$

where A1 = absorbance of aqueous layer, A_0 = Absorbance of control.

Cell adhesion of lactic acid bacteria in vitro

Ability of selected LAB isolates to adhere in 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 (HTC-116) in vitro was performed according to Duary et al. 2011 with modifications. The cell lines were obtained from the bio bank of the International Centre for Chemical and Biological Sciences (ICCBS), University of Karachi, Pakistan, and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (*v*/v), 1% l-glutamine (*v*/*v*), and 1% penicillin-streptomycin solution (*v*/*v*). The medium was sterilized by filtration and stored at 4 ± 1 °C until use. Monolayer of cell lines with 80% confluence was used in the study.

The LAB isolates were grown in MRS broth and adjusted to 0.5 Macfarland turbidity standards (1.5×10^8 CFU/ml) as described earlier. LAB cells were further, serially diluted in tubes containing sterilized PBS and adjusted the cell concentrations to 1.5×10^2 cfu/ml. from each cell line, 10^6 cells/ml in 0.5 ml of DMEM was placed in six-well tissue culture plates and incubated at 37 ± 1 °C for 24 h in 5% CO_2. The media of the flaks containing HT-29 and HCT-116 were discarded, and the new media were added every other day. After 14 days of incubation (14 days post confluence phase), monolayers were washed twice with sterile PBS. One milliliter of LAB isolate and 1 ml of DMEM media were inoculated in to the wells containing monolayers ($n = 9$). The plates were further incubated at 37 ± 1 °C for 1 h in 5% CO_2 (Nuaire NU-8700E, USA). After 1 h, the monolayers were washed five times with sterile PBS, fixed with methanol and observed under microscope (Optica B 500 i, Italy) at magnification of × 100. Subsequently, the attached LAB cells/well were counted and adhesion ability was expressed as the percentage ratio between the LAB cells initially inoculated and the LAB cells remained attached after washing with PBS.

Investigation of isolated lactic acid bacteria for in vitro bioactivity

Intracellular cell free extracts isolated lactic acid bacteria were investigated for their potential bio-activities including anti-bacteria, ant-cancer, DPPH FREE radical scavenging activity, and cholesterol assimilation ability in vitro.

Preparation of ICCE of LAB isolates

Selected LAB isolates were inoculated into sterile MRS broth and incubated at 37 ± 1 °C for18 h. The LAB cells were harvested by centrifugation at 10,000×g, 5 min at 4 °C; pellets were washed twice and re-suspended in sterile phosphate-buffered saline (PBS). The cells were adjusted to 0.5 Macfarland standard (1.5×10^8 CFU/ml) and the intracellular components of the cells were extracted by ultrasonic disruption (Sonorex RK100H, Germany) as described by Zhang et al. (2011). Debris of the LAB cells was removed by centrifugation (10,000×g, 10 min at 4 °C) to obtain the intracellular cell free extract (ICCE).

Anti-bacterial activity of lactic acid bacteria

The anti-bacterial activity was studied against drug-sensitive organisms including *Escherichia coli* ATCC 2592, *Klebsiella pneumoniae* ATCC 35594, *Staphylococcus aureus* ATCC 6571, *Streptococcus sanguinis* ATCC 10556, *Streptococcus salivarius* ATCC 13419, *Enterococcus faecalis* ATCC49532, *S. flexenari* ATCC 12022, *Acinetobacter baumannii* ATCC 17978, *Streptococcus mutans* ATCC 25175, and *Streptococcus pyogenes* ATCC 700294 and multidrug-resistant organisms including *Escherichia coli* ATCC 35218, *Staphylococcus aureus* 16 EMRSA NCTC 13143, *Klebsiella pneumoniae* ATCC 700603, *Enterococcus faecalis* ATCC 700802, and *Salmonella enterica* ATCC 700408. The abovementioned pathogenic bacterial strains were obtained from Microbial Bank of ICCBS, University of Karachi, Pakistan. Anti-bacterial activity of intracellular cell-free extracts of the selected LAB was evaluated according to the method given in Tharmaraj and Shah (2009) with modifications. Each test pathogen was inoculated in to tubes containing sterile nutrient broth and incubated at 37 ± 1 °C for 18 h. After incubation, the tubes were adjusted to 0.5 Mcfarland turbidity standards (1.5×10^8 CFU/ml). Soft agar was melted and at 45 ± 2 °C, 100 μl of each pathogenic culture was added to separate soft agar tubes and poured onto the solidified agar plates. Plates were rotated to evenly distribute the culture and allowed to solidify. By using sterile 6-mm diameter borer, wells were made on the solidified plates; wells were clearly marked. Hundred microliters of each LAB intracellular cell free extract was added to the wells. PBS was used as the control. Plates were incubated at 37 ± 1 °C for 24 h. After incubation, inhibition zones were measured using a calibrated scale and the anti-bacterial activity was interpreted as per the criteria given by Reis et al. 2016 (inhibition zones < 4 mm in diameter considered as weak activity, inhibition zones of 5–9 mm in diameter considered as average activity, inhibition zones > 10 mm in diameter considered as strong activity).

Anti-cancer activity of the intracellular cell free extract of lactic acid bacteria

The intracellular cell-free extract of selected LAB was prepared as described earlier, and the ICCE were freeze dried to evaporate de-ionized water and stored at 4 °C until further use. The freeze-dried LAB ICCE were dissolved in dimethyl sulfoxide to obtain the concentrations of 5 μg/ml, 25 μg/ml, 50 μg/ml, 250 μg/ml, and 500 μg/ml. Anti-cancer activity was evaluated using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay in *Homo sapiens* colon colorectal adenocarcinoma (HT-29) ATCC HTB-38 and *Homo sapiens* colon colorectal carcinoma (HTC-116) ATCC CCL-247 cell lines obtained from the BioBank of the ICCBS, University of Karachi, Pakistan, according to the method of Aliabadi et al. 2014. The cell lines were as described earlier. Dimethyl sulfoxide and cetuximab (Merck, UK) were used as the control and reference drug, respectively.

DPPH free radical scavenging activity of intracellular cell free extracts of lactic acid bacteria

The 2, 2, diphenyl-1-pricrylhydrazyl DPPH free radical scavenging activities of freeze-dried ICCE were dissolved in dimethyl sulfoxide to obtain the concentrations of 5 μg/ml, 25 μg/ml, 50 μg/ml, 250 μg/ml, and 500 μg/ml and evaluated the DPPH free radical scavenging activity according to the method described by Perera et al. 2016. The absorbance was recorded at 517 nm. The free radical scavenging activity was calculated as follows.

$$\text{Scavenging activity}\ (\%) = \left[\left(\text{control}^{\text{OD}} - \text{sample}^{\text{OD}}\right)/\text{control}^{\text{OD}}\right] \times 100$$

$^{\text{OD}}$ (absorbance at 517 nm).

Dimethyl sulfoxide and 6-hydroxy-2-5-7-8-tetramethylchroman-2-carboxylic acid (trolox) were used as the control and reference standard, respectively.

BSH activity and cholesterol assimilation of lactic acid bacteria

Bile salt hydrolysis screening medium was prepared by supplementing MRS agar with 0.5% porcine bile *w/v*, 0.5% sodium tauroglycocholate *w/v*, 0.5% taurodeoxycholic acid sodium salt *w/v*, and 0.37 $CaCl_2$ g/l (Singh et al. 2014). LAB cells were adjusted to 0.5 Macfarland standards (1.5×10^8 CFU/ml) and from each isolate, 100 μl was spotted on bile salt hydrolysis (BSH) screening plates and incubated at 37 °C for 48 h. After incubation, BSH activity was calculated by measuring the precipitation zones.

Cholesterol assimilation of LAB was quantified according to the method described by Duchesneau et al. (2014) with some modifications. Modified MRS broth (MRS broth containing porcine bile 0.3%, *w/v* and water-soluble cholesterol 0.1 g/l) was prepared, and absorbance was measured at 570 nm. LAB cells were adjusted to 0.5 Macfarland standards (1.5×10^8 CFU/ml) and from each isolate, 100 μl was inoculated in modified MRS broth and incubated at 37 °C, 48 h followed by centrifugation at 4500×*g* (4 °C, 5 min). Supernatant (1 ml) mixed with ethanol (3 ml, 95%, *v/v*), potassium hydroxide (2 ml, 50%, *w/v*), heated (60 °C, 10 min) and cooled. Hexane (5 ml) and distilled water (1 ml) were added to the mixture, incubated at room temperature for 10 min and separated an aliquot of hexane layer, and the solvent was evaporated under nitrogen. To these tubes, freshly prepared *O*-

Table 1 Carbohydrate fermentation pattern of lactic acid bacteria isolated from fermented flour of finger millet varieties

Isolate code	G	F	M	L	GA	MZ	MB	A	R	SU	SA	SO	MN	C	CB	D
R01	+	+	+	+	+	−	−	−	−	+	−	−	−	−	−	+
R03	+	+	+	+	+	−	−	−	−	+	−	−	−	−	−	+
R05	+	+	+	+	+	−	−	−	−	+	−	+	+	−	−	+
R06	+	+	+	+	+	−	−	−	−	+	+	+	+	−	−	+
R17	+	+	+	+	+	−	−	−	−	+	−	+	+	−	−	+
RV02	+	+	+	+	+	−	−	−	−	+	−	+	+	−	−	+
RV07	+	+	+	+	+	−	−	−	−	+	−	+	+	−	−	+
RV11	+	+	+	+	+	−	−	−	−	+	−	+	+	−	−	+
RV19	+	+	+	+	+	−	−	−	−	+	−	+	+	−	−	+
RV28	+	+	+	+	+	−	−	−	−	+	−	−	+	−	−	+
O01	+	+	+	+	+	−	−	−	−	+	+	+	−	+	−	+
O02	+	+	+	+	+	−	−	−	−	+	−	−	−	−	−	+
O06	+	+	+	+	+	−	−	−	−	+	−	−	−	−	−	+
O24	+	+	+	+	+	−	−	−	−	+	−	−	+	−	−	+
O28	+	+	+	+	+	−	−	−	−	+	−	−	+	−	−	+

The letters R, RV, and O refers to lactic acid bacteria isolated from *Ravi*, *Ravana*, and *Oshadha* varieties of finger millet. The letters G, F, M, L, GA, MZ, MB, A, R, SU, SA, SO, MN, C, CB, and D refer to glucose, fructose, maltose, lactose, galactose, melezitose, melibiose, arabinose, ribose, sucrose, salicin, sorbitol, mannitol, cellulose, cellobiose and dextrose, respectively. + ferment, − do not ferment

+ ferment, − do not ferment

phtalaldehyde in acetic acid (1 ml, 50 mg/ml) and concentrated H_2SO_4 (250 μl) was mixed and incubated at room temperature (10 min). Absorbance of reaction mixture was measured at 570 nm in UV spectrophotometer. Uninoculated MRS broth was used as the control. A standard curve of absorbance versus cholesterol concentrations was generated using the cholesterol concentrations of 0–500 μg/ml cholesterol in MRS. The cholesterol assimilated by the LAB isolates was calculated using the following formula:

$$\text{Cholesterol assimilated } (\mu g/ml) = [\text{cholesterol } (\mu g/ml)]_{0h} - [\text{cholesterol } (\mu g/ml)]_{24h}$$

Table 2 16S ribosomal RNA sequencing results of the isolated lactic acid bacteria and their GenBank accession numbers

Isolate code	Genus /Species identification	NCBI GenBank accession
R01	*Paenibacillus* species	MF480545.1
R03	*Bacillus cereus*	MF480550.1
R05	*Streptococcus lutetiensis*	MF574476.1
R06	*Brevibacillus borstelensis*	MF480552.1
R17	*Lactobacillus plantarum*	MF405176.1
RV02	*Lactobacillus fermentum*	MF033346.1
RV07	*Bacillus cereus*	MF480468.1
RV11	*Lactobacillus fermentum*	MF405134.1
RV19	*Lactococcus lactis* subspecies *lactis*	MF480428.1
RV28	*Enterococcus faecium*	MF480431.1
O01	*Bacillus cereus*	MF574478.1
O02	*Bacillus cereus*	MF574479.1
O06	*Bacillus cereus*	MF574477.1
O24	*Pediococcus acidilactici*	MF480434.1
O28	*Enterococcus lactis*	MF574475.1

The letters R, RV, and O refer to lactic acid bacteria isolated from *Ravi*, *Ravana*, and *Oshadha* varieties of finger millet

Statistical analysis

All experiments were conducted in triplicates 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 the multiple comparisons using Tukey's family error rate performed to analyze the data. Values $P < 0.05$ were considered as significant.

Results

Isolation of lactic acid bacteria from fermented finger millet flour

A total of 90 bacterial colonies were isolated from three varieties of fermented finger millet flour namely *Ravi* (28), *Ravana* (31), and *Oshadha* (31). Among them, 57 bacterial colonies were demonstrated to have typical LAB colony morphology on MRS agar as described by de Man et al. (1960) and of which 38 were gram-positive cocci/bacilli that are non-spore forming and non-motile organisms isolated from *ravi* (14), *raavana* (13), and *oshadha* (11). LAB are usually gram-positive cocci or bacilli, non-spore forming, and non-motile organisms; hence, these new isolates were assumed for their phenotypical similarity to LAB.

Biochemical characteristics of lactic acid bacteria

Biochemical characterization of the 38 phenotypically LAB identical isolates revealed that 15 isolates namely R01, R03, R05, R06, R17, RV02, RV07, RV11, RV19, RV28, O01,O02, O06, O24, and O28 were negative for catalase, oxidase, indole, voges proskauer, methyl red, citrate utilization, and urease. LAB isolates R03, R17, RV02, O01, and O02 were positive for starch hydrolysis. Isolates R03, R17, RV02, RV11, and O24 could hydrolyze arginine, while none of the LAB isolates could hydrolyze orinithine and lysine. LAB are usually negative to biochemical tests including indole, methyl red, voges proskauer, citrate utilization, catalase, and oxidase (Chowdhury et al. 2012). Amino acid hydrolysis during growth provides energy to LAB for their metabolic activities during fermentation (Pessione and Cirrincione 2016). Hence, these 15 LAB isolates were selected for further studies.

Sugar fermentation pattern of lactic acid bacteria

Lactic acid bacteria represents a group of microorganisms that are functionally related by their ability to produce lactic acid

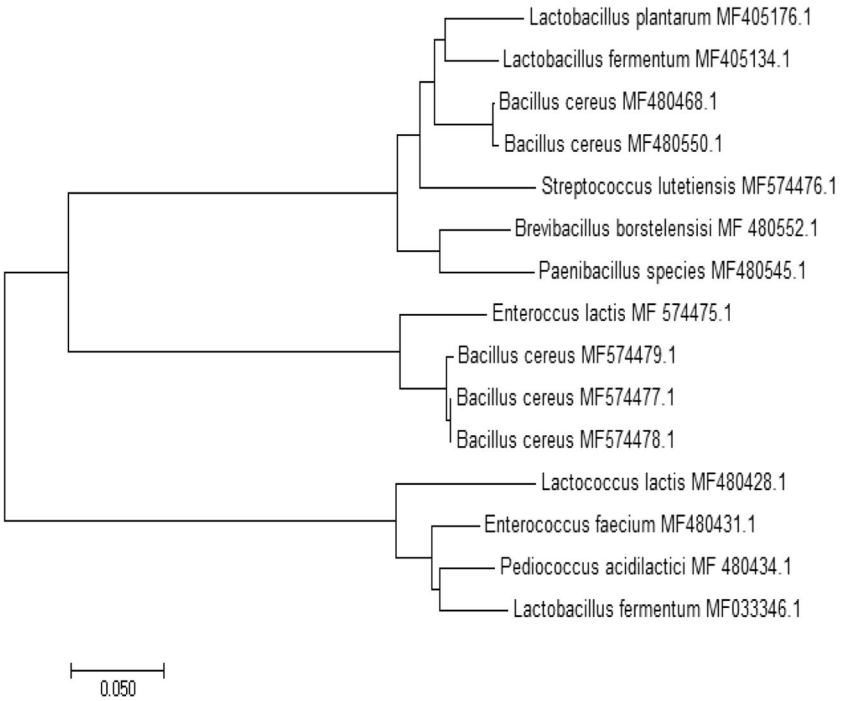

Fig. 1 Evolutionary relationships of taxa of the lactic acid bacteria isolated from fermented flour of finger millet. The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 1.16278609 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. The analysis involved 15 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + non-coding. All positions containing gaps and missing data were eliminated. There were a total of 355 positions in the final dataset. Evolutionary analyses were conducted in MEGA7*

by fermenting sugars. Therefore, the ability to ferment different carbohydrates is one of the important characteristic that makes LAB ideal for fermentation. All 15 selected LAB isolates could ferment sugars including glucose, fructose, maltose, lactose, galactose, sucrose, and dextrose. Only isolate O01 ferments cellulose. LAB strains R06 and O01 could also ferment salicin, while, except isolates R01, R03, and RV28, all others ferment sorbitol. Except isolates R01, R03, O01, O02, and O06, all other isolates ferment mannitol. None of the isolates could ferment melezitose, melibiose, arabinose, 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.

Molecular identification of newly isolated lactic acid bacteria

The LAB were identified by comparing 16S rRNA partial sequences with those present in NCBI GenBank. 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 GenBank are presented in Table 2, and the phylogenetic tree of newly isolated LAB is presented in Fig. 1.

Safety attributes of isolated lactic acid bacteria

Out of 15 LAB isolated from fermented finger millet flour, seven isolates namely R17 (*L. plantarum*), RV02 (*L. fermentum*), RV11 (*L. fermentum*), RV19 (*L. lactis*), RV28 (*E. faecium*), O24 (*P. acidilactici*) ,and O28 (*E. lactis*) were free from hemolysis, DNAse, and gelatin hydrolysis in vitro thus possessing safety attributes. 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).

Probiotic attributes of lactic acid bacteria

Seven LAB isolates R17, RV02, RV11, RV19, RV28, O24, and O28 demonstrated significant differences ($P < 0.05$) in their probiotic attributes. Except isolates RV11and O28, others tolerated acid up to pH 1.5 (Table 3). Probiotic bacteria need to survive passage through the stomach, where the pH can lie between 1.5 and 2.0 and further required to stay viable for 4 h or more before they move to the gastrointestinal 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 (Bakari et al. 2011; Dunne et al. 2001). The average

Table 3 Probiotic characteristics of isolated lactic acid bacteria

Inhibitory condition	Survival of LAB isolates						
	R17	RV02	RV11	RV19	RV28	O24	O28
pH 7	+	+	+	+	+	+	+
pH 3	+	+	+	+	+	+	+
pH 1.5	+	+	−	+	+	+	−
Gastric juice	+	+	−	+	+	+	−
0.2% bile	+	+	+	+	+	+	+
0.5% bile	+	+	+	+	+	+	+
1.0% bile	+	+	+	+	+	+	+
1.5% bile	+	+	+	+	+	+	−
2.0% bile	+	+	−	−	+	−	−
4% NaCl	+	+	+	+	+	+	+
5% NaCl	+	+	+	+	+	+	+
8% NaCl	+	+	+	+	+	+	+
12% NaCl	+	+	−	+	+	+	+
0.1% phenol	+	+	+	+	+	+	+
0.2% phenol	+	+	+	+	+	+	+
0.3% phenol	+	+	+	+	+	+	+
0.4% phenol	+	+	−	+	+	+	−
0.5% phenol	−	−	−	−	−	−	−
30 °C	+	+	+	+	+	+	+
37 °C	+	+	+	+	+	+	+
42° C	+	+	+	+	+	+	+

The letters R, RV, and O refers to lactic acid bacteria isolated from *Ravi*, *Ravana*, and *Oshadha* varieties of finger millet

+ LAB survived in the presence of inhibitory conditions, − LAB did not survive in the presence of inhibitory conditions)

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); hence, commercial probiotic bacteria need to tolerate at least up to 0.3% bile (Dunne et al. 2001). Except isolate O28, all isolates tolerated up to 1.5% bile. Isolates namely R17, RV02, and RV28 tolerated up to 2% bile (Table 3). All isolates grew at temperature between 30 and 42 °C (Table 3). Ability to tolerate normal body temperature enables the probiotic to have an active metabolism in the gut. On the other hand, ability to tolerate high temperature enables the better rate of growth as well as high yield of lactic acid production during fermentation and reduces the contaminations in fermentation processes (Ibourahema et al. 2008). Except isolate RV11, others could tolerate NaCl up to 12% (Table 3). When LAB survives 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 concentration of salts in the gut (Menconi et al. 2014). Except isolates RV11 and O28,

Table 4 Antibiotic susceptibility/resistance pattern of newly isolated lactic acid bacteria

Antibiotics	Diameter (in mm) of the inhibition zone of the LAB isolates				
	R17	RV02	RV19	RV28	O24
Cefotaxime	19.25 ± 0.75b	24.50 ± 0.29a	26.25 ± 0.25a	21.50 ± 0.87b	18.25 ± 0.25b
Gentamycin	13.00 ± 0.0c	12.50 ± 0.50c	12.50 ± 0.64c	18.50 ± 0.50a	13.75 ± 0.63c
Tetracycline	0. 0 ± 0. 0e	9.75 ± 0.48d	12.00 ± 0.0c	11.75 ± 0.48c	23.50 ± 0.50b
Chloramphenicol	14.75 ± 0.75b	16.00 ± 0.0b	20.25 ± 0.85a	0.0 ± 0.0c	15.75 ± 0.25b
Sulfamethoxazole	0.0 ± 0.0d	22.00 ± 0.41b	23.00 ± 0.0b	20.50 ± 0.29b	19.00 ± 0.0b,c
Ciprofloxacin	20.25 ± 0.25b	11.75 ± 0.48d	16.50 ± 0.50c	24.75 ± 0.48a	10.50 ± 0.50d
Amikacin	24.75 ± 0.25b	23.25 ± 0.25b	27.00 ± 0.58a	21.00 ± 0.58c	18.00 ± 0.0d
Bacitracin	34.75 ± 0.25a	29.75 ± 0.85b	16.25 ± 0.25e	21.00 ± 0.0d	16.00 ± 0.0e
Ampicillin	10.75 ± 0.63c	14.50 ± 0.50b	12.00 ± 0.0c	9.25 ± 0.25c,d	26.25 ± 0.25a
Amoxicillin	13.50 ± 0.29d	10.00 ± 0.0c	12.50 ± 0.50d	11.75 ± 0.48d	24.75 ± 0.48b
Cephalothin	23.75 ± 1.31a	16.50 ± 0.50c	17.00 ± 0.0c	18.50 ± 0.29c	15.50 ± 0.65c
Vancomycin	14.25 ± 0.85b	15.50 ± 0.50b	16.50 ± 0.29b	12.75 ± 0.48b	22.25 ± 0.85a
Erythromycin	20.75 ± 0.25b	23.00 ± 0.0a	21.25 ± 0.63b	22.75 ± 0.25a,b	16.75 ± 0.48d

Data is expresses as mean ± SEM, $n = 9$. With in a row, mean values with lowercase letters are significantly different ($P < 0.05$). The letters R, RV, and O refers to lactic acid bacteria isolated from *Ravi*, *Ravana*, and *Oshadha* varieties of finger millet. Concentration of antibiotic discs (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, and erythromycin—30 μg)

others tolerated simulated gastric juice of pH 1.5 (Table 3). Ability of potential probiotic strains to survive in the human gastric juice, which has pH between 1.5 and 2.0, is the key indication that displays the ability of the strains to survive passage through the stomach (Shewale et al. 2014). Except isolates RV11 and O28, others tolerated 0.4% phenol (Table 3). 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. Lactic acid bacterial isolates R17 (*L. plantarum)*, RV02 (*L. fermentum*), RV19 (*L. lactis*), RV28 (*E. faecium*), and O24 (*P. acidilactici*) demonstrated superior probiotic attributes and hence were selected for further studies.

Antibiotic susceptibility of lactic acid bacteria

Lactic Acid Bacterial isolates demonstrated significant differences ($P < 0.05$) in susceptibility/resistance at tested antibiotic discs concentration. Isolates RV02, RV19 and O24 were susceptible to all the antibiotics at tested concentrations (Table 4). Isolates RV 02, RV19, and O24 was susceptible to all the antibiotics at tested concentrations. Resistance or susceptibility to antibiotics alone will not cause risk in probiotic LAB candidates. Ability to transfer the respective antibiotic resistance encoding genes is the real cause of risk (Gueimonde et al. 2013) and therefore need to investigate prior to commercial applications.

Table 5 Auto-aggregation of isolated lactic acid bacteria

LAB isolates	Auto-aggregation (%) of LAB isolates with time (h)				
	1 h	2 h	3 h	4 h	5 h
R17	1.78 ± 0.26d,e	5.56 ± 0.10c,d	7.20 ± 0.11c	44.26 ± 0.56a,b	60.44 ± 1.71b
RV02	3.59 ± 0.27b,c	16.09 ± 0.48a	22.40 ± 0.27a	38.34 ± 0.59d	46.25 ± 0.55c
RV19	10.41 ± 0.12a	12.71 ± 0.75b	17.03 ± 0.30b	49.48 ± 1.06c	68.58 ± 1.55a
RV28	2.57 ± 0.29d	3.76 ± 0.86d	8.77 ± 0.49c	15.41 ± 0.55f	19.16 ± 0.62e
O24	10.53 ± 0.29a	13.91 ± 0.48a,b	15.38 ± 0.20b	27.14 ± 0.54e	36.86 ± 0.89d

Data is expressed as mean ± SEM, $n = 9$. Within a column, mean values with lowercase letters are significantly different ($P < 0.05$). The letters R, RV, and O refers to lactic acid bacteria isolated from *Ravi*, *Ravana*, and *Oshadha* varieties of finger millet

Auto-aggregation ability of lactic acid bacteria

Probiotics that are able to auto-aggregate have the potentiality to adhere in to the gut epithelium. Therefore, aggregation is considered as a prerequisite of an ideal probiotic candidate (Kos et al. 2003). In this study, isolate RV19 (*L. lactis* sub species *lactis*) demonstrated highest auto-aggregation of 68.6%, which is higher than the reported data by Li et al. (2015) of 12.49% auto-aggregation in a strain of *L. lactis* at the end of 5 h incubation. Significant difference ($P < 0.05$) in auto-aggregation was observed among the LAB strains (Table 5). Auto-aggregating ability of LAB strains was observed to be time dependent; therefore, increment of auto-aggregation was observed with the increase of the 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 R17 (*L. plantarum*), RV02 (*L. fermentum*), and RV19 (*L. lactis* sub species *lactis*) demonstrated 67.20 ± 0.50%, 51.72 ± 0.25%, and 45.2 ± 0.88%, affinity to xylene, respectively. Therefore, these strains have elevated hydrophobic properties (Giarous et al. 2009). Further, it was observed that these strains demonstrated highest auto-aggregation ability. Hence, as Del Re et al. (2000) suggested that hydrophobicity improves the auto-aggregation, observations of this study completely agree with their statement. Isolate RV28 (*E. faecium*) and O24 demonstrated 67.40 ± 0.22 and 47.16 ± 0.5% affinity for chloroform respectively, which is an acidic solvent and electron acceptor (Table 6). Based on physio-chemistry of microbial cell surfaces, several studies concluded that the presence of glycoproteinaceous materials in the cell surface of bacteria results in higher hydrophobicity, whereas presence of polysaccharides results in hydrophilic surfaces (Rojas and Conway 1996; Pelletier et al. 1997).

Adhesion to epithelial cell lines by lactic acid bacteria

Significant differences ($P < 0.05$) in adhesion of LAB isolates to HCT-116 and HT-29 cell lines were observed. The results revealed that isolate R17 (*L. plantarum*) exhibited highest cell adhesion with HCT 116 as well as HT 29 demonstrating 53.50 ± 7.3 and 61.66 ± 5.7% adhesion respectively. Whereas, RV28 (*E. faecium*) exhibited the lowest adhesion to cell lines tested (Table 7). Results of this study completely agree with Reid et al. (1988) on the relationship of auto-aggregation and cell adhesion.

Anti-bacterial activity of lactic acid bacteria

Lactic acid bacterial isolates R17 (*L. plantarum)*, RV02 (*L. fermentum*), RV19 (*L. lactis*), RV28 (*E. faecium*), and O24 (*P. acidilactici*) demonstrated potential bactericidal activity against drug-sensitive pathogens including *E. coli*, *K. pneumonia*, *S. aureus*, *S. salvarius*, and *S. flexenari* and multidrug-resistant pathogens including *E. coli*, *S. aureus*, and *S. enterica* (Table 8). This study revealed the potential antibacterial activity of newly isolated LAB strains against pathogens causing infections in gut, skin, and respiratory track.

Anti-cancer activity of intracellular cell free extracts of lactic acid bacteria

The intracellular cell free extract of isolate O24 demonstrated the lowest IC_{50} value of 151.98 ± 2.25 and 240.43 ± 2.57 μg/ml against HCT 116 and HT 29 cells, respectively. Further, IC_{50} of the intracellular cell free extracts of LAB isolates varied between 2.3- and 4-fold with comparison to standard control drug cetuximab that showed IC_{50} value of 89.43 ± 2.65 and 120.55 ± 5.79 μg/ml against HCT 116 and HT 29 cells, respectively (Table 9).

Table 6 Cell surface hydrophobicity of isolated lactic acid bacteria

LAB isolates	In vitro adhesion (%) of LAB strains to different solvents		
	Xylene	Ethyl acetate	Chloroform
R17	67.19 ± 0.50	0 ± 0	32.14 ± 0.47
RV02	51.723 ± 0.88	0 ± 0	27.93 ± 0.22
RV19	45.2 ± 0.18	5.24 ± 0.09	23.40 ± 0.54
RV28	9.7 ± 0.75	0 ± 0	67.02 ± 0.22
O24	18.43 ± 0.71	21.22 ± 0.1	47.16 ± 0.94

Data is expressed as mean ± SEM, $n = 9$. The letters R, RV, and O refers to lactic acid bacteria isolated from *Ravi*, *Ravana*, and *Oshadha* varieties of finger millet

Table 7 Cell adhesion of isolated lactic acid bacteria

LAB isolates	In vitro adhesion (%) of LAB strains to different cell lines	
	HCT-116	HT-29
R17	53.5 ± 7.38	61.66 ± 5.77
RV02	41.66 ± 4.07	48 ± 3.97
RV19	20.16 ± 2.26	27.33 ± 2.99
RV28	19.16 ± 2.26	20.66 ± 1.55
O24	22.16 ± 2.65	29.5 ± 1.87

Data is expressed as mean ± SEM, $n = 9$. The letters R, RV, and O refers to lactic acid bacteria isolated from *Ravi*, *Ravana*, and *Oshadha* varieties of finger millet

Table 8 Anti-bacterial activity of intracellular cell free extracts of isolated lactic acid bacteria

Test pathogens	Mean diameter of the zones of inhibition (in mm)				
	R17	RV02	RV19	RV28	O24
E. coli ATCC 2592	15.33 ± 0.33b	16.67 ± 1.45b	15.33 ± 0.33b	17.33 ± 0.33b	15.33 ± 0.33b
K. pneumonia ATCC 35594	15.33 ± 0.33c	14.66 ± 0.33c	15.00 ± 0.00c	18.33 ± 0.33b	18.33 ± 0.33b
S. aureus ATCC 6571	10.33 ± 0.33d	13.33 ± 0.33c	12.66 ± 0.33c	13.33 ± 0.33c	13.00 ± 0.57c
S. sanguinis ATCC 10556	0.00 ± 0.00b	0.00 ± 0.00b	0.00 ± 0.00b	0.00 ± 0.00b	0.00 ± 0.00b
S. salvarius ATCC 13419	14.66 ± 0.33d	16.00 ± 0.00c	16.00 ± 0.00c	10.66 ± 0.33e	14.00 ± 0.00d
S. flexenari ATCC 12022	14.33 ± 0.33b	15.66 ± 0.33b	16.00 ± 0.00b	12.33 ± 0.66c	15.66 ± 0.33b
E. faecilis ATCC 49532	8.33 ± 0.33d	0.00 ± 0.00e	0.00 ± 0.00e	0.00 ± 0.00c	12.66 ± 0.33e
A. baumani ATCC 17978	0.00 ± 0.00g	15.33 ± 0.33c	11.33 ± 0.33f	13.00 ± 0.00e	14.33 ± 0.33d
S. mutans ATCC 25175	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c
S. pyogenes ATCC 700294	0.00 ± 0.00e	12.00 ± 0.00c	12.33 ± 0.33c	15.00 ± 0.57b	13.66 ± 0.33b,c
E. coli ATCC35218[a]	15.33 ± 0.33c	16.00 ± 0.00c	18.33 ± 0.33b	16.66 ± 0.33b,c	15.33 ± 0.33c
S. aureus[a] NCTC13143	17.33 ± 0.33c	15.00 ± 0.00b	17.66 ± 0.66c	18.66 ± 0.66b	15.66 ± 0.33b
K. pneumonia ATCC700603[a]	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c
E. faecilis ATCC700802[a]	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c
S. enterica ATCC700408[a]	12.00 ± 0.00d	15.33 ± 0.33b	9.66 ± 0.88d	16.00 ± 0.00b	15.66 ± 0.66b

Data is expressed as mean ± SEM, $n = 9$. Within a row, mean values with lowercase letters are significantly different ($P < 0.05$). The letters R, RV, and O refers to lactic acid bacteria isolated from *Ravi*, *Ravana*, and *Oshadha* varieties of finger millet

[a] Multidrug-resistant strains

DPPH free radical scavenging activity of intracellular cell free extracts of lactic acid bacteria

Significant differences ($P < 0.05$) in DPPH free radical scavenging activity was observed in the intracellular cell free extracts of LAB isolates at 500 μg/ml concentration. Isolate RV19 exhibited the highest DPPH free radical scavenging activity of 54.33 ± 0.88% followed by R17 which demonstrated scavenging activity of 49.00 ± 2.65% (Table 10).

Table 9 Anti-cancer activity of the intracellular cell free extracts of isolated lactic acid bacteria

LAB isolate	IC_{50} values (μg ml^{-1}) of the intracellular cell free extracts of LAB against cancer cell lines	
	HCT-116	HT-29
R17	371.10 ± 26.6	327.55 ± 2.63
RV02	354.80 ± 23.20	306.27 ± 1.74
RV19	319.43 ± 3.74	247.12 ± 4.68
RV28	203.60 ± 19.90	288.42 ± 4.64
O24	151.98 ± 2.25	240.43 ± 2.57
Reference[a]	89.43 ± 2.65	120.55 ± 5.79

Data is expressed as mean ± SEM, $n = 9$. The letters R, RV, and O refers to lactic acid bacteria isolated from *Ravi*, *Ravana*, and *Oshadha* varieties of finger millet

[a] Reference drug cetuximab

BSH activity and in vitro cholesterol assimilation of lactic acid bacteria

BSH activity was reported to influence the metabolism of cholesterol in mammals. In this study, weak BSH activity was observed in all the isolates excepting RV28 which did not exhibit any BSH activity (Table 11). Except RV28, other isolates demonstrate < 10% of cholesterol assimilation in vitro. Tanaka et al. (1999) stated that BSH activity is high in genus *Bifidobacterium* compared to *Lactobacillus* and further suggested that high BSH activity is commonly present in strains isolated from gastrointestinal track of mammals, when

Table 10 DPPH free radical scavenging activity of the intracellular cell free extracts of LAB isolates

LAB isolate	DPPH free radical scavenging activity of intracellular cell free extracts of LAB at 500 μg/ml
R17	49.00 ± 2.65a
RV02	12.33 ± 0.88c
RV19	54.33 ± 0.88a
RV28	46.33 ± 0.88a,b
O24	7.00 ± 0.58d

Data is expressed as mean ± SEM, $n = 9$. Within a column, mean values with lowercase letters are significantly different ($P < 0.05$). The letters R, RV, and O refers to lactic acid bacteria isolated from *Ravi*, *Ravana*, and *Oshadha* varieties of finger millet

Table 11 Bile salt hydrolysis activity and cholesterol assimilation of isolated lactic acid bacteria

LAB isolate	BSH activity	Assimilation of cholesterol (%)
R17	++	6.28
RV02	++	9.78
RV19	+	4.42
RV28	+	5.41
O24	++	9.14

The letters R, RV, and O refers to lactic acid bacteria isolated from *Ravi*, *Ravana*, and *Oshadha* varieties of finger millet. + extent of precipitation zone around the LAB isolates

+ extent of precipitation zone around the LAB isolates

compared to strains isolated from plants. Therefore, results of our study agree with this statement (Table 11).

Discussion

Finger millet flour is reported as one of the most commonly consumed ingredients in traditionally fermented food in Africa such as *ogi*, *uji*, *togwa*, *ogi-baba*, *kwunu-zaki*, *degue*, and *mangisi* since ancient times. The microorganisms involved in fermentation of this food were identified as *L. plantarum*, *L. fermentum*, *L. brevis*, *L. delbrueckii*, *L. bulgaricus*, and *Enterococcus* species (Blandino et al. 2003). Further, the production of *Koozhu*, a finger millet-based fermented food product in India, contains *Lactobacillus* and *Pediococcus* as the starter (Kumar et al. 2010), hence indicating the safe use of fermented finger millet flour as an ideal substrate for the development of probiotic food. In this study, five potential probiotic LAB strains namely R17 (*L. plantarum*), RV02 (*L. fermentum*) and RV19 (*L. lactis* sub species *lactis*), RV28 (*E. faecium*), and O24 (*P. acidilactici*) were isolated from fermented finger millet flour, which exhibited superior probiotic attributes including tolerance to in vitro gastric conditions (acid, bile, salt, phenol, gastric juice, and temperature), hydrophobicity, and auto-aggregative properties. Previous authors have also reported to isolate LAB strains such as *L. fermentum* and *L. plantarum* (Leroy and Vuyst, 2004), *L. lactis* (Salama et al., 1995), *E. faecium* (Muyanja et al., 2002), and *P. acidilactisi* (Soro-Yao et al. 2014) from fermented finger millet-based food. According to Nomura et al. (2006), there is no difference between the strains isolated from dairy and non-dairy sources in terms of phenotypic and genotypic characteristics and enzyme profile. They further reported that plant-derived strains tolerate high salt, ferment a range of carbohydrates, and produce flavors similar to dairy derived strains.

Three isolates namely RV02, RV19, and O24 were susceptible to all the antibiotics at tested concentrations, whereas other two isolates demonstrated resistance to some. According to Teuber et al. (1999), LAB frequently harbors plasmids of different sizes, and some may contain antibiotic resistance determinants. Therefore, LAB used as starter cultures for the production of food could possibly contain antibiotic resistance genes. However, virulence is established only if the organism has the ability to transfer the resistance. Fewer physiological and molecular data are available on the antibiotic resistances of LAB in fermented foods.

Ability to adhere into gut epithelium is an important characteristic of a potential probiotic candidate. In vitro models with the use of human cell lines are extensively used for the preliminary investigations of cell adhesion characteristics of LAB strains. These models help to overcome constrains associated with in vivo models (Kimoto et al. 1999). Two colonic human intestinal epithelial cell lines namely HT-29 and HCT-116 cell, which possess morphological and physiological characteristics similar to normal human enterocytes, are therefore commonly used in in vitro models (Bernet et al. 1994). In our study, intracellular cell free extracts of LAB isolates namely R17, RV02, RV19, RV28, and O24 exhibited potential anti-cancer activity.

The anti-bacterial activity is one of the most important ways to measure the efficacy of potential probiotic candidates that makes them ideal to use in alternative therapy to treat bacterial infections. The results of the anti-bacterial activity of the LAB isolates in this study revealed that they can inhibit both drug-sensitive as well as multidrug-resistant human pathogens hence agreeing with the findings of earlier authors (Georgieva et al. 2015). Cell lines containing colorectal cancer cells are widely used to investigate the anti-cancer effects. Mechanisms proposed to demonstrate the anti-cancer activity extend from changes in the metabolic activities of gut microflora and changes in the colon physicochemical conditions by removing the carcinogens, producing anti-tumorigenic or anti-mutagenic substances and increasing the immunity of the host (Aliabadi et al. 2014). In this study, ICCE of selected LAB isolates demonstrated anti-cancer activity against both HTC-116 and HT-29 cell lines. The ICCE of LAB in our study demonstrated low DPPH free radical scavenging activity and cholesterol assimilation ability in vitro.

In conclusion, LAB namely R17, RV02, RV19, RV28, and O24 isolated from fermented flour of finger millet varieties grown in Sri Lanka demonstrated probiotic attributes and bioactive properties in vitro therefore demonstrating the potentiality to use them as probiotics.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

References

Adebiyi JA, Obadina AO, Adebo OA, Kayitesi E (2017) Fermented and malted millet products in Africa: expedition from traditional/ethnic foods to industrial value-added products. Crit Rev in Food Sci and Nutri 58:463–474

Aliabadi SH, Mohammadi F, Fazeli H, Mirlohi M (2014) Effects of *Lactobacillus plantarum* A7 with probiotic potential on colon cancer and normal cells proliferation in comparison with a commercial strain. Iran *J* Basic Med Sci 17:815–819

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Aswathy RG, Ismail B, John RP, Nampoothiri KM (2008) Evaluation of the probiotic characteristics of newly isolated lactic acid bacteria. Appl Biochem Biotechnol 151:244–255

Bakari D, Tatsadjieu NL, Mbawala A, Mbofung CM (2011) Assessment of physiological properties of some lactic acid bacteria isolated from the intestine of chickens use as probiotics and antimicrobial agents against enteropathogenic bacteria. Innov Rom Food Biotechnol 8:33–40

Bernet FM, Brassart D, Neeser RJ, Alain S (1994) Adhesion of human bifidobacterial strains to cultured human intestinal epithelial cells and inhibition of enteropathogen-cell interactions. Appl Environ Microbiol 59:4121–4128

Blandino A, Al- Aseeri ME, Pandiella SS, Cantero D, Webb C (2003) Cereal-based fermented foods and beverages. Food Res Int 36:527–543

Chandra D, Chandra S, Pallavi SAK (2016) Review of finger millet (Eleusine coracana (L.) Gaertn): a powerhouse of health benefiting nutrients. Food Sci & Human Wellness 5:149–155

Chmielewska A, Szajewska H (2010) Systematic review of randomised controlled trials: probiotics for functional constipation. World J Gastroenterol 16:69–75

Chowdhury A, Hossain MN, Mostazir NJ, Fakruddin M, Billah MM, Ahmed MM (2012) Screening of lactobacillus spp. from Buffalo yoghurt for probiotic and antibacterial activity. J Bacteriol Parasitol. https://doi.org/10.4172/2155-9597.1000156

Clinical and Laboratory Standards Institute (2012) Performance standards for antimicrobial susceptibility tenting; Twenty second informational supplement. Available at http://www.facm.ucl.ac.be/intranet/CLSI/CLSI-2017-M100-S27.pdf. Accessed June 12 2015

Collee JC, Fraser AG, Marmion BP, Simmons A (2006) Mackie and MaCartney practical medical microbiology 14th edition, 131–140. Church hill. Livingstone, London

Dasanayaka PN (2016) Characterization of some ex situ conserved finger millet (*Eleusinecoracana* (L.) germplasm accessions in Sri Lanka. IJMS 3: 141–150

Del-Re B, Sgorbati B, Miglioli M, Palenzona D (2000) Adhesion, autoaggregation and hydrophobicity of 13 strains of Bifidobacterium longum. Lett Appl Microbiol 31:438–442

De Man JC, Rogosa M, Sharpe ME (1960) A medium for the cultivation of lactobacilli. J Appl Microbiol 23:130–135

Doi K, Phuong O, Kawatou F, Nagayoshi Y, Fujino Y, Ohshima T (2013) Identification and characterization of lactic acid bacteria isolated from fermented rice bran product. Springer plus. https://doi.org/10.1186/2193-1801-2-485

Duary RK, Rajput YS, Batish VK, Grover S (2011) Assessing the adhesion of putative indigenous probiotic lactobacilli to human colonic epithelial cells. Indian J Med Res 134:664–671

Duchesneau CT, Jones ML, Shah D, Jain P, Saha S, Prakash S (2014) Cholesterol assimilation by *Lactobacillus* probiotic bacteria: an *in vitro* investigation. Bio Med Res Int. https://doi.org/10.1155/2014/380316

Dunne C, O'Mahony L, Murphy L, Thornton G, Morrissey D, O'Halloran S, Feeney M, Flynn S, Fitzgerald G, Daly C, Kiely B, O'Sullivan GC, Shanahan F, Collins JK (2001) *In vitro* selection criteria for the probiotic bacteria of human origin: correlate with the vivo findings. Am J Clin Nutr 73:386–392

Felley CP, Corthesy-Theulaz I, Rivero JL, Sipponene P, Kaufmann BP, Wiesel PH, Brassart D, Pfeifer A, Blum AL, Michetti P (2001) Favourable effect of acidified milk (LC-1) on helicobacter gastritis in man. *Eur* J Gastroenterol Hepatol 13:25–29

Food and Agriculture Organization (2007) FAO Technical Meeting on Prebiotics: Food Quality and Standards Service (AGNS). Food and Agriculture Organization of the United Nations (FAO) FAO; Rome, Italy. https://edisciplinas.usp.br/pluginfile.php/1800450/mod_resource/content/1/FAO%20%20Prebiotics_Tech_Meeting_Report.pdf. Accessed June12 2015

Georgieva R, Yocheva L, Tserovska L, Zhelezova G, Stefanova N, Atanasova A, Danguleva A, Ivanova G, Karapetkov N, Rumyan N, Karaivanova E (2015) Antimicrobial activity and antibiotic susceptibility of *Lactobacillus* and *Bifidobacterium* spp. intended for use as starter and probiotic cultures. Biotechnol Biotechnol Equip 29:84–91

Giaouris E, Chapot-Chartier M, Briandet R (2009) Surface physicochemical analysis of natural *Lactococcus lactis* strains reveals the existence of hydrophobic and low charged strains with altered adhesive properties. Int J Food Microbiol 131:2–9

Gibson GR, Fuller R (2000) Aspects of *in vitro* and *in vivo* research approaches directed toward identifying probiotics and prebiotics for human use. J Nutr 130:391–395

Gueimonde M, Sanchez B, Reyes-Gavilan GLC, Margolles A (2013) Antibiotic resistance in probiotic bacteria. Front Microbiol. https://doi.org/10.3389/fmicb.2013.00202

Guglielmetti S, Mora D, Gschwender M, Popp K (2011) Randomised clinical trial: *Bifidobacterium bifidum* MIMBb75 significantly alleviates irritable bowel syndrome and improves quality of life a double-blind, placebo-controlled study. Aliment pharm ther 33:1123–1132

Gupta H, Malik RK (2007) Incidence of virulence in bacteriocin producing enterococcal isolates. Dairy Sci Technol 87:587–601

Halder D, Mandal M, Chatterjee SS, Pal NK, Mandal S (2017) Indigenous probiotic Lactobacillus isolates presenting antibiotic like activity against human pathogenic bacteria. Biomedicine. https://doi.org/10.3390/biomedicines5020031

Homayouni A, Alizadeh M, Alikhah H, Zijah V (2012) Functional dairy probiotic food development: trends, concepts, and products. (Rigobelo E. Edi.) Immunology and Microbiology, Probiotics. doi: https://doi.org/10.5772/48797

Ibourahema C, Dauphin RD, Jacqueline D, Thonart P (2008) Characterization of lactic acid bacteria isolated from poultry farms in Senegal. Afr J Biotechnol 7:2006–2012

Kechagia M, Basoulis D, Konstantopoulou S, Dimitriadi D, Gyftopoulou K, Skarmoutsou N, Fakiri EM (2013) Health benefits of probiotics: a review. ISRN Nutrition. https://doi.org/10.5402/2013/481651

Kimoto H, Kurisaki J, Tsuji NM, Ohmomo S, Okamoto T (1999) Lactococci as probiotic strains: adhesion to human enterocyte-like Caco-2 cells and tolerance to low pH and bile. Lett Appl Microbiol 29:313–316

Kos B, Suskovic J, Vukovic S, Simpraga M, Frece J, Matosic S (2003) Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. J Appl Microbiol 94:981–987

Kumar RS, Varman DR, Kanmani P, Yuvaraj N, Paari KR, Pattukumar V, Arul V (2010) Isolation, characterization and identification of a potential probiotics from south Indian fermented foods (Kallappam, Koozhand Mor Kuzhambu) and its use as biopreservative. Probiotics Antimicrob Proteins 2:145–151

Kumar VB, Vijayendra SVN, Reddy OVS (2015) Trends in dairy and non-dairy probiotic products—a review. J Food Sci Technol 52:6112–6124

Kunchala R, Banerjee R, Datta Mazumdar SD, Durgalla P, Srinivas V, Gopalakrishnan S (2016) Characterization of potential probiotic

bacteria isolated from sorghum and pearl millet of the semi-arid tropics. Afr J Biotechnol 15:613–621
Li Q, Liu X, Dong M, Zhou J, Wang Y (2015) Aggregation and adhesion abilities of 18 lactic acid bacteria strains isolated from traditional fermented food. Int J Agric Policy Res 3:84–92
Langston CW, Bouma C (1960) A study of the microorganisms from grass silage. J Appl Microbiol 8:223–234
Leroy F, Vuyst LD (2004) Lactic acid bacteria as functional startercultures for the food fermentation industry. Trends Food Sci Tech 5:67–78
Menconi A, Morgan MJ, Pumford NR, Hargis BM, Tellez G (2013) Physiological properties and *Salmonella* growth inhibition of probiotic *Bacillus* strains isolated from environmental and poultry sources. Int J Bacteriol. https://doi.org/10.1155/2013/958408
Menconi A, Kallapura G, Latorre JD, Morgan MJ, Pumford NR, Hargis BM, Tellez G (2014) Identification and characterization of lactic acid bacteria in a commercial probiotic culture. Biosci Microbiota Food Health 33:25–30
Muyanj CM, Narvhus JA, Treimo J, Langsrud T (2002) Isolation and identification of lactic acid bacteria from bushera, a Ugandan traditional fermented beverage. Int J Food Microbiol 80:201–210
Nazari H, Khanjanzadeh A, Rezvani F (2012) Characterization of lactic acid bacteria as a probiotic. SJEM 3:49–55
Nomura M, Kobayashi M, Narita T, Kimoto-Nira H, Okamoto T (2006) Phenotypic and molecular characterization of *Lactococcus lactis* from milk and plants. J Appl Microbiol 101:396–405
Pelletier C, Bouley C, Cayuela C, Bouttier S, Bourlioux P, Bellon-Fontaine M (1997) Cell surface characteristics of *Lactobacillus casei* subsp. *casei*, *Lactobacillus paracasei* subsp. *paracasei*, and *Lactobacillus rhamnosus* strains. Appl Environ Microbiol 63:1725–1731
Perera HDSM, Samarasekera JKRR, Handunnetti SM, Weerasena OVDSJ (2016) *In vitro* anti-inflammatory and anti-oxidant activities of Sri Lankan medicinal plants. Ind Crop Prod 94: 610–620
Pessione E, Cirrincione S (2016) Bioactive molecules released in food by lactic acid bacteria: encrypted peptides and biogenic amines. Front Microbiol. https://doi.org/10.3389/fmicb.2016.00876
Reid G, McGroarty JA, Angotti R, Cook RL (1988) *Lactobacillus* inhibitor production against *Escherichia coli* and coaggregation ability with uropathogens. Can J Microbiol 34:344–351
Pyar H, Peh KK (2014) Characterization and identification of lactobacillus acidophilus using biolog rapid identification system. Int J Pharm Pharm Sci 6:189–193
Reis NA, Saraiva MAF, Duarte EAA, de Carvalho EA, Vieira BB, Evangelista-Barreto NS (2016) Probiotic properties of lactic acid bacteria isolated from human milk. J appl microbial 121:811–820
RnR Market Research (2017) Probiotics Market to Grow at 7% CAGR to 2022 Driven by Demand for Probiotic Dietary Supplements. Available at https://www.prnewswire.com/news-releases/probiotics-market-to-grow-at-7-cagr-to-2022-driven-by-demand-for-probiotic-dietary-supplements-648651313.html. Accessed 15 January 2018
Rojas M, Conway PL (1996) Colonization by lactobacilli of piglet small intestinal mucus. J Appl Bacteriol 81:474–480
Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425
Salama MS, Musafija-Jeknic T, Sandine WE, Giovannoni SJ (1995) An ecological study of lactic acidbacteria: isolation of new strains of Lactococcus including Lactococcus lactis subspecies cremoris. J Dairy Sci 78:1004–1017
Saleh ASM, Zhang Q, Chen J, Shen Q (2013) Millet grains: nutritional quality, processing, and potential health benefits. Compr Rev Food Sci Food Safety 12:281–295
Shahriar M, Rashidul MH, Kabir S, Dewan I, Bhuyian MA (2011) Effect of proteinase-K on genomic DNA extraction from gram-positive strains. Stam J Pharm Sci 4:53–57
Sharma M, Shukla G (2016) Metabiotics: one step ahead of probiotics; an insight into mechanisms involved in anticancerous effect in colorectal Cancer. Front Microbiol 7. https://doi.org/10.3389/fmicb.2016.01940
Sharma S, Arora M, Baldi A (2013) Probiotics in india: current status and future prospects. Pharm Aspire https://www.researchgate.net/profile/Dr_Ashish_Baldi/publication/257537041_Probiotics_in_India_Current_status_and_future_prospects/links/0c960525661ad870f0000000.pdf. Accessed 15 January 2018
Shewale RN, Sawale PD, Khedkar CD, Singh A (2014) Selection criteria for probiotics: a review. Int J Probiotics Prebiotics 9:17–22
Singh TP, Malik RK, Kaur G, Renuka (2014) Safety assessment and evaluation of probiotic potential of *Lactobacillus reuteri* strains under in vitro conditions. Int J CurrMicrobiol App Sci 3: 335–348
Soro-Yao AA, Brou K, Amani G, Thonart P, Djè KM (2014) The use of lactic acid bacteria starter cultures during the processing of fermented cereal-based foods in West Africa: a review. Trop Life Sci Res 25:81–100
Tanaka H, Doesburg K, Iwasaki T, Mierau I (1999) Screening of lactic acid Bacteria for bile salt hydrolase activity. J Dairy Sci 82:2530–2535
Teuber M, Meile L, Schwarz F (1999) Acquired antibiotic resistance in lactic acid bacteria from food. Antonie Leeuwenhoek 76:115–137
Tharmaraj N, Shah NP (2009) Antimicrobial effects of probiotics against selected pathogenic and spoilage bacteria in cheese-based dips. Int Food Res J 16:261–276
Vicente JL, Torres-Rodriguez A, Higgins SE, Pixley C, Tellez G, Donoghue AM, Hargis BM (2008) Effect of a selected *Lactobacillus* spp-based probiotic on *Salmonella enteritidis*-infected broiler chicks. Avian Dis 52:143–146
Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food (2002) Joint FAO/WHO, Ontario, Canada. http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf. Accessed 18 February 2018
Yadav R, Puniya AK, Shukla P (2016) Probiotic properties of *Lactobacillus plantarum* RYPR1 from an indigenous fermented beverage Raabadi. Front Microbiol. https://doi.org/10.3389/fmicb.2016.01683
Zhang S, Liu L, Su Y, Li H, Sun Q, Liang X, Lv J (2011) Anti oxidative activity of lactic acid bacteria in yogurt. Afr J MicrobiolRes5:5194–5152