Evolving Industrial Biotechnology in Developing Countries

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Chapter 16

Sri Lankan Finger Millet (*Elucine coracana*) Variety 'Raavana' as Potential Probiotic Source

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ABSTRACT

This study aims to isolate and identify probiotic potential lactic acid bacteria from fermented Sri Lankan finger millet variety "*Raavana*" and to investigate the probiotic characteristics, *in vitro* safety and efficacy. A bacterial isolate with typical lactic acid bacterial phenotypic and biochemical characteristics was isolated and identified. Partial sequence of the 16S rRNA gene of the Sri Lankan strain was deposited in the NCBI gene bank as *Lactococcus lactis* subsp. *lactis* FM_19LAB and the accession number MF480428 was obtained. It did not demonstrate hemolysis, DNase, gelatine hydrolysis activity as well as did not acquire complete resistance to any of the antibiotics tested hence indicating the safety. *Lactococcus lactis* subsp. *lactis* FM_19LAB had the capacity to tolerate different concentrations of acid, bile, phenol, salt, simulated gastric juices and range of temperatures. Further it exhibited anti-microbial, anticancer and anti-oxidant activities. Further, *Lactococcus lactis* subsp. *lactis* FM_19LAB assimilated cholesterol and produced lactic acid during the fermentation.

Keywords: *Sri Lankan finger millet, Lactococcus lactis* subsp. *lactis FM_19LAB, Probiotics, Prebiotics.*

INTRODUCTION

Probiotics are defined as "live microorganisms, which when administrated in adequate amounts confer a health benefit on the host" by the Food and Agriculture Organization/World Health Organization (FAO/WHO 2006). Lactic Acid Bacteria (LAB) are widely recognized among probiotic bacteria that are gram positive, non-spore forming and catalase negative organisms. The beneficial effects include promoting gastrointestinal and genitourinary health-improving the immune system, managing irritable bowel syndrome and inflammatory bowel diseases, reducing *H. pylori* associated gastric ulcers, and improving constipation (Bergonzelli *et al.*, 2006; D'Souza *et al.*, 2002; Whelan *et al.*, 2013; Koebnick *et al.*, 2003). Lactic acid bacteria (LAB), if functional as a probiotic it should exhibit resistance to acid, bile and gastric juices, adhere and colonize in-gastric mucosa (Kos *et al.*, 2003), exhibit co-aggregation preventing colonization by pathogens (Collado *et al.*, 2008) and efficacy to host which support their use as alternative therapy. Therefore, probiotic LABs are increasingly recognized in food industrial applications. As a result, novel bacterial strains with probiotic properties are been introduced in to food and pharmaceutical market particularly in dairy products (Penna *et al.*, 2007). Currently consumer demand exists for non-dairy probiotic products in beverages, supplements, capsules and freeze-dried preparations (Schrezenmeir and de Vrese, 2001). Therefore, cereals rich in prebiotics play a major role as substrate for many non-dairy probiotic products. Finger millet (*Eleusine coracana*) is known since ancient times and rich in carbohydrates, dietary fibre, minerals, and sulfur containing amino acids when compared with rice, the current major staple in south Asia (Sripriya *et al.*, 1994). Fermentation of finger millet using different starter cultures to develop functional foods has been extensively reported in Africa and Asia (Charalampopoulos *et al.*, 2002). Due to the presence of water-soluble fibres, oligosaccharides and resistant starch, it fulfils the prebiotic effects and therefore can stimulate the growth of probiotic bacteria. Though, plenty of research available on prebiotic and probiotic potential of finger millet varieties grown on different regions in the world, there is a paucity of data on Sri Lankan scenario. Furthermore, due to the increasing interest in the application of probiotics and their metabolites as an alternative strategy for treatment and prevention of infections, antimicrobial activity against human pathogens is an important property of a potential probiotic candidate. In addition, antimicrobial metabolites of probiotic LAB in preventing food spoilage, extending the shelf life stability of food and enriching the food with bioactive properties are the challenges facing food industry today. The objective of the research was to isolate probiotic potential organisms associated with Sri Lankan finger millet variety '*Raavana*' and to explore its' probiotic potential through series of experiments at the industrial technology institute of Sri Lanka.

Materials and Methods

Collection and Processing of Finger Millet Samples

Sri Lankan finger millet (*Elucine coracana*) variety namely *Raavana* was selected for the present study. Seeds of the variety represented the six different provinces

of the island including North, North Central, Eastern, Southern, Sabaragamuva and Uva, were obtained from the germplasm of the Field Crop Research and Development Institute (FCRDI), Mahailluppallama. The seeds were transported to the Microbiology Laboratory, Industrial Technology Institute (ITI) within 3 h by maintaining the temperature at 20 ± 2 °C. Samples were washed with sterilized distilled water inside a Biological safety cabinet (Thermoforma,USA) and dried at 35 ± 2 °C in an oven (Memmert Universal Oven U, Germany) till the moisture content reduced to 10 per cent. The moisture content of the samples was measured by drying the samples at 105 ± 2 °C in an oven (Memmert drying oven UF, Germany) for 5 h and calculated the water loss. Each dried sample was ground in a variable Speed Rotar Mill (Fritsch PULVERISETTE 14, Germany) and passed through a 0.5 mm sieve attached to the mill. The milled and sieved samples were packed separately in air tight containers and stored in cold room (Viessmann Tecto compact 80, Finland) at 12 ± 2 °C till use.

Fermentation of Finger Millet Samples

Six samples (n=6) containing 25 g of each was measured and transferred in to pre-sterilized glass beakers covered with aluminium foils. The flour samples were mixed with sterilized tap water in 1:3 (w/v) ratios to prepare the batter. The batters were allowed to ferment inside a biological safety cabinet at room temperature (24 ± 3 °C) for 18 h.

Isolation of Probiotic Potential Lactic Acid Bacteria from Fermented Finger Millet

Each fermented sample was serially diluted up 10^6 in sterilized physiological saline (0.9 per cent NaCl w/v). Isolation was carried out by following both spread plate and pour plate technique in duplicates. Both pour and spread plates were incubated at 37 ± 1 °C for 24 h.

Phenotypic Characterization of Lactic Acid Bacteria

Colonies appeared on MRS agar plates were picked carefully and streaked on fresh MRS agar plates. The plates were incubated at 37 ± 1 °C for 24 h. After incubation, colony morphology of isolates such as form, size, shape, surface, texture, color, elevation and margin were recorded. Gram staining, endospore staining and motility test was performed to differentiate the isolates based on their phenotypic characteristics (Aswathy *et al.*, 2008).

Biochemical Characterization of Isolates

Biochemical tests including indole, methyl red, vogeus prosker, citrate utilization, gelatin liquefaction, H_2S production, starch hydrolysis, urease and catalase were performed to characterize isolates. Sugar fermentation pattern of potential LAB isolates were investigated in triplicate (n=3) for sixteen different sugars; glucose, fructose, maltose, lactose, galactose, melezitose, melibiose, arabinose, ribose, sucrose, salicinsorbitol, mannitol, cellulose, cellobiose and dextrose.

In vitro Safety Attributes of the Newly Isolated Probiotic Isolates

Bile Salt Hydrolysis (BSH) Test

Bile Salt Hydrolysis screening medium was prepared by supplementing MRS agar with (0.5 per cent porcine bile w/v, 0.5 per cent sodium tauroglycocholate w/v, 0.5 per cent taurodeoxycholic acid sodium Salt w/v and 0.37 $CaCl_2$ g/l. The media was sterilized for 121 ± 1°C for 15 min and poured in to sterile Petri plates in triplicates and allowed to solidify. Cell density of each test bacterial isolate was adjusted to 10^5cfu/ml. From each culture, 10 µl were spotted on BSH screening media plates. Plates were incubated at 37 ± 1°C for 72 h. BSH activity was determined by observing and measuring the precipitation zones.

Hemolysis Test

Blood agar base (Himedia, India) was prepared and sterilized at 121 ± 2 °C for 15 min. The sterilized media was cooled up to 50 ± 1°C and supplemented with 5 per cent sheep blood (v/v) aseptically followed by mixing; the media was poured in to sterile petri plates and allowed to solidify. Six colonies (n=6) from each test bacterial isolate was inoculated in to blood agar plates followed by streaking. The plates were incubated at 37 ± 1 °C for 72 h. After incubation, the plates were observed for presence of hemolysis zones (β-hemolysis- clear zone of hydrolysis of blood cells around the colonies, α-hemolysis- green zone around the colonies, γ-hemolysis- no clear or no green zone around the colonies). Haemolysing strain *Streptococcus pyogene* ATCC 19615 was used as the positive control.

DNase Test

DNase agar was prepared and sterilized at 121 ± 2 °C for 15 min. Six colonies (n=6) from each bacterial isolate was spotted on the surface of the DNase agar plates. The plates were incubated at 37 ± 1 °C for 18 h and observed for thick plaque of growth around the colonies. Pathogenic strain *Serratia marcescens* ATCC 13880 was used as the positive control.

Gelatin Hydrolysis

TND agar containing 1.7 per cent tryptone (w/v), 0.3 per cent peptone (w/v), 0.25 per cent dextrose (w/v), 0.5 per cent NaCl (w/v), 0.25 per cent K_2HPO_4 (w/v), 1.5 per cent agar (w/v) and 0.4 per cent gelatine bacteriological (w/v) was prepared and sterilized for 121 ± 1 °C for 15 min. Sterilized media was poured in to sterile plates and allowed to solidify. Cell density of each test probiotics was adjusted to 10^5cfu/ml. From each culture, 10µl were spotted on TND agar plates in triplicates. Plates were incubated at 37 ± 1 °C for 72 h. After incubation, the plates were treated with saturated solution of ammonium sulphate and observed for the clear zones around the inoculated spot against the opaque background. Gelatine hydrolysing strain *Serratia marcescens* ATCC 13880 was used as the positive control.

Evaluation of the *In vitro* Probiotic Attributes of Newly Isolated Potential LAB

The newly isolated LAB were further investigated for its' ability to grow in

the presence of acid, bile, salt, phenol, temperature and gastric juice (Aswathy *et al.*, 2008). Isolates were inoculated into sterile MRS broth tubes and incubated at 37 ± 1 °C for 18 h. After incubation, the tubes were centrifuged at 10,000 × *g* at 4 ± 1 °C for 15 min. Subsequently the pellet was washed with sterile saline solution and centrifuged at 10,000 × *g* at 4 ± 1 °C for 15 min. Finally, the pellets were dissolved in 10 ml of MRS broth and adjusted to 0.5 Macfarl and turbidity standards to use in the tolerance tests.

For acid tolerance assay, MRS broth tubes were sterilized and pH was adjusted to 2, 3, and 4 using 1 M HCl. The pH adjusted broths were inoculated with 100 µl of each test bacteria (n=3). For bile tolerance assay, MRS broth tubes were adjusted with 0.2 per cent, 0.5 per cent, 1.0 per cent, 1.5 per cent and 2.0 per cent porcine Bile (w/v). The media were sterilized by filtering through 0.45 µm diameter syringe filters. Sterilized media were inoculated with 100 µl of each test bacteria (n=3). For simulated gastric juice tolerance assay, simulated gastric juice was formulated using 3.5 g/l glucose, 2.05 g/l NaCl, 0.60 g/l KH_2PO_4, 0.11 g/l $CaCl_2$, 0.37 g/l KCl. The pH of the mixture was adjusted to 2 by 1M HCl (Corcoran *et al.*, 2005). The mixture was sterilized for 121 ± 1 °C at 15 min. Prior to analysis 0.05 g/l porcine bile, 0.10 g/l lysozyme, and 0.10 g/l pepsin, were added to stock solution. The simulated gastric juice solution was sterilized by filtering through 0.45 µm diameter syringe filters. Subsequently 100 µl of each test bacteria were inoculated in to the tubes containing sterile simulated gastric juice (n=3). For salt tolerance assay (Menconi *et al.*, 2014) MRS broth tubes were prepared and adjusted to 4 per cent, 5 per cent, 8 per cent and 12 per cent (w/v) with NaCl. The media was sterilized at 121 ± 1 °C for 15 min. Aseptically 100 µl of each test bacteria were inoculated in to the MRS broth tubes and adjusted to different salt concentrations (n=3). For phenol tolerance assay MRS broth were prepared and adjusted to 0.1 per cent, 0.2 per cent, 0.3 per cent, 0.4 per cent and 0.5 per cent phenol (v/v) separately (Aswathy *et al.*, 2008). The media were sterilized for 121 ± 1 °C at 15 min. Inoculated 100 µl of each test bacteria in to the MRS broth tubes adjusted in to different phenol concentrations (n=3). All the assay tubes were mixed for 30 sec. Under aseptic condition, 200 µl of each test sample was loaded into 9 wells of the microplate and initial cell density was measured spectrophotometrically at 620 nm and the cell densities of the experimental wells were measured hourly at 1, 2, 3, 4, 5 and 6 h intervals.

For temperature tolerance assay, MRS broth was dispensed equally in to tubes and sterilized. The tubes were inoculated with 100 µl of each test bacteria (n=3). Under aseptic condition, 200 µl of each test sample was loaded into 3 sterile micro plates where in each plate 9 wells were loaded from same inoculums (n=9). Initial cell density was measured spectrophotometrically at 620 nm and the three plates were incubated at three different temperature 30 ± 1 °C (Memmert INC 153, G), 37±1°C (Gemmyco IN-010, France) and 42 ± 1 °C (MemmertINplus, Germany), respectively for 6 h. Cell density was measured hourly at 1, 2, 3, 4, 5 and 6 h intervals.

Among the isolates, an isolate with superior probiotic attributes was selected for molecular identification.

Molecular Characterization of Potential Lactic Acid Bacteria Isolates

Extraction of Genomic DNA

Test isolate was inoculated in to sterilized MRS broth tubes and incubated at 37 ± 1 °C for 24 h. after incubation; 2 ml of the inoculums was transferred in to 2.5 ml micro centrifuge tubes and centrifuged at 14000 × *g* at 4 ± 1 °C for 2 min. The supernatant was separated without disturbing the pellets and 200 µl of Tris EDTA buffer was added to pellet followed by centrifugation at 14000 × *g* at 4 ± 1 °C for 2 min. Supernatant was discarded and the pellet was re-suspended in 200 µl Tris EDTA buffer and added 10 µl of 100 µg/µl (w/v) Proteinase K enzyme. Subsequently 10 µl of 10 per cent Sodium Dodecyl Sulfate was added and mixed thoroughly. The reaction tube was incubated in a water bath at 50 ± 1 °C for 1 h. After incubation, 110 µl of phenol and 110 µl of chloroform were added and centrifuged at 14000 × *g* at 4 ± 1 °C for 2 min. Aqueous layer (approximately 150 µl) was separated without disturbing the organic layers. To the organic layer, 30 µl of ≥99.8 ethanol (v/v) and 15 µl of 3 M sodium acetate were added. The tube was mixed by vortexing and incubated in ice for 1 h. After incubation, the tube was centrifuged at 14000 × *g* at 4 ± 1 °C for 5 min. Supernatant was discarded and 1 ml of 70 per cent ethanol (v/v) was added to the tubes and mixed by inverting several times followed by centrifugation at 14000 × *g* at 4 ± 1 °C for 5 min. Supernatant was discarded and ethanol was evaporated and the pellet was dissolved in 40 µl of PCR water and stored at -20 ± 2 °C (Shahriar *et al.*, 2011 modified). The extracted genomic DNA was quantified using gel documentation system (Gel Doc™ XR+ BIO RAD, USA).

Polymerase Chain Reaction (PCR)

Bacterial DNA was amplified by Dr. MAX DNA Polymerase using DNA Engine Tetrad 2 Peltier Thermal Cycler (BIO-RAD, UK) at MACROGEN, South Korea (www.macrogen.com). Universal primers 27F (5c-AGAGTTTGATCCTGGCTCAG -3c) and 1492R (5c-GGTTACCTTGTTACGACTT -3c) were used as forward and reverse primers, respectively. The PCR was performed under the following thermocycler program; Initial denaturation at 95 ± 1 °C for 5 min followed by denaturing step conducted in 35 cycles at 95 ± 1 °C for 30 sec. Annealing of the primers to the single stranded DNA template was done at 55 ± 1 °C for 30 sec. Elongation was conducted at 72 ± 1 °C for 1 min; the final elongation was carried out at 72 ± 1°C for 10 min. The PCR products were purified using multiscreen filter plate (Millipore Corp, UK).

Sequencing of 16S rRNA Region

Sequencing of 16S rRNA region was performed using 96 capillary type ABI PRISM 3730XL Analyzer (UK). Forward primer 5' TGTCGTGAGATGTTGGGTTAAGTC 3' and Reverse primer 5' CGGTATTAGCATCTGTTTCC 3' were used. Purified PCR product was sequenced at MACROGEN, South Korea in a thermocycler program consisting of initial denaturation at 96 ± 1 °C for 1 min followed by 25 cycles at 96 ± 1 °C for 10 sec. Annealing was done at 50 ± 1 °C for 4 sec followed by elongation at 60 ± 1 °C for 4 min; and hold at 4 ± 1 °C. DNA sequences were obtained as FASTA format were compared with those from GenBank (http://www.ncbi.nlm.nih.gov) using the Basic Local Alignment Tool (http://www.ncbi.nlm.gov/BLAST/).

Sequence with a percentage of identity of 98 per cent or higher to those in databases were allocated to the same species. The sequence was aligned using the Clustal W multiple sequence alignment program (Thompson *et al.*, 1997). The 16S rRNA gene sequences of potential probiotic isolate was submitted to NCBI (http://www.ncbi.nlm.nih.gov) and GenBank accession number was obtained.

Investigating Antibiotic Susceptibility of Potential Probiotic by Alamar Blue Cell Viability Assay

Newly isolated probiotic strain was investigated for their susceptibility to 24 different antibiotics including netilmicin, amikacin, gentamycin, ofloxacin, cefoxitin, doxycycline, sulbactam, neomycin, ampicillin, vancomycin, oxacillin, streptomycin, penicillin, nitrofurantoin, sulfamethoxazole, chloramphenicol, trimethoprim and clindamycin. Antibiotic solutions were prepared at a concentration of 100 µg/ml (w/v) by dissolving the above antibiotics in sterilized distilled water followed by filtering through 0.22 µm syringe filters. Potential probiotic isolate was adjusted to 0.5 McFarland turbidity standards and stored at 4±1 °C until use. Antibiotic susceptibility was measured spectrophotometrically by performing Alamar blue assay. Each test antibiotic solution in a volume of 20 µl was aseptically added to the wells (n=9) of the sterile 96 well plate. Subsequently 173 µl of sterile MRS broth was added to all the wells followed by test probiotics (each 7 µl). Micro plates were sealed with parafilm and incubated at 37 ± 1 °C for 20 h. After incubation, 20 µl of Alamar blue cell viability reagent® was added to all the wells in dark environment. The plates were covered with aluminum foils and incubated in a shaker (CPS-350, Korea) at 30 ± 1 °C for 2 h at 100 × g. After incubation, the wells were sealed and absorbance were measured (Spectra Max plus, USA) at both λ=570 nm and λ=600 nm. Negative control was performed omitting antibiotics and replacing the volume with 193 µl of MRS broth.

Percentage inhibition of test probiotics by antibiotics was calculated by following formula.

$$\text{Per cent inhibition} = \frac{(\varepsilon ox)\lambda 2A\lambda 1 - (\varepsilon ox)\lambda 1A\lambda 2}{(\varepsilon red)\lambda 1A\lambda 2 - (\varepsilon red)\lambda 2A\lambda 1} x100$$

where,

(e_{ox}) = molar extinction coefficient of Alamar blue oxidized form (blue)

(e_{red}) = molar extinction coefficient of Alamar blue reduced form (pink)

A = absorbance of test wells

A2 = absorbance of negative control well

λ_1 = 570 nm, and λ_2 = 600 nm.

Investigating *In vitro* Anti-microbial Activity by Well Diffusion Assay

Ten strains of drug sensitive and five strains of Multi Drug Resistant (MDR) pathogens were obtained from Microbial Bank of PCMD, ICCBS University of Karachi, Pakistan. These strains were *Escherichia coli* ATCC 2592, *Escherichia coli* ATCC 35218 (MDR), *Staphylococcus aureus* ATCC 6571, *Staphylococcus aureus* EMRSA

17 COCR (MDR), *Staphylococcus aureus* EMRSA 16 NCTC 13143 (MDR), *Klebsiella pneumonia* ATCC 35594, *Klebsiella pneumonia* ATCC 700603 (MDR), *Enterococcus faecalis* ATCC 49532, *Enterococcus faecalis* ATCC 700802 (MDR), *Streptococcus mutans* ATCC 25175, *Streptococcus pyogenes* ATCC 700294, *Streptococcus sanguinis* ATCC 10556, *Streptococcus salvarius* ATCC 13419, *Salmonella enterica* ATCC 700408 (MDR), *Acinetobacter baumannii* ATCC 17978 and *Shigella flexneri* ATCC 12022. The well diffusion assay was conducted according to Naderi *et al.*, 2013 with modifications. Probiotic isolate was adjusted to 0.5 McFarland turbidity standards. Soft agar was melted and at 45 ± 2 °C, 100 µl of each pathogenic culture was added to separate soft agar tubes in triplicates and poured on to the solidified agar plates (n=3). 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 and 100 µl of probiotic inoculums was added to the well (n=9). The plates were sealed and incubated at 37 ± 1 °C for 24 h. Disc of 10 µg concentration of imipenem was used as the control. After incubation, the inhibition zones were measured using a calibrated scale. Antimicrobial activity was expressed as the diameter of the inhibition zones in mm around the wells.

Investigating *In vitro* Anticancer Activity by MTT Assay

Anticancer activity of potential probiotic was evaluated against two Colon cancer cell lines namely HCT 116 and HT 29.

To prepare the Cell free extract (CFE), isolate was inoculated in to sterile MRS broth followed by incubation at 37 ± 1 °C for 18 h. After incubation, the tubes were centrifuged at 11,000 × *g* for 30 min at 4 ± 1 °C. The supernatants were sterilized by passing through 0.22 µm syringe filter. The CFE was stored at 4 ± 1 °C until use. To prepare Cell Free Lyophilate (CFL), the CFS of test probiotic was freeze dried (Lablyo HSL 4, UK) by storing the CFE at -20 ± 1 ° C for 10 h initially and transferred to freeze drier for 45 h with 10 min initial freezing followed by 40 h of primary (when internal pressure reduced to -100 torr) and 5 h of secondary freezing at -80 ± 2 °C. The resulted CFL was stored at -20 ± 1 °C until use.

Dulbecco's Modified Eagle's Medium (DMEM) was used to maintain the cell lines. DMEM was prepared by supplementing DMEM F-12 with 10 per cent Fet al Bovine Serum (v/v), 1 per cent l-glutamine (v/v), 1 per cent penicillin-streptomycin solution (v/v) and 7.5 per cent $NaHCO_3$. The medium was sterilized by filtration and stored at 4 ± 1 °C until use.

The cryo vials containing two different cell lines were reviewed in DMEM flasks and incubated (Nuaire NU-8700E, USA) at 37 ± 1 °C with 5 per cent CO_2 for 24 h. The flasks were observed through microscope for the 80 per cent confluence. Cell viability was determined by MTT assay. HT 29 and HCT 116 cell lines were inoculated separately in to wells (n=9) of 96 well plate at a concentration of 15 × 10^3 cells/well. Probiotic CFE (5, 25, 50, 250 and 500 µl/ml) and CFL (750 µg/ml, 1250 µg/ml, 2500 µg/ml, 3750 µg/ml, 5000 µg/ml and 7500 µg/ml) at different concentrations were investigated for their anti-cancer assay. Plates were incubated at 37 ± 1 °C for 24 h with 5 per cent CO_2 After incubation, the media were discarded

and 100 µl of MTT solution (3-[4, 5-dimethylthiazol-2-yl]-2, 5- diphenyl tetrazolium bromide thiazolyl blue; 5 mg/ml in sterile PBS) was added to all the wells. The plates were incubated for 2 h at 37 ± 1 °C with 5 per cent CO_2 After incubation, 100 µl of Dimethyl sulfoxide was added and the plates were shaked to dissolve the blue crystals. The absorbance of plates was measured at λ=570 nm.

The percentage anticancer activity (per cent) was calculated using following formula,

$$\text{Percentage anticancer activity (per cent)} = \frac{MeanODtreatment}{MeanODcontrol} \times 100$$

The cell viability of controls were calculated using following formula,

$$\text{Percentage cell viability of controls} = \frac{MeanODcontrol}{MeanODcontrol} \times 100$$

Where,

Mean OD treatment = Mean value of the OD of treatment well at λ=570 nm

Mean OD control = Mean value of the OD of control well at λ=570 nm

Cetuximab was used as the positive control with CFE experiment (Lancet, 2011). The half maximum inhibitory concentration (IC_{50}) of both CFE and CFL of 8 probiotics were calculated by drawing individual scatter plots with linear regression.

Investigating *In vitro* Antioxidant Activity by DPPH Radical Scavenging Assay

The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was measured by mixing 140 µl of each CFE with 60 µl of freshly prepared DPPH solution (7 mg/100 ml methanol). Each probiotic was tested for series of concentrations including 5, 25, 50, 250 and 500 µl/ml CFE in PBS. Immediately the absorbance was measured at λ= 517 nm. The reaction mixture was allowed to stand for 30 min. Trolox was used as positive control. The scavenged DPPH was evaluated by measuring the reduction in absorbance at 517 nm.

Similarly, probiotic CFL was dissolved in 25 per cent acetone to prepare the concentrations of 10,000 µg/ml, 20,000 µg/ml and 50,000 µg/ml. Immediately the absorbance was measured at λ= 517 nm. The reaction mixture was allowed to stand for 1 hr. Trolox was used as positive control. The scavenged DPPH was evaluated by measuring the reduction in absorbance at 517 nm.

The scavenging ability was calculated by following formula:

$$\text{Per cent inhibition} = 1 - \frac{A517\,(sample)}{A517\,(blank)} \times 100\%$$

where,

A517 (sample) = Absorbance of sample at 517 λ

A517 (blank) = Absorbance of blank at 517 λ

Investigating the *In vitro* Cholesterol Assimilation

The cells of probiotic culture were adjusted to OD_{660} (10^{10}cfu/ml). Modified MRS broth was prepared by mixing 0.3 per cent (w/v) porcine bile and 0.1 g/l cholesterol-water soluble in to MRS broth and adjusted the final concentration of cholesterol in the media in to 100 mg/l. 1 ml (10^{10}cfu) of the probiotic culture was transferred in to tubes containing 5 ml of modified MRS broth (n=3). Tubes were incubated at 37 ± 1 °C for 24 h. After incubation, the tubes were centrifuged at 4500 × *g* at 4 ± 1 °C for 5 min. Supernatants (test) and un inoculated Modified MRS broth (control) were assayed for cholesterol.

Each supernatant of 1 ml volume was mixed with 3 ml 95 per cent ethanol and 2 ml of 50 per cent (w/v) potassium hydroxide. The mixture was heated at 60 ± 1 °C for 10 min in water. After cooling, 5 ml of hexane was added in to each tube and mixed thoroughly. Subsequently, 1 ml of distilled water was added to all the tubes followed by mixing and allowed to stand for 10 min to permit phase separation. A 3 ml aliquot of hexane layer from each tube was transferred in to clean tubes. Evaporation of hexane was done under the flow of nitrogen gas. *O*-phtalaldehyde (0.5 mg/ml of acetic acid) solution was freshly prepared and 4 ml was added to each tube. The tubes were allowed to stand for 10 min. Subsequently, 2 ml of concentrated H_2SO_4 was added to each tube and allowed to stand for another 10 min. The absorbance of reaction mixture was measured at 550 nm. A standard curve of absorbance verses cholesterol concentrations was generated using the cholesterol concentrations; 0, 2.91, 7.81, 15.63, 31.25, 62.5, 125, 250 and 500µg/mL cholesterol in MRS (R^2=0.99). The cholesterol assimilated by test probiotic was calculated using following formula;

Cholesterol assimilated (µg/mL) = [Cholesterol (µg/mL)]$_{oh}$-[Cholesterol (µg/mL)]$_{24h}$

Cholesterol assimilated by each test probiotic strain was also calculated in terms of percentage cholesterol assimilation;

$$\text{Per cent cholesterol assimilation} = \frac{[\text{Cholesterol assimilated cholesterol (µg/mL)}]}{\text{Cholesterol (µg/mL)}]_{oh}} \times 100 \text{ per cent}$$

Determination of Lactic Acid Production

Cells of probiotic culture were adjusted to OD_{660} (10^{10}cfu/ml).Flasks containing 10 ml of sterile MRS broth were inoculated with test probiotic cells at concentration of 10^{10}cfu/ml. The fermentations of flasks were carried out in a shaking incubator at 37 ± 1°C for 6 h with shaking speed of 150*g*. Aliquots of 5 ml were withdrawn from each fermentation flasks (in triplicates) at every 30 min intervals till 4.5 h. The aliquots were thermally treated at 95 ± 1 °C for 20 min in a water bath. The thermally treated samples were sealed well with parafilm and stored at 4 ± 1 °C. The samples were analysed for lactic acid using HPLC (Agilent technologies, 1260 Infinity, USA) equipped with a UV-VIS detector HPLC column, PhenomenexRezex™ ROA-Organic Acid H+ (8 per cent). The column was 50 mm x 7.8 mm with a length of

300 mm, 7.8 mm diameter and 8 µm particle sizes. The mobile phase was 0.005 M H_2SO_4 with an isocratic elution of a flow rate of 0.6 ml/min. the lactic acid content was quantified by measuring the wave length at 210 nm from the peak area at specific retention time for lactic acid and by considering the regression curve factor.

Statistical Analysis

All the experiments mentioned in this paper were repeated twice. The mean and the standard error of the data obtained from three parallel experiments were calculated using MINITAB 14.

Results and Discussion

Phenotypic Characterization

Four different bacterial strains isolated from fermented finger millet "*raavana*" variety. Among them, gram positive, cocci shaped that grow on MRS agar with typical LAB colony characteristics such as punctiform, glistening colony with entire margins and raised elevation was selected for further studies. Further, the isolate observed to be non-spore forming and non-motile.

Biochemical Characterization

The isolate demonstrates negative reactions to the biochemical tests including catalase, starch hydrolysis, Indole, Voges Proskeur, Methyl Red and citrate utilization thus exhibited typical LAB biochemical characteristics. Among the tested sugars, it ferments glucose, fructose, maltose, lactose, galactose, sucrose, salicinsorbitol, mannitol, cellulose, cellobiose and dextrose. However do not ferment melezitose, melibiose, arabinose and ribose.

Genotypic Characterization

The isolate was genotypically identified as *Lactococcus lactis* subsp. *lactis*. Partial sequence of the 16S rRNAgene of the Sri Lankan strain was deposited in the NCBI gene bank as *Lactococcus lactis* subsp. *lac*tis FM_19LAB and the accession number MF480428 was obtained.

In vitro Safety Attributes

Lactococcus lactis subsp. *lactis* FM_19LAB did not demonstrate hemolysis, DNAse and gelatin hydrolysis thus indicating its' safety to use as potential probiotic.

In vitro Probiotic Attributes

Lactococcus lactis subsp. *lactis* FM_19LAB could tolerate pH 3 and 4 not 2. Further, except 2 per cent, the strain could tolerate all the other bile concentrations tested. Moreover, it tolerate salt only up to 5 per cent. *Lactococcus lactis* subsp. *lactis* FM_19LAB could tolerate simulated gastric juice up to pH 4,wheras it did not survive in gastric juice containing pH 2 and 3. The isolate tolerate phenol up to 0.2 per cent and grow luxuriously in all the tested temperatures (Table 16.1).

Table 16.1: Probiotic Attributes of *Lactococcus lactis* subsp. *lactis* FM_19LAB

Acid (pH)			Bile (per cent)				Salt (per cent)				G.J(pH)			Phenol (per cent)					Temp (°C)		
3	4	0.2	0.5	1.0	1.5	2.0	4	5	8	12	2	3	4	0.1	0.2	0.3	0.4	0.5	30	37	42
-	+	+	+	+	+	+	-	+	+	-	-	-	-	+	+	+	-	-	-	+	+	+

+: Tolerate; -: Do not tolerate; G.J: Gastric Juice.

Investigating Antibiotic Susceptibility of Potential Probiotic by Alamar Blue Cell Viability Assay

Antibiotic sensitivity/resistance pattern of *Lactococcus lactis* subsp. *lactis* FM_19LAB is illustrated in the Table 16.2.

Table 16.2: Antibiotic Sensitivity/Resistance Pattern of *Lactococcus lactis* subsp. *lactis* FM_19LAB

Test Antibiotics (100 µg/ml)	*Percentage Inhibition (Per cent) of Lactococcus lactis subsp. lactis FM_19LAB Cells by the Antibiotic*
Netilmycin	47.56 ± 1.39
Amikacin	26.97 ±0.34
Gentamycin	14.71 ± 0.81
Ofloxacin	14.63 ± 0.55
Cefoxitin	19.29 ± 0.41
Doxycycline	12.55 ± 0.68
Sulbactum	23.66 ± 0.56
Neomycin	37.15 ± 0.37
Ampicillin	22.57 ± 0.67
Vancomycin	31.31 ± 0.42
Oxycillin	31.81 ± 0.78
Streptomycin	42.34 ± 0.15
Penicillin	72.83 ± 1.99
Nitrofurantoin	55.36 ± 0.42
Sulfamethoxazole	67.25 ±0.30
Cholramphenicol	19.05 ±0.17
Trimethoprin	22.10 ± 0.44
Clindamycin	0.04 ± 0.01
Ciprofloxacin	25.85 ± 0.78
Coistin	35.05 ± 0.84
Ceftazidime	47.09 ±1.13
Nystatin	59.42 ± 2.07
Imipanum	95.70 ±0.35
Tetracycline	24.21 ± 0.66

Data is expresses as mean ± SEM, n=9.

Results of the antibiotic susceptibility pattern of *Lactococcus lactis* subsp. *lactis* FM_19LAB obtained from the Alamar blue assay revealed that, imipanum have the highest inhibitory activity followed by penicillin and sulfamethoxazole. Furthermore, when the inhibition of test antibiotics observed to be <10 per cent,it was considered as the strain is potentially resistant to particular antibiotic. Such scenario was only observed in clindamycin that exhibit poorest inhibition of the probiotic strain.

In vitro Antimicrobial Activity of *L. lactis* subspp. *lactis* FM_19LAB

Table 16.3: Antimicrobial Activity of *Lactococcus lactis* subsp. *lactis* FM_19LAB against Sensitive and Multi Drug Resistant Pathogens

Test Pathogen	*Antimicrobial Activity of Probiotic Strains Diameter of the Zone of Inhibition (in mm)*
Drug sensitive organisms	
E. coli ATCC 2592	15.33 ± 0.33
K. pneumonia ATCC 35594	15.00 ± 0.00
S. aureus ATCC 6571	12.66 ± 0.33
S. sanguinis ATCC 10556	0.00 ± 0.00
S. salvarius ATCC 13419	16.00 ± 0.00
S. flexenari ATCC 12022	16.00 ±0.00
E. faecilis ATCC49532	0.00 ±0.00
A. baumani ATCC 17978	11.33 ± 0.33
S. mutans ATCC 25175	0.00 ± 0.00
S. pyogenes ATCC 700294	12.33 ± 0.33
Multi Drug Resistant Organisms (MDR)	
E. coli ATCC 35218	18.33 ± 0.33
S.aureus 17EMRSA COCR	14.66 ± 0.33
S.aureus 16 EMRSA NCTC 13143	17.66 ± 0.66
K.pneumonia ATCC 700603	0.00 ± 0.00
E.faecilis ATCC 700802	0.00 ± 0.00
S.enterica ATCC 700408	9.66 ± 0.88

■■■ is expresses as mean ± SEM, n=9.

Among the drug sensitive pathogens, *Lactococcus lactis* subsp. *lac*tis FM_19LAB demonstrated highest inhibition in *S. salvarius* ATCC 13419 and*S. flexenari* ATCC 12022.Whereas, it did not inhibit *S. mutans*ATCC 25175. Among the MDR pathogens, *Lactococcus lactis subsp. lac*tis FM_19LAB demonstrated highestinhibition in *E. coli*ATCC 35218 followed by *S.aureus* 16 EMRSA NCTC 13143. Whereas, it did not inhibit *K.pneumonia* ATCC 700603 and *E.faecilis* ATCC 700802.

As reported by many authors, the antimicrobial activity is one of the most important selection criteria for probiotics. Where, probiotics compete with other infectious bacteria for nutrients and cell surface (Quwehand and Vesterlund, 2004, Saavedra, 2003; Rodriguez *et al.*, 2003).Therefore, the results of the antimicrobial

activity of the *Lactococcus lactis* subsp. *lac*tis FM_19LAB revealed its'ability to inhibit both drug sensitive and multi drug resistant human pathogens that are causing infections in the gastrointestinal track, respiratory track as well as in the skin. Thus, provide information regarding it's' potentiality to use them in novel therapeutic food developments. Furthermore, the stranded drug imipeum used as the control with every experiment and it demonstrated highest antimicrobial activity against every tested pathogen.

In vitro Anti-cancer Activity of *L. lactis* subspp. *lactis* FM_19LAB

The anticancer activity of *L. lactis* subsp. *plactis* FM_19LAB observed to increase with increasing the concentration of CFE and CFL. Further, both CFE and CFL of *lactis sub spplactis* FM_19LAB inhibit HT 29 cell line than HCT 116 (Table 16.4).

Table 16.4: Results of the Anticancer Activity of different Concentrations of *Lactococcus lactis* subsp. *lactis* FM_19LABCFE and CFL Values against HT 29 and HCT 116 Cell Line

Percentage inhibition (per cent) of HT 29 cell line by test probiotic CFE at different concentrations					
5 µl/ml	25 µl/ml	50 µl/ml	250 µl/ml	500 µl/ml	IC_{50}
6.23 ± 1.62	16.20 ± 3.78	51.55 ± 3.66	65.97 ±1.13	80.11 ± 0.58	247.12 ± 4.68
Percentage inhibition (per cent) of HT 29 cell line by test probiotic CFL at different concentrations					
1250 µg/ml	2500 µg/ml	3750 µg/ml	5000 µg/ml	7500 µg/ml	IC_{50}
31.37 ± 1.73	39.89 ± 3.08	50.23 ± 0.46	76.46 ± 1.64	95.99 ± 0.49	3267.6 ± 76.2
Percentage inhibition (per cent) of HCT 116 cell line by test probiotic CFE at different concentrations					
5 µl/ml	25 µl/ml	50 µl/ml	250 µl/ml	500 µl/ml	IC_{50}
0.00 ± 0.00	13.80 ± 1.80	20.87 ± 0.77	63.19 ± 2.26	73.16 ± 0.60	319.43 ± 3.74
Percentage inhibition (per cent) of HCT 116 cell line by test probiotic CFL at different concentrations					
1250 µg/ml	2500 µg/ml	3750 µg/ml	5000 µg/ml	7500 µg/ml	IC_{50}
10.15 ± 0.45	21.15 ± 0.45	52.46 ± 1.98	73.16 ± 0.60	87.15 ± 0.71	4174.0 ± 3.03

Data is expresses as mean ± SEM, n=9.

DPPH Radical Scavenging Activity of *L. lactis* subspp. *lactis* FM_19LAB

It was observed that the DPPH radical scavenging activity of *L. lactis* subsp. *plactis* FM_19LAB increased with the increment of both CFE and CFL (Figures 16.1 and 16.2).

In vitro Cholesterol of *L. lactis* subspp. *lactis* FM_19LAB

Lactococcus lactis subsp. *lactis* FM_19LAB observed to be weak cholesterol assimilator that assimilated 4.42 per cent of cholesterol in the growth medium. As reported by many authors, the cholesterol lowering effect is one of the most important selection criteria for probiotics thus can accept as an alternative therapy for treating hyperlipidemia (Homayouni *et al.*, 2012). Although test probiotic do

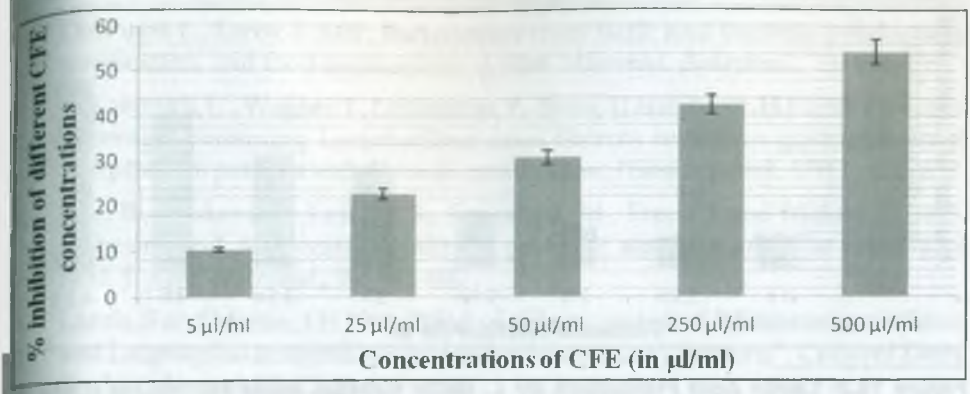

Figure 16.1: DPPH Radical Scavenging Activity of *Lactococcus lactis* subsp. *lactis* FM_19LAB CFE (Data is expresses as mean ± SEM, n=9).

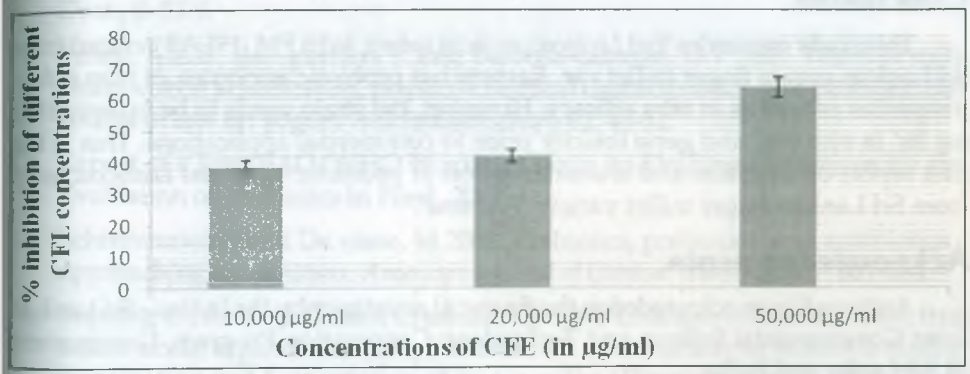

Figure 16.2: DPPH Radical Scavenging Activity of *Lactococcus lactis* subsp. *lactis* FM_19LAB CFL (Data is expresses as mean ± SEM, n=9).

not effective in removing cholesterol as a standard drug; however the results of the cholesterol assimilation of the test probiotic used in this study revealed its' ability to assimilate cholesterol at 10^{10}cfu/ml concentration. Thus, provide information regarding its' potentiality to use them in novel therapeutic food developments.

In vitro Lactic Acid Production by *L. lactis* subspp. *lactis* FM_19LAB

Lactic acid is a commercially important organic acids produced by LAB. Furthermore, lactic acid contributes food preservation as well as exerts antagonistic effect against human pathogenic microorganisms (Asmahan Ali, 2010). Therefore, ability to produce lactic acid is one of the important criteria of probiotics to become commercially successful starter cultures. It was observed that, at the end of the fermentation period, *Lactococcus lactis* subsp. *lactis* FM_19LAB produced 4.62± 0.07 mg/ml lactic acid. Further, it was observed that, the lactic acid synthesis was increased with the time (Figure 16.3).

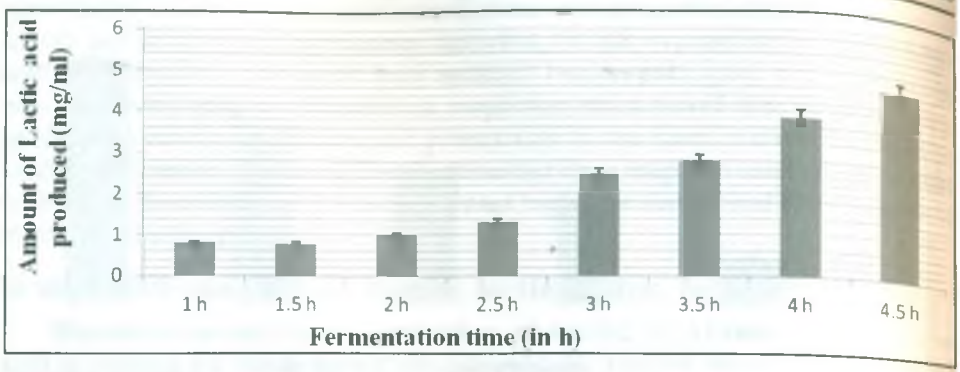

Figure 16.3: Lactic Acid Production by *L. lactis* subspp. *lactis* FM_19LAB (Data is expresses as mean ± SEM, n=9).

Conclusion

This study concludes that *Lactococcus lactis* subsp. *lactis* FM_19LAB isolated from Sri Lankan grown finger millet var. *Raavana* has probiotic attributes, *in vitro* safety properties as well as *in vitro* efficacy. However, the strain needs to be investigated for its' *in-vivo* oral and geno toxicity prior to commercial applications. This is the first report on isolation and characterisation of probiotic potential *Lactococcusspp* from Sri Lankan finger millet variety *'Raavana'*.

Acknowledgements

Authors like to acknowledge the financial assistance by the Indian - Sri Lankan Inter Governmental Science and Technology Cooperation Program, Government of Sri Lanka and India.

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