

Original Article

**ISOLATION AND CHARACTERIZATION OF POTENTIAL PROBIOTICS FROM FERMENTED RAGI
(*ELEUSINE CORACANA*)**

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ABSTRACT

Objective: The aim of this study was to isolate, characterize and identify the potential lactic acid bacteria from fermented ragi millet (*Eleusine coracana*).

Methods: A total of 177 isolates were isolated from the fermented ragi porridge. All the isolates were subjected to preliminary screening (Gram staining and catalase test). The physiological features of the probiotic isolates such as tolerance to pH (2.0, 4.0), phenol (0.3%, 0.5%) and bile salt (0.3%) were carried out. Antibiotic susceptibility test was performed using disc diffusion method. Free radical scavenging assay was conducted by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. The isolates were further characterized for their cholesterol-lowering property. Based on their survival ability and biochemical tests, the selected potent isolates were identified by 16S rRNA gene sequence by PCR and phylogenetic analysis. The 16S rRNA gene sequencing confirmed that isolates were *Lactococcus lactis* RS01, *Lactobacillus plantarum* RS09, *Lactobacillus plantarum* RS16 and *Lactobacillus plantarum* RS23.

Results: Twenty five isolates were screened from the 177 total isolates based on their morphological and biochemical characteristics (Gram staining and catalase test). All the isolates showed tolerance against acid, phenol and bile salts. However, RS09 and RS23 exhibited maximum viable count and demonstrated good tolerance at the end of 3 h. Highest antioxidant activity was recorded in RS09 ranging from 32-85%. Among the tested strains, the degradation rate of supernatants, RS09 (61.9%), RS23 (60%) and RS01 (58.97%) and RS16 (56.2%) showed the highest cholesterol assimilation rate. In addition, they also found to be resistant and sensitive to few antibiotics.

Conclusion: Based on the obtained results, these isolates are ideal *in vitro* probiotic strains and can be used further for *in vivo* evaluation.

Keywords: Ragi, Fermentation, Probiotics, Acid tolerance, Bile tolerance, Antibiotic sensitivity, DPPH

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INTRODUCTION

Probiotics are live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host. Probiotic fermented foods are now being recognized and perceived as health foods. Most of the probiotic foods that are produced now are based on dairy products however extensive research work is also available on coarse cereals and millets, which constitute the staple foods in developing countries like India. Sorghum and pearl millet are usually ground into flour and consumed either as roti or porridge with milk or it can also be prepared as beverages [1]. The probiotic potential of lactic acid bacteria from traditional fermented foods are well documented [2-5]. Lactic acid bacteria (LAB) are generally Gram-positive, non-spore forming, catalase-negative bacteria that are devoid of cytochromes and are of aero-tolerant, fastidious, acid tolerant and absolutely fermentative; lactic acid is the major end-product of sugar fermentation [6]. Lactobacilli produce various antimicrobial compounds such as bacteriocins which are a highly specific antibacterial protein that prevents food from spoilage and provide additional protection against many harmful pathogens like *Bacillus*, *Staphylococcus aureus* and *Clostridial* spores in canned foods. Lactic acid bacteria particularly *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus* and *Leuconostoc* (beneficial and non-pathogenic genera) are widely used in various fermentations [7]. LAB are considered as "generally recognized as safe" (GRAS) organisms and can be safely used for medical and veterinary applications [8]. The criteria for the *in vitro* selection of lactobacilli to be considered as health-promoting, probiotic ingredients, in several food and pharmaceutical preparations include antibiotic tolerance and also the production of an active compound, lactic acid which inhibits the growth of other microorganisms; allow them to be established in the intestinal tract [9]. Moreover, there is a great expectation that probiotic bacteria should still be alive at a certain

population number during the passage through gastrointestinal tract before it gets attached to the host lower intestinal tract and colon [10].

The objective of this study was to characterize the potential probiotics from the fermented ragi millet through acid and bile salts resistance, tolerance to phenol, and also to evaluate its antibiotic sensitivity towards the isolated strains, to check its ability to assimilate cholesterol and also to determine its antioxidant properties.

MATERIALS AND METHODS

Chemicals and reagents

All the chemicals used were of analytical grade and procured from Sigma Chemical Co., St. Louis, Mo, USA, Hi-Media, Mumbai, India and Southern India Scientific Corporation, Chennai, India.

Sample collection

A pack of ragi millet seeds (*Eleusine coracana*) were bought from the local supermarket at Maraimalai Nagar, Chennai, Tamil Nadu, India. The ragi millet seed identification was authenticated by taxonomist Dr. M. Kumar from Madras Christian College, Chennai, Tamilnadu, India. A voucher specimen was deposited in Postharvest Research Laboratory, SRM Institute of Science and Technology, Kattankulathur, Tamilnadu, India with a Voucher No: PRLRS1.

Sample preparation

Ragi millet seeds were washed and ground into fine powder and sieved to remove the husk. Two samples were prepared, wherein the first sample was prepared by mixing ragi powder with water and cooked for 10 min and the second sample was prepared by adding water and 5% starch to the ragi powder and cooked for 10 min. Both

the samples were allowed to ferment for 24 and 48 h and the pH was measured at 0, 24 and 48 h.

Isolation of probiotic strains

Samples were diluted serially to 10-fold and then inoculated on de Man, Rogosa and Sharpe (MRS) agar plates by pour plate method. MRS agar plates were incubated at 37 °C for 48 h anaerobically. Morphologically distinct colonies were picked and transferred to new MRS agar plates by streaking. Finally, pure colonies were obtained after repeated subcultures and preserved for further study.

The isolates were preliminarily screened through Gram staining characteristics (Gram-positive), catalase test (-) and cell morphology examination (Bergey's manual of systematic bacteriology).

Determination of *in vitro* characteristics of probiotics

Resistance to pH

Method of Gilliland [11] was performed. Active cultures were incubated in a shaker for 16-18 h in MRS broth at 37 °C. Tolerance of isolated strains to acidic pH was determined by growing bacteria in acidic MRS broth. MRS broth was prepared and sterilized. Separately, pH 2.0 and 4.0 was adjusted with 1 M HCl and 0.5 M NaOH. Isolated strains were inoculated in MRS broth separately. All the tubes were incubated at 37 °C and viable counts were determined by pour plate technique at every one hour gap for 3 h. And the plates were incubated at 37 °C under anaerobic condition for 48 h. Survival cell counts were expressed as log values of colony-forming units per ml (CFU/ml) [12].

Resistance to bile salt

The method of Gilliland [11] was followed. Strains were grown in MRS broth at 37 °C overnight and saturated bile solution was prepared separately by dissolving powdered bile extract at a concentration of 0.3% and without bile, served as control. The isolates were inoculated and incubated at 37 °C for 3 h and then for every one-hour viable counts were determined by pour plate method. The plates were incubated at 37 °C under anaerobic conditions for 48 h. Survival cell counts were expressed as log values of colony-forming units per ml (CFU/ml) [12].

Phenol tolerance test

The phenol tolerance of the bacterial isolates was assessed by using the protocols [15] with slight modifications. MRS broth was modified with 0.3% and 0.5% phenol to determine the phenol tolerance of the isolates. The overnight cultures (1%) of the isolates were inoculated into the freshly prepared MRS broth containing 0.3% and 0.5% phenol. After 3 h of incubation at 37 °C, OD was recorded at 600 nm [16]. The relative growth of the isolates was calculated using the following formula.

$$\text{Relative growth (\%)} = \frac{A_{(\text{control})} - A_{(\text{sample})}}{A_{(\text{control})}} \times 100$$

Antibiotic sensitivity test

Strains were evaluated for their susceptibility to antibiotics according to the technical guidelines of the European Food Safety Authority [13]. Antibiotics such as ampicillin, gentamicin, kanamycin, streptomycin, erythromycin and tetracycline were used. After culturing the isolates in MRS broth for 16 h, they were swabbed on Mueller Hinton (MH) agar plates and incubated at 37 °C for 24-48 h. The zone of inhibition was observed.

Cholesterol assimilation test

The ability of the isolates to assimilate cholesterol was determined by a modified method described by Danielson *et al.* [14]. Each freshly prepared strain culture (cell-free extract) was inoculated (1%) in MRS broth containing a water-soluble form of cholesterol from the cholesterol kit (ROBONIK, India Pvt. Ltd). The strains were incubated at 37 °C for 1 h. After one hour of incubation, the cultures were read at OD 600 nm. The efficiency of the isolates to assimilate cholesterol was calculated.

$$\text{Cholesterol assimilated (\%)} = \frac{A_{(\text{control})} - A_{(\text{sample})}}{A_{(\text{control})}} \times 100$$

DPPH radical scavenging activity

DPPH method was performed to analyze the antioxidant (stability of 2,2-diphenyl-1-picrylhydrazyl radical) activity according to [17] with slight modifications. The working solution (0.04 mmol) was prepared by diluting the stock solution with methanol. Filtrates with different concentrations were prepared for all the selected strains. The 100 µl of 0.4 mmol DPPH was added to each well containing 50 µl of the filtrate and incubated for 30 min in the dark, reading was recorded at 517 nm using a multimode reader (Multiskan Go, Japan). Gallic acid (Sigma Chemical Co., St. Louis, MO, USA) was used as a standard. The percentage of the radical scavenging activity (RSA) was calculated based on the following equation:

$$\text{DPPH scavenged (\%)} = \frac{A_{(\text{control})} - A_{(\text{sample})}}{A_{(\text{control})}} \times 100$$

(A_{control} and A_{sample} are the absorbance values for the control and sample, respectively)

Molecular identification

The isolated strains were identified according to methods described in Bergey's Manual of Determinative Bacteriology [18]. Also, 16S rRNA sequence analysis was performed for the best four potential strains. The selected isolates were identified by amplifying the 16S rRNA gene sequence through PCR (Agilent surecycler 8800, USA) using Taq 2X Master Mix Red PCR kits (Ampliqon, Copenhagen, Denmark) and the universal primer set: 27F (5'-AGAGTTTGA-TCMTGGCTC AG-3') and 1492R (5'-TACGGYTACCTTGTACGACTT-3'). PCR samples were purified using a PCR purification kit (Favorgen, Taiwan) and amplicons were sequenced by Eurofins genomics laboratory (Bangalore, India). Partial 16S rRNA gene sequences were compared to the data available in GenBank by BLAST searches of the National Centre for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov/blast>).

Statistical analysis

Statistical analyses were performed using the SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Data were subjected to a one-way ANOVA by Duncan's multiple range tests. P value < 0.05 was considered statistically significant for all analyses.

RESULTS AND DISCUSSION

Isolation of potential probiotic bacteria from fermented ragi millet

Totally 177 strains were isolated from the fermented (water and 5% starch) ragi millet. More number of colonies was observed in the sample that contained 5% starch which served as a prebiotic for the probiotic bacteria. For all the isolates, colony morphology, catalase test and Gram staining were carried out. Among the strains, 67 strains showed negative for catalase test and 25 strains were Gram positive. The selected 25 strains were screened for *in vitro* studies [19].

As probiotics are usually administered orally, they must have the ability to survive passage through the stomach and small intestine. Therefore, resistance to the bile salt in the small intestine is one of the most important selection criteria for probiotic [20]. Resistance to bile is associated to bile salt hydrolase (BSH), an enzyme that helps in hydrolyzing conjugated bile, thereby reducing its toxic effect [21].

In our study, 25 selected isolates were able to survive in the bile salt after 3 h of incubation (fig. 1). All the isolates showed significant (P < 0.05) survival rate. The highest survival was observed in RS09 isolate, while the least survival was observed in RS07 isolate. The results also showed the decrease in survival rate with increase in duration of incubation. Similar results were reported by [22]. Several reports on the survival ability of the isolates under stimulated gastric bile juice have been documented supporting the present study [23-25].

Tolerance to bile salt

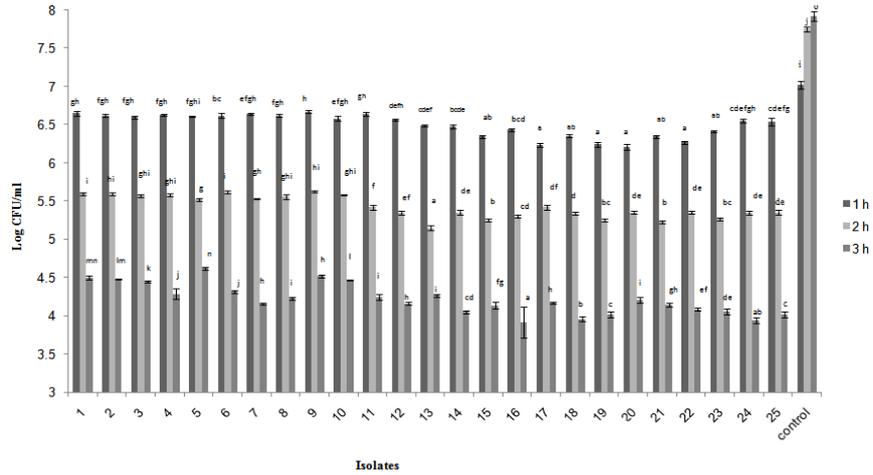


Fig. 1: Viable cell counts of isolates after 3 h of exposure to 0.3% bile salt on de Man, Rogosa Sharpe medium, error bar indicates the standard deviation (SD) of three independent experiments.

Tolerance to pH

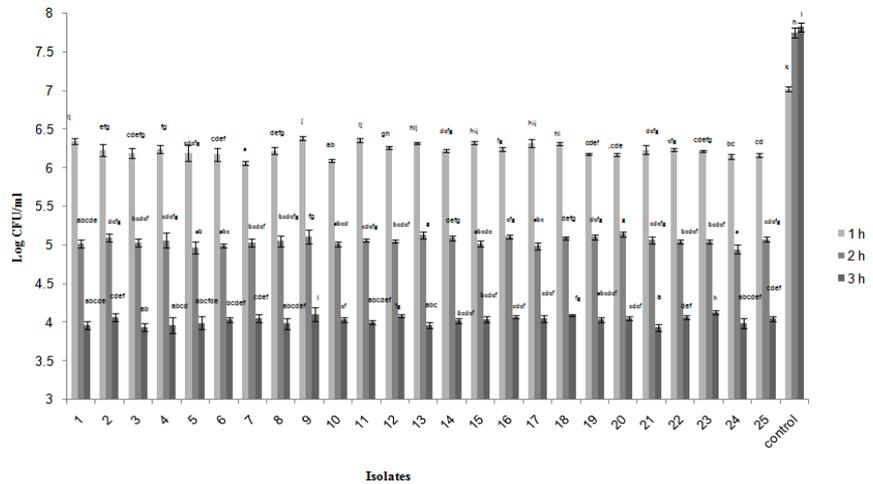


Fig. 2: Viable cell counts of isolates after 3 h of exposure to low pH 2.0 on de medium, error bar indicates the standard deviation (SD) of three independent experiments

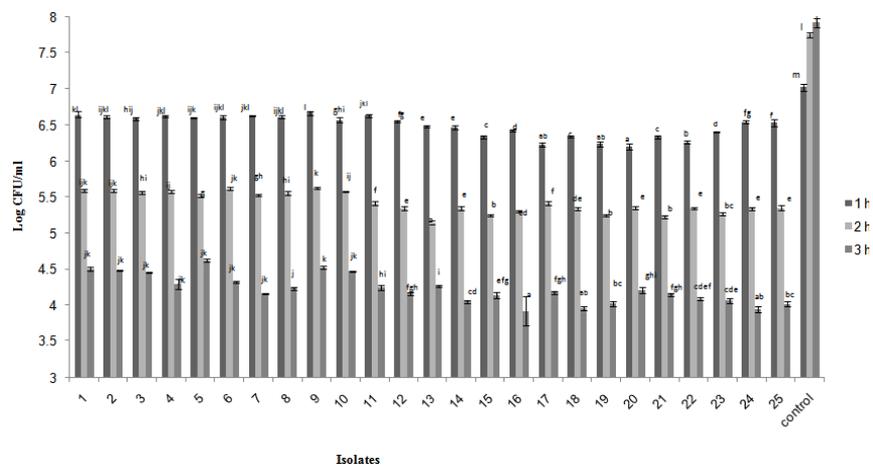


Fig. 3: Viable cell counts of isolates after 3 h of exposure to pH 4.0 on de man rogosa sharpe medium, error bar indicates the standard deviation (SD) of three independent experiments

A probiotic strain should survive transit through the stomach where the pH is low around 2 to 4. Therefore, assessing the tolerance of the isolated strains towards low pH is an important criterion. Result obtained from the acid tolerance study (fig. 2 and fig. 3) indicates that isolates which could not survive in bile salt, also failed to grow at low pH 2.0 whereas showed better survivability at pH 4.0. A similar result was observed and reported by [26]. According to [27], a hydrochloric acid that is found in the human stomach is a strong

oxidizer. Thus, it can oxidize many important biomolecular compounds in the cells. Another study concluded that viability count significantly decreased due to the high acidic pH 2.0 environments [12]. Isolates reached the maximum CFU count with a significant difference ($P < 0.05$) at pH 2 and 4. Isolate RS19 showed lower and poor tolerance after a subsequent increase in the time duration of 3 h of incubation. The highest survival was observed in RS09 isolate in both pH 2 and 4.

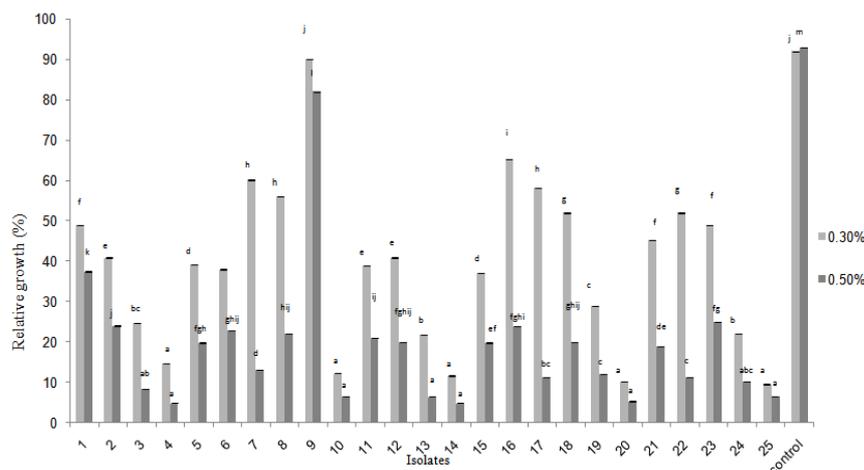


Fig. 4: Survival percentages of isolates after 3 h of exposure to phenol in de Man, Rogosa Sharpe medium, error bar indicates the standard deviation (SD) of three independent experiments

Phenol tolerance

Among the twenty-five isolates, RS01, RS09, RS16 and RS23 grew significantly better than other isolates ($P < 0.05$) in the presence of 0.3% and 0.5% phenol concentration than other isolates (fig. 4). The

isolates RS10, RS20 and RS25 showed less survival rate in the phenolic environment after 3 h of incubation at 37 °C. The above result is inconsistent with other studies [13, 30]. Based on the above *in vitro* tests, best ten isolates were chosen for the antibiotic sensitivity, cholesterol assimilation test and antioxidant activity.

Table 1: Antibiotic sensitivity of the selected isolates according to EFSA (2008)

Isolates	Kanamycin	Gentamicin	Ampicillin	Streptomycin	Erythromycin
RS01	R	S	S	R	S
RS04	S	S	R	R	R
RS06	I	S	S	R	R
RS09	R	R	S	R	R
RS11	R	S	S	R	R
RS16	I	I	S	R	S
RS17	S	S	R	I	S
RS18	S	R	S	R	R
RS22	S	R	R	R	S
RS23	R	R	S	R	R

S: Sensitive, I: Intermediate, R: Resistance.

Antibiotic sensitivity test

All the strains were evaluated for their sensitivity to antibiotics according to the technical guidelines of the European Food Safety Authority [13]. The susceptibility and resistance obtained by the selected isolates against five antibiotics is shown in the Table1. The result obtained indicates that 6 isolates were resistant to most of the antibiotics and sensitive to few antibiotics. According to [28], the antibiotic resistance found in *Lactobacillus* strains are considered to be intrinsic or natural resistance because it is chromosomally encoded and, therefore, it is non-transmissible. Resistance to antibiotics, such as gentamicin, streptomycin, kanamycin, is also considered to be intrinsic and natural in *Lactobacillus* genus [29].

Cholesterol assimilation

The assimilation rates of cholesterol by the isolated strains are shown in fig. 5. The results revealed that all examined strains were able to degrade cholesterol at high levels and also indicate that all of the strains significantly ($P < 0.05$) decreased the cholesterol level in the medium. Similarly [31], reported that probiotics rich diet significantly decreased the level of cholesterol concentrations in the plasma. Among the tested strains, the degradation rates of the supernatants of RS09 (61.9%), RS23 (60%) and RS01 (58.97%) and RS16 (56.2%) showed the highest cholesterol assimilation rate. This phenomenon of assimilating cholesterol could be due to the incorporation of cholesterol into the cellular membrane of the organism [32].

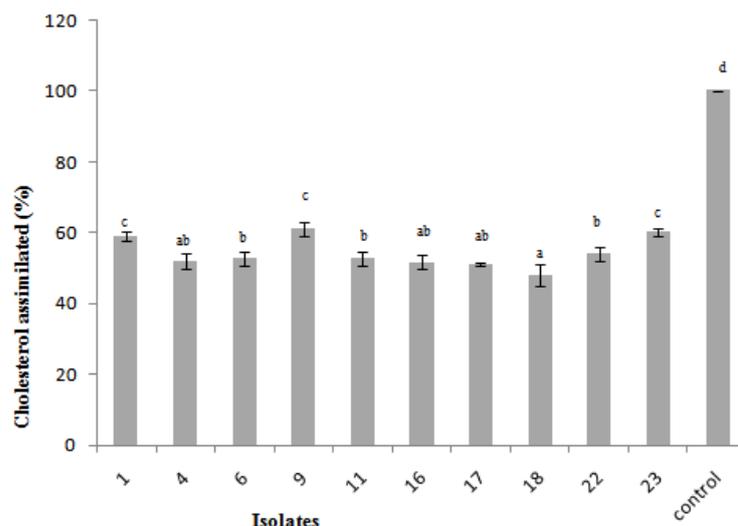


Fig. 5: Cholesterol assimilation rate of isolates in de man rogosa sharpe medium, error bar indicates the standard deviation (SD) of three independent experiments

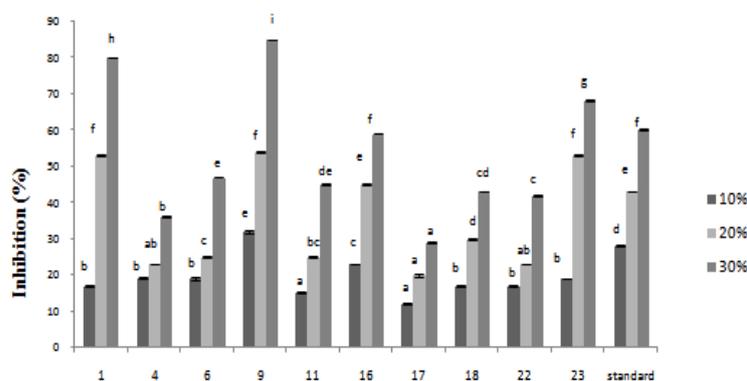


Fig. 6: 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity of selected isolates

Determination of antioxidant activity

DPPH radical scavenging activities of culture filtrate was evaluated at different concentrations. Isolate RS09 showed the highest antioxidant activity which indicates the strongest ability of the sample to act as DPPH radical scavenger. There were significant differences ($P < 0.05$) among different strains at different concentrations. Isolate RS17 showed the least inhibition percentage when compared with other isolates. The results of this study are consistent with the observation of [33]. The cell-free extract of the isolates obtained from the fermented millet showed antioxidant activity depending on the strain [34]. The antioxidant activity is concluded to be due to the hydrogen donating ability of the isolates and this can be visually noticed by the change from purple to yellow colour after 30 min of incubation in dark [35].

Molecular identification of potential isolates

Pure cultures of potential probiotic isolates were grown until log phase and genomic DNA were isolated [36, 37]. Molecular methods are important for bacterial identification [38-41] and possibly more accurate for lactic acid bacteria than the conventional phenotypic methods. The genomic DNA from promising isolates was amplified and sequenced for identification. The amplification of 16S rRNA gene was done by using universal bacterial primer 27F (5'-AGAGTTTGATCMTGGCTC AG-3') and 1492R (5'-TACGGYTACCT-TGTTACGACTT-3') according to the conditions by [42]. The PCR product was sequenced. The sequences were compared with those from GenBank using the BLAST program [43], aligned using the ClustalW software [44, 45]. The four best potential isolates were

found to be *Lactococcus lactis* RS01, *Lactobacillus plantarum* RS09, *Lactobacillus plantarum* RS16 and *Lactobacillus plantarum* RS23.

CONCLUSION

In our study, totally 177 strains were isolated from the fermented ragi millet and only twenty-five isolates were selected based on the catalase test and Gram's staining. And different *in vitro* tests such as resistance to gastrointestinal conditions (pH, 2 and 4; bile salt, 0.3%; phenol 0.3 and 0.5%) were performed and ten best strains were selected. The strains were tested to check for the ability to assimilate cholesterol. Among the isolated strains, identified as *L. plantarum* RS09 manifested the highest cholesterol removal ability and *L. plantarum* RS23 also showed a higher cholesterol removal of 60%. Accordingly, due to its potential probiotic properties, out of the four selected strains, *L. plantarum* RS09 and *L. plantarum* RS23 could be potentially used in functional food and other health products especially where cholesterol reduction in food is the main goal. It is also inferred that strains isolated from the traditionally fermented ragi millet showed antioxidant potential at different levels. This extends the application of probiotics in nutraceuticals with antioxidant potential for many degenerative diseases.

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AUTHORS CONTRIBUTIONS

All the authors are contributed equally to this work.

CONFLICT OF INTERESTS

We have no conflict of interest to declare.

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