International Food Research Journal 23(6): 2695-2701 (December 2016)
Journal homepage: *http://www.ifrj.upm.edu.my*

Identification and molecular characterization of Lactic Acid Bacteria (LAB) species from the medicinal plant *Cissus quadrangularis* (Pirandai)

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Article history

Received: 1 January 2015
Received in revised form: 4 March 2016
Accepted: 7 March 2016

Keywords

E. coli
Lactobacillus acidophilus
Lactobacillus raffinolactis
Pirandai
Raffinose

Abstract

Cissus quadrangularis (Pirandai) is well known for its ethnomedical uses and it can be taken orally for gastroprotective activity. Among various sources of isolation, traditional medicinal plants could be considered for the presence of Lactic Acid Bacteria (LAB) which can be exploited for their probiotic attributes. This study aimed at the characterization of the LAB from fermented and non-fermented Pirandai plant samples. We report the isolation of LAB (*Lactobacillus* spp. and *Lactococcus* spp.) from Pirandai. Higher percentage of LAB isolates was present in the fermented samples than the non-fermented ones. The identified LAB isolates exhibited growth at 37°C and pH 6.5. *Lactococcus* spp. preferentially utilized raffinose while *Lactobacillus* spp. fermented Lactose. Isolates were categorized as *Lactobacillus* spp. and *Lactococcus* spp. based on the partial 16-23S rRNA and partial 16S rRNA sequencing respectively. Phylogenetic Analysis revealed that the identified *Lactococcus* spp. were closely related to *Lactococcus raffinolactis* while *Lactobacillus* spp. related to *Lactobacillus acidophilus*. All isolates exhibited the inhibition of growth of *E. coli* demonstrating the probiotic activity. It was more marked with *Lactococcus* isolates than the *Lactobacillus* isolates.

Introduction

Lactic Acid Bacteria (LAB) comprise a wide range of species with *Lactobacillus* and *Lactococcus* being the most important among them. LAB are non-sporulating, non-motile, gram positive and catalase negative cocci or rods with the ability to ferment various carbohydrates to lactic acid as a major by-product of fermentative metabolism (Sieladie *et al*., 2011). They are considered as Generally Recognized As Safe (GRAS) and can be used as probiotics for medical and food applications (Patil *et al*., 2010). Probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002). The proliferation of the LAB in the food sample enhances their digestibility and promotes gut health. They are also present in the mucosal surfaces of the gastrointestinal and urogenital tract (Kandler and Weiss, 1986), where they enhance the host protection against pathogens

Extensive studies on the characterization of LAB from dairy sources were carried out (Hattingh and Viljoen, 2001; Garcia *et al*., 2008) whereas identification and characterization from plant sources have been least studied. Several strains of probiotic lactobacilli such as *Lactobacillus brevis, Lactobacillus coryniformis* and *Lactococcus lactis* subsp. *cremoris* have been incorporated in a wide range of food and dairy products (Stanton *et al*., 1998; Heller, 2001). They are commonly associated with nutritious environments like fruits, vegetables, fermented food where they mostly stay on the surface while a few of them permeate the leaves. Fermentation of fruits and vegetables with lactic acid bacteria has been popularly practiced in Asia in the form of traditional foods such as Kimchi, Sauerkraut, Koozh and Kallappam batter (Fleming and McFeeters, 1981; Sánchez *et al.*, 2003; Kumar *et al*., 2010). *Leuconostoc mesenteries, Lactobacillus brevis, Pediococcus pentosaceus* and *Lactobacillus plantarum* have been associated with vegetable fermentations (Tamminen *et al.*, 2004). The 16S rRNA sequence diversity has been extensively used as a means of identification for *Lactococcus* genus (Klijn *et al*., 1995). Due to the presence of large and closely related species among the *Lactobacillus* genus, 16-23S rRNA intergenic spacer region amplification is preferred since they exhibit greater variation than 16S rRNA structural gene (Senan *et al*., 2008).

Herbal plants might serve as the source for potential probiotic organisms. Nevertheless, there are very little efforts made in exploring the probionts

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from fermented plant samples. To the best of our knowledge, Ayurvedic sources such as Kanjika and Kutajarista were characterized for the presence of probiotics (Reddy *et al.*, 2007; Himanshu *et al*., 2011). *Cissus quadrangularis* (Pirandai) a curious climbing plant with 4-ribbed to 4-winged stems (Mishra *et al.*, 2010) reported to possess bone fracture healing, antifungal, anti-oxidant, anti-bacterial, anti-helminthic is widely used in Indian traditional medicinal system. Reports justify its beneficial effects in management of obesity and complications associated with metabolic disorders (Oben *et al.*, 2006). Also the plant extracts exhibits anti-microbial activity against *B. subtils, B. cerus* and *S. aureus* (Mishra *et al*., 2010). Further administration of Cissus quadrangularis to mice significantly increased the mucosal defensive factors thereby promoting ulcer healing (Jainu *et al*., 2006). They are usually administered as ayurvedic preparations in the form of capsules or decoction of dried stalks (Mehta *et al*., 2001; Jainu *et al*., 2006). Gastroprotective effects of soluble products of Pirandai stems might be attributed for the presence of LAB with anti-microbial potential. Thus in the present study fermented, and non-fermented Pirandai stems of Indian origin were used to assess the presence of LAB and the molecular and functional characterization of the identified isolates were performed.

Materials and Methods

Collection of plant samples

The plant material (*Cissus quadrangularis* - Pirandai) was collected from local areas of Chennai. Leaves and tendrils were removed completely, and the plant samples were washed thoroughly with sterilized water for several times and cut off to small pieces with a sterile scalpel under aseptic conditions.

Isolation of lab from plant samples

Non-fermentation

Ten gram of plant sample pieces were suspended in 90 ml of 1X PBS and kept at 37°C in a static incubator for two hours. 10 µl of the suspended solution was taken and inoculated in 3 ml of MRS broth and allowed for incubation at 37°C under anaerobic conditions. Samples from overnight-incubated MRS broths were serially diluted and plated on de Man, Rogosa and Sharpe (MRS) agar incubated at 37°C.

Fermentation

100 g of plant sample pieces were immersed in 300 ml of 5% brine solution and maintained under anaerobic conditions at room temperature (25°C). The supernatant from the pickled sample was collected after 24; 48; 72 hours plated on MRS agar maintained at 37°C for overnight.

Morphological and biochemical characterization of lab isolates

The isolated colonies were transferred to MRS broth and purified by streaking twice on MRS and M17 agar. Overnight incubated cultures were gram stained and examined microscopically for morphology and phenotype. Catalase test was performed by adding few drops of 3% H_2O_2 to the overnight culture on a glass slide. Growth of the isolates was assessed in MRS broth at different temperatures (20°C and 45°C) and distinctive pH (pH 4.4 and pH 9.6). Salt tolerances of the isolated strains were tested by incorporating 6.5% and 15% NaCl in MRS broth. Sugar fermentation was assessed by observation of color change of Andrade's peptone water accomplished by addition of single carbohydrates to the basal medium. The production of CO_2 by the isolates was tested for the presence of air bubbles in peptone water with Durham's tube. Homofermentative and Heterofermentative tests were carried out according to the method reported previously (Zuinga *et al*., 1993).

Genomic DNA extraction from lab isolates

Bacterial cultures were pelleted by centrifuging at 6000 rpm for 5 min and suspended in 570 µl of 1X TE Buffer (pH 8). 3 µl of 10mg/ml lysozyme was added with gentle mixing and incubated for 1hr at 37°C. 30 µl of SDS was added and incubated at 37°C for 30 min. 25 µl of 5M NaCl, 80 µl of CTAB were added and incubated at 65°C for 10 min. This was followed by repeated extraction of Phenol, Chloroform and Isoamyl Alcohol (25:24:1) which was centrifuged at 6000 rpm for 5 min. DNA was precipitated by the addition of equal volumes of Iso-Propyl alcohol followed by ethanol and centrifuged at 6000 rpm for 5 min. Pellet was air dried and dissolved at 50µl of TE buffer and stored at -20°C. The entire isolated DNA was tested for purity by assessment of A_{280}/A_{230} ratios.

Molecular characterization of the lab isolates

Semi-universal primers L1/L2 based on the variable loop in 16S rRNA sequence of *Lactobacillus* species 21mer forward primer coupled with 21mer universal sequences (L2) from the flanking terminal of the 16S rRNA gene were applied to determine the genera of the type strains. To identify lactococcal species semi-universal primers (PI/

P2) were designed based on the inconstant region of V1 lactococcal 16srRNA (Klijn *et al.*, 1995). Isolates were differentiated as *Lactobacillus* sp and *Lactococcus* sp by direct sequencing of respective amplicons. These corresponding regions were PCR amplified with genomic DNA as the template in (Eppendorf mini cycler9, Eppendorf International, Hamburg, Germany) using these primers (P1 GCG GCG TGC CTA ATA CAT GC, P2 TTG TTG CCT CCC GTA GGA GT, L1 CTC AAA ACT AAA CAA AGT TTC, L2 CTT GTA CAC ACC GCC CGT CA). The amplification was performed using 1µg DNA, 1U Taq polymerase, 10X Taq Buffer (Bangalore Genei, India) and 0.5 pm of both forward and reverse primers. The cycling conditions were 95°C for 5 min, followed by 36 cycles of 95°C for 1 min, 55°C for 1 min, followed by an extension at 72°C for 1 min and final extension at 72°C for 10 min. The PCR products were resolved on 1% agarose along with the 100 bp DNA ladder (Bangalore Genei, Bangalore, India) and visualized under UV-Spectrophotometer. DNA of *L. collinoides* and *L. lactis* was used as the positive control while *E. coli* as the negative control.

Multiple sequence alignment and phylogenetic analysis

Nucleotide sequence analysis of LAB amplicons was performed by BLASTN (Altschul *et al*., 1990) and subsequently; a phylogenetic tree was constructed using MEGA 4.1 software using the Neighbor-joining method, bootstrap analysis was carried out for 1000 replicates (Tamura *et al*., 2011).

Determination of anti-bacterial activity of lab isolates from pirandai

A turbidimetric assay was performed in a 96 well plate with minor modifications in the protocol (Yang *et al.*, 2012) to assess the activity of identified LAB isolates from Pirandai against growth of *E. coli.* Active *E. coli* cultures were grown in LB medium at 37°C till OD_{600} reaches 0.4. LAB isolates were grown at 37°C for 24 h as mentioned above and centrifuged for 10 min at 15000 x g. Similarly the supernatants from *L. fermentum* and *L. lactis* that served as the positive control were also obtained. These supernatants were filtered across 0.2 µm to remove the presence of any residual cells and they are the potential source of active components released by LAB. A pilot study was conducted wherein 100 µl of *E. coli* culture was mixed with 5 to 40 µl of LAB supernatants and the reaction was carried out by incubating at 37°C for 3 h at 120 rpm. The absorbance was measured at 630 nm and percentage inhibition in the *E. coli* growth was calculated by $[(OD^{Control} - OD^{Test})/(OD^{Control})]^{*}100$. Significant inhibition was noticed with 20 µl of LAB supernatant and was used for all the subsequent experiments. Three independent experiments were conducted and mean + SEM was calculated for percentage inhibition.

Statistical analysis

Statistical analysis for yield of isolates at different fermentation time period was done using by one-way ANOVA followed by Tukey's multiple comparison test using Graphpad prism software (Version 5.09. $p \leq 0.05$ was considered significant.

Results

Morphological characteristics of isolates

Isolated colonies from the fermented samples showed comparable differences in their colony morphological characteristics while the non-fermented showed similar type of colony. At 48 h of fermentation 60% isolates were identified while 16% isolates both at 24 h and 72 h of fermentation. The yield of isolates from the non-fermented samples was only 8%. Based on the morphological characteristics such as texture, size, color, form and elevation, there were 25 isolates identified and all of them were Gram positive as well as catalase negative. Among the isolates 84% cocci were classified in Group A and 16% rods under Group B. Due to the maximum yield of isolates the optimum time for fermentation was selected as 48 h for further characterization.

Effect of pH, temperature and NaCl for isolates from pirandai

At an acidic pH of 4.4 about 12% of the isolates exhibited growth whereas 20% of the isolates could grow at a pH of 9.4. About 40% of the isolates showed growth at 20°C while 12% of the isolates were tolerant at 45°C. With a medium concentration of 6.5% NaCl, 20% of the isolates could survive whereas none of the isolates grew at 15% NaCl. All the isolates grew at 37°C, pH 6.5 and in a NaCl free-medium which was considered as the optimum growth condition for characterization of LAB isolates.

Sugar fermentation of isolates from pirandai

Among Group A isolates 72% utilized Lactose and Glucose while 58% fermented sucrose. All isolates of Group B preferentially utilized Raffinose and 88% of the isolates utilized Sucrose, Lactose and Glucose. None of the isolates fermented Inulin or Arabinose. Fermenting isolates were homofermentative in nature supported by the absence of CO_2 production. Raffinose was exclusively utilized by Group B

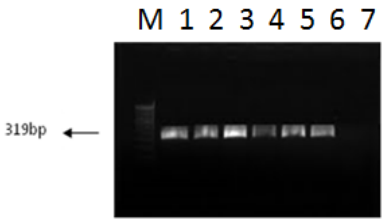

Figure 1. Gene Amplification using semi-quantitative PCR of Lactococcus spp. partial 16S rRNA with genomic DNA from Group A isolates as the template. 1% Agarose gel showing 319bp amplification by Group A isolates as Lane M – Marker, Lane 1 to 6 – Group A isolates & Lane 7 – Negative control.

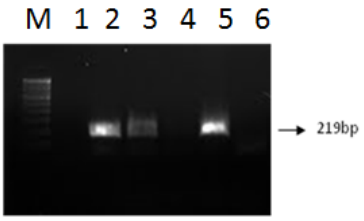

Figure 2. Gene Amplification using semi-quantitative PCR of *Lactobacillus* spp. partial 16-23S rRNA with genomic DNA from Group B isolates as the template. 1% Agarose gel showing 219bp amplification by Group B isolates as Lane M – Marker, Lane 1– Negative control, Lane 2 to 5 - Group B isolates.

isolates whereas Group A did not utilize it.

Molecular characterization of isolates

The isolated genomic DNA with A_{280}/A_{230} of 1.8-2.0 was used as the template for amplification. The primers designed for *Lactococcus* specific genus showed amplification at 319 bp (Fig 1) with Group B isolate DNA templates. Similarly, the primers for *Lactobacillus* specific genus exhibited amplification at 250 bp (Fig 2) with genomic DNA from Group A as the template. Also these primers were specific that it did not show amplification with any template from opposite groups or *E. coli* DNA.

Multiple sequence alignment and phylogenetic analysis

The pairwise alignment of *Lactococcus* isolate was performed using BLASTN, and results showed 81% sequence similarity with *Lactococcus lactis.* The phylogenetic tree of *Lactococcus* species based upon partial 16s rRNA region is shown in Fig 3. The identified *Lactococcus* isolate was closely related to *Lactococcus raffinolactis* and distantly related to *Lactococcus plantarum*. Pairwise sequence alignment of obtained *Lactobacillus* isolates (16-23S rRNA) showed high sequence similarity to *Lactobacillus fermentum* with 96% identity. For most of the isolates only representative type strains from each group was selected for sequence analysis. The phylogenetic analysis revealed that identified *Lactobacillus* isolate was closely related to *Lactobacillus fermentum* and *Lactobacillus acidophilus* while distantly related to *Lactobacillus brevis* (Figure 4).

Effect of lab isolates on E. coli *growth*

The inhibition in the growth of *E. coli* by lactococcal isolates was high compared to *Lactobacillus* isolates as shown in Figure 5a and 5b. As expected, *E. coli* growth was inhibited by *L. fermentum* and *L. lactis*. There was no significant difference in the percentage inhibition of *E. coli* growth between the lactococcal isolates and *L. lactis* while few isolates from *Lactobacillus* group (3, 4, 8 ,10) exhibited significant difference ($p < 0.05$) compared to *L. fermentum* (Figure 5). These findings suggest that LAB isolates identified from Pirandai exhibits inhibitory effect towards *E. coli* growth.

Discussion

There are many beneficial plant foods that have not been investigated in Asia for the presence of Lactic Acid Bacteria (LAB). Some fresh plant samples subsume variety of epiphytic microbiota, many spoilage microorganisms and extremely meager population of LAB (Chen *et al.*, 1983). Cissus quadrangularis has been used in ayurveda as an anti-helminthic, dyspeptic and treatment of certain bowel infections (Stohs and Ray, 2012). Though these favorable effects were attributed to the presence of advantageous phytochemicals (Adesanya *et al*., 1999; Mehta *et al*., 2001), characterization of probiotic potential microorganisms becomes necessary and important. Hence we chose Pirandai; a medicinal plant for isolation of potential LAB from both fermented and non-fermented samples since fermentation enhances the nutrient content of the food by promoting the biosynthesis of vitamins, amino acids, proteins by degrading anti-nutritional factors and also as an enrichment media (Sahlin, 1999; Zinedine *et al*., 2005). The isolated, population was dominated by gram positive cocci and not the Gram positive rods. The low population of *Bacillus* among the isolates might be due to their inability to get established at early hours of fermentation and as time proceeds bacillus population starts to take over cocci (Giraffa, 2004).

Group A organisms were homofermentative in nature fermenting raffinose and lactose while Group B isolates were homofermentative predominantly

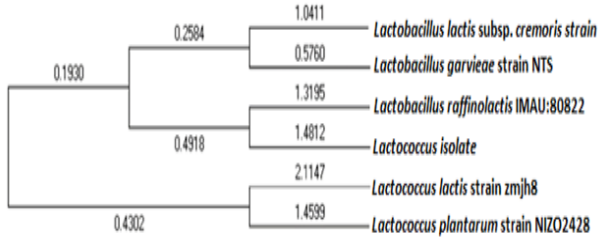

Figure 3: The Phylogenetic tree constructed using the nucleotide sequences of different partial 16S rRNA of different *Lactococcus* spp. - *L. lactis* subsp. *cremoris* (Acc No: AB598935.1), *L. garviae* (Acc No: JX875953.1), *L. lactis* (Acc No: FJ171326.1), *L. raffinolactis* (Acc No: HM058984.1), *L. plantarum* (Acc No : EU091466.1) showing the evolutionary relationship between them. One thous& bootstrap trials were run using the neighbour-joining algorithm by MEGA 5.1. Branch lengths are proportional to the evolutionary distance. Tree shows a very closer relationship of the identified *Lactococcus* spp. to *L. raffinolactis.*

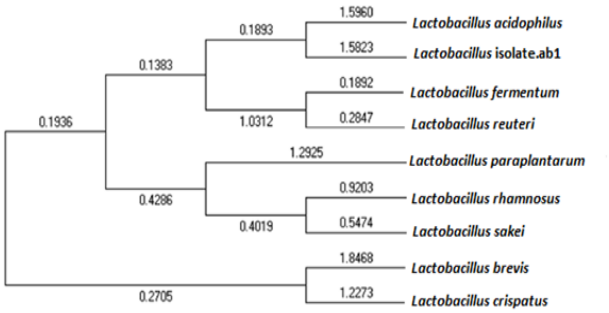

Figure 4: The Phylogenetic tree constructed using the nucleotide sequences of different partial 16-23S rRNA of different *Lactobacillus* spp. - *L. acidophilus* (Acc No: AB102855.1), *L. fermentum* (Acc No: JX118834.1), *L. reuteri* (Acc No: EU547293.1), *L. raffinolactis* (Acc No: HM058984.1), *L. paraplantarum* (Acc No : AB362736.1), *L. rhamnosus* (Acc No: JX118842.1), *L. sakei* (Acc No: HQ022862.1), *L. brevis* Acc No: JX1188839.1), *L. crispatus* (Acc No: FN692037.1) showing the evolutionary relationship between them. One thous & bootstrap trials were run using the neighbor-joining algorithm by MEGA 5.1. Branch lengths are proportional to the evolutionary distance. Tree shows a very closer relationship of the identified *Lactobacillus* spp. to *L. acidophilus*

fermenting lactose and glucose. Similar sugar fermentation patterns were also observed in *L. acidophilus* and *L. raffinolactis*, capable of utilizing Lactose and raffinose respectively (Kandler, 1983). Phytochemical analysis showed total carbohydrates content of the plant while the data on individual sugars like lactose and raffinose is not available from the literature search. This could be due to the fact that there are around 350 species of Pirandai in India implying its heterogeneity and the plant as such has been used mainly for Medicinal preparations. All isolates grew at 37°C and at pH 6.5. These results were similar with studies carried out with other LAB species (Breheny *et al*., 1975; Nannen and Hutkins, 1991; Hutkins and Nannen, 1993; Rao *et al.*, 2004). Only 20% of the isolates survived at 6.5% NaCl while no growth was observed with 15% NaCl. It has been shown that growth of *L. plantarum* is altered based on NaCl concentrations (Rao *et al.*, 2004).

PCR analysis revealed the presence of *Lactobacillus* spp. and *Lactococcus* spp. in fermented and non-fermented samples. Similarly mixed population of lactic acid bacteria was also observed in the fermentation of black olives (Sánchez *et al.*, 2003). BLAST analysis of identified *Lactococcus* spp. exhibited high degree of homology to *Lactococcus lactis* while *Lactobacillus* spp. to *Lactobacillus fermentum.* Phylogenetic Analysis revealed the close relatedness of *Lactobacillus* spp. with *Lactobacillus acidophilu*s and *Lactobacillus fermentum*. These results were interesting with respect to the presence of *L. plantarum, L. acidophilus* and *L. fermentum* in fermented vegetable products (Gonzalez *et al*., 1993; Passos *et al*., 1993; Ben and Ampe, 2000). Similarly *Lactococcus* spp. exhibited close relatedness to *Lactococcus raffinolactis* and thus the capability of the Group B organisms to ferment raffinose which is a unique characteristic property of *Lactococcus raffinolactis* (Kelly *et al.*, 1998). LAB isolates identified from Pirandai revealed their inhibitory effect towards *E. coli* growth. The percentage of growth inhibition was higher among the *Lactococcus* compared to *Lactobacillus* isolates. These findings corroborates with the previous reports where a large number of LAB isolates with different anti-microbial potentials from a variety of fermented and pickled vegetables have been identified (Kimoto *et al*., 2004; Chiu *et al*., 2008). The functional characteristics of these LAB isolates that we have examined did not show significant differences and sequencing of selected LAB isolates showed their close relatedness to *L. fermentum* and *L. raffinolactis* without many variations in the gene sequence and thus it appears that these isolates may belong to same pool. Previous studies on identification of LAB from fermented vegetables and plant extracts shows the presence of well-established probionts such a *L. fermentum, L. rhamnosus, L. reuteri, L. animalis, L. plantarum* and *L. brevis* (Visser *et al.*, 1986). There are few studies regarding the isolation and characterization of seventeen *Lactobacillus* spp. from Kanjika which is an Ayurvedic formulation (Reddy *et al*., 2007) and isolated Lactobacillus spp. (VR1) from Kutajaristha

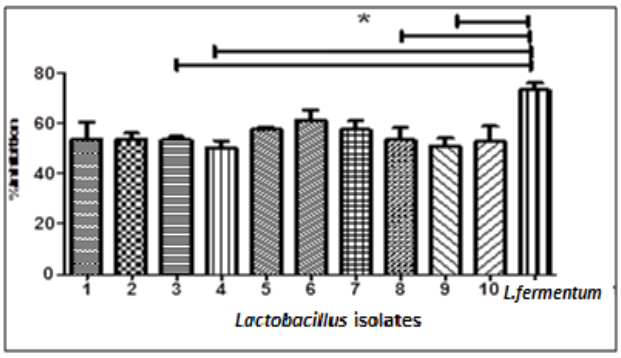

Figure 5. Antimicrobial activity of LAB isolates from Pirandai against *E. coli*. 20 µl of the cell-free LAB supernatants was mixed with overnight *E. coli* culture and the absorbance was measured at 630 nm. Percentage inhibition in growth of *E. coli* by lactobacillus isolates compared to *L. fermentum*. Values are mean + SD of three independent experiments.

showed highest homology to *L. plantarum* and exhibited probiotic characteristics such as tolerance to acidic pH, bile salts and simulated gastric juice (Himanshu *et al.*, 2011).

Conclusions

In summary we have identified the presence of both *Lactococcus* spp. and *Lactobacillus* spp. from fermented as well as non-fermented Pirandai stalks. The identified *Lactococcus* spp. is closely related to *L. raffinolactis* and *Lactobacillus* spp. to *L. acidophilus*. Also these LAB isolates exhibits an inhibitory effect on *E. coli* growth and is noticeably high with the *Lactococcus* isolates. Thus these findings suggest that Pirandai stalks could be a potent source of probiotics.

Acknowledgement

This work received financial support from the M.Tech (Food Technology) program funding by Department of Biotechnology, Government of India, New Delhi to Center for Food Technology, Anna University. U. Aarthy, T. Poonkodi and A. Ashrethalatha contributed equally for this work.

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