

Original Research Article

<https://doi.org/10.20546/ijcmas.2019.803.084>

Isolation and Characterization of Potential Probiotic Strains Isolated from Traditional Indian Fermented Foods

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ABSTRACT

Keywords

Lactobacillus,
Probiotics,
Fermented foods,
Lactic acid bacteria,
Acid tolerance

Article Info

Accepted:
07 February 2019
Available Online:
10 March 2019

The present study explored the traditional Indian fermented food samples for potential probiotic strains with superior probiotic properties to that of commercially available strain. A total of 102 food samples were collected aseptically and 200 isolates were purified using MRS media. 132 were identified as Gram-positive bacteria and 43 were identified as yeast cultures. On the basis of morphological and biochemical properties, 120 isolates were provisionally identified as *Lactobacillus* spp. These isolates were evaluated for desired probiotic properties such as acid, bile and pancreatin tolerance, and only seven isolates were found to pass these tests. These seven isolates were further evaluated for other properties, including carbohydrate fermentation, autoaggregation, haemolysis and antibiotic resistance profile, on the basis of which, the strains 4A and 2IC were found to have excellent probiotic potential and were also found superior when compared with the commercially available probiotic strain (control) *Lactobacillus casei* Shirota (LcS).

Introduction

The word probiotic comes from the Greek “pro bios”, meaning ‘for life’ and was first used by Lilly and Stillwell (1965) to describe “substances secreted by one microorganism that stimulate the growth of another (Sayes *et al.*, 2018). Probiotics are defined by WHO/FAO (2006) as “live microorganisms, which when consumed in adequate amounts

confer health benefit to the host”. The most commonly used probiotic strains belongs to heterogeneous group of lactic acid bacteria (LAB); *Lactobacillus*, *Enterococcus*, *Streptococcus*, *Leuconostoc*, *Lactococcus*, *Pediococcus*, *Bifidobacterium* and yeast *Saccharomyces boulardii*. LAB, especially *Lactobacillus*, have received much attention due to their “generally recognized as safe” (GRAS) status and because of their health

promoting benefits as probiotics (Zielinska *et al.*, 2015).

Nutritional benefits exerted by probiotics include their role in enhancing the bioavailability of various minerals, digestibility of protein and synthesis of vitamins in yogurt (Radulovic *et al.*, 2010). The reported therapeutic benefits of probiotic consumption include lipid and serum cholesterol reduction, enhancement of immune function, anticancer, anti-allergic, antioxidative and antimicrobial properties (Lee *et al.*, 2014).

The selection criteria of probiotics for human consumption suggest that they should be gastric juice and bile tolerant, safe, and viable; must be able to survive through the gastrointestinal tract in order to adhere and colonize epithelial cells (Yadav *et al.*, 2015). Moreover, probiotic strain must possess the ability to be manufactured under industrial conditions, survival and retention of their functionality during storage, heat tolerance, phage resistance and sensory properties (Nahaisi *et al.*, 2014).

Fermentation is one of the oldest food technology used worldwide for development of a diversity of aroma, flavor, texture, preservation and biological enrichment of food products by different microbial population using traditional methods. Fermented vegetable foods like 'gundruk', 'sinki'; cereal/pulse based fermented foods like 'kinema', 'tungrymbai', 'dosa'; fermented milk beverages like 'dahi', 'churpa' and alcohol like 'ghanti' are indigenous to people of India (Rawat *et al.*, 2018). Some internationally well-known fermented foods include 'kimchi', 'sauerkraut', 'kombucha', 'yogurt', 'tempeh', 'kefir', etc.

Increasing levels of multidrug resistance pathogens along with increasing demands for

natural substitutes for drugs and the emergence of scientific and clinical evidence showing the efficacy of probiotic strains has led to examine probiotics and other alternatives to pharmaceutical remedies. Due to growing consciousness about potential health benefits of LAB, there is an abundant opportunity available to explore a variety of Indian fermented foods for the isolation of new LAB strains for their potential role in probiotic research. Therefore, the present investigation was designed to isolate the best suited bacterial strain(s) from the fermented foods that can be used as a potential probiotic.

Materials and Methods

Sample collection and isolation of cultures

A total no. of 102 fermented food samples like homemade curd, butter milk, dosa batter, idli batter, jalebi batter and bhatura dough were collected aseptically and stored at 4°C till further processing. One gram or 1 ml of sample was suitably diluted and was spread on Man Rogosa Sharpe (MRS) agar and incubated at 37°C for 24-48 h. Isolated colonies of distinct morphology were sub cultured on MRS plates. All the isolates were preserved in 50% glycerol stocks at -20°C for further use (Khagwal *et al.*, 2014). Pure culture of *Lactobacillus casei* Shirota (LcS) was isolated from probiotic drink 'Yakult' and was used as control for catalase, oxidase, acid, bile, pancreatin, carbohydrate fermentation and autoaggregation tests.

Phenotypic characterization

The isolated colonies were studied for their colony morphology; provisional identification of the isolates was based on Gram's staining, endospore staining, catalase and oxidase tests. Only those isolates that were Gram-positive, endospore, catalase and oxidase negative were selected and subjected to following tests to

qualify for probiotics parameters. *E. coli* and *Pseudomonas aeruginosa* were used as positive control for catalase and oxidase test respectively whereas LcS served as negative control for both the tests. All the tests were performed in duplicate (Li *et al.*, 2014).

Acid tolerance

1% mother culture of selected isolate was transferred to MRS broth with pH 3 [adjusted using 1N hydrochloric acid (HCL)] which was then serially diluted and 100 µl was plated on MRS plates at 0, 3 and 6 h and incubated at 37°C for 48 h. The cultures which passed pH 3 test were tested for their ability to survive at pH 2.5 following the same procedure. Viability of the isolates was assessed by plate count method and expressed in terms of colony forming units (cfu) as log cfu/ml. Survival rate was given by following formula (Gonzalez-Vazquez *et al.*, 2015).

$$\% \text{ survival rate} = (\log \text{ cfu } 3^{\text{rd}}, 6^{\text{th}} \text{ h} / \log \text{ cfu } 0^{\text{th}} \text{ h}) \times 100$$

Bile tolerance

The bile tolerance of the isolates was determined by transferring 1% of seed culture to MRS broth supplemented with 0.3% bile which was then serially diluted and spread on MRS agar plates at 0, 3 and 6 h and incubated at 37°C for 48 h (Shehata *et al.*, 2016). Viability of the isolates was assessed by plate count method and expressed as log cfu/ml. Survival rate was given by following formula.

$$\% \text{ survival rate} = (\log \text{ cfu } 3^{\text{rd}}, 6^{\text{th}} \text{ h} / \log \text{ cfu } 0^{\text{th}} \text{ h}) \times 100$$

Pancreatin tolerance

1% of mother culture was inoculated in MRS broth containing 0.5% pancreatin which was then serially diluted and from this last dilution

100 µl was spreaded on MRS plates at 0, 3 and 6 h and incubated at 37°C for 48 h (Rubio *et al.*, 2014). Viability of the isolates was assessed by plate count method and expressed as log cfu/ml. Survival rate was given by following formula.

$$\% \text{ survival rate} = (\log \text{ cfu } 3^{\text{rd}}, 6^{\text{th}} \text{ h} / \log \text{ cfu } 0^{\text{th}} \text{ h}) \times 100$$

Auto aggregation test

The auto aggregation capability of bacteria helps in maintaining the bacterial population in the gastrointestinal tract. Bacterial cultures were grown for 18 h at 37°C in MRS broth. The cells were harvested by centrifugation at 5000g for 15 min, washed twice and resuspended in phosphate buffer saline (PBS).

Cell suspensions (4ml) were mixed by vortexing for 10 s and autoaggregation was determined during 5 h of incubation at room temperature. 0.1 ml of the upper suspension was transferred to another tube with 3.9 ml of PBS every hour and the absorbance (A) was measured at 600 nm. The autoaggregation percentage was expressed as: $1 - (A_t/A_0) \times 100$, where A_t represents the absorbance at time $t = 1, 2, 3, 4$ or 5 h and A_0 is the absorbance at $t = 0$. The final autoaggregation value was indicative of the isolates' ability to aggregate among themselves (Kos *et al.*, 2003; Botta *et al.*, 2014).

Acid production from carbohydrate

The production of acid and gas from glucose was determined in phenol red broth base supplemented with 1% glucose and gas produced was trapped in durham tubes at temperatures 15°C and 37°C. The isolates were categorized on the basis of fermentation group. The fermentation of glucose without gas, growth at 37°C and no growth at 15°C identifies obligately homofermentative

lactobacilli (OHOL); growth both at 15°C and 37°C without gas production is characteristic of facultatively heterofermentative lactobacilli (FHEL), whereas gas production at 37°C and variable growth at 15°C were characteristic of obligately heterofermentative lactobacilli (OHEL) (Koll *et al.*, 2010).

Haemolysis assay

For evaluating haemolytic ability of the isolates test culture was streaked onto blood agar plates containing 5% (v/v) of sheep blood and incubated at 37°C for 48 h.

Haemolysis was evaluated by examining plates for the signs of α -haemolysis (green-hued zones around colonies), β -haemolysis (clear zones around colonies) or γ -haemolysis (no zones around colonies (Borah *et al.*, 2016).

Antibiotic susceptibility

The selected strains were tested for their antibiotic susceptibility by using antibiotic disc diffusion method. 100 μ l freshly prepared broth culture was then spreaded on to Muller-Hinton agar plates and allowed to dry. Antibiotic discs were placed on the inoculated plates and were incubated for 48 h at 37 °C. The zone of inhibition diameter around each disc was measured. The results were expressed as susceptible (S), intermediate (I) and resistant (R). The test was performed according to Clinical and Laboratory Standards Institute (CLSI) standard procedure (Li *et al.*, 2014).

Molecular identification

The best isolates selected based on their probiotic properties were, identified using 16S rRNA sequencing. The purified cultures were sent for commercial sequencing and the sequence data obtained was compared using basic alignment search tool (BLAST).

Results and Discussion

Sample collection and isolation of cultures

A total of 200 microbial cultures were isolated from 102 food samples based on their colony morphology. The samples that were used for the isolation of probiotic bacteria are an indigenous part of Indian diet. Some of these fermented foods are reported to have significant medicinal properties and are consumed unknowingly as, probiotic food/drinks by local people. Zeilinska *et al.*, (2015) isolated 38 strains from six samples of cucumber pickles and cabbage pickles.

Phenotypic characterization

The isolates were provisionally identified based on the results of Gram's staining, endospore staining, catalase and oxidase test (Hammes *et al.*, 2009). Out of 200 isolates, 132 were identified as Gram-positive bacterial rods and coccobacilli and 43 were identified as yeasts. From 132 bacterial isolates, 12 were identified as endospore formers. Remaining 120 bacterial isolates gave negative results for both catalase and oxidase tests. The results of above tests suggested that these isolates belonged to the *Lactobacillus* genus according to Bergey's Manual of Bacteriology. Similar findings were also reported by Yadav *et al.*, (2016).

Acid tolerance

In order to exert beneficial effects, the probiotic bacteria must be able to survive harsh conditions of gastrointestinal tract. These bacteria must be able to withstand low pH conditions of stomach for a minimum of 90 min before being able to colonize the gut and show their health promoting benefits (Haghshenas *et al.*, 2015). pH of stomach can be as low as 1.0. Usually, in most in-vitro assays pH-3 is preferred because of the

buffering action of food matrix which has shielding effect on the ingested bacteria (Zielinska *et al.*, 2015). For this reason, the assessment of acid tolerance was performed at pH-3 and pH-2.5. Out of 120 isolates, 81 strains were able to survive pH-3 for 3 h and 57 strains survived the same pH for 6 h. The above 81 tolerant isolates were then tested for their ability to survive pH-2.5, and it was found that only 34 strains were able to survive at this pH for 3 h and none survived for 6 h. Owusu-Kwarteng *et al.*, (2015) stated that a probiotic culture with survival rate of more than 80% at pH-2.5 after 4 h incubation can be considered resistant to low pH. Similar findings were reported from previous studies, where different *Lactobacillus* species of various origins were able to retain their viability at pH values ranging from 3-7 for 3 h (Adetoye *et al.*, 2018). Comparison of percent survival rate of overall best 7 isolates and control (LcS) in pH 3 and pH 2.5 are shown in Figure 1 and 2.

Bile tolerance

Ability to tolerate bile salt is required for colonization and metabolic activity of bacteria in the small intestine and is thus considered an important parameter for evaluating the probiotic potential of the isolates. Bile salt concentration in small intestine is approximately 0.3%, therefore survival percentage of the isolates were determined after exposure to 0.3% bile salt for 3 h and 6 h (Park *et al.*, 2015). Out of 81 isolates that qualified the acid tolerance test, 29 strains were able to withstand 0.3% bile for 3 h and 19 strains survived the same bile concentration for 6 h. Bile salt resistance could possibly be due to the ability of bacteria to deconjugate bile salts, which is associated to the capability to eliminate cholesterol from the intestinal environment (Baick *et al.*, 2015). Shehata *et al.*, (2016) reported survival percentage of various *Lactobacillus* species in 0.3% bile after 3 h incubation with 85% being the

highest surviving percentage for one of their isolates. Figure 3 shows comparative % survival rate of the overall 7 best isolates and the control in 0.3% bile.

Pancreatin tolerance

Pancreatic enzymes aid in digestion of carbohydrates, proteins and fats of our food and are released through the pancreatic duct into the small intestine. Tolerance to pancreatin is considered as another important criterion for predicting the survival of a strain in the gastrointestinal environment (FAO/WHO, 2006). Out of 19 isolates that qualified bile test only 5 strains survived 0.5% pancreatin at 3 h incubation and 7 strains survived 6 h incubation. The results were supported by various studies where isolates were able to tolerate 0.5% pancreatin (Rubio *et al.*, 2014; Tallapragada *et al.*, 2018). Figure 4 shows % survival rate of the overall 7 best isolates and the control in 0.5% pancreatin.

Auto aggregation test

Auto aggregation ability indicates the adhesion ability of strains to human intestinal cells which is regarded as a beneficial characteristic for probiotics and helps providing a competitive advantage by preventing immediate elimination of the strains by peristalsis (Kos *et al.*, 2003). 21C showed the highest auto-aggregation ability of 93% followed by 4A which possessed auto-aggregation ability of 79% whereas LcS showed 59% auto-aggregation ability. The autoaggregation percentage increased with incubation time and results were consistent with previous studies (Borah *et al.*, 2016). Andrabi *et al.*, (2016) documented 80.33% auto-aggregation percentage as the highest in their study involving various LAB isolates. Figure 5 represents the autoaggregation percentage of overall 7 best isolates and control.

Table.1 Acid and gas production from phenol red glucose broth at 15°C and 37°C

S. NO.	STRAIN ID	AT 15 °C		AT 37 °C	
		ACID	GAS	ACID	GAS
1.	4A	-,-	-,-	+,+	-,-
2.	20A	-,-	-,-	+,+	-,-
3.	21C	+,+	-,-	+,+	-,-
4.	47A	+,+	+,+	+,+	+,+
5.	100A	-,-	-,-	+,+	-,-
6.	101A	-,-	-,-	+,+	-,-
7.	102A	-,-	-,-	+,+	-,-
8.	LcS	-,-	-,-	+,+	-,-

OHOL obligately homofermentative lactobacilli (- - -)

FHEL facultatively heterofermentative lactobacilli (+ - -)

OHEL obligately heterofermentative lactobacilli (+ + +)

Table.2 Antibiotic susceptibility of isolates

Antibiotics	Dose (µg/disc)	4A	21C
Chloramphenicol	30 µg	35 mm (S)	42 mm (S)
Erythromycin	15 µg	30 mm (S)	38 mm (S)
Gentamycin	10 µg	18 mm (S)	29 mm (S)
Nalidixic acid	30 µg	12 mm (I)	17 mm (I)
Penicillin G	10 µg	35 mm (S)	38 mm (S)
Streptomycin	10 µg	15 mm (I)	12 mm (I)

R, resistant (inhibition zone diameter < 12 mm); S, susceptible (inhibition zone diameter > 17 mm); I, intermediate (inhibition zone diameter between 12-17 mm)

Fig.1 Percent survival rate of 7 isolates and control at pH3

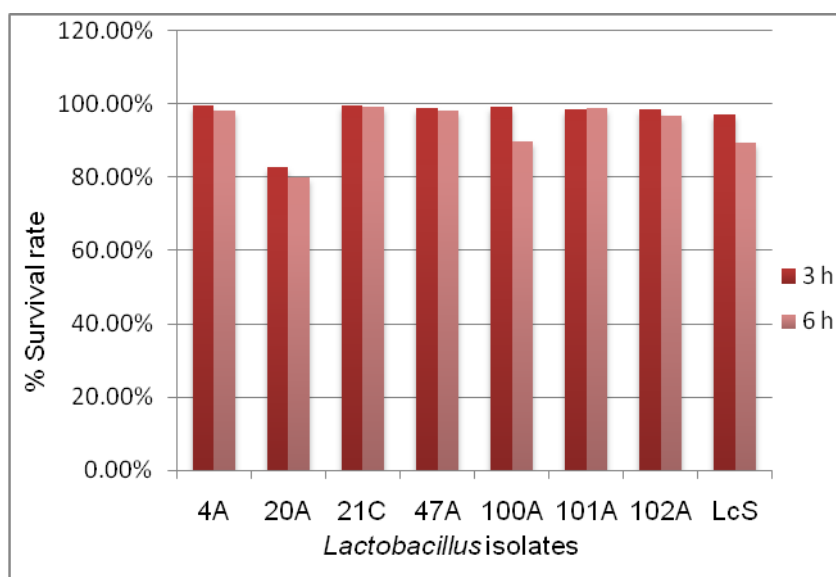


Fig.2 Percent survival rate of 7 isolates and control at pH 2.5

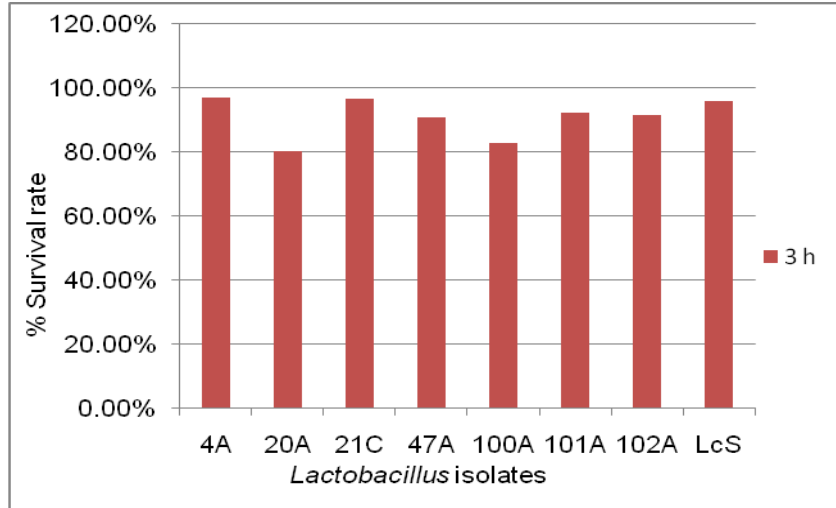


Fig.3 Percent survival rate of 7 isolates and control at 0.3% bile

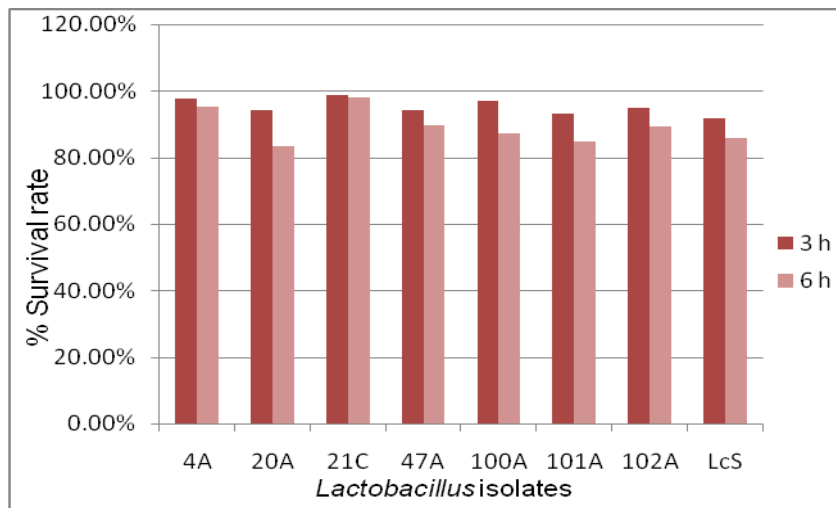


Fig.4 Percent survival rate of 7 isolates and control at 0.5% pancreatin

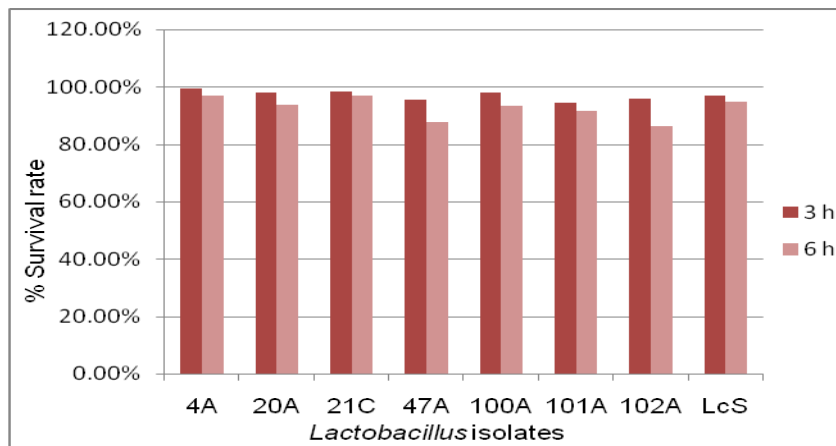
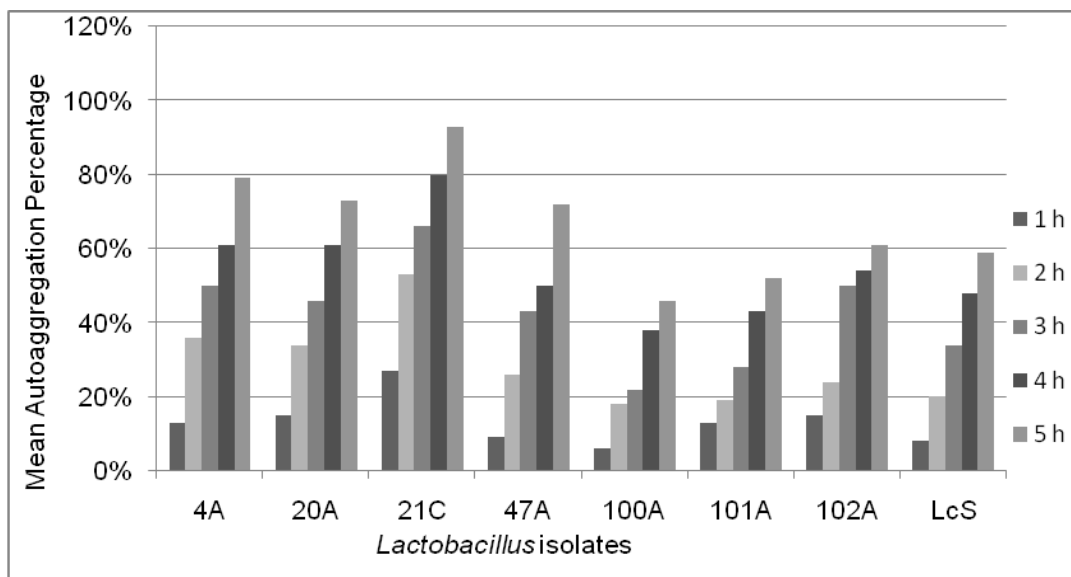


Fig.5 Autoaggregation percentage of 7 isolates and control



Acid production from carbohydrate

To determine fermentation group, some physiological properties were assessed using glucose as the test sugar. From final selected 7 isolates, 5 were identified to be OHOL, 1 was identified to be FHEL and 1 was OHEL (Table 1). LcS was identified as OHOL. Koll *et al.*, (2010) also categorized their isolates into different fermentation groups.

Haemolysis assay

The two isolates 4A and 21C with best results were tested for their non-pathogenic character by streaking them on blood agar plates. Tested strains showed no haemolysis (γ -haemolysis). Evaluation of haemolytic activity is an important safety requirement frequently used to assess potential probiotic strains. Usually no haemolysis by the test strain is the indicative of its safety for human use (Owusu-Kwarteng *et al.*, 2015).

Antibiotic susceptibility

The results of susceptibility test (Table 2) of isolates 4A and 21C against six antibiotics

demonstrated that the strains were susceptible to chloramphenicol, erythromycin, gentamycin and penicillin. These isolates showed intermediate sensitivity to nalidixic acid and streptomycin only. None of the isolates showed resistance to any antibiotic drug (inhibition zone diameter < 12 mm).

Molecular identification of strains

Finally selected isolates 4A and 21C were subjected to BLAST analysis based on their 16S rRNA sequences. The results showed that strain 4A shared 99% identity with *Lactobacillus fermentum* and the strain 21C shared 99% identity with *Lactobacillus plantarum*. Sequences of both the strains have been submitted to GenBank database of NCBI with accession no. MK367582 for 4A and MK360767 for 21C.

Acknowledgments

This research was supported by the Department of Microbiology, Chaudhary Charan Singh University, Meerut, India. All the authors of the research paper and books cited are highly acknowledged.

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How to cite this article:

Neha Khagwal, D.C. Sharma and Sharma, P.K. 2019. Isolation and Characterization of Potential Probiotic Strains Isolated from Traditional Indian Fermented Foods. *Int.J.Curr.Microbiol.App.Sci.* 8(03): 680-689. doi: <https://doi.org/10.20546/ijcmas.2019.803.084>