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Screening of Probiotic Potential of *Lactobacillus* Strains Isolated using Homemade Curd from Mysore District, Karnataka

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Received: March 29, 2018; **Published:** June 27, 2018

Abstract

Background: Investigation of *Lactobacillus* spp. with potential probiotic traits is prerequisite for the preparation of probiotics for human use and their applications in food and dairy industries.

Methods: Homemade curd samples were collected from Mysore District. India, Isolation was performed using Rogosa Sharpe agar and identification was done by conventional methods. Further species level identification was performed by HiLacto (Himedia, KB020) commercial kit for *Lactobacillus* spp. Further, screened for probiotic potential by MTT assay method.

Results: The seven isolates were identified as *Lactobacillus fermentum* (MSL1 and MSL15), *Lactobacillus mucosae* (MSL3 and MSL22), *Lactobacillus paracollinoides* (MSL4 and MSL21) and *Lactobacillus reuteri* (MSL8). The *L. fermentum, L. reuteri* and *L. paracollinoides* were highly tolerant to pH 2, bile (1%), salt (7.5%). The *L. fermentum*, and *L. reuteri* exhibited highest coaggregation ability with *Escherichia coli* and *Salmonella ebony* and more antagonistic activity towards *Staphylococcus aureus* and *Pseudomonas aeruginosa* with inhibition zone of 10.3 ± 0.57 and 10.2 ± 1.52, also showed highest cholesterol assimilation ability with 45.33 ± 0.57 and 42.33 ± 2.55% respectively. *L. fermentum* susceptible for six antibiotics tested and remaining strains showed variable results.

Conclusion: Results of the present study indicated that, the Lactobacillus isolates contained potential probiotic trait and perhaps promising isolates as probiotics.

Keywords: *Curd; Lactobacillus; Cholesterol Assimilation; Coaggregation; Antagonistic Activity*

Introduction

Curd is one of the products among pancahgavya (Milk, curd, ghee, Cow dung and urine) [1], prepared by the fermentation of milk of cow or buffalo traditionally using the previously retained curd as starter in most households and constitutes a significant part of the daily diet [2]. Curd containing beneficial bacteria such as *Lactobacillus* sp. *Bifidobacteria* sp, *Lactococci* sp considered as probiotics [3] and can be defined as biopreparations containing living bacteria given in adequate quantity confers health benefits in human and animals [4-6]. Mankind have been utilized these beneficial bacteria for thousands of year for the production of fermented food and dairy products with sensory qualities [7].

Lactobacillus (LBs) is a gram positive bacteria isolated from various sources and normally found in the human adult gastrointestinal (GI) tract [8-10]. It is one of the predominant bacteria found in curd and considered as major probiotic bacillus [11]. *Lactobacillus* spp possess many health benefits such as, stimulation of intestinal mucosal immune response in human, consumption of probiotics containing these bacteria could increase the number of peripheral blood B lymphocytes thereby reducing inflammation of intestine [12]. It was also reported that the long term consumption of probiotic drinks increases interferon-γ production by T lymphocytes and thereby decreases the allergic symptoms [13]. In addition, LBs could enhance intestinal immune response by stimulating the dendritic and natural killer cells thereby modulating the secretion of certain proinflammatory cytokines [14].

Many species of LBs, including *L. casei, L. fermentum, L. plantarum, L. reuteri* and *L. acidophilus* have been characterized for probiotic properties such as, acid and bile tolerance, salt tolerance, antimicrobial property, bacteriocin production, adhesion to intestinal epithelia and immunomodulatory activity [11,15]. Additionally these bacteria possess lot of health benefits and were helpful in the treatments of gastrointestinal disorders [16], Notwithstanding, a complex mixture of these beneficial bacteria present in curd or yoghurt reported to exhibits anti-tumor effect in human and animals [17].

With this background, the present study has been undertaken to investigate LBs with potential probiotic traits and to determine their coaggregation ability with pathogenic bacteria and cholesterol assimilation. Moreover, dairy sector is prominent in Mysore District, Karnataka State and curd constitutes the regular part of their diet. Therefore, the study has been carried out to identify and safety usage of the indigenous bacteria for probiotic potentiality.

Materials and Methods

Bacteria and growth conditions

Curd samples were collected from Mysore District, Karnataka State, India and transported in an ice pack container. The samples were suspended in a 0.85% saline and serially diluted and the 50 µl aliquots were pour plated on De Man Rogosa Sharpe agar (MRS, Himedia, India) and incubated at an anaerobic condition (Anaerobic gas jar, Sigma-Aldrich, USA) for 24h at 30 ± 2ºC. After the incubation, single col-

onies were randomly picked and purified by repetitive streaking on MRS [18]. Further cultures were maintained on agar slant at 4ºC. The single colonies were suspended in 20% glycerol and preserved at -80ºC for long term storage. Pathogenic bacteria used in this study were previously characterized and maintained at Environmental Microbiology Laboratory, Indian Institute of Technology, New Delhi, India.

Identification of *Lactobacillus*: Phenotypic and biochemical tests

The genus level identification of LBs isolates was performed by colony characteristics on MRS agar medium and through morphological, physiological and biochemical tests as per the standard procedures [19]. Further, species level identification was carried out using HiLacto kit (Himedia, KB020).

Probiotic properties of *Lactobacillus* isolates

Acid and base tolerance (pH)

The tolerance of LBs in different pH was conducted as described by Yuksekdag., *et al*. [20], with modifications. The 10 ml each MRS broth was prepared and autoclaved and inoculated with 50µl overnight active culture of LBs followed and incubated anaerobically at 30 ± 2ºC for 16h. The cell pellets were collected after centrifugation at 8000 rpm for 10 minutes and washed twice with PBS (130 mM NaCl and 10mM sodium phosphate, pH 7.0). The cell suspension was prepared with 10 ml PBS and adjusted pH levels into 2.0., 3.0., 4.0., 7.0.and 8.0., using 1.0M hydrochloric acid (HCl) and 1.0M sodium hydroxide (NaOH) chased with incubation for 4 h. The bacterial viability was tested by MTT assay.

MTT assay

In this procedure 50 µl of MTT solution (5 g/l) was added to the 500 µl of cell suspension and incubated for 45 minutes. Cell pellets were collected after centrifugation at 8000 rpm for 10 minutes and washed twice with PBS and dissolved in one ml of DMSO (Dimethyl sulfoxide) for development of purple colour product (Fornazon) produced by viable bacteria. The colour product can read at 570 nm (OD_{570}) using spectrophotometer (Multiscan MCC 340; Labsystem). The viability percentage was calculated by determining one MTT reduction unit (MRU)/ml corresponds to 5.8 x 10^6 CFU/ml.

Salt tolerance

The salt tolerance (NaCl) of LBs was determined as explained earlier by subjecting them at different concentration of NaCl, 1.0, 2.5, 5.0, 7.5 v/v, chased with incubation for 4h. The survivability percentage was calculated.

Bile sensitivity test

Bile tolerance was determined by growth of LBs in a bile salt (oxgall, Himedia) at the range of 0.1, 0.2, 0.3, 0.5 and 1.0% amended in PBS chasing with incubation for 4h. The bacterial growth was monitored as explained earlier.

Curdling of milk

The pasteurized milk was distributed to 10 ml sterilized tubes and 30 µl of fresh culture of LBs were inoculated and incubated at room temperature for 12h. The tubes were observed for coagulation of milk.

Growth at different temperature

Survival of LBs isolates at elevated temperature was determined with modified methods of Corcoron., *et al*. [21] and Collins., *et al* [22]. The 10 ml MRS broth was inoculated with aliquot of 50 µl active cultures LBs isolates. The tubes were incubated at different temperature (15ºC, 30 ± 2ºC, 45ºC and 50ºC) for 24h. After the incubation the bacterial growth was monitored by turbidity assessment.

Assimilation of cholesterol

Cholesterol lowering capacity of LBs was determined according to the method of Guo., *et al* [23]. The MRS-Thio broth was supplemented with 0.3% (w/v) bile salt meanwhile alcohol-soluble cholesterol was filtration sterilized using 0.22 µm filter and added to the broth at a final concentration of 100 µg/ml. The 1.0 ml aliquot of overnight broth culture was inoculated and incubated in a shaker (100 rpm) at 37°C for 24h. After the incubation, bacterial cells were harvested by centrifugation at 8000 rpm at 4°C for 10 minutes and cell free supernatant (CS) was collected. The cholesterol left over was then calculated using the following formula:

CS = (C–C*)/C×100, where C and C* were the concentrations of cholesterol present in the supernatant of control and tests respectively.

Antibiotic resistance

Antibiotic resistance test was conducted for six LBs isolates according to the method described by Zhang B., et al [24], against Chloramphenicol, Vancomycin, Cefoperazone, Ampicillin, Ciprofloxacin and Gentamycin antibiotics and the diameters of inhibition zones were measured using calipers. The interpretation of inhibition zones was according to the method of CLSI.

Coaggregation assay

Coaggregation experiment was performed as described by Kizerwetter-swida M and Binek M [25]. The test bacteria (*E. coli, S. ebony*) and *Lactobacillus* isolates were grown for 16h in broth and cell pellets were collected by centrifugation at 8000 rpm for 10 minutes. The pellets were washed twice in coaggregation buffer ($CaCl_2$ 0.1 mM, $MgCl_2$ 0.1 mM, NaCl 0.15 mM, NaN_3 3.1 mM in 1 mM Tris buffer, pH 7.0) and resuspended in the same buffer. The 2 ml of each *Lactobacillus* cell suspension was mixed with 2 ml of test bacteria and the OD_{600} was measured. The set up was incubated for 4h at room temperature and OD_{600} was measured. The percent of coaggregation was calculated:

% coaggregation = (OD1 – OD2 / OD1) x 100%

Statistical Analysis

Statistical evaluation was performed using IBM SPSS 20 (SPSS, Inc., Chicago) with statistical significance determined at $P \leq 0.05$. One-way ANOVA tests was used to compare the experimental results and expressed as standard error mean (± SEM), standard deviation (mean ± SD).

Result

Isolation and identification of *Lactobacillus* isolates

A total of 28 LBs strains were isolated from 20 curd samples. Seven out of twenty eight isolates were promising with good probiotic traits and were chosen for further characterization.

On MRS agar media, the colony appeared creamy white, smooth, circular, elevated colonies and they were Gram positive bacilli, except MSL22 isolate which was coccobacillus. All isolates were non-spore forming, nonmotile, KOH positive, nitrate reduction negative, catalase negative and showed variable results for IMViC tests. The isolates MSL4 and MSL8 are inconspicuous for esculin hydrolysis, whereas MSL3 and MSL22 were utilized the same. Four out of seven isolates positive for arginine hydrolysis and five isolates were positive for citrate utilization (Figure 1, Table 1). The species level identification was performed for all seven isolates, based on sugar utilization pattern and were categorized into *Lactobacillus fermentum* (MSL1, MSL15), *Lactobacillus mucosae* (MSL3, MSL22), *Lactobacillus paracollinoides* (MSL4, MSL21) and *Lactobacillus reuteri* (MSL8) (Table 2).

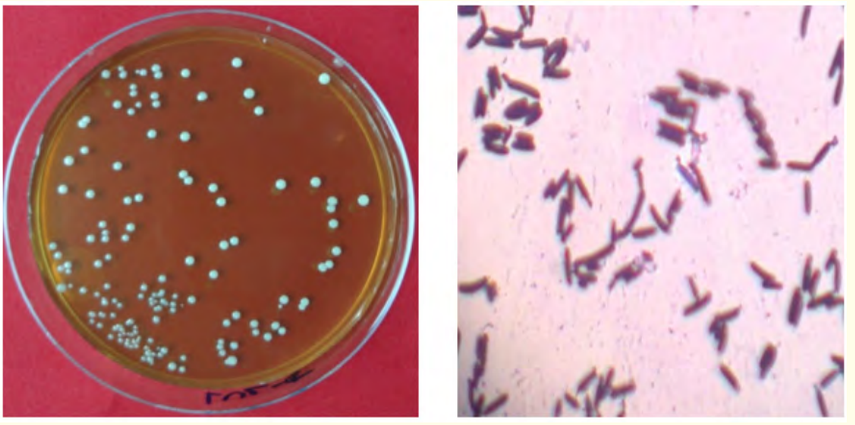


Figure 1: *Typical colonies of Lactobacillus on MRS agar and its microscopic observation (100x oil immersion).*

Isolates	MSL1	MSL3	MSL4	MSL8	MSL15	MSL21	MSL22
Morphology							
Gram's staining	+	+	+	+	+	+	+
Negative staining	*Bacillus*	*Bacillus*	*Bacillus*	*Bacillus*	*Bacillus*	*Bacillus*	*Coccobacillus*
Endospore staining	-	-	-	-	-		-
Biochemical and physiological tests							
KOH	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	-	-
Esculin hydrolysis	-	+	ND	ND	-	-	+
Nitrate reduction	-	-	-	-	-	-	-
Methyl red test	+	-	-		+	+	+
Voges Proskauer test	-	-	-	+	-	-	+
Citrate utilization test	+	+	+	V	+	+	V
Indole Production	-	-	-	-	-	-	-
NH3 from Arginine	+	ND	-	+	+	+	ND

Table 1: *Determination of morphological, biochemical and physiological parameters for Lactobacillus isolates.*

+: Positive; - : Negative; V: Variable; ND: Not Differentiated

Sugar	*Lactobacillus* isolates						
	MSL1	MSL3	MSL4	MSL8	MSL15	MSL21	MSL22
Xylose	D	D	+	N	D	+	D
Cellobiose	D	N	N	N	D	N	N
Arabinose	-	D	D	+	-	D	D
Maltose	+	+	+	+	+	+	+
Galactose	+	D	ND	+	+	ND	D
Mannose	D	N	N	N	D	N	N
Millibiose	+	D	+	+	+	+	D
Raffinose	+	D	-	+	+	-	D
Sucrose	+	+	-	+	+	-	+
Trehalose	D	N	-	-	D	-	N

Table 2: *Identification of Lactobacillus sp. based on utilization of sugar the isolates were identified as L. fermentum (MSL1/MSL15), L. mucosae (MSL3/MSl22), L. paracollinoides (MSL4) and L. reuteri (MSL8).*

+: Positive; - : Negative; D: Variable and ND: Not Differentiated

Probiotic properties of *Lactobacillus* isolates

It was observed that all seven strains survived at pH 3.0 and pH 8.0 for 4h. However isolate *L. paracollinoides* and *L. reuteri* observed to survive in acidic pH with 0.72 and 0.68 OD610 and survival percentage of 68.0 and 58.06 followed by *L. mucosae* and *L. fermentum* with 0.67 and 0.66 OD610 with survival percentage of 54.03 and 53.22 at pH 3. The *L. reuteri L. paracollinoides, L. mucosae* survived at pH 8.0 for 4h with survival percentage of 54.0, 51.6 and 41.2 respectively, but the *L. fermentum* is least survived at alkali pH. None of the isolates survived at pH 2 beyond 4h incubation period (Figure 2).

On salt tolerance test, all the isolates exhibited turbid growth at NaCl concentration of 1 to 2.5% v/v for 4h. The isolate *L. mucosae* and *L. fermentum* survived maximum at 7.5% NaCl with 39.33 ± 2.33 and 35.51 ± 2.93% for the incubation period of 4 h, followed by *L. paracollinoides, L. fermentum, L. reuteri,* and *L. mucosae* with 25.96 ± 1.15, 25.5 ± 0.018, 22.44 ± 1.09 and 22.44 ± 1.09% respectively (Figure 3).

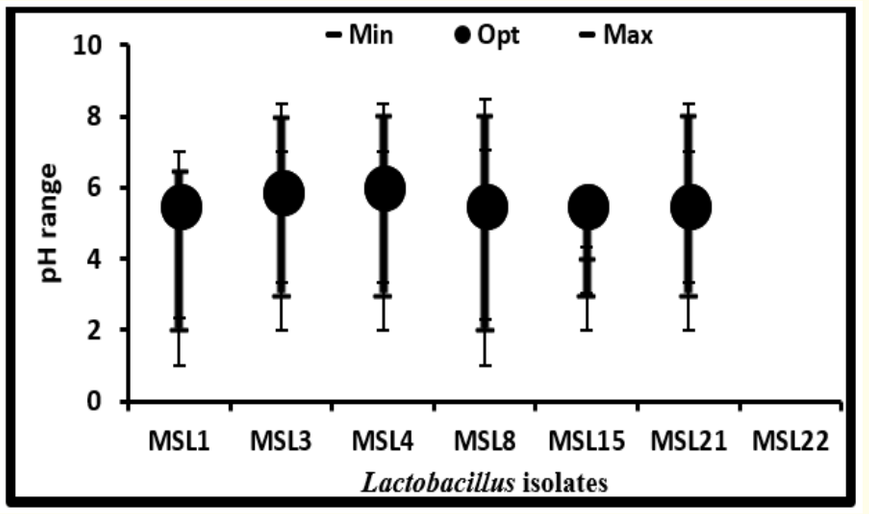
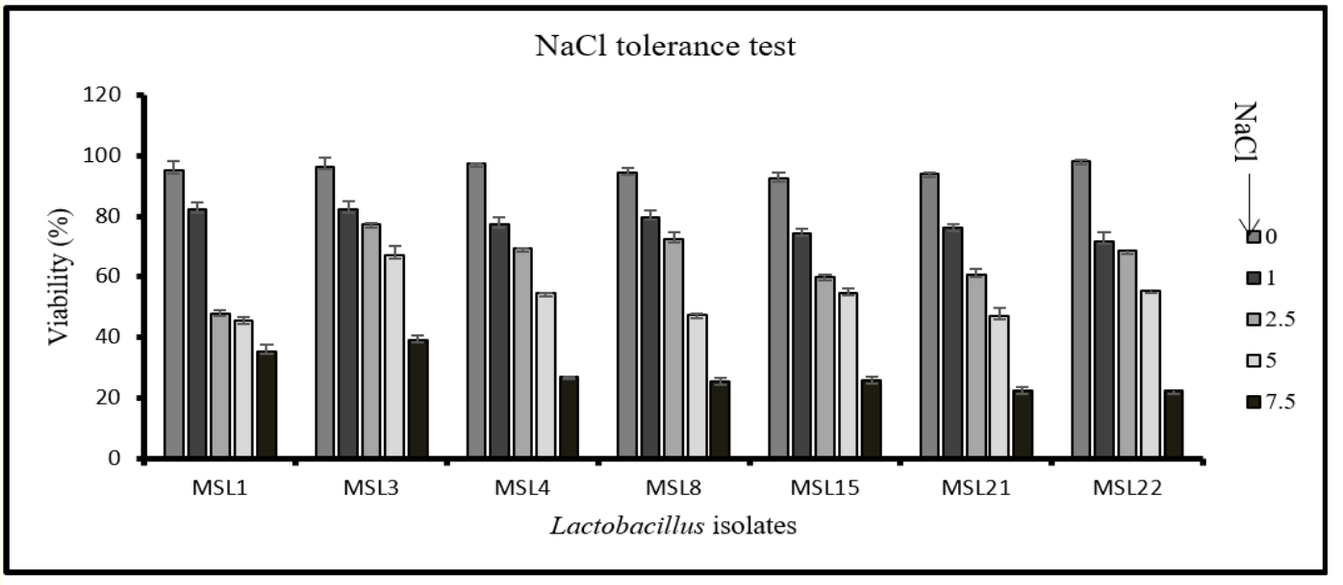


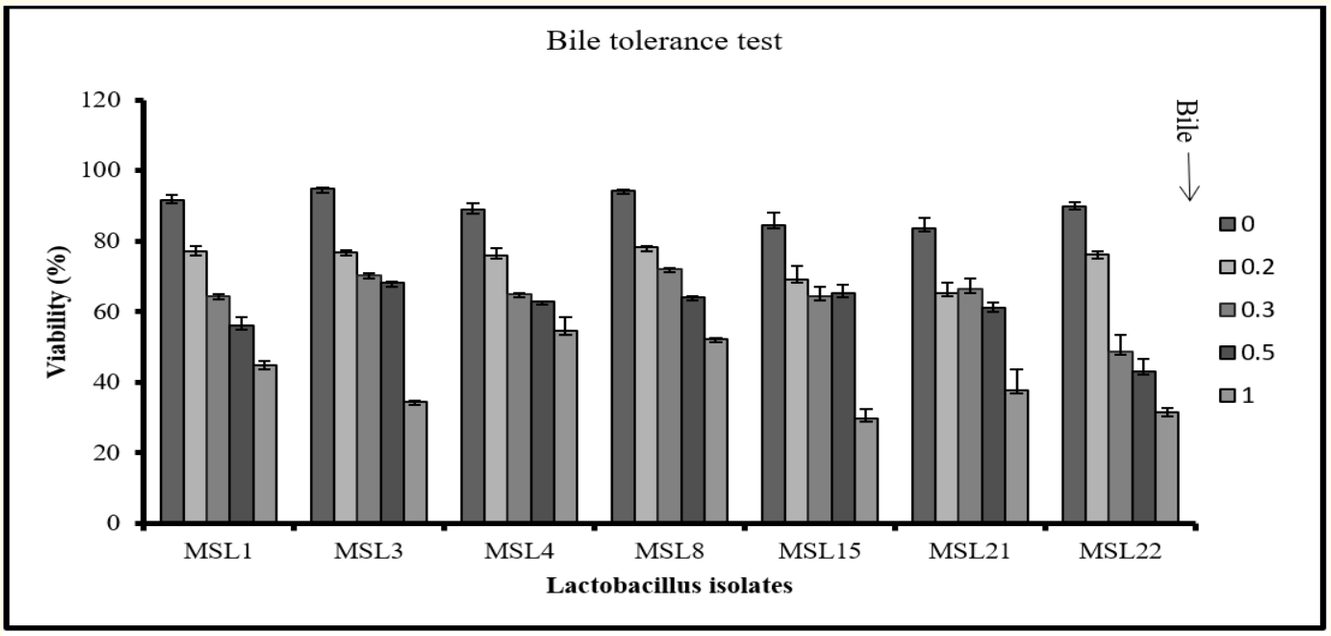
Figure 2: *Tolerance of Lactobacillus isolates in different pH. The error bar represent standard error of the mean values of results from triplicate experiment and significantly different ($p \leq 0.05$).*



NaCl %	Survivability of *Lactobacillus* isolates in different NaCl concentration (%)						
	MSL1	**MSL3**	**MSL4**	**MSL8**	**MSL15**	**MSL21**	**MSL22**
0	95.2 ± 2.96	96.5 ± 2.84	97.3 ± 0.29	94.6 ± 1.24	92.5 ± 2.0	93.9 ± 0.6	98.1 ± 0.6
1	82.32 ± 2.15	82.32 ± 2.76	77.39 ± 2.27	79.8 ± 2	74.5 ± 1.52	76.2 ± 1.13	71.8 ± 3.07
2.5	47.99 ± 1.11	77.33 ± 1.2	69.48 ± 0.28	72.3 ± 0.086	60.04 ± 2.65	60.87 ± 0.87	68.52 ± 1.76
5	45.66 ± 3.38	67.15 ± 1.08	54.75 ± 2.89	47.4 ± 0.029	54.85 ± 0.45	47.14 ± 1.48	55.51 ± 2.76
7.5	35.51 ± 2.93	39.33 ± 2.33	27.17 ± 1.48	25.5 ± 0.018	25.96 ± 1.15	22.44 ± 1.09	22.44 ± 1.09

Figure 3: *Tolerance of Lactobacillus isolates in different salt concentration (NaCl). The error bar represent standard error of the mean values of results from triplicate experiment and significantly different ($p \leq 0.05$).*

All the species showed turbid growth at 0.2 to 0.4% bile salt, as the concentration increased beyond 0.4%, growth rate was reduced for all species except *L. paracollinoides* and *L. reuteri* exhibited maximum tolerance to 1% bile salt with survival percentage of 54.66 ± 4.0 and 52.21 ± 0.21 respectively, followed by *L. fermentum, L. paracollinoides, L. mucosae, L. mucosae* and *L. fermentum* with 44.66 ± 1.4, 37.83 ± 5.9, 34.46 ± 0.28, 31.35 ± 1.35 and 29.69 ± 2.6%. In addition all strains were fermented milk in to curd and showed turbid growth at 30 ± 2°C and moderate turbidity at 40oC. None of the bacteria survived beyond 45°C (Figure 4).



Bile (%)	Survivability of *Lactobacillus* isolates in different bile concentration (%)						
	MSL1	MSL3	MSL4	MSL8	MSL15	MSL21	MSL22
0	91.66 ± 1.45	94.73 ± 0.57	88.86 ± 1.98	94.31 ± 0.315	84.53 ± 3.6	83.63 ± 2.9	89.96 ± 1.1
0.2	77.0 ± 0.5	76.86 ± 0.57	76.04 ± 0.038	78.22 ± 0.22	69.17 ± 2.7	65.22 ± 3.1	76.05 ± 4.8
0.3	64.33 ± 2.33	70.4 ± 0.57	65.08 ± 3.58	72.08 ± 0.087	64.29 ± 2.1	66.31 ± 2.3	48.71 ± 1.2
0.5	56.0 ± 2.51	68.06 ± 0.33	63.0 ± 0.0	64.19 ± 0.19	65.11 ± 2.4	60.97 ± 1.7	43.0 ± 3.5
1	44.66 ± 1.4	34.46 ± 0.28	54.66 ± 4.0	52.21 ± 0.21	29.69 ± 2.6	37.83 ± 5.9	31.35 ± 1.35

Figure 4: *Tolerance of Lactobacillus isolates in different bile concentration. The error bar represent standard error of the mean values of results from triplicate experiment and significantly different (P ≤ 0.05).*

The *L. fermentum* and *L. reuteri* recorded highest cholesterol lowering property of 45.33 ± 0.57, 42.33 ± 2.51, compare to their counterparts and all of them inhibited human pathogens involved in this study. However *L. mucosae* and *L. reuteri* recorded highest, 10.2 ± 1.52 and 10.3 ± 0.52 against P. aeruginosa and S. aureus. Again *L. paracollinoides* and *L. reuteri* recorded high level of co-aggregation with 56.66 ± 2.8 and 53.6 ± 3.21%, followed by *L. fermentum, L. mucosae* with 48.33 ± 3.0, 43.57 ± 5.7% respectively (Table 3), and *L. fermentum* susceptible to all six antibiotics whereas *L. paracollinoides* showed resistance to four antibiotics and *L. reuteri* showed resistance to three of the six antibiotics (Table 4).

Isolates	MSL1/MSL15	MSL3/MSL22	MSL4	MSL8
Antimicrobial activity				
Pseudomonas aeruginosa	5.2 ± 1.15	10.2 ± 1.52	6.2 ± 1.0	9.2 ± 0.28
Salmonella ebony	4.6 ± 2.08	8.8 ± 2.08	5.1 ± 1.15	8.1 ± 1.0
Escherichia coli	5.4 ± 1.52	7.6 ± 2.08	5.3 ± 1.52	8.5 ± 0.57
Staphylococcus aureus	6.3 ± 1.0	9.5 ± 1.52	6.4 ± 2.08	10.3 ± 0.57
Co-aggregation (%)	48.33 ± 3.0	43.3 ± 5.7	56.66 ± 2.8	53.6 ± 3.21
Growth at different temperature (°C)				
15	+	-	-	+
30 ± 2	++	++	++	++
45	+	+	+	+
50	-	-	-	-
Cholesterol Assimilation	45.33 ± 0.57	34 ± 2.0	30.66 ± 3.78	42.33 ± 2.51

Table 3: *Characterization of Lactobacillus isolates. The results are expressed as mean ± SD, each value is the average of triplicate experiments and significantly different (P≤ 0.05), ++: Turbid Growth;+: Growth; -: No Growth.*

	***Lactobacillus* isolates**			
Antibiotics	**MSL1/MSL15**	**MSL3/MSL22**	**MSL4**	**MSL8**
CPZ	S	S	R	R
AMP	S	S	R	S
VAN	S	S	S	S
GEN	S	R	S	S
CIP	S	S	R	R
CHL	S	S	R	R

Table 4: *Antibiotic sensitivity of Lactobacillus isolates. CPZ: Cefoperazone (30 μg); AMP: Ampicillin (10 μg); VAN: Vancomycin (30 μg); GEN: Gentamycin (10 μg); CIP: Ciprofloxacin (5μg); CHL: Chloramphenicol (30μg); R: Resistance; S: Susceptibility.*

Discussion

A number of *Lactobacillus* spp. have been isolated and identified from various sources using MRS medium, supplemented with 0.05% cysteine [26,27]. They were also isolated from curd and other fermented dairy products [28]. *Lactobacillus brevis* was isolated from milk and were characterized for technological properties [29] and *L. acidophilus* isolated from yoghurt [30]. In the present study, we aimed isolate indigenous lactic acid bacteria for their probiotic potentiality from Mysore jurisdiction, India. In this study four species of *Lactobacillus* including *L. fermentum, L. reuteri, L. paracollinoides* and *L. mucosae* isolated from curd samples, with promising probiotic traits such as high tolerance to bile, slat, and acidic pH, cholesterol assimilation and coaggregation abilities.

It had been suggested that microbes should have a minimum set of characteristics that could predict probiotic potential such as, ability to resist acid in stomach and to colonize the small intestine in presence of bile salts [11]. The *L. fermentum* and *L. reuteri* identified in this study were resistance to acidic pH 2 for more than 2h incubation period, results were in contrast with the previous reports [31,32], where *L. fermentum* showed tolerance to pH 2.5. However it was also determined, survival rate of *L. fermentum* at pH 2.5 for 3 h upto 80% [33] *in vitro*. Merely probiotic bacteria in curd had advantage with the protective effect of casein in an acidic condition of the stomach as that of *in vivo* [34].

Salt tolerance is another important probiotic traits of LBs. It has been reported that brines with 5 - 6% NaCl is generally used for olive fermentation by *L. plantarum* [35]. The current results showed, *L. mucosae* and *L. fermentum* viable at the NaCl concentration of 7.5% and similar with the findings of Hoque MZ., *et al.* [36], notwithstanding *L. plantarum* to 6% NaCl concentration [37].

Bile juice is essential for digestion and killing up of pathogenic bacteria that enters in the body and its concentration was 0.3% during the digestion process [38]. The probiotic bacteria must resist the bile in order to survive and colonize the intestine. In this context *L. collinoides, L. reuteri* used in this study exhibited greater survivability at 1% bile concentration compared to remaining species and the results were correlated with Tulumoglu S., *et al* [6]. As the concentration of bile increased affects the survival rate and the bacteria tolerate bile by the activity of hydrolase enzyme or food itself reduces its impact [39].

Even though all chosen strains recorded high temperature tolerance, more cholesterol removal and inhibition against test pathogenic bacteria, however the efficacy varied between strains. *L. reuteri, L. fermentum* and *L. paracollinoides* were more promising isolates than the other tested isolates. In addition they showed inhibition effect against tested human pathogens and may be bacteriocin or metabolites perhaps produced during the test [40].

Antibiotic susceptibility is another important trait of probiotic lactobacilli, as antibiotic resistance in bacteria through horizontal gene transfer [41,42]. The *L. fermentum* identified in this study, showed susceptibility to all tested antibiotics followed by *L. reuteri* indicating less possibilities of antibiotic resistant gene transfer.

Bacterial co-aggregation is another important nature of probiotic lactobacilli and inhibits colonization of pathogenic bacteria by dislodging it from epithelial cell competitively [43]. The variable co-aggregation was observed among the tested isolates and bacterial cellular surface components may be involved in the interaction, leads to cellular aggregation [44,45].

Conclusion

Assigning any bacteria as probiotics, it must fulfill all the criteria required to be considered as potential probiotic candidate. The *L. reuteri, L. fermentum* and *L. paracollinoides* are appropriate probiotic species identified in this study. Further study in this line is on progress for evaluating these strains as probiotic supplement for human.

Authors' Contributions

All the authors have contributed equally in this research work.

Conflict of Interest

The authors declared that there are no conflicts of interest.

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