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Probiotic potential assessment and enzyme profiling of indigenous Lactic Acid bacteria#

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Abstract

The present study focuses on evaluating the probiotic potential and enzyme profiling of indigenous lactic acid bacteria. The cultures used were Lactiplantibacillus plantarum, two Lacticaseibacillus rhamnosus, Lactobacillus acidophilus, and Limosilactobacillus fermentum. In vitro analysis of acid and bile tolerance, cell surface hydrophobicity, aggregation potential, bile salt hydrolase activity, antimicrobial activities, and safety assessment were done for the isolates. All the cultures exhibited varying degrees of probiotic attributes confirming the strain specificity. The isolate Lactobacillus acidophilus, displayed the highest antimicrobial activity against both E. coli and S. aureus with zones of clearance measuring 16.50±0.50 mm and 19.00±1.00 mm, respectively. Enzyme profiling of the isolates was done for 19 enzymes and the results revealed the presence of four distinct enzymes in all cultures: leucine arylamidase, valine arylamidase, β-galactosidase, and β-glucosidase.

Keywords: *Lactic acid bacteria, GRAS status, probiotics, API ZYM profile, enzymes*

Human gut microbiota contains Lactic Acid Bacteria (LABs), which are widely used as probiotics because of their intriguing nutritional profile, impact on host immunity, GRAS (Generally Recognised as Safe) status, and capacity to prevent the growth of hazardous foodborne and other pathogens. The selection of probiotic strains in the food industry is crucial, as they must survive processing and storage under severe conditions while retaining their beneficial qualities. To improve host health, recent emphasis has been placed on isolating probiotics from native sources. Thus, it is fascinating to develop cost-effective indigenous probiotic starter cultures for food fermentations.

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An important part of many food fermentation processes is played by the LAB which produces a variety of enzymes and participates in robust metabolic activities. The assessment of the wide range of enzyme activity is an essential procedure that aids in identifying LAB strains with the most favourable attributes for use in food production, ultimately leading to the improvement of the final product's quality (Nemska *et al*., 2016). Also, to safeguard the safety of probiotics, their enzymatic activities need to be evaluated to ensure that they do not produce potentially toxic substances (Kim *et al*., 2021). In this regard, the current study aimed to isolate LAB from various indigenous sources and offer a more comprehensive report on their enzymatic potential along with the assessment of probiotic characteristics.

Materials and methods

Selection of bacterial cultures

In the current research, a total of five indigenous lactic acid bacterial cultures were employed, with four obtained from diverse sources such as cow dung, appam batter and sweet potato and one obtained from MTCC. The isolation of the three isolates was carried out by selective enrichment in de Man, Rogosa and Sharpe (MRS) broth. Appropriate dilutions of these samples were pour-plated in de Man, Rogosa and Sharpe (MRS) agar (Hi-Media) and incubated at 37°C for 48 h to isolate discrete colonies. Preliminary characterisation of the five selected cultures was carried out based on catalase test and Gram staining. All the LAB cultures were streaked on MRS (de Man Rogosa Sharpe) agar for purification and were propagated in MRS broth. For long-term storage, the cultures were maintained in 70 per cent glycerol at -18°C. The molecular level identification by 16s rRNA sequencing, of the isolates was conducted at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram.

Acid and bile tolerance

The acid tolerance of the isolates was assessed by inoculating cultures into sterile MRS broth tubes at pH 2.0 and 3.0. The number of survivors was determined by measuring absorbance at 600 nm every hour for four hours after incubation at 37°C (Pundir *et al*., 2013). Similarly, to assess the bile tolerance, the isolates were exposed to sterile MRS broth tubes containing 0.3 and 0.6 percent bile salts. The number of survivors was quantitatively assessed by measuring the absorbance at 600 nm at hourly intervals for four hours after the incubation at 37°C (Shehata *et al*., 2016). The experiments were conducted in triplicates.

Bile salt hydrolase activity (BSH)

BSH activity was assessed using a modified approach reported by Daliri *et al*. (2022). The MRS agar plates were made by adding 0.5 per cent (w/v) bile salt and 0.37 g/L $CaCl_2$ to the medium. About 50 µL of overnight-grown LAB cultures were inoculated into the MRS agar wells. After 24 hours of incubation at 37 °C, the plates were inspected for the presence of precipitated bile acid around the wells.

Bacterial cell surface hydrophobicity

The bacterial cell surface hydrophobicity of bacterial strains was determined using xylene extraction (Li *et al*. 2015). The bacterial strains' affinity for hydrocarbons (hydrophobicity) was estimated as adhesion percentage using the formula:

Cell surface hydrophobicity = [(Ao – A)/ Ao]×100, where Ao and A are the absorbances before and after extraction with organic solvent (xylene), respectively. The experiments were conducted in triplicates.

Auto-aggregation assay

Following Abushelaibi *et al*. (2017), the auto-aggregation assay was conducted with minor modifications. The absorbance (A_0) at 600 nm was measured, and then the sample absorbance (A_1) was measured after 1 and 5 h of incubation at 37°C. The auto-aggregation percentage was expressed as:

Auto-aggregation % = $[(A_0 - A_1)/ (A_0) \times 100]$, where A_0: initial optical density, A_1: optical density after incubation. The experiments were conducted in duplicate.

Co-aggregation

The co-aggregation was evaluated against *E. coli* following the methodology of Abushelaibi *et al*. (2017), with some necessary modifications. The absorbances of the bacterial suspensions alone and their combination were measured at different time points (0 h and 2 h). The co-aggregation percentage was calculated as follows:

$$\text{Co-aggregation \%} = \frac{\frac{A_{Path} + A_{LAB}}{2} - A_{Mix}}{\frac{A_{Path} + A_{LAB}}{2}} \times 100$$

where A_{Path} and A_{LAB} represent absorbances at 600 nm of the separate bacterial suspensions in control tubes, A_{Mix} represents the absorbance of the mixed bacterial suspension. The experiments were conducted in duplicate.

Antimicrobial activity

To assess the antimicrobial activity of the LAB strains, the agar well diffusion method described by Balouiri *et al.* (2016) was employed. The strains were evaluated for their ability to produce antimicrobial substances against *Staphylococcus aureus* and *Escherichia coli*. Subsequently, wells with a diameter of 5 mm were created in the agar plates and loaded with 50-100 µL of the tested strain cultures. Following incubation at 37°C for 24-48 h, the clear zones of inhibition were measured in millimetres.

Antibiotic susceptibility testing

All bacterial isolates were subjected to antibiotic susceptibility testing using a panel of antibiotics, including Vancomycin (10 mcg), Amoxicillin (10 mcg), Penicillin (10 units), Gentamicin (50 mcg), Tetracycline (10 mcg), Chloramphenicol (25 mcg), Ciprofloxacin (30 mcg), and Streptomycin (25 mcg). Freshly grown cultures of LAB were cultured in MRS agar at 0.5 McFarland units, corresponding to a concentration of 10^{10} colony-forming units (CFU) per millilitre. Antibiotic discs were then placed on the inoculated agar plates and incubated at 37°C for 24-48 h. The diameters of the zones of inhibition around the antibiotic discs were measured, and the sensitivity conditions were defined using the NCCLS/ CLSI criteria (Mohammed and Çon, 2021)

API ZYM profile of isolates

The enzymatic profiles of LAB isolates were investigated using the API ZYM kit (Bio-Mérieux, France). For the test, each isolate was cultured overnight in MRS broth at 37 °C and the cells were harvested through refrigerated centrifugation. The cells were resuspended for inoculation into the microcupules of the kit. Fifty microliters of the suspension, equivalent to the No.3 McFarland standard, were then added to the microcupules, which were covered and incubated at 37°C for 4 h. Following incubation, a drop of both ZYM A and ZYM B reagents was added to each microcupule and allowed to incubate for an additional 5 min. The results were recorded by employing a visual colour intensity scale ranging from 0 to 5. A rating of 0 indicates no change in colour, which signifies a negative reaction. A rating of 5 represents the highest intensity of staining, while values of 3, 4, and 5 are considered indicative of a positive reaction (Fadl and Kamel, 2022).

Statistical analysis

Statistical analysis was performed using SPSS 23.0 software. All data were presented as Mean ± Standard Error (SE). Significant differences between treatments were tested with analysis of variance (ANOVA), followed by Duncan's post-hoc test and paired t-tests, with significance levels set at $p < 0.05$ and $p < 0.01$.

Results and discussion

Lactic acid bacteria were isolated from various indigenous sources as mentioned in Table 1. All the cultures purified by streaking on MRS agar displayed a rod-shaped morphology, were Gram-positive, and tested negative for catalase. The isolates were genotypically identified by 16s rRNA sequencing (Table 1).

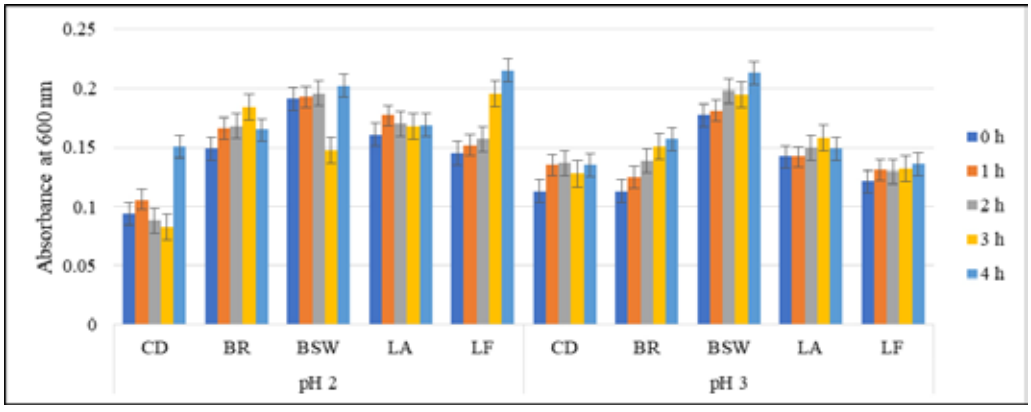
Acid and bile tolerance

In this study, the LAB isolates exhibited growth in the pH range of 2–3 and demonstrated resilience to bile salt concentrations between 0.3-0.6 per cent. This is crucial for their selection

Table 1: Lactic acid bacteria cultures used in the study

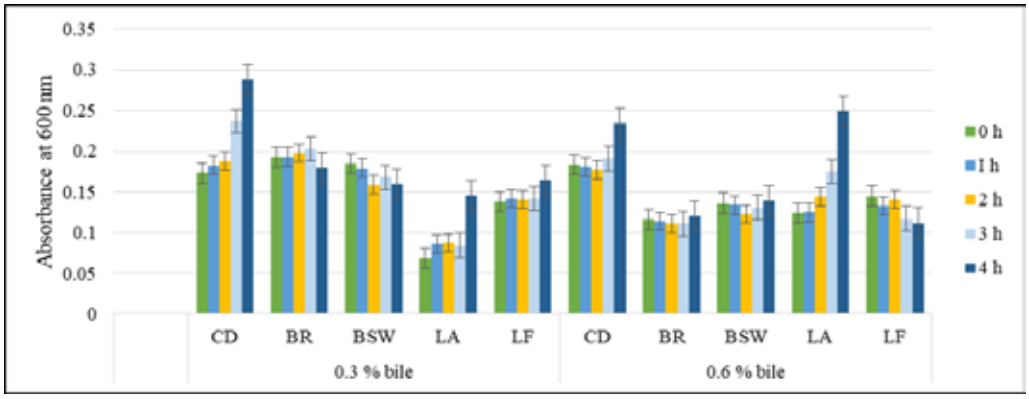
Code	Source	Organism
CD	Cow dung	*Lactiplantibacillus plantarum* DMCD03
BR	Appam batter	*Lacticaseibacillus rhamnosus* DMBR05
BSW	Sweet potato	*Lacticaseibacillus rhamnosus* DMAN06
LA	MTCC	*Lactobacillus acidophilus* 307
LF	Chilly	*Limosilactobacillus fermentum* 13

as probiotics, as they must endure the harsh conditions of the stomach during gastric transit for 2-4 h (Montoro *et al*., 2016). Following a 4-hour incubation at 37°C, it was observed that the isolates CD and LF exhibited a significant increase in growth at pH 2. Moreover, at pH 3, BSW displayed a notable growth surge after 4 h of incubation, as outlined in Fig. 1. Higher endurance to acid (Dhundale *et al*., 2018) and bile environments (Argyri *et al*. (2013) have been reported for LAB which is consistent with our findings (Fig. 2). Tolerance to 0.3 per cent and 0.6 per cent bile salts has been reported earlier by Rappai (2020) in the LAB isolated from human milk.



Figures are the OD_{600} nm Mean ± Standard error of three replications, *-significant at five percent level (p<0.05), **- significant at one percent level (p<0.01), ns- non significant (p≥0.05)

Fig. 1. Acid tolerance of isolates at pH 2 and pH 3



Figures are the OD_{600} nm Mean ± Standard error of three replications, *-significant at five percent level (p<0.05), **- significant at one percent level (p<0.01), ns- non significant (p≥0.05)

Fig. 2. Bile tolerance of isolates at 0.3 per cent and 0.6 per cent bile

Bile salt hydrolase activity

LAB possessing bile salt hydrolase (BSH) activity can transform conjugated bile acids into their deconjugated counterparts. This conversion reduces the efficiency of fat absorption in the small intestine, prompting the liver to produce more bile acids from cholesterol and thereby resulting in a decrease in overall serum cholesterol levels (Vijayalaksmi *et al*., 2020). In this study, none of the isolates exhibited a prominent BSH activity which is similar with the results of Motey *et al*. (2021).

Cell surface hydrophobicity

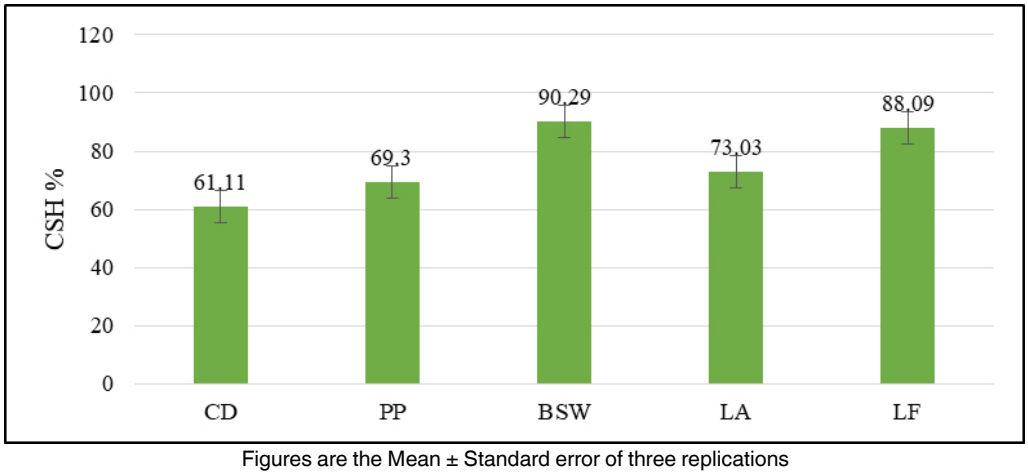
When selecting potential probiotics, it is crucial to consider a minimum hydrophobicity level of 40 per cent, as it plays a key role in influencing adhesion and interactions with host cells (Del Re *et al*., 2000). In our study, the cell surface hydrophobicity (CSH) percentage of the isolates ranged from 36.94 to 90.29 per cent, with BSW having the highest and BR the lowest adhesion potential (Fig. 3). According to Serrano-Niño *et al*. (2016), a CSH value exceeding 50 percentage signifies a high level of hydrophobicity which is in agreement with our results except for BR. Vijayalakshmi *et al*., (2020) found a notably high hydrophobicity (64.98 ± 0.01%) in *L. plantarum* BNH17, which is confirmed by our findings.

Auto-aggregation

In this study, all isolates demonstrated varying degrees of auto-aggregation, which showed an upward trend at 5 h than the first hour. This is likely due to the accumulation of aggregation-promoting elements, as previously observed (Goh and Klaenhammer, 2010). In the present study, the highest observed auto-aggregation value was for LF (39.65 %) followed by LA (34.31 %), and CD (30.91 %) after 5 h of incubation (Fig. 4). Prolonged incubation times enhance auto-aggregation capabilities, as demonstrated by Dias *et al*. (2013). Li *et al*. (2015) proposed that surface proteins play a role in the auto-aggregation capabilities of the strains.

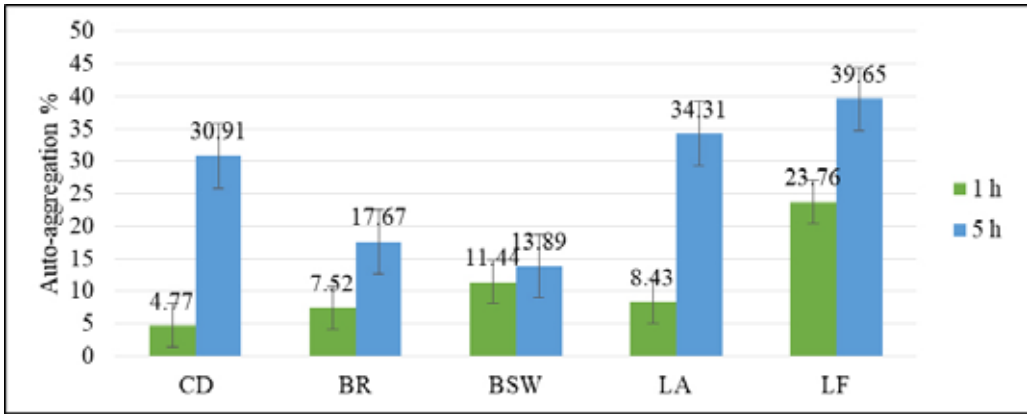
Co-aggregation

Co-aggregation tests are essential for selecting probiotic bacteria, emphasising their ability to interact with pathogenic bacteria. Effective probiotics should suppress pathogens while displaying strong adherence to the gut epithelium. Co-aggregation is linked to lactobacilli's ability to interact with pathogens (Jena *et al*., 2013). Campana *et al*. (2017) found that co-aggregation features vary by strain, highlighting its strain-specific nature and beneficial impact. In this study, the co-aggregation of the isolates was assessed against *E. coli*. The highest co-aggregation was obtained for BSW with 23.27 per cent and the lowest was for LA with 9.25 per cent as shown in Fig. 5. According to Solieri *et al*. (2014), coaggregation values below 20 per cent



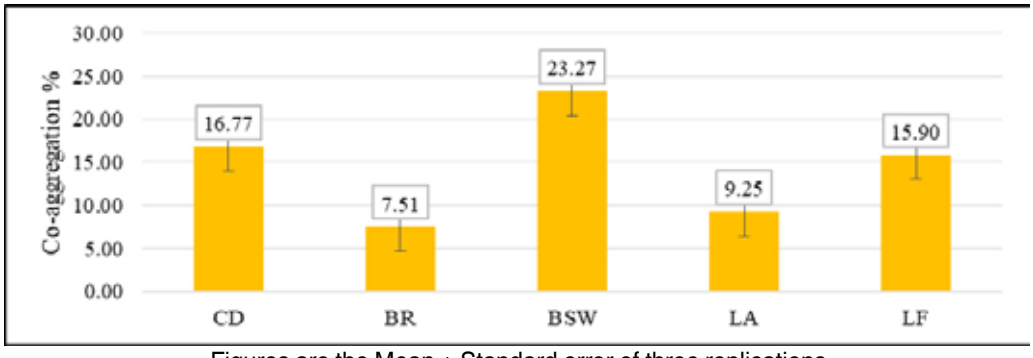
Figures are the Mean ± Standard error of three replications

Fig. 3. Cell surface hydrophobicity percentage of isolates with xylene



Figures are the Mean ± Standard error of two replications

Fig. 4. Auto-aggregation percentage of isolates



Figures are the Mean ± Standard error of three replications

Fig. 5. Co-aggregation percentage of isolates against *E. coli*

suggest weak coaggregation ability, and in this context, isolate BSW demonstrated significant coaggregation with *E. coli*.

Antimicrobial activity

In this study, we evaluated the antibacterial activity of *Lactobacillus* isolates against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria as detailed in Table 2. The results indicated that isolates BR and LA displayed superior antimicrobial activity against *E. coli*, with a zone of inhibition measuring 16.50±0.50 mm. Conversely, isolate LA demonstrated heightened activity against *S. aureus*, yielding a zone of inhibition measuring 19 mm. This result corroborates with Klimko *et al*. (2020) who found that *L. acidophilus* CM MSU 146 exhibited the highest effectiveness against Gram-positive bacteria, while *L. rhamnosus* CM MSU 528 showed maximum inhibitory action against Gram-negative bacteria. The antimicrobial efficacy of LABs arises from the generation of bioactive metabolites during their growth, encompassing organic acids, fatty acids, hydrogen peroxide, and bacteriocins (Gaspar *et al*., 2018).

Table 2. Antimicrobial activity- Zone of clearance against *E. coli* and *S. aureus*

Isolates	Zone of clearance (mm)	
	E. coli	***S. aureus***
CD	16.00±1.00	16.50±0.50
BR	16.50±0.50	16.00±1.00
BSW	15.00±0.00	16.50±0.50
LA	16.50±0.50	19.00±1.00
LF	14.00±0.00	16.00±1.00

Figures are the Mean ± Standard error of three replications

Antibiotic susceptibility test

Antibiotic resistance may limit the effectiveness of probiotics due to the potential for genetic transmission of resistance (Borriello *et al*., 2003). The susceptibility of the LAB strains was evaluated against eight distinct antibiotics belonging to six diverse classes by using disc diffusion assay on MRS agar plates. The strains displayed a consistent resistance pattern to β-lactams and glycopeptides but were notably susceptible to aminoglycosides, chloramphenicol, tetracycline, and fluoroquinolones, suggesting they are not multi-drug resistant (Table 3). Antibiotics like Chloramphenicol and Tetracycline, which inhibit protein synthesis, are generally effective in suppressing the growth of lactobacilli (Shehata *et al*., 2020). Our results are consistent with those of Ammor *et al*. (2007), who documented that *Lactobacillus* spp. has intrinsic resistance to vancomycin. In contrast to our findings, Klimko *et al*. (2020) revealed that most *Lactobacillus* strains displayed resistance to gentamicin and kanamycin while demonstrating lower resistance to tetracycline, chloramphenicol, and ampicillin.

API ZYM profile

The enzymatic profile could be employed to make inferences about the probable type of LAB present in the sample, as suggested by Humble *et al*. (1977). The enzymatic activities of the isolated LAB strains are shown in Table 4. All of the isolates displayed Leucine arylamidase, Valine arylamidase, β-galactosidase activity, and β-glucosidase activities. β-galactosidase in probiotic bacteria facilitates lactose breakdown beneficial for lactose-intolerant individuals (de Vrese *et al*., 2003). Aminopeptidases like Leucine arylamidase and valine arylamidase play a major role in the hydrolysis of bitter peptides, leading to the release of amino acids which results in the improved flavour of the cheese. Phosphohydrolase activity was detected in the majority of the strains, but notably absent in the case of LA. According to Mene'ndez *et al*. (2001), the lack of alkaline phosphatase activity appears to be a prevalent finding in LAB which correlates with our study. Likewise, Gonza´lez *et al*. (2015) reported the absence of Trypsin and α-chymotrypsin enzymatic activities in LAB isolates and found that these enzymes can act as virulence factors in certain bacteria. Therefore, the absence of these enzymatic activities is considered advantageous in isolates intended for use in food applications. Furthermore, it is noteworthy that none of the isolates exhibited lipase activity. All the tested lactobacilli strains were devoid of adverse enzymes such as trypsin, α-chymotrypsin, β-glucuronidase, and N-acetyl-β-glucosaminidase (Nemska *et al*., 2019). The enzyme β-glucuronidase is linked to the production of toxins, mutagens, and carcinogens (Dabek *et al*., 2008). Our results are consistent with the report of Georgieva *et al*. (2009). None of the isolates examined in our study displayed any detectable α-mannosidase or α-fucosidase activities. This finding aligns with previous research findings by Gonza´lez *et al*. (2015). These enzymes, α-mannosidase and α-fucosidase, are critical for the breakdown

Table 3. Antibiogram of isolates represented as the zone of clearance in mm

Antibiotics	Zone of clearance of the isolates (mm)				
	CD	BR	BSW	LA	LF
Vancomycin (10 mcg)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Chloramphenicol (25 mcg)	32.33±0.33	30.33±0.88	32.00±0.00	29.67±0.88	31.00±0.58
Tetracycline (10 mcg)	30.00±0.58	20.00±0.00	33.33±0.67	22.00±0.00	31.00±0.58
Streptomycin (25 mcg)	21.00±0.00	18.67±0.33	20.33±0.33	15.00±0.00	20.00±0.00
Amoxicillin (10 mcg)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Ciprofloxacin (30 mcg)	24.67±0.33	10.33±0.33	26.67±0.33	12.00±0.58	25.00±0.00
Penicillin (10 units)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Gentamicin (50 mcg)	19.00±0.58	22.00±0.00	20.67±0.33	18.00±0.00	19.33±0.88

Figures are the Mean ± Standard error of three replications

Table 4. Enzymatic profiling of the isolates using API ZYM kit

Sl. No.	Enzyme assayed for	Isolates				
		CD	BR	BSW	LA	LF
1	Control					
2	Alkaline phosphatase	-	-	-	-	-
3	Esterase (C 4)	+	+	-	-	+
4	Esterase Lipase (C 8)	-	+	-	-	-
5	Lipase (C 14)	-	-	-	-	-
6	Leucine arylamidase	+	+	+	+	+
7	Valine arylamidase	+	+	+	+	+
8	Cystine arylamidase	+	+	-	-	-
9	Trypsin	-	-	-	-	-
10	α-chymotrypsin	-	-	-	-	-
11	Acid phosphatase	-	-	-	-	-
12	Naphthol-AS-BI-phosphohydrolase	+	+	+	-	+
13	α-galactosidase	-	-	-	-	-
14	β-galactosidase	+	+	+	+	+
15	β-glucuronidase	-	-	-	-	-
16	α-glucosidase	-	+	-	+	+
17	β -glucosidase	+	+	+	+	+
18	N-acetyl-ß-glucosaminidase	-	-	-	-	-
19	α-mannosidase	-	-	-	-	-
20	α-fucosidase	-	-	-	-	-

(+): positive reaction, (-): negative reaction

of complex carbohydrates containing mannose and fucose.

It is important to highlight that, although the API ZYM system offers a semi-quantitative analysis, these findings hold considerable significance. They facilitate the discernment and choice of LAB strains that exhibit valuable technological and probiotic characteristics. The various enzymes produced by LAB can significantly impact both the composition and flavor of food.

Conclusion

The probiotic characteristics of the five lactic acid bacteria isolates from various indigenous origins were assessed through various in vitro experiments, encompassing tests for acid and bile tolerance, bile salt hydrolase activity, cell surface hydrophobicity, auto-aggregation and co-aggregation capabilities, and antibiotic susceptibility. Although none of the isolates displayed all the tested characteristics, five isolates exhibited some of the properties, highlighting the strain-specific nature of these probiotic attributes. Additional research is essential to assess the effectiveness of the suggested probiotic strains *in vivo*, which should encompass an examination of the health benefits conferred to the host. The utilization of the API ZYM assay kit revealed diverse enzyme activities among all the LAB strains. The predominant enzymes produced by these strains included leucine arylamidase, valine arylamidase, β-galactosidase, and β-glucosidase. The diverse enzymes exhibited unique features, signifying the variances in stability among the LAB strains.

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Conflicts of interest

There were no conflicts of interest reported by the authors

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