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METABOLISM AND FUNCTIONAL HETEROGENEITY OF FERMENTED MILK ORIGIN LACTIC ACID BACTERIA FOR LACTOSE INTOLERANCE

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ABSTRACT

Lactic acid bacteria (LAB) are cosmopolitan in distribution with multiple ecological niches. LAB shows diverse applications in improvement of health by biochemical interference or imuno modulation to overcome several clinical circumstances. Lactose intolerance (LI) is one such situation, where individual show lactose maldigestion after the consumption of dairy products. It is necessary to overcome such a condition by employing indigenous beneficial bacteria or their products. Therefore, in this study we have isolated and characterized LAB from fermented milk samples, from remote villages of districts of south India. Traditionally fermented milk samples (68) were collected, cultivated on MRS medium, identified by biochemical and carbohydrate metabolic activity and correlated with Bergey's manual of systematic bacteriology. When matched with other LAB, *Lactiplantibacillus* isolates were able to reduced pH of medium significantly and reached pH of 4.6 in 48 hours. On MRS agar, 450 different bacterial isolates were isolated, recognized as presumptive LAB and classified up to the level of genera as *Lactiplantibacillus* (285), *Lactococcus* (70), *Pediococcus* (19), *Streptococcus* (20), and *Enterococcus* (16). Later, β-galactosidase screening was carried out using MRS/X-gal agar medium. Out of 450 LAB isolates only *Lactiplantibacillus* isolates were potential β-galactosidase producers. GRAS organisms such as LAB are multifaceted diverse group of bacteria localized in varieties of fermented foods/in the intestine and recognized as probiotics. Distinct contribution of LAB in health care and disorder management made this organism as a choice for alternate therapy; hence functionality of LAB can be promoted for LI management.

Keywords: Probiotic, β-galactosidase, LAB, Lactose intolerance

INTRODUCTION

Fermented foods are conventionally used in routine diet in many parts of India. Curd is classically prepared by fermenting milk with previously prepared curd and making up a sizable portion of the daily diet (**Balamurugan *et al.*, 2014; Rezac *et al.*, 2018; Castellone *et al.*, 2021**). There is no standard starter culture used to make curd at domestic level, therefore each household's LAB that ferment the milk may vary significantly. The curd is presumed to contain LAB with probiotic qualities and therefore requires scientific confirmation. This research was done to assess the probiotic qualities of the LAB from home-made curd in southern India. Rural areas represent the ethnicity of food culture and provide customers with access to native microorganisms that enhance their overall health. The purpose of collecting samples from rural areas is to isolate indigenous bacteria which are prototrophs. Although there is no much significance with regard to homes or locations but the consumers particularly elderly of the region showed longevity with minimum health problems and to study biochemical diversity of LAB may result in the production of a novel enzyme called β-galactosidase.

Lactose intolerance (LI) is a condition of inability to digest lactose with lack of deficiency of lactase (β-galactosidase) enzyme. Typical clinical symptoms include stomach discomfort and distension, borborygmi, flatulence and diarrhea that appear between 30 and 120 min after the consumption of lactose (**Harrington and Mayberry, 2008; Gayathri and Vasudha, 2018; Vasudha *et al.*, 2023b**). Prevalence of LI was estimated to be 80–100% in Asian and African countries where prevalence of lactase non-persistence is reported to be quite low in Northern European countries **(Ingram *et al.*, 2009; Gayathri and Vasudha, 2018).** In contrast, prevalence of LI was estimated to be 48% per 200 participants on the Indian subcontinent, notably in the northern area, while it was found to be higher (66%) in the southern region **(Babu *et al.*, 2010).** One of the causes of lactase persistence features in northern India is Indo-Aryan population migration, which was later disseminated by the intermixing of native populations (**Gayathri and Vasudha, 2018**). Therefore, it is important to investigate how the genetic marker for the lactase persistence trait is distributed among the populations of northern and southern India.

Lactose is a disaccharide molecule, hydrolyzed by the enzyme β-galactosidase to produce monosaccharides like glucose and galactose, which are then absorbed by the small intestine. During lactose intolerant due to the lack of β-galactosidase, unabsorbed lactose molecules passed into the bowel lumen, resulting in increase in the volume and intestinal fluid content by converting lactose molecules into short-chain fatty acids and gas; hydrogen (H_2), carbon dioxide (CO_2) and methane (CH_4). The gut microbiota found in intestinal content offers a salvage pathway for lactose digestion, which may cause a variety of gastrointestinal symptoms **(Swagerty *et al.*, 2002; Perino *et al.*, 2009).** The amount of lactose consumed, expression of β-galactosidase, intestinal microbiota and sensitivity of the digestive system, all these affect the symptoms after ingesting lactose. In order to diagnose β-galactosidase deficiency a number of diagnostic practices are available, *viz.*, hydrogen breath test, lactose tolerance test, fecal reducing sugar test, stool acidity test, biopsy of small intestine and tests to measuree the direct activity of the β-galactosidase enzyme **(Yang *et al.*, 2013; Gayathri and Vasudha, 2018; Vasudha and Gayathri, 2023).**

The beneficial LAB in the gut are capable of producing endogenous β-galactosidase and observed to assist in lactose hydrolysis or natural therapy for LI **(Lomer *et al.*, 2009; Savaiano *et al.*, 2013; Vasudha *et al.*, 2023a).** LAB are strictly fermentative, Gram-positive, catalase-negative, microaerophilic, and acid-tolerant organisms. In addition, lactic acid-producing, non motile and non-sporeformer and they were classified either homofermentative or heterofermentative micoorganisms based on how they metabolize carbohydrates **(Dimidi *et al.*, 2019; Ayivi *et al.*, 2020)**. The homo-fermentative group uses EMB (Embden-Meyerhof-Parnas or glycolytic pathway) to convert a carbohydrate into lactic acid and using phosphoketolase pathway, hetero-fermentative bacteria may convert glucose to produce equimolar amounts of lactate, CO_2, ethanol, or acetate **(Vinderola and Reinheimer, 2003; Kakelar *et al.*, 2019; Vasudha and Gayathri, 2023).** *Lactiplantibacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, and *Enterococcus* are the few genera of LAB serving as main starters in fermentation, particularly for dairy products and some of them naturally make up the intestinal microbiota. They are regarded as probiotics and generally recognized as safe (GRAS) organisms **(Bin Masalam *et al.*, 2018; Reuben *et al.*, 2020; Gayathri *et al.*, 2022a).** Recent research have revealed that the potentiality of probiotic LAB are used for treating intestinal problems and effective in mouse model for various clinical intestinal diseases or disorders **(Chen *et al.*, 2002; Gayathri, 2016**; **Gayathri *et al.*, 2022b).** As indigenous bacteria would aid in managing LI, the current investigation was carried out to isolate and characterize potential LAB from samples of conventionally fermented milk.

MATERIALS AND METHODS

Collection and isolation of fermented milk samples

A total of 68 traditional fermented milk samples were collected from individual households from rural villages of districts of south India (Table 1) and the chemicals used for the present study were procured from Himedia Laboratories Pvt, Ltd. India. 1g of sample was weighed and homogenized in an aseptic condition and tenfold serial dilution was made with 0.85% physiological saline. To distinguish the acid-producing bacteria from other bacteria, under microaerophilic conditions, 0.1 mL of the dilution sample was inoculated on MRS medium (de Man, Rogosa, Sharpe agar) with 1% $CaCO_3$ and incubated at 37 °C for 24 to 48 hours and typical colonies were selected and maintained in MRS agar slants at 4 ℃.

Morphological and physiological characterization of LAB

LAB isolates were identified with distinctive morphological characters, such as form, elevation, margin, color and texture. Following morphological characteristics study, isolates were further subjected to Gram's staining, motility, catalase test, endospore staining and characterized for NaCl tolerance test at different concentration of 2%, 3%, 4%, 6% and 10%, as well as their ability to grow at various pH levels of 2, 4, 6, 7 and 8 and different temperatures of 10, 15, 37, and 45 °C. Further, characterized based on Bergey's manual of systematic bacteriology **(Vos *et al*., 2011; Ismail et al., 2018; Alharbi, & Alsaloom, 2021; Goa et al., 2022; Huligere et al., 2023).** While the carbohydrate fermentation was performed using different carbohydrates *viz*,. sucrose, lactose, dextrose, maltose, fructose, galactose, mannitol, arabinose, cellobiose, maltose, sorbitol, rhamnose, mannose, and xylose.

Acidification activity

Selected LAB isolates were inoculated to a 10% w/v solution of skim milk powder in a conical flask and incubated at 37 °C for 48 to 72 hours to acidify the milk. Samples were taken out at various points throughout incubation (0, 12, 24, 48, and 72 hours) to measure the change in pH of the medium. Based on their ability to produce acid, LAB isolates were used to categorize into rapid acid producers (pH 4.6 in less than 12 hours), moderate acid producers (pH 4.6 in between 12 and 48 hours) and slow acid producers (pH 4.6 in more than 48 hours) **(Attia *et al*., 2001**; **Yi et al., 2011; Akabanda *et al*., 2014; Fguiri *et al*., 2016; Alharbi, & Alsaloom, 2021).**

Screening of β-galactosidase producing LAB

Screening of β-galactosidase producing LAB was performed by using X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as a substrate which is, in fact, analogue of lactose and IPTG (isopropyl β-D-1 thiogalactosidase) was used as an inducer. Selected LAB were inoculated into MRS medium containing 60 µL of X-gal (20 mg/ml dissolved in DMSO) and 10 µL of IPTG solution and incubated at 37 °C/ 48 hours **(Gheytanchi *et al*., 2010; Deng *et al*., 2020**; **Kolev *et al*., 2022; Vasudha *et al*., 2023b)**. Blue-colored colonies that developed during incubation were considered as β-galactosidase producing bacteria.

Determination of β-galactosidase activity

Selected LAB cultures were adjusted to 1.0 OD (560 nm) and centrifuged (12,000 x g/5 min/4°C) and washed 2X/PBS. 50 µL of toluene/acetone (1:9 v/v) was used for bacterial cells permeabilize and 100 µL of an aliquot of cell permeabilized was added to a tube containing 900 µL of phosphate buffer, then add 200 µL of ONPG (4 mg/mL) solution and incubated at 37°C for 15 min. 0.5 mL of 1 M Na_2CO_3 solution was added to stop the reaction. Absorbance values at 420 and 560 nm was recorded using a NanoDrop 2000C UV-Spectrophotometer (Thermo Fisher Scientific, Inc. USA). The activity of β-galactosidase was assessed in Miller units **(Vinderola and Reinheimer, 2003; Li *et al*., 2012; Gomaa, 2018; Deng *et al*., 2020; Kolev *et al*., 2022; Vasudha *et al*., 2023b).**

$$\beta - \text{galactosidase activity} = 1000 \frac{(A1_{420} - 1.75 \times A2_{560})}{(15\ \text{min} \times 1\text{mL} \times A1_{560})}$$

Where, $A1_{560}$ represents the absorbance before the test and $A2_{560}$ represents the absorbance of the reaction mixture.

Statistical analysis

Each experiment being carried out in triplicate, all results of each experiment were presented as mean ± standard errors of mean. One-way ANOVA was done to compare data using GraphPad Prism 9 while differences was significant at $P<0.05$.

RESULTS AND DISCUSSION

Comprehensively, 450 LAB isolates were obtained from 68 fermented milk samples collected from remote villages of districts of south India (Table 1). Majority of LAB isolates showing distinguishable morphological characteristics on MRS agar plate and colonies, appeared small, circular, creamy white, opaque (Fig 1A; Table 2) and microscopic observation showed typical bacilli (Fig. 1B) and cocci. **Sharma & Bajwa (2021)** isolated a total of nine isolates from Kaladhi sample, which were identified using biochemical analysis and morphological traits. **Taye *et al*., (2021)** isolated total 41 bacterial isolates from raw milk, cheese and yoghurt samples, which they then classified into five different genera of LAB and *Bifidobacteria* spp.

Table 1 Collection of fermented milk samples from different remote villages of south districts of India

Sample type	Region/District	Sampling place/village	Sample code
Fermented milk	Davangere 14.23°N 75.9°E	Tholahunse	C1-C3
		Kakkaragolla	C4-C7
		Avaragolla	C8-C10
		Kodaganur	C11-C13
		Kondajji	C14-C16
	Tumkur 13.34°N 77.1°E	Lakshmisagara	C17-C21
		Kadajjana playa	C22-C26
		Veeraganahalli	C27-C30
		Devarahalli	C31-C33
		Chikkadasarahalli	C34-C38
		Venkatapura	C39-C41
		Ponnasamudra	C42,C43
		Thondagere	C44,C45
		Kaidala	C46,C47
	Chitradurga 14.00°N 76.50°E	Ajjanahalli	C48-C50
		Hirehalli	C51,C52
		Mannekote	C53
		Kondlahalli	C54,C55
		Maskal	C56,C57
		Javagondanahalli	C58-C60
	Ballari 15.1500°N 76.9333°E	Emmiganur	C61,C62
		Kottur	C63,C64
	Bijapura 16.82°N 75.72°E	Ukumanal	C65,C66
		Katakanalli	C67,C68

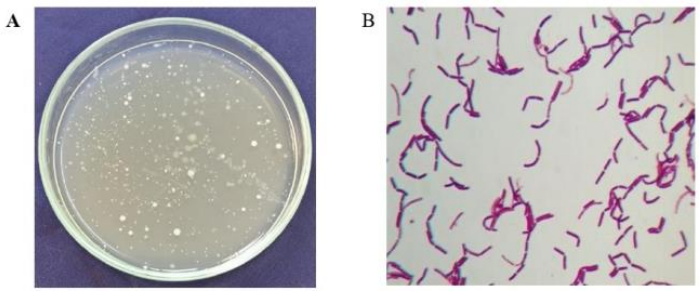

Figure 1 (A) Colony morphology of LAB isolates grown on MRS agar medium (B) Microscopic view of Gram's stained LAB isolates (100 X) (Olympus. USA)

Table 2 Colony morphology and microscopic observation of LAB isolates

Group	Colony morphology							Microscopic observation
	Size	Form	Color	Elevation	Margin	Texture	Opacity	
1	Small	Round	Cream	Convex	Entire	Smooth	Opaque	Bacillus
2	Small	Round	Cream	Convex	Entire	Smooth	Opaque	Long bacillus
3	Small	Round	Cream	Convex	Entire	Smooth	Opaque	Short bacillus
4	Small	Round	Cream	Convex	Entire	Smooth	Opaque	Rods in chain
5	Large	Round	Cream	Convex	Entire	Slime	Translucent	Long bacillus
6	Large	Irregular	Cream	Flat	Undulate	Rough	Opaque	Diplococci
7	Large	Round	White	Convex	Entire	Smooth	Opaque	Cocci in chain
8	Large	Irregular	Colourless	Flat	Undulate	Smooth	Translucent	Cocobacilli
9	Large	Irregular	Cream	Convex	Entire	Smooth	Opaque	Cocci
10	Small	Round	Cream	Convex	Entire	Smooth	Opaque	Cocci in chain
11	Large	Irregular	White	Flat	Entire	Rough	Opaque	Cocci
12	Small	Round	White	Convex	Entire	Smooth	Opaque	Cocci

In the present study, presumptive tests supported the findings that a few isolated bacteria were Gram-positive bacilli and cocci, immobile, and non-endospore-forming organisms. Growth at different NaCl concentration conditions were diverse, at 2% NaCl concentration 67% growth was observed, likewise, at 3% NaCl (68%), 4% NaCl (56%), 6% NaCl (48%) and 10% NaCl (0%) varied bacterial density was observed. Ability to grow at various pH levels were also determined, at different pH 2 (0%), pH 4 (35%), pH 6 (91%), pH 7 (84%) and pH 8 (78%) percentage of tolerance to varied pH was varied. While at different temperatures such as 10°C (7%), 15°C (24%), 37°C (98%), and 45 °C (28%), rate of temperature tolerance was differing (Fig. 2). Overall, selected LAB isolates showing maximum growth rate at 2 and 3 % NaCl concentration (67% and 68% respectively), at pH 4 (91%), temperature at 37°C (98%) were found to be ideal condition for maximum growth parameters. **Wassie & Wassie (2016)** reported that, a total of 83 LAB isolates except one could grow in 2% NaCl except some genera like *Lactococcus*, on the contrary, none of the isolates grew at 6.5% NaCl. **Sangwan *et al.*, (2015)** reported that, 200 LAB were isolated and tested for morphological and physiological characteristics with similar results.

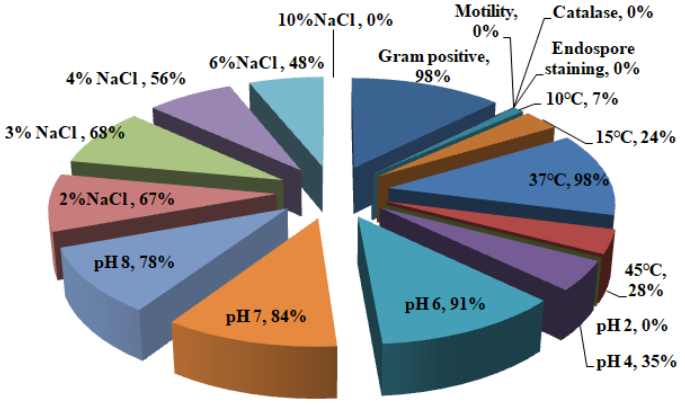

Figure 2 Physiological characterization of LAB isolates and growth at different physical and nutritional condition

LAB are known for utilizing diverse carbon forms and transforming into organic acids along with other metabolites. LAB showing diverse attributes of health promotion, hence used as starter culture for technological applications. It has been highly evident that autochthonous LAB are preferred as they adapt quickly to the native substrate. In the present study, LAB isolates exihited different rates of glucose fermentation (Fig. 3), majority of the isolates reduced glucose, lactose, sucrose, maltose, fructose, galactose, mannitol, arabinose, cellobiose, maltose, sorbitol, rhamnose, mannose, and xylose within 24 to 48 h and were considered as homofermentative producing only acid with no gas. In an another study, 5% of the *Lactiplantibacillus* isolates fermented xylose, 45% of the isolates fermented sorbitol and trehalose, and none of the isolates decreased mannitol **(Asha *et al.*, 2012).** These findings demonstrated the LAB isolates are able to use diverse carbohydrates as a source of carbon for their development in *in vitro* experiments perhaps favorable in *in vivo* environment.

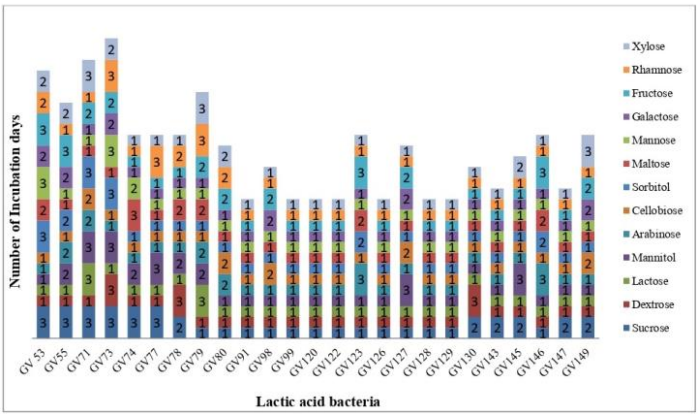

Figure 3 Carbohydrate fermentation of LAB isolates

LAB were charactersised at the generic level, with *Lactococcus* spp, were creamy white, yellowish, small, large colony and with a circular edge and *Lactiplantibacillus* spp. were whitish, small or large in size. *Leuconostoc* spp. were more often found in pairs, *Pediococcus* spp., which were found in tetrads and *Streptococcus* spp. were in chains. Additionally, LAB were classified into six genera including *Leuconostoc*, *Lactococcus*, *Lactiplantibacillus*, *Enterococcus*, *Streptococcus* and *Pediococcus* spp. **(Savadogo *et al.*, 2004, Harun-ur-Rashid *et al.*, 2007).** In an another study, **Abd El Gawad *et al.*, (2010)** reported five distinct genera of LAB *viz. Aerococcus* (18%), *Leuconostoc* (26%), *Enterococci* (20%), and *Lactiplantibacillus* (30%) spp. Abdullah and Osman (2010), also reported that *Lactiplantibacillus* (69.23%), *Lactococcus* (19.23%) and *Pediococcus* (11.53%) diversity. **Taye *et al.*, (2021)** isolated 41 bacterial isolates, which were classified into five distinct genera of LAB and *Bifidobacteria* spp. Based on biochemical tests and carbohydrate fermentation test, bacterial genera grouped into *Lactiplantibacillus* (24.38%), *Lactococcus* (21.94%), *Streptococcus* (19.51%), *Leuconostoc* (14.64%), *Bifidobacteria* (12.19%) and *Pediococcus* (7.31%). **Wassie & Wassie (2016)** identified a total of 83 LAB isolates from raw cow milk samples, which classified into six genera: *Lactococcus* (21.69%), *Leuconostocs* (18.07%), *Streptococcus* (9.64%), *Pediococcus* (12.05%) and *Entrococcus* (9.64%). **Mohammed & Çon (2021)** isolated 12 LAB from 25 white cheese samples and nine of the isolates were identified as *Enterococci* (*E. durans*, *E. faecium*, *E. faecalis* and *E. gallinarum*). Other identified strains were *Lactiplantibacillus pentosus*, and *Loigolactiplantibacillus coryniformis subsp. torquens*. **Goa *et al.*, (2022)** isolated 12 LAB from fermented milk samples and identified using primary screening of LAB**,** as *Lactococcus lactis subsp. lactis*, *Lactiplantibacillus acidophilus*, *Lactiplantibacillus plantarum*, *Limosilactiplantibacillus fermentum* and *Leuconostoc lactis*. In the present study, based on morphological and physiological parameters, the isolates were categorized into five genera of LAB, namely, *Lactiplantibacillus* (69%), *Lactococcus* (17%), *Leuconostocs* (5%), *Streptococcus* (5%) and *Entrococcus* (4%) (Fig. 4). Compared with other studies, our fermented milk samples contained diverse group of LAB, amongst *Lactiplantibacillus* genera (69 %) was predominant.

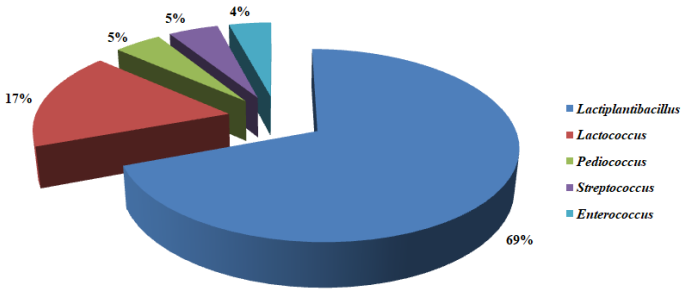

Figure 4 Different genera of LAB isolated from fermented milk samples (%)

LAB have strong acidifying activity, they produce more acid than other bacteria, compared to other LAB species, *Lactiplantibacillus* species reduced medium pH more quickly and reached target pH of 4.6 in 48 hours. **Seifu *et al.*, (2012)** reported that among LAB, *Lactiplantibacillus salivarius* was able to lower the pH of the skim milk culture medium from 6.78 to 4.38 within 2 days incubation period. After this incubation period, *Lactiplantibacillus plantarum* and *Lactiplantibacillus delbrueckii* subspecies bulgaricus could reduce the pH 6.77 and 6.76 to 4.57 and 4.58 respectively. In the current investigation, more than 24 hours to produce enough acid to lower the growth medium pH to 4.6. Comparatively *Lactiplantibacillus* spp. decreased the medium pH and reached the desired pH of 4.6 in 24 hours quicker than the other LAB (Fig. 5).

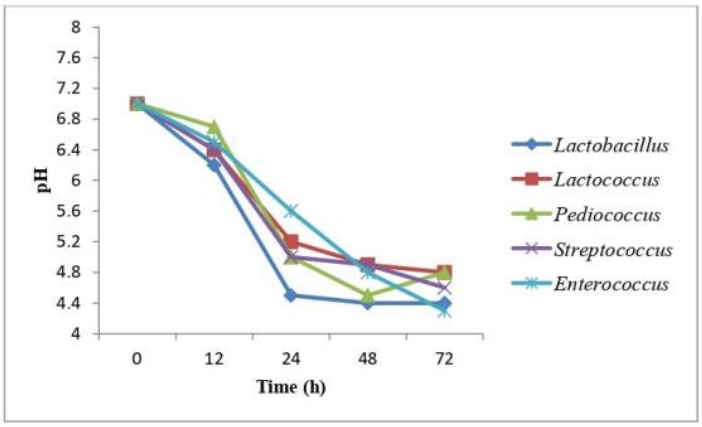

Figure 5 Skim milk powder fermented by LAB isolates changes its pH at various incubation times

In the present study, LAB isolates were examined for the presence of β-galactosidase using a qualitative test on X-gal plates. Production of β-galactosidase was detected by the development of blue colonies (Fig. 6A). ONPG substrate was used for β-galactosidase activity estimation spectrophotometrically and recorded in miller units (Fig.6B). Amongst various isolates, GV126 showing maximum activity (984.58±4.72 Miller units) and GV99 showing minimum activity (431.92±6.24 Miller units) were compared with other LAB isolates (Table 3). **Vinderola and Reinheimer (2003)** analysed the β-galactosidase activity of many *Lactoplantibacillus* stains, including *L. acidophilus*, *L. acidophilus* and other bacteria with enzyme activity ranging from 518±40 to 2,053±25 Miller units. **Gheytanchi *et al.* (2010)** reported that the β-galactosidase enzyme activity of *L. delbrueckii* substrains bulgaricus and *L. casei* isolated from cheese ranged from 867 to 1,966 U/ml. In a recent study, **Kolev *et al.*, 2022** reported that, *Enterococcus faecium* OSU-PECh-27A and *L. helveticus* OSU-PECh-4A showed specific activity of 3331 and 1110 mU/min/mg, respectively. Considering all the above aspects, LAB were successfully isolated in the present investigation from fermented milk, which is an excellent source of LAB. Probiotic supplements that include β-galactosidase-producing *Lactiplantibacillus* genus potentially help those with lactose intolerance. Thus, diverse lactic acid bacterial that are possessing distinct functional attributes such as β-galactosidase production, could be used as starter culture for the formulation of milk-based food for LI management.

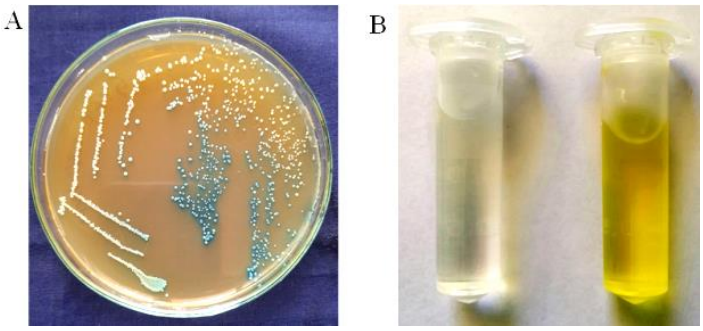

Figure 6 (A) LAB isolates in blue color on X-gal supplemented MRS agar plate indicating β-galactosidase production (B) ONPG test for β-galactosidase production

Table 3 Determination of β-galactosidase activity (Miller units)

Isolate Name	β-galactosidase activity (Miller units)
GV53	523.85±3.60
GV71	570.85±2.00
GV73	623.33±3.05
GV80	445.72±1.00
GV99	431.92±6.24
GV120	655.10±4.04
GV122	448.70±0.99
GV126	984.58±4.72
GV129	440.81±2.00
GV145	511.28±0.57

CONCLUSION

LAB live in a wide range of biological niches and are extensively dispersed. LAB has a wide range of uses in improving health through biochemical interference to overcome disease/allergy. In the current study, potential LAB isolated from fermented milk from rural southern areas of India. LAB generally recognized as safe (GRAS) for consumption showing biochemical diversity with functional attributes. The productions of starter cultures and for the manufacture of fermented milk perhaps utilize these isolates. Further, evaluation on consortium of isolates could yield multidimensional benefits with diversified collection probiotics, perhaps help in development of therapeutic foods with other biomedical applications. At present an array of LAB are used in health care products and these are helpful to improve LI symptoms by increasing lactose breakdown in the colon. In this approach, probiotic LAB for LI might lead to a potential strategy for the disease management or prevention.

Authors' contributions: M Vasudha, executed the experimental work, prepared the manuscript and was involved in the interpretation and analysis of the data. Devaraja Gayathri conceptualized and designed the study, and was involved in the acquisition of data, analysis and interpretation of data for the study.

Conflict of Interest: The authors declare that they have no conflict of interest.

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