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RESEARCH ARTICLE

Growth, Acidification profile and Proteolytic activity of thermophilic *Lactobacillus* spp. isolated from traditional sweet curd of West Bengal

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Abstract: Microbial fermentation of proteins by lactic acid bacteria offers a promising way to produce bioactive peptides with diverse health benefits. This study evaluated 10 Lactobacillus spp., previously isolated and characterized from traditional Indian sweet curd of West Bengal, for acidification profile, viable counts, proteolytic activity, and free amino acid content. Strains were cultured in skim milk at 42 °C for 24 h and stored at 7 ± 2 °C for 7 days. Peptide production, measured by the o-phthaldialdehyde (OPA) method, showed significant changes during storage. *Lactobacillus delbrueckii* subsp. *indicus* (JSL_2P_2) had the highest peptide release (2.63 ± 0.04 mg leucine/ml), while *Lactobacillus acidophilus* (KM_4) and *Lactobacillus salivarius* subsp. *salivarius* (BM_6) had the highest free amino acid concentrations (0.42 ± 0.01 mg leucine/ml) by day 7. These results show proteolysis in fermented milk is dynamic and strain-dependent, making starter selection vital for desired nutritional and functional qualities.

Keywords: Fermentation, Proteolysis, Bioactive peptides, *Lactobacillus* spp., Traditional Indian sweet curd.

Fermentation is a cost-effective, accessible biotechnological process used to generate bioactive peptides, enhance nutrition, and improve food sensory qualities. In dairy, fermented milk products result from milk acidification by lactic acid bacteria (LAB) during fermentation. Milk is an excellent substrate for LAB, supporting growth and release of health-promoting peptides (Estruch & Lamuela-Raventós, 2023). In India, dahi (sweet and sour), lassi, yoghurt, kefir, shrikhand, koumiss, and leben are popular fermented dairy products, rich in nutrients and functional peptides typically produced by single or mixed LAB strains (Wang, Bai, & Peng, 2020).

Bioactive peptides are protein fragments with diverse physiological benefits, including antihypertensive, antioxidant, antimicrobial, anti-inflammatory, anticancer, antithrombotic, hypolipidemic, hypocholesterolemic, and mineral-binding effects. Their activity depends on amino acid sequence and structure (Chai et al. 2020; Ricci-Cabello et al. 2012;). Bioavailability after ingestion is critical, as peptides must survive digestion and reach target tissues (Vermeirssen et al. 2004). Fermentation with highly proteolytic LAB strains effectively increases peptide content, with strain selection being crucial for enzymatic specificity and potent bioactivity (Padhiyar et al. 2025).

This study evaluated growth, acidification kinetics (pH, titratable acidity, viable LAB counts), and levels of peptides and free amino acids from 10 *Lactobacillus* spp. strains isolated from traditional sweet curd of West Bengal, India. Strains were used to ferment skim milk at 42 °C for 24 h, followed by storage at 7 ± 2 °C for 7 days to monitor biochemical changes. The investigation revealed strains with high fermentation proficiency and notable peptide-releasing ability suitable for subsequent applications.

All chemicals used in this study were procured from Nice Chemicals Pvt. Ltd. and Sigma-Aldrich (USA). Microbial cultures were previously isolated from traditional sweet curd at the Department of Dairy Microbiology, West Bengal University of Animal and Fishery Sciences. The isolates were characterized based on their phenotypic traits, morphological features, physiological properties, and sugar fermentation profiles. For the present study, ten *Lactobacillus* spp. were selected, with their respective isolate codes and culture names as follows:

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The particular *Lactobacillus* spp. were activated in sterilised MRS broth and 12% skim milk at 42ÚC for 12 h and subsequent sub-culturing were done. Their microscopy (100 X) was observed by simple and negative staining. Then 100 ml skim milk was fermented for 24 h at 42ÚC with the specific *Lactobacillus* spp. at the rate of 2% (v/v) of inoculum and was checked for various chemical parameters at 0^{th}, 3^{rd}, 5^{th} and 7^{th} day of storage at 7±2ÚC. During the course of study, its purity and morphology were examined before sub-culturing.

The pH of fermented product was determined by using digital pH meter following the procedure stated in IS: 1479 (Part II), 1961. The titratable acidity was measured by the method given by Bandyopadhyay et al. (2016) of the fermentates.

The fermented milk product was analysed for viable lactic counts using modified skim milk agar plates and incubated at 42ÚC for 24 h. Typical yellow-coloured colonies were counted for determining the total lactic count.

Peptide content was measured using the OPA (o-phthaldialdehyde) method of Church *et al.* (1983). A 150 µl sample aliquot was added to 3 ml OPA reagent in a cuvette and assayed spectrophotometrically at 340 nm after 2 min incubation at room temperature. A control was prepared with inoculated skim milk at 0 h of fermentation. Peptide content was expressed as leucine equivalents from an L-leucine standard curve (Fig. 1).

Free amino acids were measured by the cadmium–ninhydrin method (Tsai et al. 2006) using TCA extracts (section 2.5.1). Reagent was prepared from 0.8 g ninhydrin in 80 ml ethanol (99.5%) + 10 ml acetic acid, with 1 g CdCl, in 1 ml water. Samples (0.5 ml) were mixed with 1 ml reagent, incubated at 84 °C for 5 min, cooled, and read at 570 nm (Mapada UV-6100 PCS). Results were calculated from an L-leucine standard curve (Fig. 2).

All ten skim milk fermentates of *Lactobacillus* spp. were stored at 7 ± 2 °C for 7 days. Chemical parameters—pH, titratable acidity, viable counts, peptide, and free amino acid content—were analyzed on days 0, 3, 5, and 7.

During storage, the pH of the product decreased significantly ($p < 0.05$), from 3.67 ± 0.02 to 3.49 ± 0.02 (Table 1). This trend is consistent with observations reported for other fermented milk and cereal-based beverages, including koumiss, boza, ayran, and jarma (Yadav et al. 2007; Zhumakadyrova & Mazhitova, 2024). Correspondingly, titratable acidity increased from 0.98 ± 0.02 to 1.47 ± 0.00% lactic acid, reflecting sustained metabolic activity of *Lactobacillus* spp. even under refrigerated conditions. Such post-fermentation acidification not only compromises product stability but also influences the survival of viable cells, aligning with previously described pH–acidity interactions in lactic acid fermentations (Mishra & Sharma, 2014; Tomovska et al. 2016).

Table 1: Changes in pH and titratable acidity (% lactic acid) of *Lactobacillus* fermentates during storage at 7±2ÚC

Sl. No.	Isolate	pH				Titratable acidity (% lactic acid)			
		0^{th} day	3^{rd} day	5^{th} day	7^{th} day	0^{th} day	3^{rd} day	5^{th} day	7^{th} day
1.	BM_6	$3.78±0.01^{cBC}$	$3.72±0.03^{bcBCD}$	$3.69±0.02^{abCD}$	$3.65±0.02^{aCD}$	$1.25±0.02^{cB}$	$1.32±0.01^{bB}$	$1.35±0.01^{bB}$	$1.40±0.01^{aB}$
2.	DMK_2	$3.74±0.02^{cBC}$	$3.68±0.02^{bcD}$	$3.64±0.00^{bD}$	$3.60±0.01^{aD}$	$1.19±0.02^{cDE}$	$1.28±0.02^{bC}$	$1.31±0.00^{bCD}$	$1.36±0.01^{aC}$
3.	JSL_2P_2	$3.67±0.02^{cC}$	$3.60±0.02^{bcE}$	$3.54±0.02^{bE}$	$3.49±0.02^{aE}$	$1.25±0.01^{cBC}$	$1.28±0.02^{cBC}$	$1.33±0.02^{bBC}$	$1.40±0.01^{aB}$
4.	KM_4	$3.84±0.02^{cB}$	$3.77±0.00^{bcBC}$	$3.72±0.02^{bC}$	$3.68±0.02^{aC}$	$1.17±0.01^{cDE}$	$1.19±0.02^{cD}$	$1.28±0.02^{bDE}$	$1.39±0.02^{aBC}$
5.	AS_1	$4.02±0.01^{cA}$	$3.96±0.02^{bcA}$	$3.92±0.01^{abAB}$	$3.88±0.02^{aAB}$	$0.98±0.02^{cF}$	$1.02±0.01^{bcE}$	$1.05±0.01^{abG}$	$1.09±0.01^{aF}$
6.	DRA_5	$3.74±0.02^{bBC}$	$3.71±0.01^{bCD}$	$3.65±0.01^{aD}$	$3.60±0.02^{aD}$	$1.16±0.02^{bDE}$	$1.20±0.00^{bD}$	$1.26±0.01^{aE}$	$1.28±0.02^{aDE}$
7.	CH_1	$3.85±0.02^{cB}$	$3.78±0.01^{bcB}$	$3.71±0.03^{abC}$	$3.69±0.03^{aC}$	$1.20±0.00^{bD}$	$1.28±0.02^{aBC}$	$1.29±0.00^{aDE}$	$1.31±0.00^{aD}$
8.	K_5	$3.67±0.02^{cC}$	$3.61±0.02^{bcE}$	$3.57±0.03^{abE}$	$3.52±0.00^{aE}$	$1.32±0.01^{cA}$	$1.39±0.01^{bA}$	$1.44±0.01^{aA}$	$1.47±0.00^{aA}$
9.	MSS_1	$4.07±0.01^{bA}$	$3.97±0.04^{bA}$	$3.96±0.01^{bA}$	$3.92±0.03^{aA}$	$1.16±0.01^{cE}$	$1.18±0.01^{bcD}$	$1.21±0.01^{bF}$	$1.26±0.01^{aE}$
10.	$BS_3AR_4P_1$	$3.99±0.11^{aA}$	$3.91±0.02^{aA}$	$3.87±0.01^{aB}$	$3.83±0.02^{aB}$	$1.21±0.00^{cCD}$	$1.26±0.01^{bC}$	$1.29±0.01^{aDE}$	$1.31±0.00^{aD}$

* Values are the mean of three replicates along with standard error (Average±SE).
* Different superscripts (a, b, c) in a row denotes significant ($p<0.05$) differences for a specific parameter between storage days of each isolate.
* Different superscripts (A, B, C, D, E, F, G) in a column denotes significant ($p<0.05$) differences for a specific parameter between isolates at different storage day.

Fig. 1 Standard curve of L-leucine by OPA method

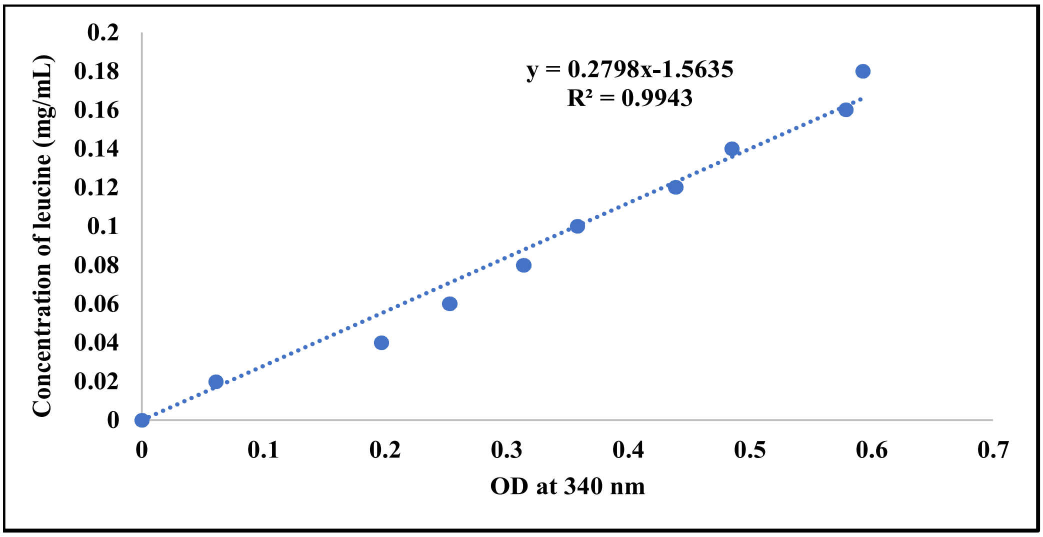

Fig. 2 Standard curve of L-leucine for quantification of free amino acids

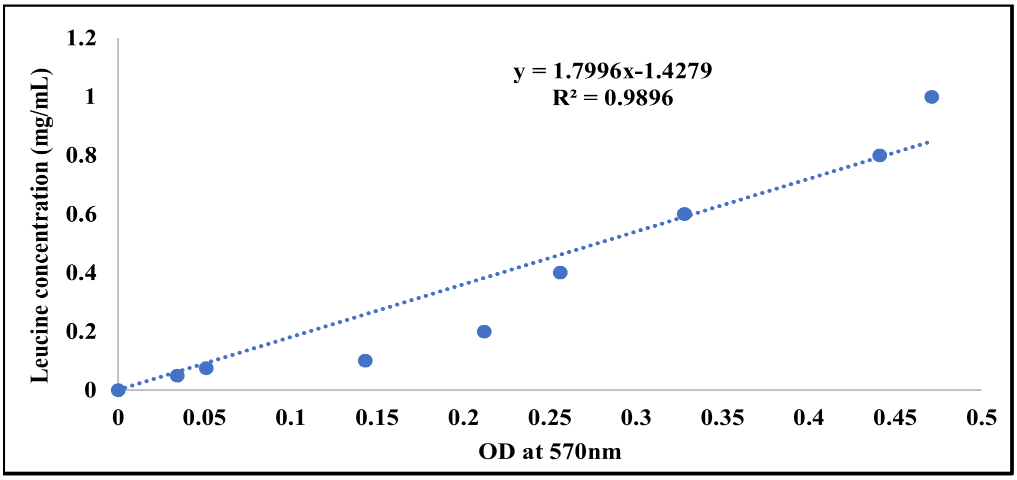

Table 2: Changes in viable counts (log CFU/ml) of *Lactobacillus* fermentates during storage at 7±2 °C

Sl. No.	Isolate	Storage days			
		0^{th} day	3^{rd} day	5^{th} day	7^{th} day
1.	BM_6	8.46±0.57	8.40±0.54	8.36±0.58	8.33±0.56
2.	DMK_2	8.41±0.54	8.39±0.52	8.34±0.54	8.28±0.51
3.	JSL_2P_2	8.24±0.46	8.16±0.46	8.16±0.46	8.16±0.46
4.	KM_4	8.39±0.53	8.26±0.52	8.26±0.52	8.12±0.48
5.	AS_1	9.01±0.20	9.01±0.20	8.90±0.21	8.89±0.20
6.	DRA_5	8.10±0.42	7.80±0.37	7.77±0.41	7.71±0.39
7.	CH_1	8.04±0.42	7.91±0.40	7.80±0.41	7.70±0.40
8.	K_5	8.19±0.44	8.06±0.45	7.95±0.45	7.88±0.45
9.	MSS_1	8.81±0.16	8.65±0.16	8.55±0.17	8.44±0.16
10.	$BS_3AR_4P_1$	8.81±0.16	8.71±0.15	8.61±0.15	8.54±0.12

* Values are the mean of three replicates along with standard error (Average±SE).
* The total lactic count of isolates varies non-significantly (p>0.05) at each storage days and also the total lactic count of each isolates decreased non-significantly (p>0.05) with storage day.

Lactobacillus counts decreased during storage at 7 ± 2/ °C, declining from 9.01 ± 0.20 to 7.70 ± 0.40 log CFU/ml (Table 2). Although the observed changes were not statistically significant (p/ >/ 0.05), prolonged storage, increased acidity, and reduced pH appeared to contribute to the reduction in viability. Comparable trends have been reported in *Lactobacillus* fermentates (Patil et al. 2015) and in rice bran–fortified yoghurt,

Table 3: Changes in peptide and free amino acid content (equivalent to mg leucine/ml) of *Lactobacillus* fermentates during storage at 7±2ÚC

Sl. No.	Isolate	Peptide content (equivalent to mg leucine/ml)				Free amino acid content (equivalent to mg leucine/ml)			
		0^{th} day	3^{rd} day	5^{th} day	7^{th} day	0^{th} day	3^{rd} day	5^{th} day	7^{th} day
1.	BM_6	$2.36±0.01^{aAB}$	$2.43±0.04^{aAB}$	$2.46±0.07^{aAB}$	$2.53±0.07^{aAB}$	$0.34±0.01^{bA}$	$0.36±0.03^{abAB}$	$0.40±0.01^{aA}$	$0.42±0.01^{aA}$
2.	DMK_2	$2.21±0.04^{bABC}$	$2.26±0.07^{abBC}$	$2.35±0.03^{abABC}$	$2.39±0.04^{aB}$	$0.28±0.03^{bAB}$	$0.31±0.02^{bBC}$	$0.39±0.01^{aA}$	$0.41±0.01^{aAB}$
3.	JSL_2P_2	$2.47±0.07^{aA}$	$2.50±0.06^{aA}$	$2.55±0.08^{aA}$	$2.63±0.04^{aA}$	$0.33±0.03^{aA}$	$0.34±0.03^{aABC}$	$0.37±0.01^{aAB}$	$0.40±0.00^{aAB}$
4.	KM_4	$2.36±0.07^{aAB}$	$2.42±0.07^{aAB}$	$2.46±0.06^{aAB}$	$2.51±0.06^{aAB}$	$0.35±0.03^{bA}$	$0.40±0.00^{aA}$	$0.42±0.01^{aA}$	$0.42±0.01^{aA}$
5.	AS_1	$1.87±0.34^{aBCD}$	$1.91±0.12^{aDE}$	$1.94±0.12^{aEF}$	$1.97±0.09^{aDE}$	$0.11±0.00^{cE}$	$0.14±0.01^{bD}$	$0.16±0.01^{abE}$	$0.17±0.01^{aD}$
6.	DRA_5	$1.54±0.34^{aD}$	$1.56±0.09^{aF}$	$1.58±0.10^{aG}$	$1.61±0.06^{aF}$	$0.11±0.01^{bE}$	$0.13±0.01^{bD}$	$0.17±0.01^{aE}$	$0.19±0.01^{aD}$
7.	CH_1	$1.66±0.06^{aCD}$	$1.70±0.03^{aEF}$	$1.73±0.05^{aFG}$	$1.76±0.04^{aEF}$	$0.13±0.00^{cE}$	$0.17±0.01^{bD}$	$0.19±0.00^{aDE}$	$0.21±0.01^{aD}$
8.	K_5	$2.11±0.14^{aABC}$	$2.19±0.03^{aC}$	$2.26±0.07^{aBCD}$	$2.34±0.13^{aBC}$	$0.21±0.01^{cCD}$	$0.27±0.03^{bcC}$	$0.32±0.03^{abC}$	$0.38±0.01^{aAB}$
9.	MSS_1	$2.12±0.08^{aABC}$	$2.13±0.08^{aC}$	$2.13±0.14^{aCDE}$	$2.15±0.07^{aCD}$	$0.24±0.03^{bBC}$	$0.28±0.04^{abC}$	$0.34±0.01^{aBC}$	$0.36±0.03^{aBC}$
10.	$BS_3AR_4P_1$	$1.91±0.12^{aABCD}$	$2.04±0.06^{aCD}$	$2.07±0.08^{aDE}$	$2.15±0.08^{aCD}$	$0.15±0.01^{cDE}$	$0.18±0.01^{bcD}$	$0.22±0.00^{bD}$	$0.33±0.03^{aC}$

* Values are the mean of three replicates along with standard error (Average±SE).
* Different superscripts (a, b, c) in a row denotes significant ($p<0.05$) differences for a specific parameter between storage days of each isolate.
* Different superscripts (A, B, C, D, E, F, G) in a column denotes significant ($p<0.05$) differences for a specific parameter between isolates at different storage day

where *Streptococcus thermophilus* populations similarly declined during storage (Darwish et al. 2023).

Peptide content increased significantly (p/ </ 0.05) during storage, albeit at a gradual rate (Table 3). Among the fermentates, JSL_2P_2 exhibited the highest peptide levels, starting at 2.47 ± 0.07/ mg leucine/ml on day 0 and rising to 2.63 ± 0.04/ mg/ml by day 7, significantly exceeding those of other strains. This difference is likely attributable to variations in strain-specific proteolytic enzyme activity (Donkor et al. 2007). Similarly, amino acid concentrations increased significantly during storage, with BM_6 and KM_4 reaching 0.42/ mg leucine/ml, followed by DMK_2 at 0.41/ mg/ml on day 7, indicative of ongoing protein hydrolysis and α-amino group release, as measured by OPA absorbance (Shihata & Shah, 2000). These observations are consistent with previous reports documenting enhanced proteolysis during refrigerated storage in various fermented milk products (Darwish et al. 2023; Teichert et al. 2021; Lee et al. 2011; Zhang et al. 2024).

Conclusion

This study evaluated the effects of 10 Lactobacillus spp. on physical and chemical parameters during skim milk fermentation. *Lactobacillus delbrueckii* subsp. *indicus* (JSL_2P_2) showed the highest proteolytic activity during storage at 7 ± 2 °C, highlighting its potential for producing health-promoting bioactive peptides. Other thermophilic strains *Lactobacillus salivarius* subsp. *salivarius* (BM_6), *Lactobacillus delbrueckii* subsp. *indicus* (DMK_2, K_5), and *Lactobacillus acidophilus* (KM_4) also displayed good stability and similar chemical traits. As bioactive peptides are latent within parent proteins, their release through proteolysis during digestion or fermentation underscores the industrial potential of these Lactobacillus cultures.

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Conflicts of interest

All authors have declared that they don't have any conflict of interest for publishing this research.

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