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Native *Lactococcus lactis* strains as dual-purpose cultures: Comparing enzymatic activity and technological performance for fermented dairy applications

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Abstract

Technological variability among indigenous dairy bacteria offers opportunities for starter culture development, although strain selection requires consideration of multiple functional characteristics beyond milk acidification. This study evaluated the technological properties of three *Lactococcus lactis* isolates (HBM-IAUF-2, LBM5, and AN17) recovered from traditional Iranian yogurt. Milk acidification, growth performance, proteolytic and lipolytic activities, intracellular enzyme profiles, crude exopolysaccharide (EPS)-associated carbohydrate recovery, apparent autolysis, and tolerance to preservation and oxidative stresses were investigated. Results were expressed as mean ± standard deviation from three independent replicates and analyzed for significant differences ($p < 0.05$). LBM5 demonstrated the greatest acidification capacity, reaching a milk pH of 4.08 ± 0.04 and titratable acidity of 1.02 ± 0.03% after 24 h. This isolate also exhibited the highest OPA-reactive amino-group concentration (1.82 ± 0.06 mmol/L serine equivalents), β-galactosidase activity (21.30 ± 0.55 U/mL), and crude EPS-associated carbohydrate recovery (213.50 ± 7.80 mg glucose equivalents/L). Its survival after freeze-drying was 81.50 ± 1.50%, with 93.20 ± 1.30% viability retention during 28 days of refrigerated storage. In contrast, AN17 showed the greatest apparent autolysis after 48 h (36.10 ± 1.50%). Integrated heatmap analysis distinguished the broad fermentative and preservation-related characteristics of LBM5 from the stronger autolytic response of AN17. These findings highlight complementary technological profiles among indigenous lactococci, supporting further evaluation of LBM5 as a starter candidate and AN17 for ripening-oriented applications.

Keywords: Indigenous isolates, *Lactococcus lactis*, Enzymatic activity, Starter cultures, Technological properties, Traditional yogurt.

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Introduction

Traditional fermented dairy products are valuable sources of indigenous lactic acid bacteria (LAB), particularly strains adapted to local fermentation conditions (Ezzatpanah, 2023). The microbial processes involved in dairy fermentation also influence product quality, safety, and bioactive composition, as reviewed by Nwadi et al. (2026). Among these bacteria, *Lactococcus lactis* is widely employed in dairy fermentation because of its acidifying capacity and contribution to product development. However, strains belonging to the same species may differ considerably in their technological performance. Li et al. (2020) reported substantial variation in fermentation characteristics among 227 dairy-derived isolates, while Sun et al. (2026) examined the relationship between ecological diversification and acidification behaviour in 147 isolates. These findings indicate that species identification alone is insufficient for selecting suitable starter cultures. Studies on indigenous dairy isolates have also documented differences in functional characteristics (Mousavi et al., 2026; Namazi et al., 2026). In a related investigation, Goudarzi Shams Abadi et al. 2026 examined the antimicrobial and antibiofilm properties of the yogurt-derived *L. lactis* PAN1 isolate, addressing functional traits beyond fermentation performance. Growth performance and milk acidification are among the first technological characteristics examined when assessing dairy isolates for starter culture applications (Douwenga et al., 2021). The technological suitability of dairy isolates is also influenced by their enzymatic characteristics and tolerance to processing conditions. Proteolytic and peptidolytic enzymes are involved in protein degradation and peptide metabolism, both of which are important in flavour development during dairy fermentation and ripening (Huang and Kok, 2020; Shi et al., 2023). Activities associated with carbohydrate and lipid metabolism also vary among dairy isolates and contribute to differences in their technological characteristics (Nicosia et al., 2023). Autolysis facilitates the release of intracellular enzymes, while bacterial exopolysaccharides can influence the structural properties of fermented dairy products (Zhao et al., 2023; Surber et al., 2021; Ng et al., 2022).

The performance of selected cultures also needs to be considered in relation to the food matrix. Turhan et al. (2025) examined the effects of probiotic cultures and product formulation on dairy product quality. Similarly, Matan and Khunjan (2025) investigated the effects of limonene pretreatment on the safety and prebiotic functionality of fresh amaranth sprouts, highlighting the role of food-matrix conditions in supporting beneficial microorganisms.

Indigenous *L. lactis* strains have also been investigated for applications beyond dairy fermentation. For example, Goudarzi Shams Abadi et al. (2026c) incorporated the cell-free supernatant of LBM15 into chia mucilage-based coatings to improve the preservation of

refrigerated ostrich meat. Freezing and freeze-drying can reduce bacterial viability, although the extent of cell damage varies with strain characteristics and processing conditions (Ge et al., 2024; Wang et al., 2025). Despite the documented technological diversity of dairy-associated lactococci, the combined fermentative, enzymatic, and preservation-related characteristics of the three yogurt-derived isolates HBM-IAUF-2, LBM5, and AN17 have not been comparatively characterized. Accordingly, this study evaluated milk acidification, growth performance, hydrolytic and enzymatic activities, apparent autolysis, exopolysaccharide-associated carbohydrate recovery, and tolerance to preservation-related stresses in these isolates to assess their potential for further development as starter or adjunct cultures.

Materials and methods

Bacterial strains and culture conditions

Traditional yogurt sampled in Tashan (Behbahan County, Khuzestan, Iran) provided the three lactic acid bacterial isolates investigated here. The isolates were identified by comparing their 16S rRNA gene sequences against NCBI reference sequences, following the approach used by Astuti et al. (2025) for dairy-associated isolates. Sequence similarity was approximately 99% to the respective reference entries for *L. lactis* HBM-IAUF-2 (MG760433), LBM5 (PV652829), and AN17 (PP657517). These names are used throughout this study to designate the corresponding isolates based on their closest sequence matches. The accession numbers belong to the reference sequences rather than the isolates examined here.

Stock cultures of HBM-IAUF-2, LBM5, and AN17 were stored at −80 °C; the storage medium was de Man–Rogosa–Sharpe (MRS) broth supplemented with glycerol at 20% (v/v). Before experimental use, each culture underwent two consecutive transfers into fresh MRS broth, with incubation for 18–24 h at 37 °C per transfer. Culture purity was assessed by colony morphology, Gram staining, and catalase activity. Inocula were standardized to 0.5 McFarland turbidity. Where washed cells were required, pellets were obtained from overnight cultures by centrifugation (6,000 × g, 15 min, 4 °C). Two washes with sterile phosphate-buffered saline (PBS; pH 7.2) preceded resuspension in the medium or buffer specified for each assay (Barzegar et al., 2021).

Milk acidification and titratable acidity

Milk acidification by HBM-IAUF-2, LBM5, and AN17 was examined using an adaptation of Barzegar et al. (2021). Standardized bacterial suspensions (100 µL) were added to individual 10-mL portions of sterile reconstituted skim milk (10%, w/v). Incubation lasted 24 h at 37 °C, with uninoculated milk maintained under the same conditions as the negative control. A calibrated

digital pH meter was used to record milk pH immediately after inoculation and at 24 h. For each replicate, the final reading was subtracted from the initial reading to obtain the pH decrease. An adapted titration based on AOAC Official Method 947.05 was used to determine titratable acidity. A 10-mL aliquot of fermented milk was titrated against standardized NaOH (0.1 mol/L), with phenolphthalein indicating the endpoint. Titration ended when a faint pink colour remained visible for approximately 30 s. The volume of alkali consumed was converted to lactic acid equivalents as follows:

$$\textit{Titratable acidity (\%, w/v)} = (V \times N \times 0.09008 \times 100) / Vs$$

In this expression, the titrant volume and normality are denoted by V (mL) and N, respectively, while Vs denotes the volume of milk analysed (mL). The conversion factor, 0.09008, corresponds to the grams of lactic acid equivalent to 1 mL of 1 N NaOH.

Quantitative determination of proteolytic activity

OPA-reactive amino groups in fermented milk were quantified as an indicator of proteolysis, adapting the procedure of Nielsen et al. (2001). For sample preparation, 2.5 mL of fermented milk received 0.5 mL of distilled water and 5 mL of trichloroacetic acid (0.75 N). After vortex mixing for 1 min and standing for 10 min at room temperature, the mixture was passed through Whatman No. 2 filter paper. The OPA reagent contained sodium tetraborate (25 mL, 100 mmol/L), sodium dodecyl sulfate (2.5 mL, 20%, w/v), OPA (40 mg dissolved in 1 mL of methanol), and β-mercaptoethanol (100 µL). Distilled water was used to bring the final volume to 50 mL. A 20-µL portion of the filtrate was combined with 400 µL of freshly prepared OPA reagent. The absorbance at 340 nm was recorded after a 2-min reaction at room temperature. Unfermented milk subjected to the same extraction and OPA reaction procedures served as the matrix blank to account for background amino-group reactivity. The measured responses were corrected for the corresponding blank values before quantification using the serine calibration curve. Results were expressed as mmol serine equivalents/L of fermented milk.

Qualitative proteolytic and lipolytic activities

Skim-milk agar was used to screen extracellular proteolysis by an adaptation of the well-diffusion assay described by Ebadi Nezhad et al. (2020). Each well (6 mm in diameter) received 50 µL of cell-free culture supernatant. After 24 h at 37 °C, the well diameter was subtracted from the measured clearing diameter to report proteolytic activity in mm. Lipolytic screening followed Syrokou et al. (2021), using agar supplemented with tributyrin (1%, v/v). A 10-µL aliquot of each fresh standardized culture was spotted onto the surface. Following 72 h of incubation at 37 °C, the diameter of the clearing surrounding each spot was measured in mm.

Exopolysaccharide production

The crude EPS fraction was obtained by adapting the procedure of de Souza Sigali et al. (2026). Separate MRS broth cultures of HBM-IAUF-2, LBM5, and AN17 were established with 1% (v/v) inoculum and incubated for 24 h at 37 °C. Cells were first removed by centrifugation (6,000 × g, 15 min, 4 °C). The supernatants underwent treatment with trichloroacetic acid (5%, w/v) for 30 min at 4 °C, followed by centrifugation at 8,000 × g for 15 min. Two volumes of cold absolute ethanol were then added to the clarified liquid. After 24 h at 4 °C, the precipitated fraction was recovered at 8,000 × g for 20 min and redissolved in distilled water. Carbohydrates in the recovered fraction were quantified by an adapted phenol–sulfuric acid assay (DuBois et al., 1956). To 1 mL of sample, 0.5 mL of phenol solution (5%, w/v) and 2.5 mL of concentrated sulfuric acid were added. Colour development proceeded for 20 min at room temperature before the absorbance at 490 nm was recorded. Uninoculated MRS broth processed identically served as the blank. A glucose calibration curve was used to convert the assay response into carbohydrate recovery, reported as mg glucose equivalents per litre of culture. These values represent carbohydrates recovered in the crude EPS fraction rather than purified EPS mass.

Esterase activity

Crude intracellular extracts were prepared from overnight cultures of HBM-IAUF-2, LBM5, and AN17 (Sangwan et al., 2015). Cells were harvested at 6,000 × g for 15 min at 4 °C, washed twice, and suspended in sodium phosphate buffer (50 mmol/L, pH 7.0). Sonication under chilled conditions disrupted the cells. Subsequent centrifugation (10,000 × g, 15 min, 4 °C) removed cellular debris, and the supernatants were retained for the enzyme assays. The esterase assay used p-nitrophenyl acetate (pNPA) and an adaptation of Pavarina et al. (2021). Each reaction combined 100 µL of crude intracellular extract with 900 µL of a 1 mmol/L pNPA solution prepared in sodium acetate buffer (100 mmol/L, pH 6.5). Absorbance at 405 nm was recorded following a 15-min incubation at 37 °C.

An enzyme-free control, prepared by replacing the extract with buffer, was used to correct for spontaneous substrate hydrolysis. The amount of p-nitrophenol released was obtained from its calibration curve and used to calculate activity in U/mL.

Enzyme activity (U/mL) = Q / (t × Ve)

Q denotes the product formed in the entire reaction after blank correction (µmol), including any analytical dilution correction. The denominator comprises the reaction duration, t (min), and the volume of crude extract used, Ve (mL).

Autolytic activity

The decline in cell-suspension turbidity was used to estimate apparent autolysis through an adaptation of Hazır Dalca et al. (2018). Harvested cells underwent two washes with potassium phosphate buffer (50 mmol/L, pH 7.0), which also served as the resuspension medium. Suspensions were adjusted to an initial absorbance of approximately 1.0 at 600 nm and maintained at 37 °C, with measurements taken at 24 and 48 h. Apparent autolysis was calculated as:

$$Autolysis\ (\%) = [(A_0 - A_t) / A_0] \times 100$$

where A_0 and A_t represent the absorbance at the beginning of incubation and at the specified sampling time, respectively.

Growth performance in different culture media

A comparative culture-based assessment was conducted in MRS broth, M17 broth, and sterile reconstituted skim milk (10%, w/v) to examine the growth performance of HBM-IAUF-2, LBM5, and AN17, following the general approach of Valenzuela et al. (2024). Washed-cell suspensions were added to each medium at 1% (v/v), giving an initial population of approximately 6 log CFU/mL. Cultivation proceeded for 24 h at 37 °C. Serial dilutions were prepared in sterile saline (0.85%, w/v) and spread-plated on M17 agar. Following 48 h of plate incubation at 37 °C, colony counts were used to calculate viable populations in log CFU/mL. The same enumeration procedure was used for preservation and oxidative-stress assays, with results reported on the appropriate sample basis.

Survival after freezing

An adaptation of Ge et al. (2024) was used to assess freezing tolerance, with sterile reconstituted skim milk (10%, w/v) serving as the suspension medium for washed cells. The suspensions underwent 24 h of freezing at −20 °C, followed by thawing at room temperature.

Percentage survival was obtained by dividing the post-freezing viable count by the pre-freezing count and multiplying by 100; both counts were expressed as untransformed CFU/mL.

Freeze-drying and viable-cell enumeration

Freeze-drying tolerance was examined using an adaptation of Ge et al. (2024). Washed cells were dispersed in sterile reconstituted skim milk (10%, w/v) before processing. A 12-h prefreezing step at −80 °C preceded vacuum lyophilization for 30 h. The resulting powders were kept in sterile, airtight containers. Before viable-cell enumeration, 1 g of powder was rehydrated in 9 mL of sterile saline for 15 min at room temperature. Counts were reported as log CFU/g.

For survival calculations, viable counts before and after drying were expressed on the same original-suspension volume basis without logarithmic transformation. The post-drying count was divided by the pre-drying count and multiplied by 100.

Storage stability of freeze-dried cultures

Freeze-dried cultures were stored at 4 °C for 28 days, following the general approach of Ge et al. (2024), with modifications. Viable counts were determined immediately after drying and after storage using the rehydration and enumeration procedures described above. Storage survival (%) was calculated as the ratio of viable counts on day 28 to those on day 0, multiplied by 100, using untransformed CFU/g values.

Oxidative-stress tolerance

Oxidative-stress tolerance was evaluated by exposing washed cells to hydrogen peroxide, following the general approach of Lee et al. (2023), with modifications. Cell suspensions were prepared in PBS (pH 7.2) at approximately 8 log CFU/mL and treated with 0.4 mmol/L H_2O_2 for 30 min at 37 °C. Untreated suspensions served as controls. Following exposure, cells were washed to remove residual peroxide and enumerated by plating as described above. Survival (%) was calculated from the ratio of viable counts in treated and control suspensions using untransformed CFU/mL values.

β-Galactosidase activity

β-Galactosidase activity was determined using o-nitrophenyl-β-D-galactopyranoside (ONPG) according to Prasad et al. (2013), with modifications. Crude enzyme extract (100 µL) was mixed with 900 µL of ONPG solution in 50 mmol/L sodium phosphate buffer (pH 7.0) and incubated at 37 °C for 10 min. The reaction was stopped by adding 1 mL of 1 mol/L sodium carbonate. Absorbance was measured at 420 nm against an enzyme-free blank. Released o-nitrophenol was quantified using a calibration curve, and activity was expressed as U/mL of crude enzyme extract. One unit was defined as the amount of enzyme releasing 1 µmol of o-nitrophenol per minute under the assay conditions.

Aminopeptidase activity

Aminopeptidase activity was assessed using L-leucine-p-nitroanilide as the substrate, following the general approach of Nicosia et al. (2023), with modifications. Crude enzyme extract (100 µL) was mixed with 100 µL of 2.5 mmol/L substrate and 100 µL of 50 mmol/L Tris–HCl buffer (pH 7.6). After incubation at 37 °C for 15 min, the reaction was terminated with 100 µL of 30%

(v/v) acetic acid. Absorbance was measured at 405 nm against a substrate blank, and released p-nitroaniline was quantified using a calibration curve. One unit (U) was defined as the amount of enzyme releasing 1 µmol of p-nitroaniline per minute under the assay conditions. Activity was expressed as U/mL of crude enzyme extract.

General protease activity

General protease activity was determined using the casein hydrolysis assay described by Li et al. (2019). Crude enzyme extract (1 mL) was mixed with 1 mL of 1% (w/v) casein prepared in 50 mmol/L sodium phosphate buffer (pH 7.0). After incubation at 37 °C for 20 min, the reaction was stopped by adding 2 mL of 0.4 mol/L trichloroacetic acid. The mixture was centrifuged at 5,000 × g for 15 min at 4 °C.

An aliquot (1 mL) of the supernatant was combined with 5 mL of 0.4 mol/L sodium carbonate and 1 mL of Folin–Ciocalteu reagent. Following incubation at 37 °C for 20 min, absorbance was measured at 660 nm.

Proteolytic activity was quantified using an L-tyrosine calibration curve and expressed as U/mL of crude enzyme extract. One unit (U) was defined as the amount of enzyme releasing 1 µg of tyrosine equivalents per minute under the specified assay conditions.

Statistical analysis

Results are presented as mean ± SD of three independent replicates (n = 3). Data were analysed using one-way ANOVA and Tukey's HSD test, except growth data, which were assessed by two-way ANOVA, and autolysis, which was analysed using repeated-measures ANOVA with strain as the between-subject factor and time as the within-subject factor. Analyses were performed in SPSS 22 at $p < 0.05$. Different lowercase superscripts within table rows indicate significant differences among strains. Heatmap values were standardized row-wise as Z-scores across the three isolates.

Results and Discussion

Milk acidification and titratable acidity

After 24 h of milk fermentation, LBM5 showed a significantly greater pH decrease (2.57 ± 0.05) than HBM-IAUF-2 and AN17 ($p < 0.05$), reaching 4.08 ± 0.04 (Fig. 1a,b). Its titratable acidity (1.02 ± 0.03%, as lactic acid equivalents) was also significantly higher.

The relationship between acidification and growth may involve both carbohydrate utilization and nitrogen acquisition. In dairy lactococci, lactose can be transported through the LacE/LacF phosphotransferase system and hydrolyzed by LacG phospho-β-galactosidase (Kleerebezem et

al., 2020; Karlsen et al., 2021). Growth in milk also depends on proteolytic release and uptake of nitrogenous compounds, involving extracellular casein degradation, peptide transport, and intracellular peptidase activity (Huang and Kok, 2020). Differences in these pathways could partly explain the observed variation, although their activities were not directly measured in the three isolates. The final pH range (4.08–4.47) partly overlaps that reported by Dahou et al. (2026) for cheese-derived lactococci after 24 h (4.17–4.36), although differences in fermentation matrices and experimental conditions limit direct comparison. Alizadeh Behbahani et al. (2026) reported a decrease in milk pH from 6.49 to 4.85 after 24 h for cheese-derived Lactobacillus acidophilus TMPC 41127. Vasiee et al. (2026a) reported a 24-h pH reduction of 1.48 for *Lacticaseibacillus casei* ANNC 26 isolated from traditional Khiki cheese, indicating a moderate acidification capacity.

Sun et al. (2026) further observed, among 147 isolates, that acidification performance was predominantly isolate-specific rather than consistently associated with ecological lineage. Importantly, titratable acidity reflects neutralizable acidity within the buffered milk matrix rather than the concentration of lactate alone. In their study of yogurt-derived LBM15, Goudarzi Shams Abadi et al. (2026a) separately quantified lactic, acetic, and formic acids by HPLC. Further measurements of acidification over time and organic acid production would help explain the performance of LBM5 in milk.

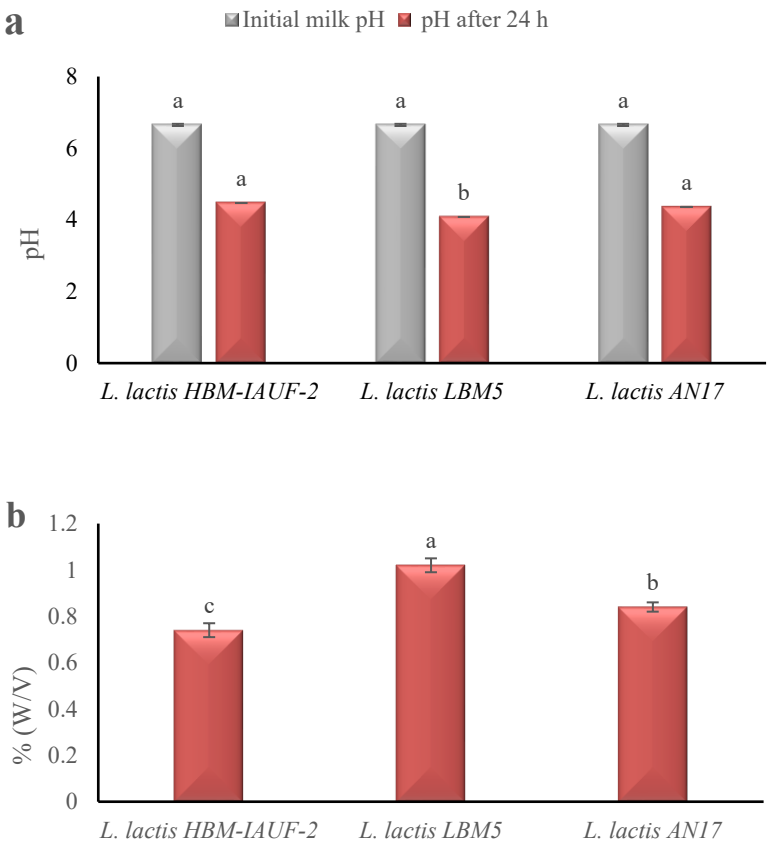


Fig. 1. Acidification performance of *Lactococcus lactis* strains HBM-IAUF-2, LBM5, and AN17 in reconstituted skim milk: (A) pH values before and after 24 h of fermentation and (B) titratable acidity expressed as lactic acid after 24 h. Data show means ± SD from three independent experiments (n = 3). Within each measurement, strains bearing different lowercase letters differ significantly (Tukey's test, $p < 0.05$).

Proteolytic activity

Proteolytic activity differed among HBM-IAUF-2, LBM5, and AN17 in both assays (Fig. 2a, b). LBM5 exhibited significantly higher OPA-reactive amino-group concentrations (1.82 ± 0.06 mmol/L serine equivalents) and a larger casein-hydrolysis zone (16.20 ± 0.40 mm) than the other isolates ($p < 0.05$). The corresponding responses of AN17 were intermediate, suggesting differences in the capacity of these isolates to hydrolyze milk proteins under the conditions examined. Casein-derived peptides provide an important nitrogen source for dairy lactococci. In *L. lactis*, the cell-envelope proteinase PrtP initiates casein hydrolysis, while the Opp transport system and intracellular peptidases participate in subsequent peptide utilization. Huang and Kok (2020) demonstrated that modifying the proteolytic machinery of *L. lactis* alters the composition and biological potential of casein-derived peptides. Differences in proteolytic capacity may influence both nitrogen availability during fermentation and the pool of peptide precursors involved in later biochemical transformations. Evidence from dairy isolates also points to considerable variability in this characteristic. Barzegar et al. (2021) observed strain-dependent

proteolytic activity among LAB recovered from Iranian raw-milk cheeses, with *Lactiplantibacillus plantarum* B20 showing a pronounced response. More recently, Rubak et al. (2026) reported detectable OPA-reactive amino groups but no clear casein-hydrolysis zones among several *L. lactis* isolates. In the present study, all three isolates showed measurable proteolytic activity in fermented milk and on skim-milk agar, with the highest values recorded for LBM5. Differences in proteolytic activity are particularly relevant to starter and adjunct culture selection, as peptide availability and amino-acid metabolism can affect fermentation and cheese ripening. Iqbal et al. (2025) also examined how probiotic cultures influence bioactive peptides and sensory characteristics in yogurt, providing a product-level context for the technological relevance of proteolytic activity.

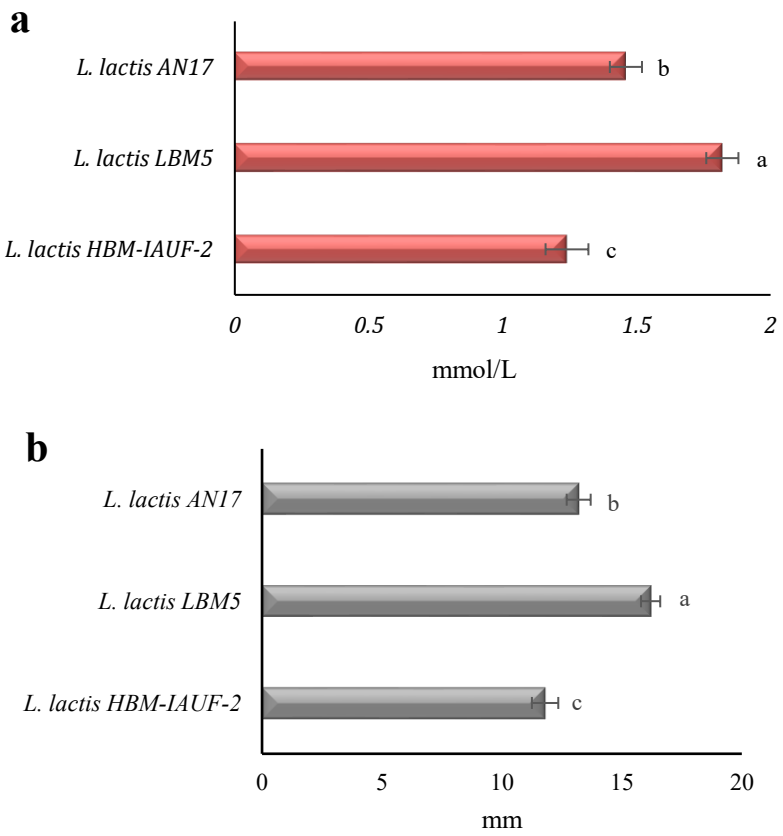


Fig. 2. Proteolytic activity of *Lactococcus lactis* strains HBM-IAUF-2, LBM5, and AN17: (A) quantitative proteolytic activity determined by the *o*-phthaldialdehyde (OPA) assay and expressed as mmol/L serine equivalents and (B) qualitative proteolytic activity expressed as the diameter of the casein-hydrolysis zone on skim-milk agar. Results summarize three independent experiments as means ± SD (n = 3). For each assay, different lowercase letters identify significant differences among strains, as determined by Tukey's HSD test ($p < 0.05$).

Qualitative lipolytic activity

LBM5 showed the largest tributyrin-hydrolysis zone, followed by AN17 and HBM-IAUF-2 (Fig. 3). The technological relevance of this finding is particularly interesting in light of the results of Nicosia et al. (2023), who detected no lipolytic activity among 26 lactic acid bacterial isolates

from traditional cheeses, despite observing considerable variation in other technological traits. Tributyrin hydrolysis provides a screening phenotype; its contribution to milk-fat hydrolysis and flavour development requires evaluation in dairy products.

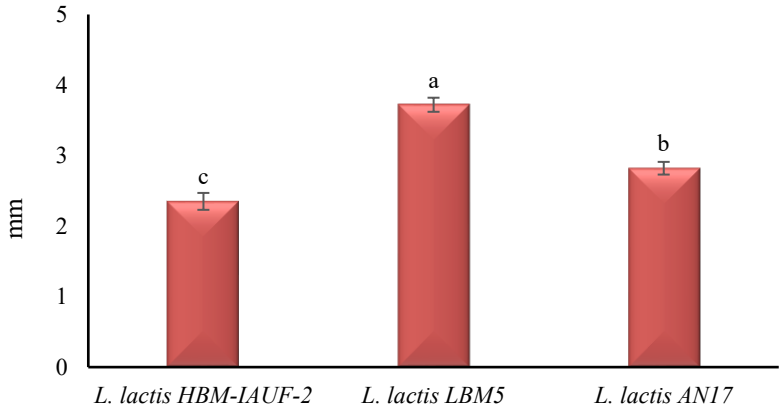


Fig. 3. Qualitative lipolytic activity of *Lactococcus lactis* strains HBM-IAUF-2, LBM5, and AN17 on tributyrin agar. Values are expressed as mean ± standard deviation of three independent experiments (n = 3). Different lowercase letters indicate significant differences among strains according to Tukey's HSD test ($p < 0.05$).

Exopolysaccharide production

The technological significance of bacterial exopolysaccharides (EPS) extends beyond their thickening effect to their interactions with the milk protein network. Brüls et al. (2024) demonstrated that EPS structure influences casein interconnectivity, pore distribution, and syneresis in yogurt, while Ramos et al. (2023) highlighted the contribution of EPS-producing cultures to water retention in non-fat yogurt. LBM5 showed the highest carbohydrate recovery in the crude EPS fraction (Fig. 4). However, this measurement does not establish EPS purity or its effects on dairy product texture. Mucoid EPS production was also reported for Lactobacillus acidophilus TMPC 41127 by Alizadeh Behbahani et al. (2026), although this qualitative observation cannot be directly compared with the carbohydrate recovery measured here.

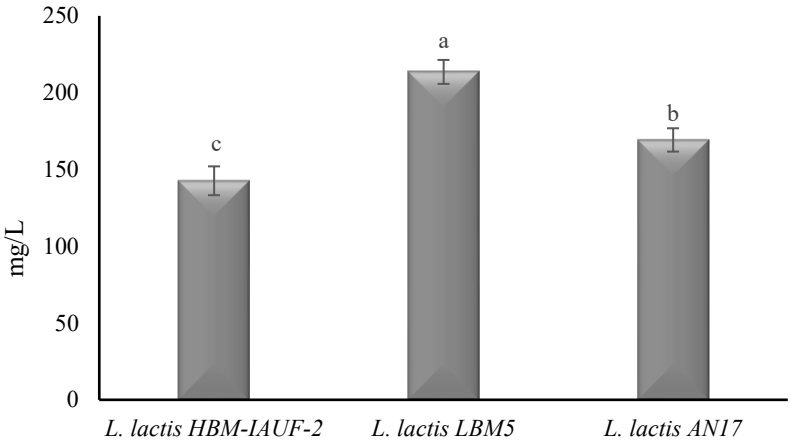


Fig. 4. Crude exopolysaccharide (EPS)-associated carbohydrate production by *Lactococcus lactis* strains HBM-IAUF-2, LBM5, and AN17, expressed as mg glucose equivalents/L of culture. Results summarize three independent experiments as means ± SD (n = 3). For each assay, different lowercase letters identify significant differences among strains, as determined by Tukey's HSD test ($p < 0.05$).

Time-dependent autolysis

The apparent autolytic response increased between 24 and 48 h in all three isolates, although the magnitude of this change differed among strains (Fig. 5). At 24 h, AN17 and HBM-IAUF-2 exhibited comparable responses, whereas LBM5 showed the lowest apparent autolysis. By 48 h, AN17 displayed the highest value (36.10 ± 1.50%), followed by HBM-IAUF-2 and LBM5, revealing a clearer separation in their time-dependent profiles. This variation is technologically relevant because autolysis can govern the timing and availability of intracellular enzymes during cheese ripening. Cell-envelope disintegration is influenced by autolysin activity, cell-wall architecture, and environmental conditions, and the release of intracellular peptidases can facilitate further peptide hydrolysis and amino-acid availability. Dorau et al. (2021) demonstrated that enhanced autolysis in *L. lactis* RD07 was associated with faster accumulation of free amino acids in model cheese. In subsequent laboratory- and industrial-scale trials, Zhao et al. (2023) reported 31–34% higher free-amino-acid concentrations in cheeses produced with highly autolytic lactococcal starters than with reference cultures. Vasiee et al. (2026b) reported 51.11% autolytic activity at 37 °C for *Levilactobacillus brevis* M3R3 isolated from traditional Khiki cheese.

The higher apparent autolysis of AN17 after 48 h suggests that these isolate warrants further investigation for cheese-ripening applications where intracellular enzyme release is desirable. In contrast, the lower apparent autolysis of LBM5, alongside its favourable growth and acidification characteristics, illustrates how distinct isolates may offer different technological advantages.

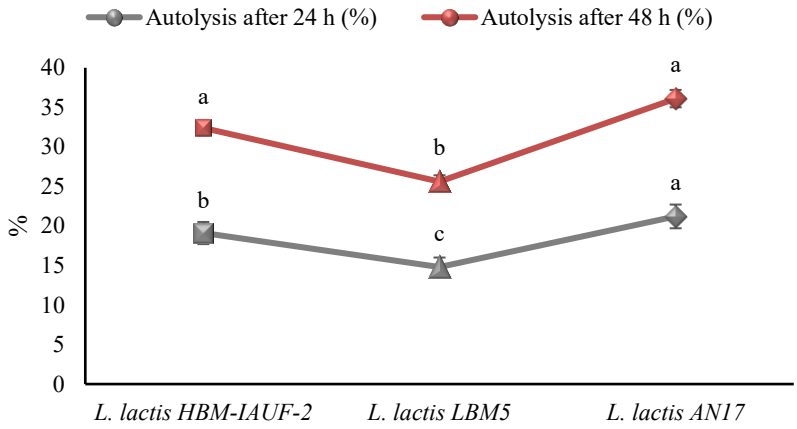


Figure 5. Time-dependent autolysis of *Lactococcus lactis* strains HBM-IAUF-2, LBM5, and AN17 after 24 and 48 h of incubation. Values are expressed as the mean ± standard deviation of three independent experiments. Different lowercase letters indicate significant differences among strain–time combinations based on pairwise comparisons with Tukey's adjustment following the two-factor repeated-measures analysis ($p < 0.05$).

Final viable counts in different growth media

LBM5 showed significantly higher viable counts than HBM-IAUF-2 and AN17 within each tested medium, including 9.46 ± 0.06 log CFU/mL in MRS broth (Fig. 6). These endpoint counts indicate differences in population size after 24 h, rather than differences in growth rate.

For all isolates, viable counts were numerically highest in MRS and lowest in M17, although statistical significance between media cannot be inferred from the within-medium comparisons. Medium-dependent growth differences also require consideration. Alfano et al. (2025) reported substantial changes in growth and lactate production when the carbohydrate supplementation of M17 was modified for *L. lactis* I7, illustrating the influence of substrate availability on fermentation performance.

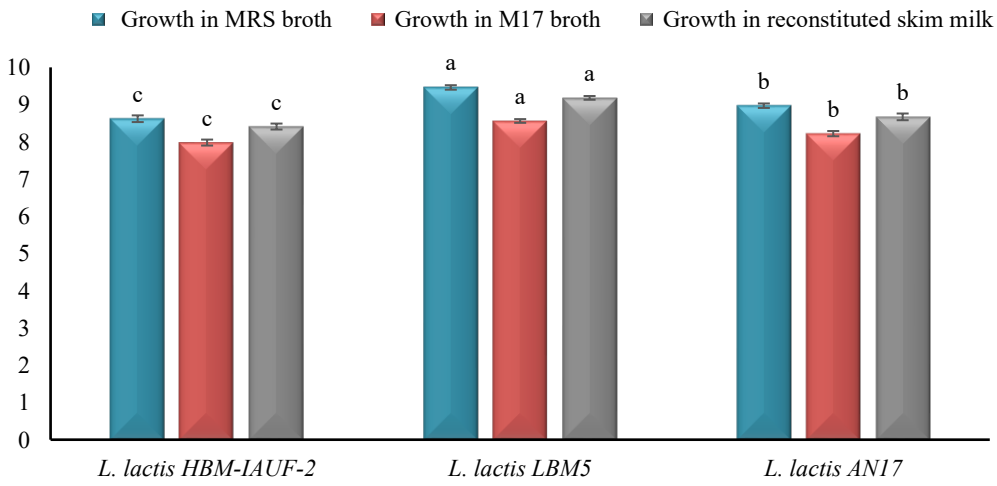


Figure 6. Final viable counts of *Lactococcus lactis* strains HBM-IAUF-2, LBM5, and AN17 after cultivation in MRS broth, M17 broth, and reconstituted skim milk. Values are expressed as mean ± standard deviation of three independent experiments. Data were analyzed by two-way ANOVA considering strain and culture medium as fixed factors. Different lowercase letters indicate significant differences among strains within the same growth medium according to Tukey's test ($p < 0.05$).

Preservation stability and oxidative-stress tolerance

Preservation-related stresses can compromise bacterial viability through membrane dehydration, osmotic imbalance, and oxidative damage. Ge et al. (2024) reported 81.02% freeze-drying survival for *L. lactis* ZFM559 using an optimized protective formulation. Matrix composition also influences probiotic–protein interactions in dairy products (Chaiwong et al., 2026). Survival differed significantly among the three isolates (Table 1), with LBM5 maintaining the highest viability ($p < 0.05$). Its freeze-drying survival reached 81.50 ± 1.50%, while 93.20 ± 1.30% of the post-drying population remained viable after 28 days at 4 °C. Differences in membrane stability and cellular redox regulation may contribute to the observed variation in preservation and H_2O_2 tolerance. Related functional diversity has also been documented in yogurt-derived LBM15 and HBM-IAUF-8 (Goudarzi Shams Abadi et al., 2026a,b) and indigenous cheese isolates (Namazi et al., 2026).

Table 1. Preservation stability and oxidative-stress tolerance of *Lactococcus lactis* strains HBM-IAUF-2, LBM5, and AN17.

Test	Unit	HBM-IAUF-2	LBM5	AN17
Survival after freezing	%	69.80 ± 1.8^{c}	87.60 ± 1.4^{a}	76.20 ± 1.20^{b}
Viable count after freeze-drying	log CFU/g	7.42 ± 0.14^{c}	8.35 ± 0.09^{a}	7.78 ± 0.03^{b}
Survival after freeze-drying	%	61.30 ± 1.90^{c}	81.50 ± 1.5^{a}	68.40 ± 1.7^{b}
Survival after 28-day storage	%	79.60 ± 1.80^{c}	93.20 ± 1.3^{a}	85.20 ± 1.7^{b}
Oxidative-stress tolerance	% Survival	43.70 ± 1.80^{c}	64.50 ± 1.50^{a}	51.30 ± 1.20^{b}

Values are mean ± SD of three independent replicates (n = 3). Different lowercase superscript letters within each row indicate significant differences among isolates (one-way ANOVA followed by Tukey's HSD test, $p < 0.05$). Storage survival was calculated relative to the viable count immediately after freeze-drying (day 0).

Enzymatic activity profiles

The enzyme profiles differed among the isolates (Table 2), with LBM5 exhibiting the highest β-galactosidase, aminopeptidase, esterase, and general protease activities. These enzymes contribute to carbohydrate hydrolysis, peptide degradation, and the formation of amino-acid precursors involved in dairy flavour development. The metabolic diversity of indigenous lactococci was also documented by Goudarzi Shams Abadi et al. (2026b) in their characterization of HBM-IAUF-8. Zhao et al. (2024) and Li et al. (2024) further demonstrated that increased availability of intracellular peptidases can enhance peptide breakdown and aroma-related transformations during cheese ripening.

LBM5 also showed significantly higher esterase activity ($p < 0.05$), consistent with its tributyrin-hydrolysis profile. Shen et al. (2022) identified distinct lipid- and ester-associated metabolites among 17 *L. lactis* strains, while Li et al. (2025) reported differences in metabolite profiles and sensory characteristics of Cheddar cheeses produced with different lactococcal starters.

Table 2. Enzymatic activities of *Lactococcus lactis* strains HBM-IAUF-2, LBM5, and AN17

Enzyme activity	Unit	HBM-IAUF-2	LBM5	AN17
β-Galactosidase	U/mL	14.70 ± 0.65^{c}	21.30 ± 0.55^{a}	17.20 ± 0.55^{b}
Aminopeptidase	U/mL	61.50 ± 2.00^{c}	81.60 ± 1.60^{a}	69.20 ± 1.70^{b}
Esterase	U/mL	37.60 ± 1.70^{c}	53.80 ± 1.40^{a}	43.10 ± 1.50^{b}
General protease	U/mL	25.40 ± 1.30^{c}	37.10 ± 0.10^{a}	29.10 ± 1.10^{b}

Values are expressed as mean ± standard deviation of three independent experiments. Statistical comparisons were performed separately within each row. Different lowercase superscript letters indicate significant differences among strains according to one-way analysis of variance followed by Tukey's test ($p < 0.05$). For general protease activity, one unit was defined as the amount of enzyme releasing 1 µg of tyrosine equivalents per minute under the assay conditions; activity was expressed per mL of crude enzyme extract.

Integrated comparative profiling of technological and functional traits

The row-standardized heatmap revealed distinct technological profiles among the three indigenous isolates (Fig. 7). LBM5 exhibited the highest relative values in 18 of the 20 evaluated traits, whereas AN17 showed the highest apparent autolysis at both 24 and 48 h.

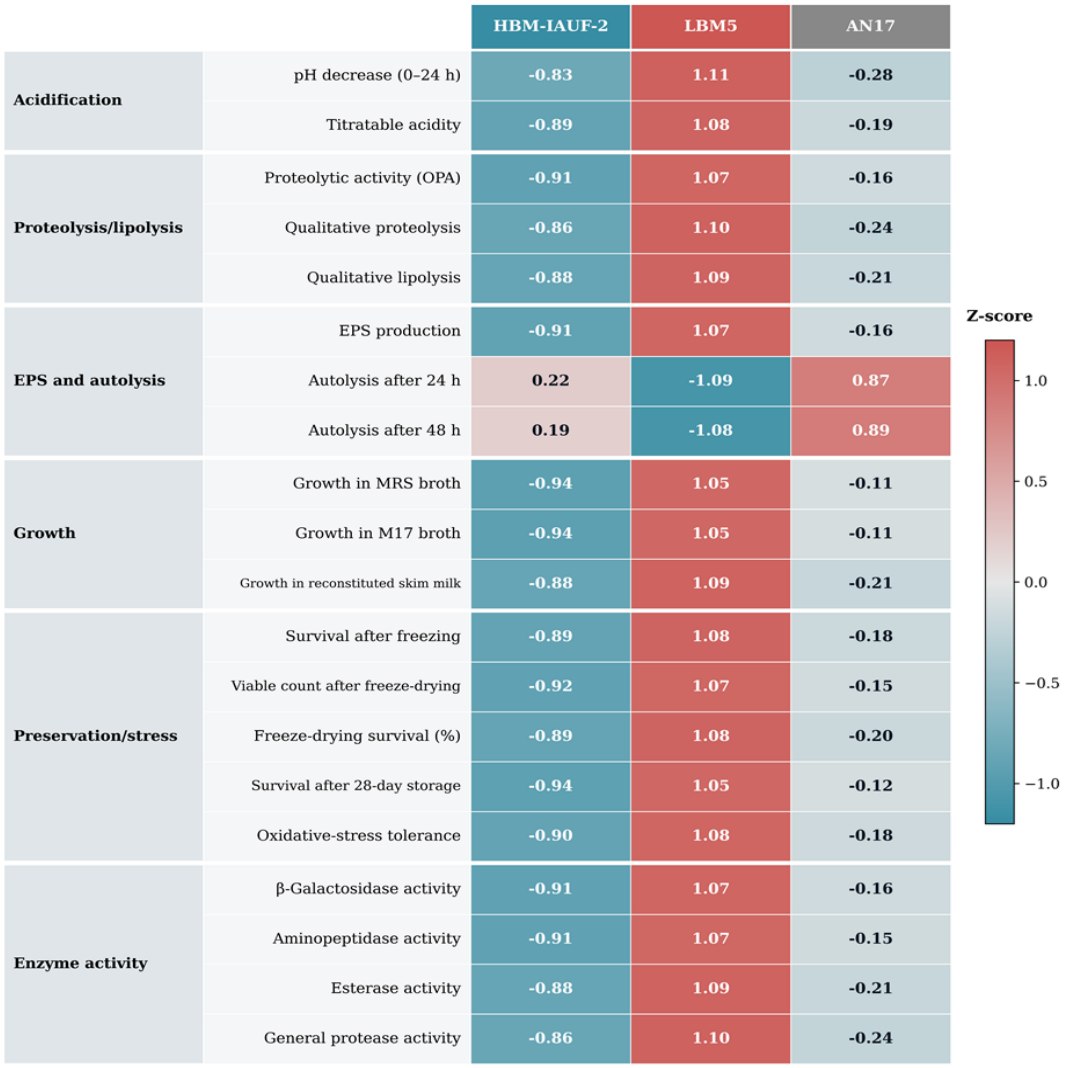


Figure 7. Comparative Z-score heatmap of the technological and functional characteristics of *Lactococcus lactis* strains HBM-IAUF-2, LBM5, and AN17. Milk acidification was expressed as the decrease in pH during 24 h of fermentation. Positive and negative Z-scores represent values above and below the mean of the three strains, respectively.

This distribution reveals an important distinction between fermentative performance and autolytic potential. The combination of acidification capacity, enzymatic activity, and preservation tolerance observed in LBM5 may be advantageous for starter culture development, whereas the greater autolytic response of AN17 may be relevant to biochemical transformations during cheese ripening. Rather than identifying a universally superior isolate, the integrated profile highlights how different technological characteristics can inform strain selection according to the requirements of specific dairy products.

Conclusions

The technological characterization of three indigenous *L. lactis* isolates revealed marked strain-dependent differences in fermentation performance, enzymatic activities, and stress tolerance. LBM5 combined efficient milk acidification with greater 24-h hydrolytic activities, crude EPS-associated carbohydrate recovery, and preservation stability, whereas AN17 exhibited a more pronounced time-dependent autolytic response. Most traits showed a similar ranking among isolates, whereas apparent autolysis followed a different pattern, supporting the joint consideration of fermentative performance and autolytic potential in culture selection. The findings distinguish LBM5 as a candidate for further starter culture development and highlight the potential relevance of AN17 for ripening-associated applications. Evaluation in representative dairy products will be essential to establish their technological performance under practical fermentation conditions.

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سویه‌های بومی *Lactococcus lactis* به‌عنوان کشت‌های دومنظوره: مقایسه فعالیت آنزیمی و عملکرد فناورانه برای کاربردهای لبنی تخمیری

بهاره گودرزی شمس‌آبادی، بهروز علیزاده بهبهانی، حسین جوینده، محمد حجتی، علیرضا وصیعی، و مرتضی تاکی

چکیده

تنوع فناورانه در میان باکتری‌های بومی لبنی فرصت‌هایی برای توسعه کشت‌های آغازگر فراهم می‌کند، هرچند انتخاب سویه، مستلزم در نظر گرفتن ویژگی‌های عملکردی متعددی فراتر از اسیدی‌کردن شیر است. در این پژوهش، ویژگی‌های فناورانه سه جدایه *Lactococcus lactis* (شامل HBM-IAUF-2، LBM5 و AN17) که از ماست سنتی جداسازی شده بودند، ارزیابی شد. اسیدی‌شدن شیر، عملکرد رشد، فعالیت‌های پروتئولیتیکی و لیپولیتیکی، آنزیم‌های درون‌سلولی، بازیابی کربوهیدرات خام مرتبط با اگزوپلی‌ساکارید (EPS)، خودتجزیه‌ای و تحمل در برابر تنش‌های نگهداری و اکسیداتیو بررسی شد. نتایج به‌صورت میانگین ± انحراف معیار از سه تکرار مستقل بیان و از نظر وجود تفاوت‌های معنی‌دار ($p < 0/05$) تحلیل شدند. سویه LBM5 بیشترین ظرفیت اسیدسازی را نشان داد، به‌طوری‌که پس از ۲۴ ساعت pH شیر به 4/08 ± 0/04 و اسیدیته قابل تیتراسیون به 1/02 ± 0/03 درصد رسید. این جدایه همچنین بالاترین غلظت گروه‌های آمینی واکنش‌دهنده با OPA (1/82 ± 0/06 میلی‌مول بر لیتر معادل سرین)، فعالیت β-گالاکتوزیداز (21/30 ± 0/55 واحد بر میلی‌لیتر) و بازیابی کربوهیدرات خام مرتبط با (EPS) 213/50 ± 7/80 میلی‌گرم معادل گلوکز بر لیتر) را داشت. بقای آن پس از انجمادخشک 81/50 ± 1/50 درصد بود و 93/20 ± 1/30 درصد از زیستایی خود را طی ۲۸ روز نگهداری در یخچال حفظ کرد. در مقابل، AN17 بیشترین خودتجزیه‌ای ظاهری را پس از ۴۸ ساعت نشان داد (36/10 ± 1/50 درصد). تحلیل یکپارچه نقشه حرارتی (Heatmap) ویژگی‌های گسترده تخمیری و مرتبط با نگهداری در LBM5 را از پاسخ خودتجزیه‌ای قوی‌تر AN17 متمایز کرد. این یافته‌ها بیانگر ویژگی‌های فناورانه مکمل در لاکتوکوکوس‌های بومی است و ارزیابی بیشتر LBM5 را به‌عنوان کاندیدای آغازگر و AN17 را برای کاربردهای معطوف به رسیدگی (Ripening) پشتیبانی می‌کند.