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Assessment of the Technological Properties of *Lactobacillus acidophilus* TMPC 41127 Isolated from Traditional Khiki Cheese

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ABSTRACT

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In this study, the technological characteristics of the probiotic strain *Lactobacillus acidophilus* TMPC 41127, isolated from Khiki cheese, were investigated to evaluate its potential for industrial applications. The assessed characteristics included thermal resistance, exopolysaccharide (EPS) production, acidifying ability, proteolytic activity, and autolytic activity. The results showed that the *L. acidophilus* TMPC 41127 strain exhibited acceptable thermal resistance, with the highest survival rate (53.74%) recorded at 50°C. The strain was also classified as a strong acidifier, as it was able to reduce the initial pH of 6.49 to 4.85 within 24 hours. Furthermore, it demonstrated a significant capacity for producing mucoid EPS. Its proteolytic activity was confirmed by the formation of a halo with a diameter of 22.03 mm. Finally, the strain's autolytic activity was at a moderate level (20.21%) at 4°C and a weak level (14.07%) at -20°C. These findings collectively confirm that the native *L. acidophilus* TMPC 41127 strain possesses desirable technological traits, making it a strong candidate for use as a starter culture in the fermented food industry and as a potential probiotic in functional foods.

1- Introduction

Probiotics are living microorganisms that, when consumed in adequate amounts, confer a health benefit on the host and help balance the gastrointestinal microbiota. This concept was first introduced by Lilly and Stillwell in 1965 to describe factors that promote the growth of other microorganisms [1]. The lactic acid bacteria (LAB) are a foundational group within this category, characterized as Gram-positive, non-sporulating, and anaerobic bacteria that primarily produce lactic acid through carbohydrate metabolism [2,3]. This inherent ability for acidification makes LAB essential to food fermentation processes, as it effectively inhibits the growth of spoilage and pathogenic microorganisms. The core genera of LAB, including *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, and *Streptococcus*, are widely used as starter cultures for products like yogurt and cheese, and their "Generally Recognized as Safe" (GRAS) status from regulatory bodies like the FDA underscores their importance in food supplements and functional foods [4,5].

A key species among LAB is *Lactobacillus acidophilus*, a rod-shaped bacterium that plays a vital role in maintaining the microenvironmental stability of the mammalian gastrointestinal tract [6]. As an obligate homofermentative bacterium, it excels under anaerobic conditions and effectively regulates microbiome composition through extensive gut colonization [7]. The probiotic efficacy of *L. acidophilus* extends to modulating the host's immune response through direct interaction with immune cells, bolstering the body's defense system [8]. These functional traits, combined with a confirmed safety profile, have established *L. acidophilus* as a valuable biotechnological agent with significant commercial and clinical importance, leading to its widespread application in dairy, dietary supplements, and even cosmetic formulations [9]. The industrial utility and clinical efficacy of LAB are underpinned by a suite of robust technological characteristics. A critical property is their thermotolerance, which allows them to withstand extreme conditions such as high temperatures

during feed pelleting and pasteurization, thus ensuring strain viability [10]. Furthermore, their capacity for rapid acidification not only enhances microbial stability but also facilitates syneresis in cheese production and activates ripening enzymes crucial for flavor and texture development (Ebadi Nezhad et al., 2020). [11]. Beyond acidification, LAB contribute to product quality through casein proteolysis, where intracellular proteases and peptidases released upon cell lysis improve texture and flavor profiles [12]. Other key traits include the production of exopolysaccharides (EPS), which provide structural stability and resistance to environmental stressors [13] while also offering functional benefits like antioxidant and immunomodulatory properties [14]. Finally, cellular autolysis is a pivotal mechanism in which the spontaneous breakdown of cells under suboptimal conditions releases key intracellular enzymes, accelerating cheese ripening and reducing bitterness [15,16].

Given the critical importance of a suite of technological characteristics, including thermotolerance, rapid acidification, proteolysis, EPS production, and autolysis, in determining the industrial and clinical utility of LAB, a comprehensive evaluation is essential for strain selection. While the general functions of these properties are known, their specific expression can vary significantly between individual strains. Therefore, this study was conducted with the aim of systematically evaluating these properties in the *Lactobacillus acidophilus* TMPC 41127 strain, which was isolated from Khiki cheese. By comprehensively analyzing these key technological traits, this research seeks to validate the strain's suitability as a robust starter culture for fermented products and a promising probiotic candidate for various food and health applications.

2- Materials and Methods

2.1. Materials

To assess the technological characteristics of the *L. acidophilus* TMPC 41127 strain, MRS broth, MRSC agar, and MRS agar from the Quelab

(Canada) brand were used as culture media. All other materials utilized in this research were of laboratory grade.

2.2. Probiotic Strain Activation

In this study, the probiotic strain *Lactobacillus acidophilus* TMPC 41127, which had been isolated from Khiki cheese in previous research, was utilized. For activation, 100 μ L of the lyophilized culture was transferred to MRS broth and incubated anaerobically at 37 °C for 48 hours. Following successful activation, this culture was used to evaluate the desired technological characteristics.

2.3. Thermal resistance

To evaluate the resistance of the *L. acidophilus* TMPC 41127 strain to high temperatures, the method of Noshad et al. (2021) was used [17]. First, the strain was activated in MRS broth. After 48 hours of anaerobic incubation at 37 °C, the culture was centrifuged for 5 min at 6000 rpm and 4 °C. The resulting pellet was washed with PBS and then diluted in the same buffer. Subsequently, the samples were treated in a water bath at temperatures of 50, 60, 70, and 80 °C for 20 min. Following this heat treatment, the samples were incubated for 16 hours at 37 °C, and their optical absorbance was finally measured at a wavelength of 600 nm.

2.4. Exopolysaccharide production

Exopolysaccharide (EPS) production by the *L. acidophilus* TMPC 41127 strain was investigated according to the method of Ebadi Nezhad et al. (2020) [11]. For this purpose, the *L. acidophilus* TMPC 41127 strain was first activated and then cultured on MRSC agar (MRS agar supplemented with 0.25% L-cysteine). The plates were incubated anaerobically for 72 hours at 37 °C. To evaluate the effect of various carbon sources, 2% of either glucose, fructose, lactose, or sucrose was individually added to the culture media.

2.5. Acidifying ability

To evaluate the acidifying activity of the strain, 100 μ L of the bacterial culture was added to 10 mL of a 10% (w/v) skim milk powder solution. The mixture was then incubated at 30 °C for 24 hours. The pH was measured using a digital pH meter (Hanna Instruments, São Paulo, Brazil) at the initial time point, and again after 6 hours and 24 hours [18].

2.6. Proteolysis activity

The proteolytic activity of the bacterial strain was evaluated using skim milk agar medium. For this purpose, 50 μ L of the CFS was aseptically dispensed into a 5-mm diameter well created in the center of each agar plate. The plates were then incubated for 24 hours at 37 °C. The formation of a clear halo around the well indicated proteolytic activity, and its diameter was measured in millimeters [11].

2.7. Autolytic activity

The autolytic activity of the *L. acidophilus* TMPC 41127 strain was evaluated according to the method of Laslo et al. (2024) [16]. First, the strain was cultured in MRS broth and then centrifuged for 5 min at 6000 rpm and 4 °C. The resulting pellet was washed twice with phosphate buffer and then resuspended in the same buffer until its optical absorbance at 600 nm reached 1. Finally, the suspension was subjected to temperatures of 37, 4, and -20 °C for 24 hours, after which the samples were incubated at 30 °C. The autolytic activity of the strain was expressed as the percentage of the initial change in optical density (1):

$$\text{Autolytic activity (\%)} = \frac{\text{OD}_0}{\text{OD}_t} \times 100$$

where, OD_0 and OD_t represent the optical density measured at the initial time point and the optical density measured at the time under study, respectively.

2.8. Statistical analysis

Data from this research were analyzed using One-Way Analysis of Variance (ANOVA) in SPSS statistical software version 26. To determine significant differences among the means, Duncan's test was applied at a significance level of $P < 0.05$. All experiments were conducted in triplicate.

3-Results and Discussion

3.1. Thermal resistance

Thermophilic properties and thermal stability are considered desirable technological traits in LAB, particularly in industrial processes like pasteurization. These characteristics indicate a strain's ability to withstand high temperatures, which is essential for survival during various production stages and for maintaining viability in final products. Therefore, evaluating thermal

stability serves as a crucial criterion for selecting probiotic strains for industrial applications [17]. The thermal stability of the studied strain was evaluated by exposing it to temperatures of 50, 60, 70, and 80°C for 20 minutes. As shown in Fig. 1, increasing the temperature had a significant and negative effect on cell survival. The highest survival rate was recorded at 50°C (53.74%), while a considerable decline in cell viability was observed with a gradual increase to 60, 70, and 80°C (38.34%, 30.99%, and 24.81%, respectively). These findings indicate that while the studied strain exhibits a notable degree of thermal resistance, temperatures above 50°C are increasingly lethal to its survival. This is consistent with previous reports. Assari et al.

(2023) observed that *L. acidophilus* typically experiences a sharp decrease in cell viability at temperatures of 65°C or higher, although the strain showed acceptable survival of approximately 7 Log CFU/ml after 10 minutes of exposure to 55°C [19]. In contrast, some studies have achieved different results through strain engineering methods. Jeon et al. (2021) demonstrated that the *L. acidophilus* EG008 strain, developed using the ALE method, showed better thermal resistance than the initial EG004 strain. The EG008 strain was able to improve its survival rate by an average of 39.11% at 66°C, which represents a statistically significant advancement [10].

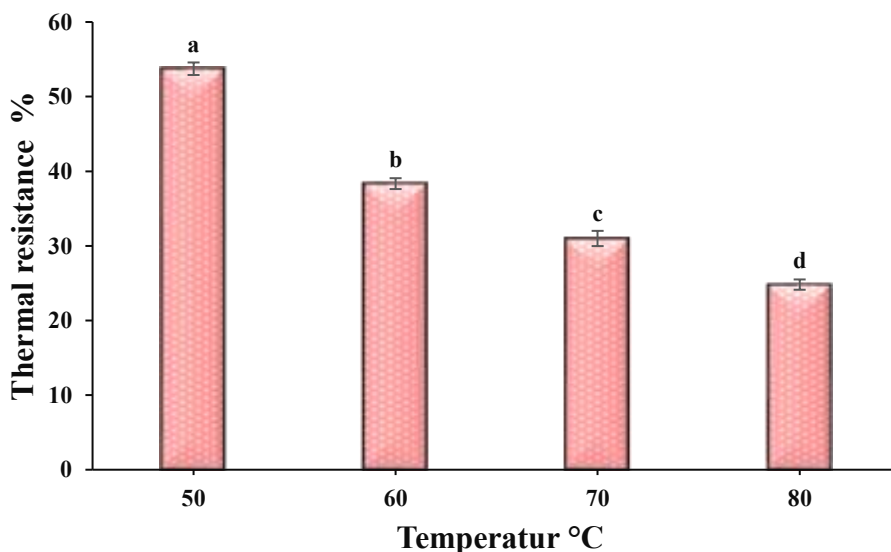


Fig. 1. Results of thermal resistance for the *L. acidophilus* TMPC 41127 strain at 50°C, 60°C, 70°C, and 80°C. Statistically significant differences between samples are indicated by different letters at the $p < 0.05$ probability level.

3.2. Exopolysaccharide production

Many strains of LAB are capable of EPS, which are extracellular polymers classified into various forms based on their attachment to the bacterial cell (Zommara et al., 2023)[20]. These forms include capsular EPS (tightly bound to the cell), loosely bound EPS, and released EPS that appears as a slimy substance in the extracellular environment. To visually assess EPS production, one can examine colony morphology. Distinct phenotypes such as mucoid or ropy on the culture medium are indicative of a strain's ability to synthesize these polymers [11]. Our results on the *L. acidophilus* TMPC 41127 strain showed that it

is capable of producing significant amounts of EPS in a mucoid form, which was observed as a gelatinous halo around the colonies. This phenotypic expression was highly dependent on the carbon source in the culture medium. The highest EPS production and the most prominent mucoid appearance were observed in media containing lactose and sucrose. This indicates a metabolic preference of this strain for using these disaccharides as the primary precursors for EPS synthesis. In comparison, the amount of production in media containing monosaccharides like glucose and fructose was significantly lower. These findings are consistent with previous

reports by Kong et al. (2022) and Farid et al. (2021), emphasizing the potential of *L. acidophilus* as an EPS producer [21,22]. In a study, EPS produced by LAB during sourdough fermentation exhibit technofunctional aspects related to their ability to bind water and retain moisture [23]. EPS produced by *Lactobacillus* species are widely used in the food industry due to their Generally Regarded As Safe (GRAS) status. These biopolymers provide desirable rheological properties such as increased viscosity, improved texture, and reduced syneresis, acting as both texturizers and stabilizers. The multifaceted properties of EPS, which depend on their chemical composition, molecular size, and three-dimensional structure, enable diverse applications including gelling agents, crystallization inhibitors, and drug delivery carriers [24].

3.3. Acidifying ability

LABs are essential in the fermentation process, as their acidifying activity directly impacts microbial safety and enhances the sensory attributes of fermented products by lowering pH. This process is influenced by key factors such as temperature, strain type, and carbon source concentration [25]. In this study, to evaluate the technological potential of the selected strain, changes in pH were investigated in a reconstituted skim milk medium after 6 and 24 hours of incubation. The results showed that the initial pH of 6.49 decreased by 0.72 units after 6 hours and by 1.64 units after 24 hours (Fig. 2). Based on the classification criteria proposed by Mohammed & Çon (2021) [26], the strain is clearly categorized as a strong acidifier due to this significant reduction in pH. This finding not only highlights the strain's potential as a suitable

starter culture for fermentation but also aligns with previous research. For instance, Barzegar et al. (2021) reported a strong acidifying potential for *L. acidophilus* strains isolated from traditional Iranian cheeses, which corroborates our findings [12]. However, it is important to note that some studies, such as the work by Zommara et al. (2023), have reported a different distribution among *Lactobacillus* strains, with the majority (51.5%) classified as slow acidifiers [20]. In a study investigating the effect of acidification on sourdough bread, the results showed that LAB strains, through their production of lactic acid, lead to an increase in the bioavailability of mineral elements and a reduction in phytate content in the final product. These findings underscore the key role of acidification in improving the nutritional value of bread and other fermented products [23]. Also, past studies have revealed that the *L. acidophilus* strain increases the rate of acidification during the fermentation of probiotic dough, resulting in a significantly higher initial acidity compared to a control sample. This strain also maintains a high final acidity throughout refrigerated storage, which leads to a noticeable drop in pH and improves the product's overall stability, shelf life, and quality [7]. This discrepancy in results may be attributed to factors like the geographical origin of the strains, the type of culture medium, and specific fermentation conditions. Additionally, LABs produce key organic acids like acetate and propionate, which improve food preservation by reducing pH and enhancing sensory qualities such as flavor and texture. These compounds also possess antimicrobial properties and beneficial health effects, including metabolic support [25].

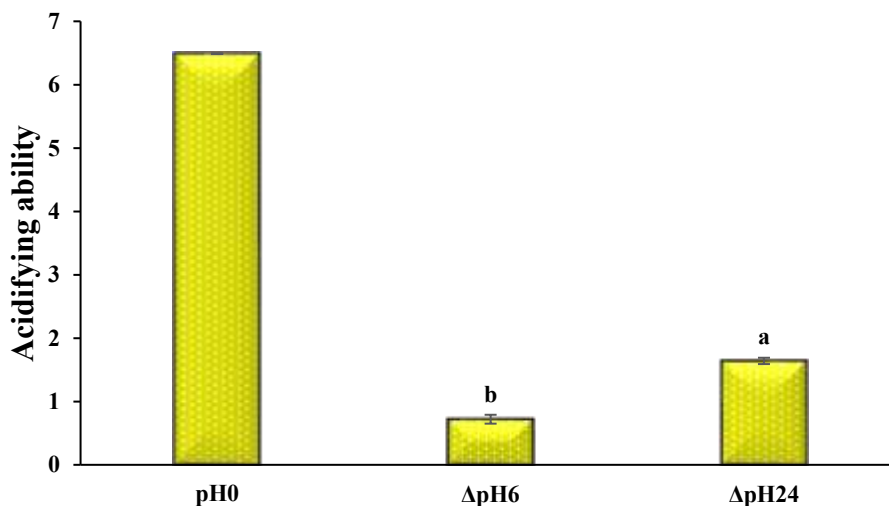


Fig. 2. Results of changes in the acidifying ability of *L. acidophilus* TMPC 41127 after 6 and 24 hours of incubation at 37°C. Statistically significant differences between samples are indicated by different letters at the $p < 0.05$ probability level.

3.4. Proteolysis activity

LABs are considered a suitable candidate for industrial applications in the production of functional foods due to their consumption safety and health benefits [28]. The proteolytic system of LABs, a two-stage process, first hydrolyzes high-molecular-weight proteins into large peptides via extracellular proteinases. These peptides are then broken down into free amino acids by intracellular peptidases, such as aminopeptidases and dipeptidases [29]. The present study's results demonstrated that the *L. acidophilus* TMPC 41127 strain showed significant proteolytic activity on skim milk agar, evidenced by a casein hydrolysis halo with a mean diameter of 22.03 ± 0.22 mm (Fig. 13, D). This finding is consistent with Nezhad et al. (2020), who reported halo diameters ranging

from 19 to 30 mm for various LAB strains [11]. However, our findings differed from those of Phupaboon et al. (2023), who observed a halo diameter ranging from 11 to 12 mm [30]. These differences are likely due to the unique genetic and metabolic characteristics of each strain and their impact on industrial performance. Ultimately, the proteolytic system of LABs plays a crucial role in enhancing the quality of food products. This is achieved by improving sensory properties like flavor and texture in dairy and meat products, increasing bread volume in the baking industry, and reducing haze in brewing. This capability establishes LABs as a safe and efficient alternative to chemical enzymes, contributing to the production of healthier, higher-quality products [28].



Fig. 3. Results of Proteolytic Activity for *L. acidophilus* TMPC 41127.

3.5. Autolytic activity

Autolytic activity, as a crucial technological trait in LAB, is the process of spontaneous cell degradation driven by enzymes like peptidoglycan hydrolase. This mechanism leads to the release of intracellular enzymes, particularly lipase and protease, which play a vital role in improving the sensory and textural qualities of fermented foods, including the cheese ripening process [31]. A common classification system categorizes autolytic strains into three groups based on their rate of cellular degradation: good (25-65% autolysis), moderate (15-24%), and poor (below 14%) [12]. Based on these criteria and the data presented in Fig. 4, the autolytic activity of the studied strain was significantly affected by temperature. The autolysis rate at 37°C was 41.05%, classifying it as good. This was significantly higher than the rates observed at lower temperatures: 20.21% at 4°C, which was classified as moderate, and 14.07% at -20°C, which was considered poor.

While our findings for this specific strain demonstrate a strong temperature dependency, the scientific literature emphasizes that autolysis is fundamentally a strain-dependent property (M'hamed et al., 2022; Bentahar et al., 2024)[32,12]. This is consistent with the results of other studies. For instance, Assari et al. (2023) reported the highest autolytic activity (38.69%) for a *L. acidophilus* strain isolated from goat milk lactic cheese at 4°C [19]. Similarly, M'hamed et al. (2022) recorded an autolysis rate of 29.51% for the *Lacticaseibacillus paracasei* L2 strain isolated from a traditional fermented food [32]. Based on previous studies that investigated the autolytic activity of lactic acid bacteria (LAB) strains, the results show that 56% of the *Lactobacillus* isolates tested exhibited high autolysis, 32% showed moderate autolysis, and the remaining 12% demonstrated low autolysis [33]. These differences in autolysis percentages across isolates confirm the wide diversity in the autolytic mechanisms of various strains.

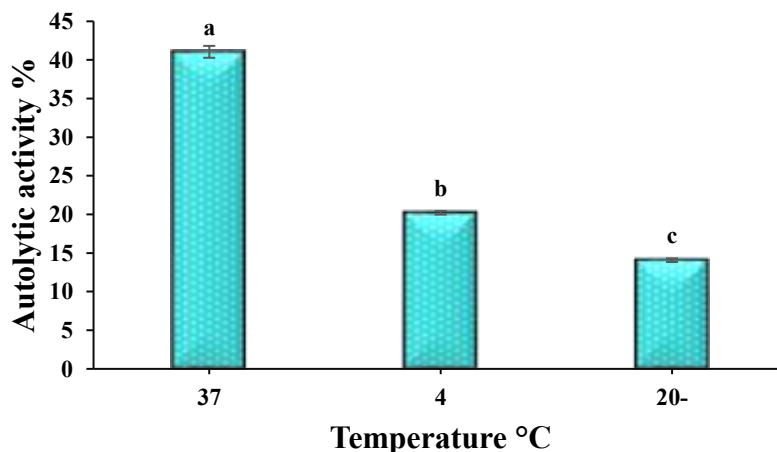


Fig. 4. Evaluation of the autolytic activity of *L. acidophilus* TMPC 41127 under Various temperature conditions (37°C, 4°C, and -20°C). Statistically significant differences between samples are indicated by different letters at the $p < 0.05$ probability level.

3.6. Data analysis using heatmap charts

The capacity of lactic acid bacteria and related strains to reduce pH is a critical determinant of their technological functionality in food fermentations. In the present study, the strain demonstrated an initial pH of 6.49, with reductions of $\Delta\text{pH}_6 = 0.72$ and $\Delta\text{pH}_{24} = 1.64$. This pattern reflects the organism's ability to gradually produce organic acids, particularly lactic acid, over time. The relatively modest acidification observed within the first 6 h indicates that the strain cannot be categorized as a fast acidifier. Instead, its performance aligns more closely with that of slow acidifying cultures, which are often preferred in specific fermentation contexts where controlled pH reduction is advantageous. Gradual acidification ensures the maintenance of microbial balance and enhances structural and sensory development during ripening, especially in cheese and meat fermentations. Furthermore, such controlled acidification may minimize defects such as excessive sourness or protein destabilization that are frequently associated with rapid acidifiers. On the other hand, in applications where rapid pathogen suppression is required—such as in fresh dairy products or minimally processed meats—the relatively slow pH decline may limit its safety contribution, necessitating co-cultivation with more acidifying strains to achieve the desired inhibitory effect.

The strain exhibited a remarkably high level of autolytic activity at 37 °C (41.05%), whereas

activity was markedly reduced at lower or suboptimal temperatures, with values of 20.21% at 4 °C and 14.07% at 20 °C. High autolysis at physiological temperature is indicative of active enzymatic self-degradation of the cell wall, resulting in the release of intracellular enzymes, such as proteases, peptidases, and glycosidases, into the surrounding environment. These enzymes play a pivotal role in proteolysis, flavor formation, and the release of bioactive peptides, which collectively contribute to the sensory and nutritional quality of fermented products. From a technological standpoint, strong autolytic activity at the fermentation stage is particularly beneficial in cheese production, where intracellular enzymes accelerate casein degradation and flavor compound release during ripening. Conversely, reduced activity at cold storage temperatures suggests that enzymatic release is minimized under refrigeration, which may enhance culture stability during distribution and extend product shelf life. This dual characteristic—high autolysis under optimal fermentation conditions and reduced activity under stress—provides a balance between functionality and preservation, making the strain suitable for a wide range of industrial processes.

The evaluation of thermal tolerance revealed that strain survival decreased progressively as the temperature increased, with viability falling from 53.74% at 50 °C to only 24.81% at 80 °C. These results point to a moderate degree of thermotolerance, suggesting that the strain is

capable of partially withstanding sublethal thermal treatments, such as those encountered during pasteurization or certain cooking processes. Thermal resistance is a critical parameter for both starter and probiotic cultures, as survival through processing is essential to ensure viability in the final product. The ability of this strain to survive up to moderate heat stress enhances its potential for use in fermented foods and probiotic formulations subjected to mild heat processing. However, the sharp decline in survival above 70 °C highlights its limited resilience under severe thermal stress, which could compromise its functional contribution in products requiring high-temperature processing. This limitation may necessitate protective strategies, such as microencapsulation, co-formulation with protective agents, or optimization of processing conditions, to maximize strain viability in industrial applications.

Taken together, the strain's functional profile suggests a versatile microorganism with distinct advantages in food fermentation. Its gradual but steady acidification supports microbial stability and textural development without excessive acid production, while its high autolytic activity at fermentation temperatures provides a valuable enzymatic contribution to flavor formation and proteolysis. At the same time, its moderate heat resistance ensures partial survival under sublethal stress, enabling its persistence in a variety of food systems. These combined traits are particularly relevant for fermented dairy and meat products, where controlled acidification, enzymatic activity, and survival under processing conditions are crucial determinants of product quality and microbial performance.

From a broader perspective, the functional profile observed in this strain mirrors the desirable balance often sought in starter or adjunct cultures: controlled acidification for texture and safety, sufficient autolysis for flavor development, and moderate stress resistance for survival under processing. While its limited acidification rate and reduced thermal tolerance at higher temperatures may restrict its stand-alone use in certain applications, these limitations can be addressed through co-cultivation with complementary strains, formulation with

protective carriers, or optimization of processing parameters.

In conclusion, this strain exhibits a unique combination of technological properties that make it a promising candidate for biotechnological and food-related applications. Its slow acidification, high autolytic activity at fermentation temperatures, and moderate thermal resistance provide a solid foundation for its application in dairy, meat, and potentially probiotic systems. To fully exploit its potential, future research should focus on genomic and proteomic analyses to elucidate the molecular mechanisms governing acidification, autolysis, and stress tolerance. Additionally, pilot-scale and industrial trials are required to validate its performance in real food matrices and to evaluate its contribution to product quality, sensory attributes, and safety.

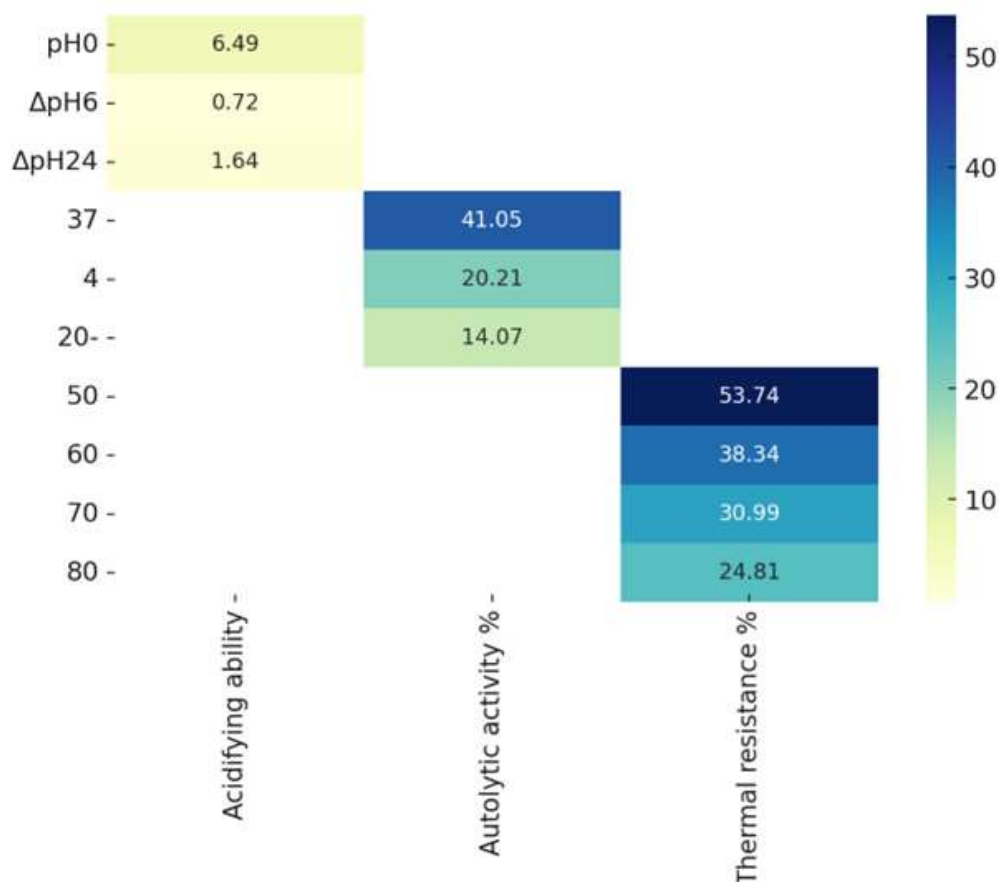


Fig. 5. Heatmap of Acidifying ability, Autolytic Activity, and Resistance.

4- Conclusion

Based on the results of this study, the native *Lactobacillus acidophilus* TMPC 41127 strain, isolated from Khiki cheese, possesses desirable technological characteristics for industrial applications. Despite its sensitivity to high temperatures, it exhibited acceptable thermal resistance, which is important for processes like pasteurization. Its strong acidifying ability led to a significant decrease in pH, confirming its potential as an effective starter culture for improving sensory attributes and shelf life. Furthermore, the strain's ability to produce mucoid exopolysaccharides (EPS) can enhance the texture and stability of food products. Its proteolytic and autolytic activities also contribute positively to flavor development and accelerating the ripening process. Collectively, the findings indicate that the *L. acidophilus* TMPC 41127 strain is not only a potential probiotic but, due to its outstanding technological traits, can be a

powerful tool for formulating new and functional food products.

5- Acknowledgements

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ارزیابی ویژگی‌های تکنولوژیکی سویه *Lactobacillus acidophilus* TMPC 41127 جداسازی شده از پنیر سنتی خیکی

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چکیده

اطلاعات مقاله

در این مطالعه، ویژگی‌های تکنولوژیکی سویه پروبیوتیک *Lactobacillus acidophilus* TMPC 41127، جداسازی شده از پنیر خیکی، به منظور ارزیابی پتانسیل آن برای کاربردهای صنعتی مورد بررسی قرار گرفت. ویژگی‌های مورد نظر شامل مقاومت حرارتی، تولید اگزوپلی ساکارید (EPS)، توانایی اسیدی سازی، فعالیت پروتئولیتیکی و فعالیت اتولیتیکی بودند. نتایج نشان داد که سویه *L. acidophilus* TMPC 41127 مقاومت حرارتی قابل قبولی از خود نشان می‌دهد؛ بالاترین نرخ بقا (۵۳/۷۴٪) در دمای ۵۰ درجه سانتی‌گراد ثبت شد. این سویه به عنوان یک اسیدی‌کننده قوی نیز طبقه‌بندی شد، زیرا توانست pH اولیه ۶/۴۹ را در مدت ۲۴ ساعت به ۴/۸۵ کاهش دهد. علاوه بر این، توانایی قابل توجهی در تولید EPS به شکل موکوئید از خود نشان داد. فعالیت پروتئولیتیکی آن با تشکیل هاله‌ای به قطر ۲۲/۰۳ میلی‌متر تأیید شد. در نهایت، فعالیت اتولیتیکی سویه در دمای ۴ درجه سانتی‌گراد در سطح متوسط (۲۰/۲۱٪) و در دمای ۲۰- درجه سانتی‌گراد در سطح ضعیف (۱۴/۰۷٪) قرار گرفت. این یافته‌ها به صورت جامع تأیید می‌کنند که سویه بومی *L. acidophilus* TMPC 41127 دارای ویژگی‌های تکنولوژیکی مطلوبی است که آن را به یک کاندیدای قوی برای استفاده به عنوان کشت آغازگر در صنایع غذایی تخمیری و یک پروبیوتیک بالقوه در محصولات فراسودمند تبدیل می‌کند.

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