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Evaluation of the potential of starter culture from Indonesian kefir grains on the characteristics of fermented milk during storage

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Abstract. *Wulansari PD, Rahayu N, Kusmayadi A, Kusuma RJ. 2025. Evaluation of the potential of starter culture from Indonesian kefir grains on the characteristics of fermented milk during storage. Biodiversitas 26: 2368-2374.* This study aims to evaluate the physicochemical quality of fermented milk produced using starter cultures derived from Indonesian kefir grains that were subcultured in goat's milk. Cow's milk was used as the raw material. The starter cultures consisted of a single strain: *Lactobacillus plantarum* (Orla-Jensen, 1919) Bergey et al., 1923 strains BP and DI, and *Lactiplantibacillus plantarum* A strain ES. Fermentation was carried out by inoculating the pasteurized milk with the cultures, followed by storage at 4°C for up to 20 days. The parameters observed during storage included pH value, total Lactic Acid Bacteria (LAB), Free Fatty Acid (FFA), acidity, and moisture content. The results showed that both the type of starter culture and the storage duration significantly affected all measured parameters. *Lactobacillus plantarum* strain DI produced fermented milk with the lowest pH value, and this value decreased over time. The total LAB count increased in samples inoculated with strain BP and DI but slightly decreased in strain ES during storage. FFA levels increased for all strains over time; acidity rose in samples with strains BP and DI but decreased in samples with strain ES; moisture content declined across all samples during storage. This study confirms that starter cultures derived from Indonesian kefir grains, when combined with appropriate storage conditions, significantly influence the physicochemical quality of fermented milk.

Keywords: Fermented milk, grain kefir, lactic acid bacteria, starter culture

INTRODUCTION

The consumption of fermented milk products has increased significantly worldwide, reflecting the growing public awareness of the health benefits and probiotic content of these products. Statistical data show that the global market for fermented milk products is projected to reach around USD 100 billion by 2027, with an estimated annual growth rate of between 6-8% (Tita et al. 2021). This increase in consumption of fermented milk is closely related to the various benefits offered by these products. Regular consumption of fermented milk products, such as yoghurt and kefir, can relieve the symptoms of lactose intolerance, making these products more accessible to individuals who are sensitive to lactose (Erkmen and Bozoglu 2016). Fermented milk products also contain Conjugated Linoleic Acid (CLA) and butyric acid, which show potential anti-cancer properties (Veiga et al. 2014; Lang et al. 2019).

However, the control of the fermentation process remains a critical yet challenging aspect, influenced by factors such as temperature, pH levels, and inhibitory compounds that can significantly affect Lactic Acid Bacteria (LAB) activity during fermentation. The diversity of starter cultures is an important factor in determining the characteristics of fermented milk products. Although commercial starter cultures are widely used, they may not always provide the expected sensory or health benefits when compared to authentic traditional starters. The use of local Lactic Acid Bacteria (LAB) strains can improve fermentation efficiency and product quality, as well as display unique flavors that suit local consumer preferences (Utami et al. 2020; Li et al. 2022). Local starters have specific adaptations that can increase fermentation yields, improve functional properties, and contribute to better sensory attributes in the final product (Nikitina et al. 2022). Fermentation using local *Lactobacillus* starters can produce superior products in terms of titratable acidity and flavor profile, making them more suitable to local tastes (Adedayo and Abdulkareem 2024).

This is supported by the widespread presence of *Lactobacillus* species, which were first isolated from milk and have since been identified in cow's milk and other dairy products (Taye et al. 2021), various fermented foods (Vasyliuk et al. 2014), fermented beverages (Susan et al. 2020), as well as kefir grains (Zanirati et al. 2015; Yerlikaya 2019; Plessas et al. 2020). Their ubiquity highlights their important role in fermentation processes across diverse dairy matrices. Milk, or milk-related products, especially fermented milk products, are a good source of probiotics and are GRAS (Generally Recognized as Safe). Kefir contains a symbiotic mixture of complex LAB and yeast

(Zanirati et al. 2015). The microbial diversity in grain kefir is influenced by several factors, including the origin of the microbiota, its maintenance, and storage conditions (Schwan et al. 2016). Milk is the main ingredient in grain kefir fermentation. The bacterial and yeast communities in grain kefir produced from two different types of milk show that the genus *Lactobacillus* is the most dominant bacterium in both samples. However, the bacterial diversity in goat milk grain kefir is higher (Sumarmono et al. 2023).

However, studies specifically investigating the unique characteristics and potential of LAB isolated from Indonesian kefir grain remain limited. The isolates obtained by Wulansari et al. (2023), namely *Lactobacillus plantarum* (Orla-Jensen, 1919) Bergey et al. 1923 strains BP and DI, and *Lactiplantibacillus plantarum* A strain ES, represent indigenous starter cultures with promising probiotic properties adapted to local conditions. This highlights the importance of exploring Indonesian kefir grains as a valuable source of novel starter cultures that may offer distinct advantages in fermented milk production compared to internationally recognized species.

MATERIALS AND METHODS

Research material

Fresh cow milk from Tasikmalaya City's As-Salam Agrobusiness Farming Group was used in this study. LAB derived from kefir grains subcultured with goat milk used in this study were *Lactobacillus plantarum* strains BP and DI, and *Lactiplantibacillus plantarum* strain ES, which were selected based on the previous research outcomes (Sumarmono et al. 2023; Wulansari et al. 2023).

LAB purification

The LAB purification process was meticulously carried out. One ose of bacterial culture was transferred into a test tube containing MRSB (Merck, Germany) medium that had been sterilized using an autoclave at 121°C with 15 psi pressure for 15 minutes. The LAB culture was then inoculated into MRSB medium and incubated at 37°C for 12 hours (Oh et al. 2000).

Procedure for the preparation of starter culture

A total of 10% (v/v) of LAB inoculum was inoculated into 100 mL of 18% (b/v) skim milk (Lactona, PT. Mirota). This specific concentration of skim milk was chosen for its ability to support the growth of the LAB cultures. The mixture was then incubated at 37°C for 12-18 hours until curd formed. The skim milk was sterilized first at 110°C with 13 psi pressure for 10 minutes. The curd produced after incubation was called a mother starter. Mother starter is inoculated into sterile 18% (b/v) skim milk as much as 10% (v/v), then incubated for 12-18 hours to produce bulk starter. The bulk starter is then inoculated into the milk to be fermented, or if it is not used immediately, it can be stored at 10°C until it is used (Ustunol et al. 2014).

Fermentation of milk

The fermentation process was conducted with utmost care and thoroughness. To achieve 18% total solids, skim milk powder was added to fresh milk and pasteurized for 10 minutes at 85°C. Following chilling, a 10% (v/v) culture of *Lactobacillus plantarum* strain BP, a 10% (v/v) culture of *Lactobacillus plantarum* strain DI, and a 10% (v/v) culture of *Lactiplantibacillus plantarum* strain ES were injected into 1 liter of heat-treated milk. After eight hours of fermentation at 37°C, the products were kept at 4°C. The product was stored and tested at 0, 10, and 20 days of storage.

Assessment of pH value

The pH value was measured with a Hanna pH-meter with calibration in pH 7 and pH 4 buffers (Hanna Instruments, Romania).

Total LAB

The total LAB count in kefir products was carried out by the Total Plate Count (TPC) method. This method was chosen for its ability to provide a comprehensive count of all viable bacteria in the sample. The media used was MRSA (Merck, Germany) at as much as 68.2 g/L, and then ketoconazole (OGBdexa) was added as an anti-yeast. A NaCl solution was made using 0.85% NaCl. Dilution was done using 1 mL of sample put into 9 mL of NaCl solution.

FFA analysis

FFA analysis was done by putting 15 mL of the sample into a 250 mL Erlenmeyer tube. A neutralized ethanol solvent is added to the Erlenmeyer. The sample solution is then added with 1 mL of phenolphthalin indicator. The sample is titrated with 0.1 N NaOH until the color is pink and lasts 30 seconds (Sudarmadji and Haryono 1984).

Acidity

The acidity level was measured using the titration method with NaOH (Merck, Germany) and phenolphthalein as an indicator (Merck, Germany), and the results were expressed as the percentage of lactic acid.

Water content analysis

Water content analysis using the drying method (Thermogravimetric). Water content testing starts with 2 mL of kefir sample put into a cup weighed in mass, then oven at 105°C for 18 hours. After the oven, the cup was put into a desiccator until the initial room temperature. Moisture content is calculated by the mass lost divided by the initial mass multiplied by 100% (AOAC 1995)

Data analysis

Multivariate analysis of variance was performed on the data, and using SPSS 16.0, Duncan's new multiple range test was used to check for differences ($\alpha = 0.05$). Graphic production was carried out using GraphPad Prism v9 (GraphPad Software, San Diego, California, USA).

RESULT AND DISCUSSION

The main finding of this study is that the pH of fermented milk significantly decreases during storage. The data presented reflect the pH values of three strains, namely *Lactobacillus plantarum* strains BP and DI, and *Lactiplantibacillus plantarum* strain ES, at three storage time intervals: 0, 10, and 20 days. The results showed that the pH value of fermented milk tended to decrease with increasing storage duration, with the highest pH value recorded at 0 days (4.35 ± 0.05), which decreased to 3.68 ± 0.5 at 20 days. *Lactobacillus plantarum* strain BP showed the highest pH value at all measurement times, followed by *Lactiplantibacillus plantarum* strain ES and *Lactobacillus plantarum* strain DI. Statistical analysis shows that the starter cultures used have significant differences (P<0.05) that affect changes in pH. In addition, the duration of storage also contributes significantly (P<0.05) to the decrease in pH, where the pH value at 20 days of storage is lower than at 0 and 10 days of storage. These pH changes over the storage periods of 0, 10, and 20 days are illustrated in Figure 1, with detailed data provided in Table S1.

Figure 2 shows the variation in total LAB in fermented milk produced using various starter cultures during storage (Table 1). The total LAB, measured in log CFU/g, showed significant differences (P<0.05) based on the starter culture type and storage duration. In *Lactobacillus plantarum* strain BP, the initial number of LAB was recorded at 7.73 ± 0.24 log CFU/g and increased to 8.85 ± 0.04 log CFU/g after 20 days of storage. The ES strain of *Lactiplantibacillus plantarum* maintained a relatively constant number of LAB, with a total of 8.36 ± 0.2 log CFU/g after 10 days of storage and only a slight decrease to 7.87 ± 0.53 log CFU/g after 20 days of storage. Meanwhile, the DI strain of *Lactobacillus plantarum* increased from 8.11 ± 0.36 log CFU/g at the beginning of storage to 8.89 ± 0.03 log CFU/g at the end of the storage period.

The FFA content in fermented milk produced using various starter cultures during the storage period is shown in Figure 3 and Table 1. The data obtained show a significant difference (P<0.05), which is influenced by the type of culture and duration of storage. All strains show an increase in FFA levels during the storage period. The BP strain of *Lactobacillus plantarum* increased FFA from 5.61 ± 0.02% on day 0 to 5.83 ± 0.03% on day 20. The ES strain of *Lactiplantibacillus plantarum* experienced an increase from 4.82 ± 0.03% at the beginning of storage to 5.33 ± 0.03% at the end of the storage period. Meanwhile, the DI strain of *Lactobacillus plantarum* increased from 4.44 ± 0.04% to 5.72 ± 003% during the same period.

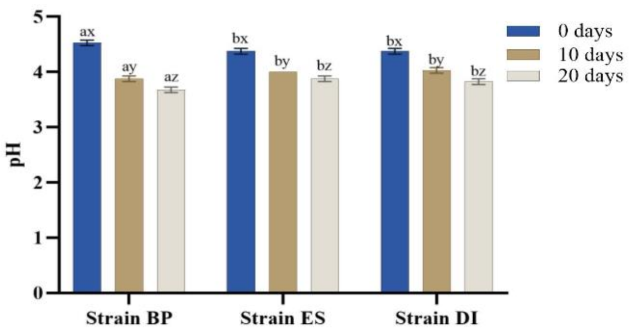

Figure 1. Changes in pH of fermented milk using different starter cultures during storage. Values are expressed as mean ± standard deviation (P<0.05) for triplicate analysis. [a,b] Different superscript indicated P<0.05 for the type of starter used; [x, y, z] Different superscript indicated P<0.05 for the storage duration

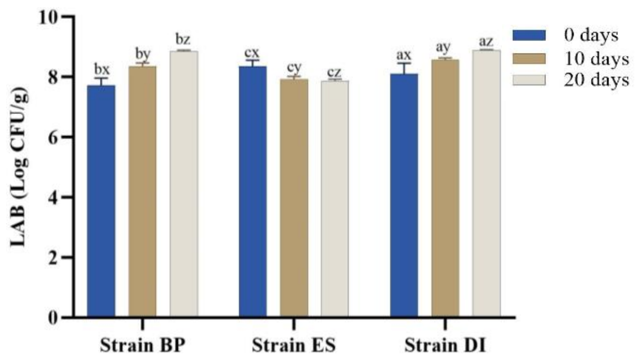

Figure 2. LAB counts (log cfu/g) of fermented milk using different starter cultures during storage. Values are expressed as mean ± standard deviation (P<0.05) for triplicate analysis. [a,b,c] Different superscript indicated P<0.05 for the type of starter used; [x, y, z] Different superscript indicated P<0.05 for the storage duration

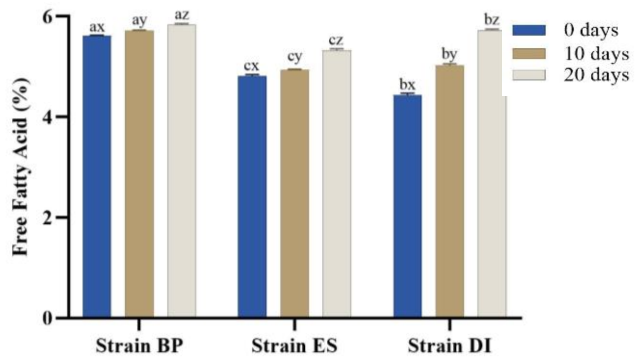

Figure 3. Free fatty acids (%) of fermented milk using different starter cultures during storage. Values are expressed as mean ± standard deviation (P<0.05) for triplicate analysis. [a,b,c] Different superscript indicated P<0.05 for the type of starter used; [x, y, z] Different superscript indicated P<0.05 for the storage duration

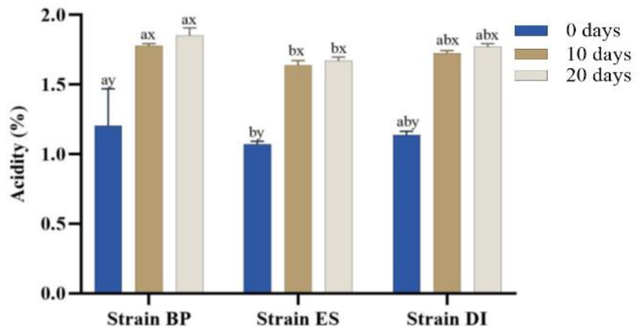

Figure 4. Acidity (%) of fermented milk using different starter cultures during storage. Values are expressed as mean ± standard deviation (P<0.05) for triplicate analysis. [a,b,c] Different superscript indicated P<0.05 for the type of starter used; [x, y, z] Different superscript indicated P<0.05 for the storage duration

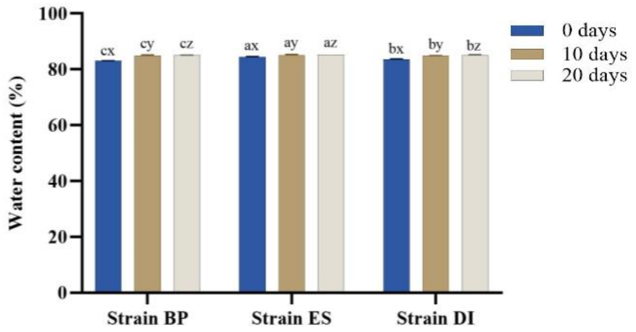

Figure 5. Water content (%) of fermented milk using different starter cultures during storage. Values are expressed as mean ± standard deviation (P<0.05) for triplicate analysis. [a,b,c] Different superscript indicated P<0.05 for the type of starter used; [x, y, z] Different superscript indicated P<0.05 for the storage duration

Table 1. Physicochemical properties of fermented milk using different starter cultures during storage

Starter	Storage (days)	pH	LAB Log (CFU/g)	Free fatty acid (%)	Acidity (%)	Water content (%)
Lactobacillus	0	4.53 ± 0.05^{ax}	7.73 ± 0.24^{bx}	5.61 ± 0.02^{ax}	1.21 ± 0.26^{ay}	83.01 ± 0.13^{cx}
plantarum strain	10	3.88 ± 0.05^{ay}	8.36 ± 0.11^{by}	5.71 ± 0.02^{ay}	1.78 ± 0.02^{ax}	84.91 ± 0.32^{cy}
BP	20	3.68 ± 0.5^{az}	8.85 ± 0.04^{bz}	5.83 ± 0.03^{az}	1.85 ± 0.06^{ax}	85.05 ± 0.12^{cz}
Lactiplantibacillus	0	4.38 ± 0.05^{bx}	8.36 ± 0.2^{cx}	4.82 ± 0.03^{cx}	1.07 ± 0.02^{by}	84.48 ± 0.13^{ax}
plantarum strain	10	4 ± 0^{by}	7.92 ± 0.1^{cy}	4.94 ± 0.02^{cy}	1.64 ±0.03^{bx}	85.03 ± 0.31^{ay}
ES	20	3.88 ± 0.05^{bz}	7.87 ± 0.53^{cz}	5.33 ± 0.03^{cz}	1.67 ± 0.03^{bx}	85.27 ± 0.02^{az}
Lactobacillus	0	4.38 ± 0.05^{bx}	8.11 ± 0.36^{ax}	4.44 ± 0.04^{bx}	1.14 ± 0.03^{aby}	83.64 ± 0.12^{bx}
plantarum strain	10	4.03 ± 0.05^{by}	8.57 ± 0.07^{ay}	5.03 ± 0.03^{by}	1.73 ± 0.02^{abx}	84.88 ± 0.11^{by}
DI	20	4.75 ±0.24^{bz}	8.89 ± 0.03^{az}	5.72 ± 0.03^{bz}	1.77 ± 0.02^{abx}	85.52 ± 0.25^{bz}

Note: Values are expressed as mean ± standard deviation (p<0.05) for triplicate analysis. Values are expressed as mean ± standard deviation (p < 0.05) for triplicate analysis. [a,b,c] Different superscript indicated P<0.05 for the type of starter used; [p, q] Different superscript indicated P<0.05 for the storage duration

The data show that the type of starter culture and the duration of storage have a significant effect (P<0.05) on the acidity of fermented milk products (Figure 4, Table 1). The BP strain of *Lactobacillus plantarum* has an initial acidity level of 1.21 ± 0.26% at 0 days, which increases to 1.85 ± 0.06% at 20 days. *Lactiplantibacillus plantarum* strain ES showed the same pattern, with an initial acidity of 1.78 ± 0.02%, which decreased to 1.67 ± 0.03% at the end of the storage period. Meanwhile, the DI strain of *Lactobacillus plantarum* showed the highest acidity level, starting at 1.14 ± 0.03% on day 0 and increasing to 1.77 ± 0.02% on day 20.

The results of the analysis show that the type of starter culture and the duration of storage have a significant effect (P<0.05) on the stability of the moisture content of fermented milk (Figure 5; Table 1). On day 0, all starter cultures showed the highest water content. After 10 days of storage, fermented milk using *Lactobacillus plantarum* BP strain and *Lactiplantibacillus plantarum* ES strain starter cultures experienced a slight decrease in water content, while *Lactobacillus plantarum* DI strain remained stable. However, after 20 days of storage, fermented milk with *Lactobacillus plantarum* BP and DI strain starter cultures showed a more significant decrease.

Discussion

The results of this study showed that the type of starter culture and the duration of storage have a significant effect on the physicochemical characteristics of fermented milk products. These findings directly answer the research question posed in the introduction, namely, how the variation in starter culture and length of storage affect the quality of fermented milk. The studies collectively demonstrate that starter culture type and storage duration significantly influence the physicochemical, microbiological, and sensory properties of fermented milk products. Different starter combinations affect acidification, proteolysis, and lipolysis in yogurt-like products (Li et al. 2020). Storage time impacts texture, pH, and water-holding capacity (Ziarno et al. 2023). Local and commercial starters show similar viability during fermentation and cold storage (Pato et al. 2020). The addition of prebiotics like fructose and oligofructose can enhance bacterial populations and sensory qualities (Zielińska et al. 2021). Starter cultures improve microbiological quality and sensory characteristics in white cheese (Salih and Abdalla 2020). The type of milk used also affects the final product's properties (Septianti et al. 2020). Furthermore, different starter cultures influence the fatty acid profiles and rheological properties of fermented goat milk products (Shunekeyeva 2021). These findings provide valuable insights for optimizing fermented milk product quality and shelf life.

The data obtained distinct performance patterns among the starter cultures (strain BP, strain ES, and strain DI) with respect to the key parameters such as pH stability, total LAB counts, FFA levels, acidity, and moisture content during storage. Specifically, strain DI exhibited the lowest pH values, indicating higher acid production and better fermentation activity; strains BP and DI showed an increase in total LAB over time, suggesting robust bacterial growth; meanwhile, strain ES maintained relatively stable but lower LAB count. Additionally, FFA levels increased progressively in all strains but were most pronounced in samples fermented with strain DI. Acidity trends followed similar patterns, with strains BP and DI showing increased acidity during storage, while strain ES showed a slight decrease. Moisture content decreased gradually across all samples regardless of the starter culture used. This emphasizes the importance of choosing the right starter culture to ensure flavor development and microbiological stability (Ayivi and Ibrahim 2022). Flavor development, especially in the sensory aspects, including taste and aroma, is closely related to the composition and characteristics of the selected starter culture (Tian et al. 2017; Ayivi and Ibrahim 2022).

Starter cultures play a crucial role in food fermentation, influencing flavor development, sensory properties, and microbiological stability (Vinicius De Melo Pereira et al. 2020; Lee et al. 2024). The selection of appropriate starter cultures is essential for enhancing taste, aroma, and overall quality of fermented products (Zang et al. 2020; Dan et al. 2022). Metabolite profiling and sensory analysis techniques can help assess the impact of starters on flavor perception (Lee et al. 2024). Microbial succession during fermentation contributes to the formation of various flavor compounds, with different microorganisms responsible for specific metabolic roles (Yang et al. 2021). The use of mixed-species starter cultures can improve flavor profiles in fermented foods (Dan et al. 2022). Additionally, starter culture development for novel fermented foods requires integrating traditional knowledge with scientific approaches, considering safety, metabolic properties, and potential health benefits (Gänzle et al. 2024). Optimizing starter culture performance can lead to improved flavor formation and product quality (Akpi et al. 2020; Nugroho et al. 2021).

In evaluating the research results, most of the findings aligned with our initial expectations; however, some outcomes deviated from predicted trends. For instance, the BP strains of *Lactobacillus plantarum* exhibited a higher FFA concentration compared to other strains. This increase may be attributed to enhanced enzymatic activities such as lipase production, that accelerate lipid hydrolysis during fermentation and storage. Such intensified metabolic processes can lead to greater release of FFA from milk fat components, thereby influencing both flavor development and physicochemical properties of fermented milk. Negative changes in several parameters with starter culture after 20 days of storage indicate that environmental factors and storage conditions have a significant role in product stability. In addition, the composition and type of starter culture used can also substantially affect the pH of the product, thus affecting storage stability. Cultures that produce higher levels of lactic acid tend to significantly lower the pH of the product, which in turn improves flavor safety and stability (Vogado et al. 2018). The pH value at the start of fermentation is another environmental factor affecting milk fermentation's stability. A lower pH value during fermentation can ensure a longer shelf life because acidic conditions can inhibit the growth of spoilage bacteria and pathogens (Vieira et al. 2015). Lactic Acid Bacteria (LAB) play a crucial role in milk fermentation, producing metabolites that enhance flavor, safety, and stability of dairy products (Rajta et al. 2023). LAB fermentation not only preserves milk but also enhances its nutritional value and potential health benefits (Gemechu 2015). The addition of specific probiotic strains, such as Lactobacillus plantarum, can further lower pH and increase lactic acid bacteria counts in fermented milk products (Mani-López et al. 2014; Melia et al. 2024). This finding indicates the need for stricter control of storage conditions to maintain product quality.

The results of this study are consistent with previous research findings that show that starter cultures significantly affect milk quality. Various starter cultures can substantially affect the fatty acid profile in fermented milk products. The study noted that certain LABs can even synthesize folate during fermentation, increasing fermented milk's nutritional value (Czarnowska-Kujawska and Paszczyk 2021). These findings show that the presence of starter cultures affects sensory properties and the functional benefits offered. The selection of different culture strains in this research was based on their unique advantages relevant to fermented milk production. Specifically, the *Lactobacillus plantarum* strains BP and DI, as well as *Lactiplantibacillus plantarum* strain ES, were chosen due to their proven probiotic potential and ability to enhance fermentation efficiency. These strains exhibit strong acidification activity, which contributes to desirable pH reduction and flavor development. Additionally, they demonstrate resilience during storage by maintaining viable cell counts and producing beneficial metabolites such as organic acids and free fatty acids that improve the sensory qualities and shelf life of fermented milk products. Therefore, utilizing these specific starter cultures offers both functional health benefits and improved product stability. Different culture strains can impact the texture and flavor profile of the product, so the selection of strains must consider the desired final characteristics of the product (Priyashantha et al. 2019). This finding is in line with the hypothesis that the production of exopolysaccharides (EPS) by certain strains can improve taste and viscosity in the mouth, leading to more attractive products to consume (Han et al. 2016; Asensio-Vegas et al. 2018). In addition, culture starters contribute to the fermentation process and can improve the nutritional profile of dairy products by increasing the availability of essential nutrients and producing beneficial metabolites (Roopashri et al. 2023).

Based on the results obtained, the hypothesis that starter culture and storage duration significantly influence the acceptable quality of milk fermentation has been confirmed. This study demonstrates that *Lactobacillus plantarum* strain DI consistently exhibited superior performance across key parameters (e.g., lower pH, higher total LAB, lower FFA) throughout the 20-day storage period, indicating its suitability for producing high-quality fermented milk with extended shelf life. Therefore, we recommend strain DI as the optimal starter culture for this application. The findings highlight the critical importance of selecting appropriate starter cultures and implementing optimal storage management to achieve high-quality fermented milk products with enhanced stability and sensory attributes.

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