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# Effect of Refrigerated Fermentation on Lactic Acid Bacteria Growth in Milk Kefir

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## HIGHLIGHTS

- Low-temperature fermentation maintained LAB viability above probiotic threshold throughout 72-hour incubation.
- Kefir fermented at 10 °C showed reduced acidification and improved sensory characteristics.
- The 50% grain concentration resulted in the lowest pH and highest LAB growth.
- The Gaussian kinetic model best represented the LAB growth curve during low-temperature fermentation.
- Controlled fermentation offers potential for enhancing the safety and probiotic quality of milk kefir.

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***Abbreviations***
CFU=Colony Forming Unit
DPPH=2,2-diphenyl-1-picrylhydrazyl
$IC_{50}$=Half Maximal Inhibitory Concentration
LAB=Lactic Acid Bacteria
MRS= de Man, Rogosa and Sharpe
MUI=Indonesian Ulema Council
ppm= parts per million
SNI=Indonesian National Standard
TPC=Total Plate Count
UHT=Ultra-High Temperature

## ABSTRACT

**Background**: Kefir, a probiotic-rich fermented milk product, offers significant health benefits. However, its ethanol content remains a concern for halal compliance. This study assesses the impact of low-temperature fermentation on ethanol content, Lactic Acid Bacteria (LAB) viability, and antioxidant activity of milk kefir as key quality indicators relevant to halal compliance and potential health benefits.

**Methods**: Milk kefir was fermented at 2–10 °C using varying starter concentrations (5–50%) and analyzed for pH, LAB viability, ethanol content (determined by iodometric titration), and antioxidant activity (using the 2,2-diphenyl-1-picrylhydrazyl [DPPH] method). Sensory properties were assessed using a hedonic quality test with 15 semi-trained panelists. Statistical analysis was conducted using analysis of variance (ANOVA) with Tukey's post hoc test ($p<0.05$), and LAB growth kinetics were modeled using Gaussian regression.

**Results**: Fermentation at low temperatures (2–10 °C) for 72 h reduced ethanol levels (<0.5%), ensuring compliance with halal certification standards while maintaining optimal LAB growth ($10^7$ Colony Forming Unit [CFU]/ml) and strong antioxidant activity (Half Maximal Inhibitory Concentration [$IC_{50}$] = 1.7586 parts per million [ppm]). The Gaussian kinetic model best represented LAB growth dynamics ($R^2$ = 0.948). The optimized kefir formulation with 50% starter concentration exhibited enhanced sensory properties and favorable probiotic characteristics.

**Conclusion**: In summary, low-temperature fermentation offers a practical approach to better control ethanol formation while maintaining LAB viability and enhancing antioxidant activity. These improvements support the production of kefir with more favorable quality attributes relevant to halal compliance and product acceptability; however, broader health-related claims should be confirmed in future studies through targeted metabolite profiling and expanded functional assays.



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## Introduction

Fermented dairy products are widely recognized for their probiotic benefits, offering modulation of the gut microbiota, enhancement of immune function, and improvements in metabolic health (Kaur *et al.*, 2022; Okoniewski *et al.*, 2023; Widyastuti *et al.*, 2021). Milk kefir, a fermented milk product rich in Lactic Acid Bacteria (LAB) and yeasts, is commonly consumed as a probiotic beverage with documented health-promoting properties. These products are associated with therapeutic effects and a range of functional attributes (Egea *et al.*, 2022; Ganatsios *et al.*, 2021; John and Deeseenthum, 2015; Yilmaz *et al.*, 2022).

The biodiversity of microorganisms in kefir produces various types of probiotics that are beneficial to health. In addition, milk kefir contains a variety of chemical components, including metabolites from fermentation reaction products, such as lactic acid, amino acids, Short-Chain Fatty Acids (SCFAs), butyric acid, ethanol, and several minerals and vitamins (Apalowo *et al.*, 2024; Tingirikari *et al.*, 2024). It is well established that the large number and variety of probiotics and chemical components in kefir directly or indirectly determine its properties and benefits (Apalowo *et al.*, 2024; Egea *et al.*, 2022). In the present study, we did not quantify individual fermentation metabolites in detail; instead, we focused on ethanol content, antioxidant activity, and LAB counts as measurable indicators related to kefir quality, halal acceptability, and potential probiotic benefits.

Fermented milk produced by a consortium of microorganisms exhibits therapeutic properties due to its composition, which contains probiotic bacteria such as Lactobacillus and Bifidobacterium. Probiotics contained in kefir are beneficial for people with lactose intolerance. These bacteria inhibit pathogenic microorganisms, exhibit antibacterial, antifungal, and antiviral properties, have anticarcinogenic effects, and modulate the immune system (Nampoothiri *et al.*, 2017; Tingirikari *et al.*, 2024; Windayani *et al.*, 2020).

However, one challenge in kefir production is its ethanol content, which results from yeast-driven fermentation (Awasti and Anand, 2020). The ethanol concentration varies and can exceed the permissible limit of 0.5%, posing a concern for halal certification and certain dietary restrictions. Optimizing fermentation conditions to reduce ethanol levels while maintaining kefir's probiotic and functional properties is therefore crucial. This is quite reasonable because the Indonesian Ulema Council (MUI) fatwa No. 10 of 2018 on food and alcoholic beverages states that only fermented drinks with an ethanol content of less than 0.5% can be declared halal. In contrast, studies on kefir have reported alcohol levels varying between 0.59-2% (Fitrianingsih *et al.*, 2022; Windayani *et al.*, 2019). Although there have never been reports that kefir is intoxicating, kefir cannot be categorized as khamr.

Naturally, in the milk fermentation process using kefir probiotics, ethanol is always produced due to the presence of yeast of the *Saccharomyces* spp. type, a probiotic component of kefir, which functions to process glucose resulting from the breakdown of lactose by LAB, such as Lactobacillus (Kurniadi and Frediansyah, 2017; Sharma *et al.*, 2020). This is one of the critical points for the halalness of fermented products using microbes (Atma *et al.*, 2018). Although the product's halal status can be declared through self-declaration, for consumer peace of mind, a halal certificate from the authorized regulator, in this case, BPJPH (Halal Product Guarantee Organizing Agency), serves as a guarantee of the product's halal status based on a halal fatwa from the MUI. Therefore, efforts are needed to reduce the ethanol content of fermented kefir products in accordance with the MUI fatwa. This effort can be carried out biologically (Gökırmaklı *et al.*, 2023), physically (Abou Ayana and Saber, 2016), or chemically (Güzel-Seydim *et al.*, 2016).

Although kefir is widely described as a functional food in the literature, the current work specifically examines low-temperature fermentation effects on ethanol reduction, LAB viability, and antioxidant capacity, without a comprehensive profiling of other fermentation metabolites. Several experiments on making milk kefir with the aim of limiting the amount of alcohol produced have been reported using variations in the type of milk, the amount of starter, the length of fermentation time, and variations in the temperature of kefir during post-harvest storage (Abou Ayana and Saber, 2016; Setiawati *et al.*, 2021; Windayani *et al.*, 2019). However, to date, there have been no reports on the results of fermentation at low temperatures or post-harvest heating treatments. Therefore, this study investigates how fermenting milk kefir at temperatures ranging from 2 to 10 °C affects its microbial stability, ethanol content, antioxidant capacity, and sensory attributes, with the aim of developing a nutrient-dense, halal-compliant functional food.

## Materials and methods

### *Collection of raw milk*

The study consisted of two experimental phases. The first phase was a preliminary optimization step using Ultra-High Temperature (UHT) milk to determine the ideal kefir grain concentration for fermentation at low temperatures. Kefir was prepared with varying starter concentrations (5%, 10%, 25%, and 50%) and evaluated for pH reduction and sensory acceptability. The 50% concentration was identified as optimal and used in the second phase.



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In the second phase, fresh milk from four different local dairy farms (Cijambu, Cilengkrang, Cilembu, and Pangalengan) was fermented using 50% kefir grains. The effects of milk source and storage duration on LAB viability were evaluated. Additionally, pH and total titratable acidity were measured to explore their correlation with LAB counts.

In the preliminary phase of the study, UHT cow's milk conforming to Indonesian National Standards (SNI) was used to determine the optimal kefir grain concentration. This was done to ensure a consistent milk matrix and minimize compositional variability during starter optimization.

The raw material for cow's milk obtained from cattle farms around Mount Manglayang, Bandung Regency, is selected according to the quality criteria of cow's milk, as specified in SNI for fresh cow's milk. Before use, fresh cow's milk was characterized for pH, temperature, alcohol test, and organoleptic properties. It was then pasteurized using the Bain- Marie method at 65 °C for 15 s. Milk samples were collected from four farms: Cijambu Farm, Pangalengan Farm, Cilembu Farm, and Cilengkrang Farm.

*Collection of kefir grains*

Kefir grains were obtained from the Integrated Laboratory UIN Sunan Gunung Djati Bandung's culture collection. To determine the type and quantity of bacteria that comprise the grain, this kefir grain was previously identified at the Microbiology Laboratory of Padjajaran University, Jatinangor, Sumedang, Indonesia.

*Preliminary test determination of the addition of kefir grain starter in milk kefir production at low temperature*

Milk samples were pasteurized using the Bain-Marie High-Temperature Short Time (HTST) method at 72 °C for 15 s and then cooled to approximately 25–27 °C before inoculation. All glassware, fermentation jars, physiological NaCl solution (Merck, Germany), stir bars, and de Man, Rogosa and Sharpe (MRS) agar media (Merck, Germany) were sterilized in an autoclave at 121 °C for 15 min prior to use.

The preliminary fermentation was adapted from Windayani *et al*. (2019) with several modifications. A total of 100 ml of cow's milk was inoculated with kefir grains at concentrations of 5%, 10%, 25%, and 50% and incubated at 4–10 °C for 24, 48, and 72 h, with stirring every 12 h. Based on preliminary optimization, the 50% kefir grain concentration was selected for further analysis. For this treatment, 50 g of kefir grains were mixed with 100 ml of cow's milk in a sterile fermentor jar and fermented at 10 °C for 72 h. The experiment was conducted in triplicate. After fermentation, kefir grains were separated using a sterile stainless-steel sieve with a 2 mm pore size, and the filtrate was used as milk kefir for subsequent analyses.

*Measurement of the degree of acidity, pH*

pH was recorded using a calibrated pH meter (Hanna Instruments, USA) to measure the acidity of kefir throughout the fermentation process, and after the results had been stored for a specific amount of time. pH was recorded at the 2, 10, 18, 24, 48, and 72 h using a calibrated pH meter.

*Organoleptic test and hedonic quality of milk kefir*

The hedonic test was conducted using the method described in (Lestari *et al.*, 2018), where three-digit codes were randomly assigned to minimize subjective judgments. A total of 15 semi-trained panelists participated in the sensory evaluation. The panelists were final-year undergraduate students (10 females and 5 males, aged 20–22 years). All panelists were familiar with fermented dairy products and had no history of lactose intolerance or dairy allergies. Twenty ml of the kefir sample were used for the test, and the sensory characteristics evaluated were taste, aroma, color, texture, and overall acceptance of the optimal milk kefir, resulting in a preference scale of 1 to 4. Randomized, single-blind testing was conducted to prevent panelist influence.

*Determination of the alcohol content of low-temperature-fermented milk kefir*

The alcohol content test was conducted using the iodometric titration method (Halim *et al.*, 2023), with the following materials used: a solution of $Na_2S_2O_3$ (Merck, Germany), $K_2Cr_2O_7$ (Merck, Germany), KI (Merck, Germany), starch (Sigma-Aldrich, USA), and $H_2SO_4$ (Merck, Germany). Samples were collected at the end of each fermentation period (24, 48, and 72 h) for ethanol analysis. The iodometric titration method for alcohol determination was validated using a 0.3% (v/v) ethanol standard solution (Merck, Germany) prior to sample analysis. The validation test yielded an average measured value of 0.28±0.01%, corresponding to a recovery rate of 93.3%, which was considered acceptable for the purposes of this study.

Ethanol content was determined by iodometric titration method using the AOAC Official Method 920.57, which has been validated for alcohol quantification in fermented beverages (AOAC International, 2019).

*Determination of the number of LAB using the Total Plate Count (TPC) method*

LAB growth was monitored at 6-hour intervals, with sampling performed at 0, 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, and 72 h of fermentation. Bacteria were enumerated using the pour plate method on MRS agar. One ml of kefir



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filtrate was serially diluted ($10^{-1}$ to $10^{-6}$) using 0.9% physiological NaCl solution. One ml from each dilutions $10^{-3}$, $10^{-4}$, $10^{-5}$, and $10^{-6}$ was transferred into sterile petri dishes, followed by the addition of 10 ml of warm MRS agar media. The dilution process was repeated three times, and the samples were incubated at 37°C for 24 h.

After incubation, colonies were counted using a colony counter (Labtron, UK). Plates with 25–250 colonies were selected for calculation. The bacterial count was expressed as Colony Forming Unit (CFU)/ml using the following formula:

Number of bacteria (CFU/ml) = number of colonies × 1/dilution factor

All TPC calculations were performed in triplicate to ensure data accuracy (Joni *et al.*, 2018).

*Antioxidant activity test*

2,2-Diphenyl-1-Picrylhydrazyl (DPPH, D9132-1G, Sigma-Aldrich, USA) is used in vitro to measure antioxidant activity. Milk kefir's activity on the DPPH radical was reported by Liu *et al*. (2005), where 200 mg of kefir was dissolved in 5 ml of methanol, thoroughly vortexed, and centrifuged. Then, 1.0 ml of the supernatant, 1.0 ml of 0.16 mM DPPH, and 3.0 ml of methanol were added. After vigorously shaking the mixture and allowing it to stand in a dark room for 30 min, the absorbance was measured at 517 nm. The ability to inhibit DPPH radicals was calculated as follows:

% Inhibition= (1-[absorbance of sample at 517 nm])/(absorbance control at 517 nm) ×100

Ascorbic acid was used as the positive control, generating a standard curve (Figure 1) with linear regression equation y = 0.696x + 36.286 ($R^2$ = 0.9749). The Half Maximal Inhibitory Concentration ($IC_{50}$) value, defined as the concentration required to inhibit 50% of DPPH radicals, was calculated from the linear regression of percent inhibition versus sample concentration.

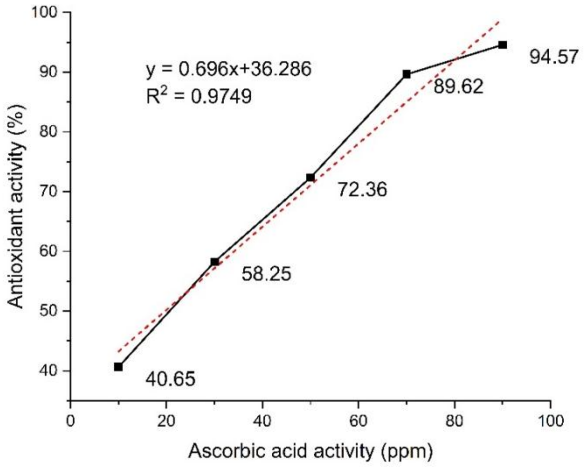


**Figure 1:** Ascorbic acid standard curve for 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant activity assay

*Statistical analysis*

All experiments were performed in triplicate, and the results are presented as mean ± Standard Deviation (SD). One-way analysis of variance followed by Tukey's post hoc test was used to determine significant differences among treatments ($p$<0.05). Analyses were performed using SPSS Statistics version 26 and Microsoft Excel 2021.

To model the fermentation kinetics of LAB and TPC, data were fitted using CurveExpert Professional version 2.7. Several nonlinear regression models (exponential, logistic, and Gaussian) were evaluated, and model selection was based on the coefficient of determination ($R^2$) and visual inspection of residuals. The Gaussian model, which yielded the highest $R^2$ (0.948) and best agreement between predicted and observed data, was selected to describe LAB growth dynamics during low-temperature fermentation.

## Results and discussion

*Effect of kefir grain concentration on pH changes during low-temperature fermentation*

The pH value is a critical parameter in assessing the quality and safety of fermented dairy products. Kefir develops its characteristic sour taste through lactose hydrolysis by LAB during fermentation. In this study, pH was measured using a pH meter at 2, 10, 18, 24, 48, and 72 h of fermentation. The pH test was conducted to evaluate the acidification rate of kefir fermented at low temperature (10 °C) using varying starter concentrations: 5%, 10%, 25%, and 50% (w/v). UHT cow's milk conforming to SNI standards was used as the fermentation substrate. The pH changes during fermentation are presented in Figure 2.

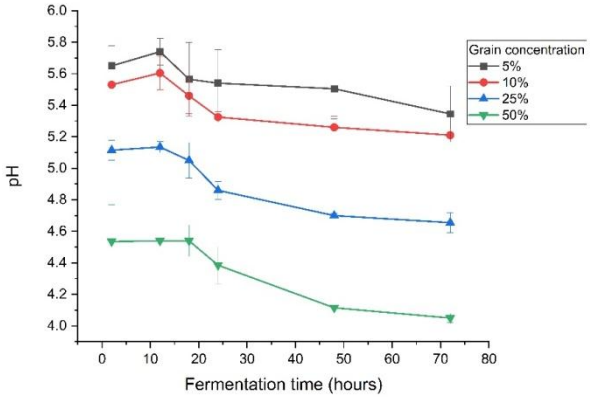


**Figure 2:** Changes in pH during fermentation at low temperatures at grain concentrations of 5%, 10%, 25%, and 50%

The results showed a decreasing pH trend across all treatments, with final pH values ranging from 5.7 (5% starter) to 4.07 (50% starter) at 72 h (Figure 2). According to Ilyin *et al*. (2023), the optimal pH range for fermented



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beverages that support intestinal microflora is 3.65–4.4. Based on this criterion, the 50% starter concentration at 72 h of incubation produced kefir with the most suitable acidity level for consumption.

The pH reduction observed in this study (from 6.5 to 4.07 at 50% starter) aligns with previous findings reporting that kefir pH typically stabilizes between 4.0–4.5 during fermentation (Gökırmaklı *et al.*, 2023; Plessas *et al.*, 2017). However, the acidification rate in the present study was notably slower compared to conventional mesophilic fermentation (20–25 °C), where similar pH values are typically achieved within 24 h (Alves *et al.*, 2021). This slower acidification at low temperature (10 °C) may be attributed to reduced metabolic activity of LAB, which require longer incubation periods to achieve comparable acidity levels.

Furthermore, the higher starter concentration (50%) compensated for the reduced fermentation rate at low temperature, resulting in adequate acidification within 72 h. This finding is consistent with Gul *et al.* (2015), who reported that increasing kefir grain concentration accelerates pH reduction due to higher initial microbial load. The gradual acidification observed in low-temperature fermentation may contribute to improved sensory attributes and sustained LAB viability compared to rapid acidification at higher temperatures (De Sainz *et al.*, 2025).

The 72 h fermentation period was selected to capture the complete acidification profile under low-temperature conditions, as the reduced metabolic activity of LAB at refrigerated temperatures (10 °C) necessitates longer incubation compared to conventional mesophilic fermentation. Nevertheless, for industrial applications, shorter fermentation times (24–48 h) may be feasible depending on targeted product specifications, as key quality parameters approached acceptable levels before 72 h in the present study.

*Organoleptic test and hedonic quality of milk kefir*

The organoleptic test results and hedonic quality of milk kefir fermented at low temperature are presented in Table 1. Statistical analysis showed that color scores were not significantly different ($p>0.05$) among all treatments, indicating that starter concentration did not affect the visual appearance of kefir. All samples exhibited a white color typical of fermented milk products. In contrast, taste, aroma, texture, and overall acceptability scores differed significantly ($p<0.05$) among treatments, with scores increasing proportionally to starter concentration. Kefir fermented with 50% starter received the highest scores for all sensory parameters, characterized by balanced acidity, distinctive kefir aroma, and thick texture.

**Table 1:** Organoleptic test results for milk kefir products at low-temperature fermentation

| Parameter | Mean | | | |
|---|---|---|---|---|
| | 5% Grains | 10% Grains | 25% Grains | 50% Grains |
| Color | 2.13±0.143 [a] | 2.00±0.577 [a] | 1.93±0.542 [a] | 2.00±0.153 [a] |
| Taste | 1.60±0.193 [a] | 2.20±0.862 [b] | 2.80±0.640 [c] | 3.87±0.638 [d] |
| Aroma | 1.20±1.74 [a] | 1.87±0.810 [b] | 2.73±0.707 [c] | 3.80±0.781 [d] |
| Texture | 1.13±0.926 [a] | 1.93±0.862 [b] | 2.60±0.781 [c] | 4.00±0.490 [d] |
| Acceptability | 2.33±0.455 [a] | 2.80±0.569 [b] | 2.80±0.569 [b] | 3.73±0.490 [c] |

Values are expressed as mean ± Standard Deviation (SD). Different superscript letters (a, b, c, d) within the same row indicate significant differences ($p<0.05$).

The higher acceptability of kefir fermented with 50% starter concentration can be attributed to several factors. First, the higher initial microbial load accelerates fermentation even at low temperatures, resulting in adequate acidification and flavor compound production. Second, the thick texture observed in the 50% treatment may be due to increased Exopolysaccharide (EPS) production by LAB, which is known to improve mouthfeel and viscosity in fermented dairy products. These findings align with recent studies by Soutelino *et al.* (2025), who demonstrated that starter concentration significantly influences texture and mouthfeel in kefir beverages. Similarly, Abou Ayana *et al.* (2025) reported that controlled fermentation conditions enhance overall sensory acceptance by achieving an optimal balance between sourness and creaminess.

It should be noted that this study did not include a direct comparison with kefir fermented at conventional mesophilic temperatures (20–25 °C). Therefore, while the 50% starter treatment at low temperature produced kefir with acceptable sensory characteristics, further comparative studies are needed to determine whether low-temperature fermentation specifically contributes to improved sensory attributes compared to standard processing conditions.

*Determination of alcohol content of low-temperature fermented milk kefir*

Alcohol content was measured to evaluate the halal compliance of milk kefir produced through low-temperature fermentation. During kefir fermentation, yeasts metabolize sugars through alcoholic fermentation, producing ethanol as a by-product. According to halal certification standards, the permissible ethanol content in



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fermented beverages is ≤0.5%. Table 2 presents the alcohol content of milk kefir fermented at different temperatures (2 °C, 10 °C, and 25 °C) over 24, 48, and 72 h.

**Table 2:** Alcohol content of milk kefir at several fermentation temperatures

| No | Fermentation Time (h) | Alcohol Content (%) | | |
|---|---|---|---|---|
| | | 2 (°C) | 10 (°C) | 25 (°C) |
| 1. | 24 | 0.35 [a] | 0.37 [a] | 0.59 [b] |
| 2. | 48 | 0.35 [a] | 0.34 [a] | 0.64 [b] |
| 3. | 72 | 0.35 [a] | 0.40 [a] | 0.73 [b] |

Values represent single measurements. Different superscript letters within the same row indicate significant differences among fermentation temperatures at $p<0.05$. Halal compliance threshold: ≤0.5% ethanol.

The results showed that fermentation at low temperatures (2 °C and 10 °C) produced milk kefir with alcohol content ranging from 0.34–0.40%, which remained significantly below the 0.5% halal threshold throughout the 72 h fermentation period. In contrast, fermentation at 25 °C resulted in significantly higher ethanol content (0.59–0.73%), exceeding the halal limit after 24 h. Statistical analysis confirmed that alcohol content at 2 °C and 10 °C was significantly lower ($p<0.05$) than at 25 °C across all fermentation periods.

Ethanol formation in kefir is primarily determined by yeast metabolism, which varies according to fermentation conditions. The present study confirms that low-temperature fermentation (2–10 °C) effectively suppresses yeast metabolic activity, resulting in ethanol levels consistently below 0.5%. This finding represents a notable improvement compared to conventional kefir fermentation at 25–30 °C, where ethanol levels frequently exceed 0.7% (Magalhães *et al.*, 2011). Simova *et al*. (2002) reported that yeast activity intensifies at higher temperatures, leading to increased ethanol accumulation. The results of this study suggest that lowering the fermentation temperature effectively minimizes yeast metabolism while still supporting LAB proliferation, a crucial factor in maintaining kefir's probiotic function without compromising its halal status.

The implications of reduced ethanol content extend beyond halal compliance. Lower ethanol levels make kefir more appealing to broader consumer groups, including individuals who avoid alcohol for dietary or health reasons. This aligns with the findings of Liu *et al*. (2014), who explored alternative fermentation strategies to produce alcohol-free dairy probiotics without diminishing their functional benefits. Additionally, the reduction in ethanol content enhances the microbiological stability of kefir, as excessive yeast activity can lead to over-carbonation and spoilage during storage.

Low-temperature fermentation (2–10 °C) significantly reduces yeast metabolic activity by inhibiting key glycolytic and fermentative enzymes, including pyruvate decarboxylase (PDC), which is essential for converting pyruvate to acetaldehyde in the ethanol production pathway (Ciani *et al.*, 2016). At psychrotrophic temperatures, yeast cell membrane fluidity decreases, leading to reduced substrate uptake and enzyme efficiency (Kourkoutas and Proestos, 2020). Furthermore, low temperatures favor the growth of psychrotrophic LAB over mesophilic yeasts, thereby altering the LAB/yeast ratio in favor of LAB dominance (Blasche *et al.*, 2021). This microbial shift results in enhanced lactic acid production and reduced ethanol synthesis, which is beneficial for producing low-alcohol or halal-compliant fermented beverages while maintaining probiotic functionality.

It is acknowledged that direct enumeration of yeast populations was not performed in this study, which represents a limitation in fully understanding the microbial dynamics during low-temperature kefir fermentation. The low ethanol content observed at 2–10 °C indirectly suggests suppressed yeast metabolic activity, as ethanol is the primary end-product of yeast fermentation (Plessas *et al.*, 2017). Future studies should incorporate yeast enumeration using selective media such as Yeast Extract Glucose Chloramphenicol (YGC) agar to provide a more comprehensive understanding of the LAB/yeast population dynamics and their correlation with ethanol production in low-temperature fermented kefir (Juan *et al.*, 2022).

*Determination of the number of LAB using the TPC method*

The viability of LAB in kefir is a crucial factor in maintaining its probiotic properties. The TPC data were processed to determine the growth kinetics of LAB, and the results are shown in Figure 2.

To analyze the fermentation kinetics of LAB in milk kefir, the TPC data were fitted using CurveExpert Professional version 2.7. Given the non-linear nature of bacterial growth over 72 h at low temperatures, several curve models were evaluated. Among them, the Gaussian model provided the best fit for the observed data, particularly in capturing the peak bacterial count at 48 h, followed by a decline. This model was selected because it effectively represents the growth and decline pattern typical of microbial fermentation, corresponding to the biological phases of lag, exponential growth, and death.

While the Gompertz and logistic models are traditionally used in microbial kinetics, the Gaussian model applied in this study better captured the biphasic growth pattern observed during low-temperature fermentation. This finding aligns with recent perspectives on microbial growth modeling (Fernández-Martínez *et al.*, 2024), which emphasize that non-monotonic growth patterns in stressed environments often require alternative mathematical



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approaches beyond classical sigmoidal models (Ribeiro and Cunha, 2025). The superior fit of the Gaussian model in our study demonstrates the importance of selecting appropriate kinetic models based on specific fermentation conditions rather than relying solely on conventional approaches.

The predicted curve aligned well with experimental data, as shown in Figure 2. This approach provides a more realistic representation of bacterial dynamics than a simple monotonic model

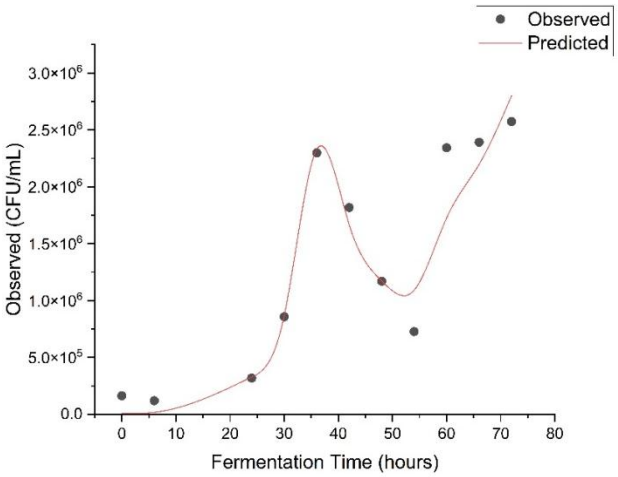


**Figure 2:** Growth kinetics of Lactic Acid Bacteria (LAB) in fermented cow's milk kefir
CFU=Colony Forming Unit

Figure 2 shows the growth dynamics of LAB during 72 h of fermentation. An initial lag phase occurred between 0–6 h, where bacterial growth was minimal as cells adapted to the new environment. This was followed by an exponential growth phase from 6–24 h, characterized by a rapid increase in cell density due to favorable nutrient and environmental conditions. From 24–42 h, growth entered the stationary phase, indicating a balance between cell division and death, likely due to nutrient depletion and accumulation of inhibitory metabolites. A decline in viable cells was observed between 42–54 h, suggesting the onset of the death phase.

Interestingly, between 60–72 h, a secondary increase in viable LAB counts was observed. This atypical resurgence may result from the reactivation of previously stressed or dormant cells, release of nutrients from lysed cells, or adaptive responses to prolonged stress conditions (Kim *et al.*, 2024; Papadimitriou *et al.*, 2016). The fitted prediction model (red line) was able to capture these complex dynamics, including the late-stage increase, suggesting that LAB behavior under extended fermentation may not strictly follow classical growth patterns.

Importantly, LAB counts remained consistently above $10^7$ CFU/ml throughout fermentation, which exceeds the minimum threshold ($10^6$ CFU/ml) for probiotic functionality, as reported by Garofalo *et al*. (2015). This suggests that kefir fermented under cold conditions still provides beneficial microbial content (Garofalo *et al.*, 2015).

Additionally, the results support previous findings by Hsieh *et al*. (2022), indicating that low fermentation temperatures slow down metabolic activity, minimizing rapid acidification that would otherwise lead to decreased bacterial viability. Fermentation conditions and subsequent storage strongly influence acid accumulation and probiotic survival in milk kefir; lactic acid levels up to around 2.4 g/100 ml and pH near 4.3 still support high cell viability, whereas excessive acidification can reduce probiotic survival and sensory quality (Pourbaba *et al*., 2022). In contrast, low-temperature fermentation helps preserve the viability of LAB, resulting in a milder, less sour sensory profile. Dong *et al*. (2020) similarly observed that probiotic dairy products fermented at lower temperatures showed greater microbial stability and improved gastrointestinal survival.

The biphasic (bimodal) growth pattern observed in LAB populations during low-temperature kefir fermentation can be attributed to several interacting factors. Firstly, cold stress induces a lag phase adaptation where LAB undergo physiological adjustments, including the expression of Cold Shock Proteins (CSPs) and modifications in membrane fatty acid composition to maintain fluidity at low temperatures. Following this adaptation period, LAB resumes active growth, resulting in the observed secondary growth phase. Secondly, microbial interactions between LAB and yeast play a significant role in shaping population dynamics. In kefir ecosystems, LAB and yeast exhibit both competitive and mutualistic relationships. Yeasts produce vitamins, amino acids, and hydrolytic enzymes that support LAB growth, while LAB acidification creates favorable conditions for certain yeast species (Blasche *et al.*, 2021). The initial decline in LAB populations may correspond to nutrient competition with yeast, followed by a resurgence as yeast activity diminishes at low temperatures and secondary metabolites become available. Additionally, the pH drop during fermentation may initially stress LAB populations before acid-tolerant strains dominate the second growth phase (Wang *et al.*, 2021)

These findings highlight the potential advantages of low-temperature fermentation, including maintaining microbial viability and improving consumer acceptance. Future studies should investigate how different fermentation temperatures impact the long-term survivability of LAB during storage and their persistence in the gastrointestinal tract after consumption.

This study focused primarily on the viability of LAB and pH dynamics during low-temperature fermentation. However, yeast populations and key metabolites such as



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acetic acid and propionic acid, which contribute significantly to alcohol formation and flavor development, were not analyzed. Future studies should include these parameters to provide a more comprehensive understanding of the fermentation process and its influence on sensory attributes.

*Antioxidant activity test*

The antioxidant activity of low-temperature fermented milk kefir was evaluated using the DPPH radical scavenging assay, with ascorbic acid as the positive control. The $IC_{50}$ values, representing the concentration required to inhibit 50% of DPPH radicals, are presented in Table 3. Lower $IC_{50}$ values indicate stronger antioxidant activity.

**Table 3:** Antioxidant activity half maximal inhibitory concentration ($IC_{50}$) of milk kefir from four farms

| Samples | Milk Source | Antioxidant Activity (ppm) | Antioxidant Strength |
|---|---|---|---|
| P1 | Cijambu Farm | 0.5862 | Very Strong |
| P2 | Pangalengan Farm | 8.9310 | Very Strong |
| P3 | Cilembu Farm | 2.0344 | Very Strong |
| P4 | Cilengkrang Farm | 1.7586 | Very Strong |

Values represent single measurements. $IC_{50}$<50 ppm = very strong antioxidant; 50–100 ppm = strong; 100–150 ppm = moderate; >150 ppm = weak (Molyneux, 2004)
ppm=parts per million

All kefir samples exhibited very strong antioxidant activity, with $IC_{50}$ values ranging from 0.59 to 8.93 Parts per million (ppm, Table 3). Notably, all samples demonstrated stronger antioxidant activity than ascorbic acid ($IC_{50}$=19.07 ppm), which served as the positive control. Among the four milk sources, kefir produced from Cijambu Farm milk (P1) showed the lowest $IC_{50}$ (0.59 ppm), indicating the highest antioxidant capacity, while Pangalengan Farm milk (P2) showed the highest $IC_{50}$ (8.93 ppm). Despite this variation, all values remained within the "very strong" antioxidant category ($IC_{50}$<50 ppm) according to the classification by Molyneux (2004).

The variation in antioxidant activity among milk sources may be attributed to differences in the initial composition of raw milk, including protein content, fatty acid profile, and endogenous antioxidant compounds such as vitamins and minerals. However, as noted by Priyashantha *et al.* (2021) and Vitali *et al.* (2025), variations in milk origin generally have minimal impact on antioxidant capacity when fermentation conditions are standardized, which is consistent with our observation that all samples exhibited very strong antioxidant activity regardless of milk source.

The strong antioxidant activity observed in low-temperature fermented kefir can be explained by several mechanisms. First, during fermentation, LAB produce bioactive peptides through proteolysis of milk proteins, particularly caseins, which have been shown to exhibit radical scavenging properties (Guzel-Seydim *et al.*, 2011). Second, organic acids produced during fermentation, including lactic acid and acetic acid, contribute to the overall antioxidant capacity by chelating pro-oxidant metal ions (Fiorda *et al.*, 2017). Third, low-temperature fermentation may preserve heat-sensitive antioxidant compounds that would otherwise degrade at higher temperatures. Jiang *et al.* (2021) reported similar findings, demonstrating that low-temperature fermentation in dairy products preserves functional peptides and their associated antioxidant effects more effectively than conventional fermentation processes.

Compared to previous studies, the antioxidant activity of low-temperature fermented kefir in this study ($IC_{50}$=0.59–8.93 ppm) was considerably stronger than that reported for conventionally fermented kefir. Sadewi *et al.* (2023) reported an $IC_{50}$ value of 43.99 ppm for goat milk kefir curd fermented at room temperature, which was categorized as a very strong antioxidant. Similarly, Azizi *et al.* (2021) in their comprehensive review reported $IC_{50}$ values around 52.71 µg/ml for rice milk kefir. The substantially lower $IC_{50}$ values observed in the present study suggest that low-temperature fermentation may contribute to enhanced antioxidant compound preservation; however, direct comparison is limited by differences in milk sources, kefir grain composition, and assay conditions across studies.

The results of the DPPH assay ($IC_{50}$=1.7586 ppm) suggest that low-temperature fermented kefir exhibits enhanced antioxidant activity, indicating the preservation of bioactive compounds including peptides, organic acids, and polyphenols. This finding aligns with the research of Guzel-Seydim *et al.* (2011), who reported that kefir contains a range of antioxidant compounds that contribute to reducing oxidative stress. The enhanced antioxidant potential observed in this study may be attributed to the higher retention of peptides, as lower temperatures slow proteolysis, allowing bioactive peptides to accumulate over time. Similar observations were made by Jiang *et al.* (2021), who demonstrated that low-temperature fermentation in dairy products preserves functional peptides and their associated antioxidant effects more effectively than conventional fermentation processes.

It is acknowledged that the specific bioactive compounds responsible for the observed antioxidant activity were not individually quantified in this study. Future research should employ techniques such as HPLC-MS to identify and quantify peptides, organic acids, and other antioxidant compounds in low-temperature fermented kefir to better understand the mechanisms underlying its antioxidant potential.

*Limitations*

This study has several limitations that warrant



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acknowledgment. First, the sensory evaluation used a 4-point hedonic scale, which may provide lower sensitivity compared to the more commonly used 5-point or 9-point scales. Although this scale was sufficient to detect relative preference differences among treatments, future studies should employ a broader hedonic scale to enhance the precision of sensory data. Although enumeration was carried out using MRS agar, which is a selective medium favoring the growth of LAB, it is important to acknowledge that colony counts on MRS agar may still include occasional non-LAB microorganisms able to tolerate the medium (Blasche *et al.*, 2021; Wang *et al.*, 2021). Therefore, while the results better reflect LAB populations compared to TPC on general media, they may not represent the absolute numbers of all LAB species present

## Conclusion

This study demonstrates that low-temperature fermentation at 2–10 °C using a 50% kefir grain inoculum can produce milk kefir with ethanol content below the halal compliance limit of 0.5%, while maintaining LAB viability above the probiotic threshold and providing acceptable sensory characteristics. The fermentation process gradually reduced pH, supported dynamic LAB growth, and limited ethanol accumulation more effectively than fermentation at 25 °C. Milk kefir produced under low-temperature conditions also showed very strong antioxidant activity, with $IC_{50}$ values ranging from 0.59 to 8.93 ppm, suggesting the potential preservation of bioactive compounds during refrigerated fermentation. Overall, low-temperature fermentation enables the production of milk kefir with high functional value, clear halal compliance, and superior antioxidant properties compared to standard fermentation processes. Future research should investigate in more detail how low temperatures affect the composition and function of bioactive components, as well as compare these outcomes directly with traditional kefir to further support these findings.

## Author contributions

R.B.S., N.W., and T.C. contributed to the conception and design of the study; R.B.S. was responsible for data collection; R.N.H. performed data analysis and interpretation; N.W. and T.J. drafted the manuscript; All authors critically revised the article for important intellectual content, approved the final version to be submitted, and agreed to be accountable for all aspects of the work.

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## Conflicts of interest

The authors declare that there is no conflict of interest.

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## Ethical consideration

Not Applicable.

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