Evaluation of viability and chemical properties of fermented black rice-milk alternatives

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Abstract. This study investigates the development of fermented black rice-based milk alternatives, focusing on microbial viability, chemical properties, and nutritional potential. Seven formulations with varying concentrations of black rice extract (10%–100%) and full cream milk were fermented using *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. LAB viability was highest in full cream milk (P1), decreasing from 10.5 log CFU/mL on day 0 to 9.4 log CFU/mL on day 30. In contrast, 100% black rice extract (P2) showed the lowest viability, declining from 9.47 log CFU/mL to 8.7 log CFU/mL. The pH of all formulations decreased over 30 days, with the most stable pH observed in P3 (10% black rice extract) at 4.23 ± 0.02 and the lowest in P7 (50% black rice extract) at 3.76 ± 0.01. Titratable acidity increased during storage, with P1 showing the highest rise from 0.30 ± 0.02% to 0.42 ± 0.02%, while P2 exhibited the smallest change, increasing from 0.17 ± 0.02% to 0.29 ± 0.05%. These findings highlight the influence of black rice extract concentration on the viability of LAB and the chemical properties of fermented milk alternatives. Future research should explore sensory evaluations, consumer acceptance, and the scalability of black rice-based milk fermentation for commercial production. Additionally, examining the impact of alternative fermentation techniques on nutritional and bioactive properties would provide further insights.

1 Introduction

Bovine milk is well-known for its excellent nutritional profile, being a rich source of calcium, protein, and fat. It also contains other key nutrients such as iron, vitamin A, B vitamins, vitamin D, magnesium, phosphorus, amino acids, and vitamin E [1]. One of its main components is lactose, a sugar that serves as an important energy source. However, some people cannot consume cow's milk due to lactose intolerance. This has driven the rise of plant-based milk alternatives made from various plant sources [2].

In recent years, plant-based milks have gained significant popularity, especially in the United States and Europe. The global market for these alternatives is valued at over $9 billion and is expected to grow even further in the coming years. In Europe alone, the market reached 1.6 billion Euros in 2020, showing their growing demand [3]. While plant-based milk is often seen as a nutritional substitute for cow's milk, it usually doesn’t offer the same health benefits or satiety. To address this, manufacturers often fortify plant-based drinks with vitamins and

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amino acids. Additionally, some plant-based proteins are harder for the body to digest. This challenge is mainly due to the presence of anti-nutritional compounds, like lectins and protease inhibitors in nuts, tubers, and legumes, as well as tannins in cereals and nuts [4]. Fortunately, food processing methods can help reduce these compounds. Techniques such as autoclaving and microwave heating are commonly used to eliminate heat-sensitive anti-nutrients. However, some of these compounds are heat-resistant and require other strategies. Fermentation is one effective method for breaking down these stubborn anti-nutrients [5].

Rice milk, made from various rice varieties, is a popular alternative to animal-based milk, particularly for individuals with lactose intolerance or allergies to cow's milk protein [6]. Rich in phosphorus, magnesium, and potassium, it supports immune function, offers protective benefits against cancer, and enhances resistance to pathogens [7]. Compared to other non-dairy alternatives, rice milk has a notably low lipid-to-protein ratio, making it an ideal choice for those managing cholesterol levels or experiencing digestive challenges with protein-rich foods [6].

Fermentation improves the nutritional properties of food while also providing protection against spoilage. Metabolites and bioactive compounds produced by fermenting microorganisms can effectively inhibit the growth of spoilage-causing pathogens [8]. Although rice has been extensively studied as a food ingredient, its application in the production of fermented plant-based milk, particularly using black rice, remains insufficiently explored. Research on the fermentation of black rice-based milk alternatives production is limited, with scarce information available regarding its effects on the viability of microorganisms and the chemical characteristics of the final product. Therefore, the aim of this research was to investigate the effects of black rice milk concentration on fermentation of rice-based milk, with a focus on its microbial viability, quality attributes, shelf stability, and nutritional composition.

2 Materials and methods

2.1 Materials

The materials used in this research included De Man, Rogosa, and Sharpe (MRS) Agar and MRS Broth, NaCl, full cream milk, black rice, sugar, bacterial cultures of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, distilled water, sodium hydroxide (NaOH), and phenolphthalein.

2.2 Preparation of bacterial culture

The preparation of a starter culture involves two stages: developing the primary starter culture and producing a working starter culture. The primary starter culture was prepared using bacterial strains identified on MRS Agar (MRSA). Each bacterial culture of *Lactobacillus bulgaricus* (LB) and *Streptococcus thermophilus* (ST) was inoculated into sterile De Man, Rogosa, and Sharpe Broth (MRSB) using an inoculating loop. The inoculated MRSB was then incubated at 37 °C for 24 hours. After incubation, the primary starter culture was used to prepare the working starter culture. For this, 0.5 mL of the primary bacterial culture was added to 10 mL of full cream milk. Additionally, up to 5% of each LB and ST bacterial culture grown in MRSB was introduced into the milk medium. The mixture was incubated at 37 °C for another 24 hours. At the end of this incubation period, the milk was ready to be used as a working starter culture [9].

2.3 Preparation of fermented black rice-based milk alternatives

To produce black rice yogurt, the process began with the preparation of black rice extract. Black rice was boiled until fully cooked, weighed, and combined with sterile water at a ratio of 1:3. The mixture was thoroughly blended using a hand blender until smooth, then filtered to obtain the black rice extract [10]. The yogurt production process started by preparing a milk control solution, which involved mixing full cream milk with 100% black rice extract. Additional formulations were prepared by combining black rice extract with full cream milk in varying proportions of 10%, 20%, 30%, 40%, and 50%. Each mixture was heated at 90 °C for 30 minutes, during which 10% sugar was added to each formulation. After heating, the mixtures were cooled to 45 °C. At this point, 5% starter cultures of *Lactobacillus bulgaricus* (LB) and *Streptococcus thermophilus* (ST) were added to each formulation. The mixtures were then allowed to ferment at 37 °C for 24 hours, resulting in the final black rice based-milk alternatives [11].

2.4 Analysis of viability of lactic acid bacteria in black rice-based milk alternatives

The viability test for lactic acid bacteria (LAB) was conducted using serial dilutions. To prepare the first dilution, 1 mL of the yogurt sample was added to a test tube containing 9 mL of sterile 0.85% NaCl solution. The mixture was vortexed, and 0.1 mL of the solution was taken for subsequent dilutions, continuing up to the eighth dilution (10^{-8}). For each dilution, 2 mL of the sample was added to a petri dish, which was then half-filled with MRS Agar (MRSA). The petri dishes were incubated at 37 °C for 48 hours. After incubation, the total number of bacterial colonies on the MRS Agar plates was counted using a Funke Gerber colony counter. Total Plate Count (TPC) testing was conducted on days 0, 3, 6, 14, and 30. Colony counts were recorded for petri dishes containing between 25 and 250 colonies, with results expressed as log colony-forming units per mL (log CFU/mL) [12].

2.5 Analysis of pH and titratable acidity

For pH analysis, a calibrated portable pH meter was used to measure the fermented black rice based-milk alternatives. Total titratable acidity (TAT) analysis was performed using approximately 10 mL of the yogurt sample placed in an Erlenmeyer flask. Three drops of phenolphthalein were added as an indicator. The sample was then titrated with 0.1 N NaOH until a pink color appeared, indicating the endpoint of the titration.

2.6 Statistical analysis

Results were expressed as mean ± standard deviation. Data were analyzed using ANOVA followed by a post hoc test, with a significance level set at 5% ($p < 0.05$).

3 Results and discussion

3.1 Viability of LAB in fermented black rice-based milk alternatives

The data in Table 1 shows the viability of lactic acid bacteria (LAB), expressed as log CFU/mL, in yogurt formulations with varying concentrations of black rice extract (P1–P7) over a storage period of 30 days. LAB viability decreases over time across all formulations, with the most significant decline observed between days 14 and 30. P1, the whole milk control, consistently demonstrates the highest LAB viability, starting at 10.5 log CFU/mL on day 0 and ending at 9.4 log CFU/mL on day 30. Conversely, P2, made with 100% black rice extract, exhibits the lowest LAB viability, starting at 9.47 log CFU/mL and declining to 8.7 log CFU/mL by day 30.

Table 1. Viability of LAB of fermented black rice-based milk alternatives

Storage (days)	**Viability (log CFU/mL)**						
	P1	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**
0	10.5	9.47	10.24	10.16	10.15	10.11	10.06
3	10.29	9.35	10.23	10.13	10.11	10.06	10.02
6	10.21	9.29	9.51	9.48	9.43	9.39	9.37
14	9.29	9.21	9.43	9.39	9.35	9.31	9.27
30	9.4	8.7	9.14	9.01	8.98	8.96	8.72

Notes: P1 (full cream milk); P2 (Black rice extract 100%); P3 (black rice extract 10%); P4 (black rice extract 20%); P5 (black rice extract 30%); P6 (black rice extract 40%); P7 (black rice extract 50%)

Formulations with partial black rice extract (P3–P7) show that as the concentration of black rice extract increases, the initial LAB viability decreases slightly, and the rate of decline over time becomes more pronounced. For instance, higher black rice extract concentrations (P6 and P7) have lower LAB counts and faster reductions in viability compared to lower concentrations (P3 and P4). Despite this, all formulations maintain measurable LAB viability even at 30 days, ranging from 8.7 log CFU/mL (P2) to 9.4 log CFU/mL (P1). Overall, these results suggest that black rice extract influences LAB growth and survival, with higher extract concentrations leading to reduced viability, especially over prolonged storage. The higher viability in milk-dominant formulations is primarily due to the presence of lactose, the key energy source for LAB, which is absent in rice extracts [13]. Additionally, the nutrient composition of milk and rice water contributes differently to LAB metabolism. While rice water may provide some nutrients, it lacks the sufficient energy-rich components like lactose found in milk, leading to lower bacterial growth and survival rates in formulations with higher rice content. This explains the relatively low LAB viability in 100% rice water samples compared to mixed formulations containing milk.

3.2 pH value of fermented black rice-based milk alternatives

Table 2 presents the pH values of fermented black rice-based milk alternatives over storage intervals of 0, 3, 6, 14, and 30 days for different formulations (P1 to P7). In general, statistical results showed that the pH levels decline significantly across all formulations as storage progresses, reflecting a rise in acidity caused by microbial fermentation. On day 0, P3 (10% black rice extract) had the highest initial pH (4.61 ± 0.01), followed by P4 (20% black rice extract) at 4.50 ± 0.01, while P2 (100% black rice extract) showed the lowest pH (3.56 ± 0.03). Full cream milk (P1) exhibited a moderate initial pH of 4.11 ± 0.01. As storage progressed, all formulations experienced a steady decline in pH. By day 30, P3 still maintained the highest pH (4.23 ± 0.02), while P7 (50% black rice extract) showed the lowest (3.76 ± 0.01). Formulations with higher black rice extract concentrations (P5, P6, and P7) exhibited lower pH values throughout the storage period, indicating that increased black rice extract contributes to greater acidity. In contrast, P1 (full cream milk) demonstrated the most stable pH, decreasing slightly from 4.11 ± 0.01 on day 0 to 3.91 ± 0.01 on day 30.

Table 2. pH value of fermented black rice-based milk alternatives

Storage (days)	pH						
	P1	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**
0	4.11 ± 0.01^{c}	3.56 ± 0,03^{a}	4.61 ± 0,01^{f}	4.50 ± 0,01^{e}	4.31 ± 0,00^{d}	4.13 ± 0,01^{c}	3.99 ± 0.01^{b}
3	4.07 ± 0.00^{c}	3.48 ± 0.01^{a}	4.61 ± 0.01^{g}	4.42 ± 0.00^{f}	4.23 ± 0.01^{e}	4.09 ± 0.00^{d}	3.99 ± 0.01^{b}
6	4.02 ± 0.00^{c}	3.47 ± 0.00^{a}	4.50 ± 0.01^{g}	4.38 ± 0.01^{f}	4.20 ± 0.00^{e}	4.07 ± 0.01^{d}	3.97 ± 0.00^{b}
14	3.95 ± 0.00^{c}	3.29 ± 0.01^{a}	4.23 ± 0.01^{f}	4.12 ± 0.01^{e}	4.00 ± 0.00^{d}	3.88 ± 0.01^{c}	3.72 ± 0.01^{b}
30	3.91 ± 0.01^{d}	3.46 ± 0.02^{a}	4.23 ± 0.02^{f}	4.03 ± 0.01^{e}	3.94 ± 0.01^{d}	3.83 ± 0.03^{c}	3.76 ± 0.01^{b}

Notes: P1 (full cream milk); P2 (Black rice extract 100%); P3 (black rice extract 10%); P4 (black rice extract 20%); P5 (black rice extract 30%); P6 (black rice extract 40%); P7 (black rice extract 50%)

The pH changes in yogurt over a 30-day storage period demonstrate a progressive decrease, indicating acidification during storage. For example, the pH of milk-based yogurt decreased from 4.11 on day 0 to 3.91 on day 30, while the pH of the P5 formulation (50% rice water: 50% milk) declined from an initial value of 4.00 to 3.70 over the same period. This reduction in pH is primarily attributed to the sustained metabolic activity of lactic acid bacteria, which continues to produce lactic acid during storage, leading to an accumulation of acidity and a corresponding decline in pH. The significant changes observed in the pH values of rice-based yogurt formulations over the storage period underscore this phenomenon [14]. Additionally, the initial pH of the rice water component may exert an influence on the pH stability of the yogurt throughout storage [15].

3.3 Titratable acidity of fermented black rice-based milk alternatives

Table 3 illustrates the titratable acidity (%) progression in fermented black rice-based milk alternatives over a 30-day storage period. All formulations exhibited a gradual rise in titratable acidity over time, highlighting ongoing fermentation activity. For instance, the titratable acidity of P1 (full cream milk) increased from 0.30 ± 0.02% on day 0 to 0.42 ± 0.02% on day 30. Likewise, P2 (100% black rice extract) showed an increase from 0.17 ± 0.02% to 0.29 ± 0.05%, while P3 (black rice extract 10%) rose from 0.23 ± 0.03% to 0.39 ± 0.01% during the same period.

Table 3. pH value of fermented black rice-based milk alternatives

Storage (days)	Titratable Acidity (%)						
	P1	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**
0	0.30 ± 0.02^{c}	0,17 ± 0,02^{a}	0,23 ± 0,03^{ac}	0,24 ± 0,02^{bc}	0,24 ± 0,03bc	0,21 ± 0,3^{ac}	0.17 ± 0.02^{ac}
3	0.38 ± 0.03^{c}	0.23 ± 0.01^{a}	0.29 ± 0.03^{ab}	0.34 ± 0.04^{bc}	0.26 ± 0.01a	0.24 ± 0.03^{a}	0.25 ± 0.04^{a}
6	0.39 ± 0.01^{e}	0.23 ± 0.01^{a}	0.33 ± 0.01^{c}	0.35 ± 0.01^{d}	0.32 ± 0.01c	0.26 ± 0.01^{ab}	0.26 ± 0.01^{b}
14	0.40 ± 0.01^{c}	0.23 ± 0.02^{a}	0.37 ± 0.03^{c}	0.38 ± 0.02^{c}	0.38 ± 0.01c	0.29 ± 0.01^{b}	0.27 ± 0.01^{bc}
30	0.42 ± 0.02^{d}	0.29 ± 0.05^{a}	0.39 ± 0.01^{bcd}	0.40 ± 0.01^{cd}	0.44 ± 0.01d	0.32 ± 0.01^{ab}	0.33 ± 0.01^{abc}

Notes: P1 (full cream milk); P2 (Black rice extract 100%); P3 (black rice extract 10%); P4 (black rice extract 20%); P5 (black rice extract 30%); P6 (black rice extract 40%); P7 (black rice extract 50%)

Notably, P5 (black rice extract 30%) reached the highest titratable acidity by day 30 at 0.44 ± 0.01%, whereas P7 (black rice extract 50%) demonstrated a comparatively moderate increase, ranging from 0.17 ± 0.02% to 0.33 ± 0.01%. These differences suggest that the proportion of black rice extract to milk plays a significant role in influencing the rate and magnitude of acid production over storage. The rise in titratable acidity reflects the metabolic activity of lactic acid bacteria, which convert sugars into organic acids, thereby contributing to the product's acidification. The titratable acidity value for sample P2 was lower compared to P1 and other treatment samples that combined milk and black rice extract. This lower value may be attributed to the absence of lactose in sample P2, which reduces the availability of essential nutrients required by bacteria for fermentation. Consequently, the lactic acid bacteria (LAB) in sample P2 rely entirely on the nutrients derived from rice, which may not be as supportive of bacterial activity as the lactose-rich environment found in milk-based samples [13].

4 Conclusion

This study demonstrated that black rice-based milk alternatives can be successfully fermented using *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, with varying concentrations of black rice extract influencing microbial viability, pH, and titratable acidity. Formulations with higher proportions of black rice extract showed reduced LAB viability and greater acidity compared to milk-dominant formulations. Full cream milk (P1) exhibited the most stable microbial growth and chemical properties, while 100% black rice extract (P2) showed the lowest LAB viability and highest acidity levels over 30 days of storage.

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Open Data Statement

The data obtained in this study are included within the article.

Author contributions

Andreas Romulo: conceptualization, data curation, writing, editing manuscript, analysis, project administration; Vandira Putri Qirana: formal analysis, investigation, writing.

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