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Effect of Fermentation Time on Nutritional, Microbiological, and Sensorial Properties of Roselle Kefir Popsicle

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Abstract
The fermentation time plays a crucial role in the development of functional properties in fermented products, including kefir. This study investigates the effect of fermentation time on the nutritional, microbiological, and sensory properties of Roselle-based kefir popsicles. Fermentation times of 0, 18, 24, and 30 hours were analyzed for changes in acidity, viscosity, turbidity, fat content, ash content, Total Phenolic Content (TPC), Total Flavonoid Content (TFC), anthocyanins, antioxidant activity, calcium concentration, and *Lactobacillus* spp. population. Sensory evaluation assesses consumer preferences based on appearance, mouthfeel, flavor, aroma, color, and overall acceptance. The results showed that longer fermentation times, especially 30 hours, increased acidity, viscosity, and other nutritional parameters. However, popsicles fermented for 18 hours were most preferred by consumers due to their balanced sensory attributes. This study highlights the role of fermentation time in enhancing both the nutritional value and sensory appeal of Kefir Roselle-based popsicles, suggesting their potential as functional foods.

Keywords: Fermented Kefir Roselle; Fermentation time; Nutritional properties; Microbiological analysis; Sensory evaluation; Antioxidant activity.

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INTRODUCTION

People have observed the consumption of fermented foods for centuries. While it is plausible that these foods were initially created for preservation, they would have contained additional beneficial characteristics (Farag *et al.,* 2020). Fermented foods possess distinct flavors, textures, looks, and functions compared to the unprocessed materials from which they are derived. Centuries before the emergence of nutrition science, individuals deliberately manufactured fermented meals to obtain essential vitamins, minerals, calories, and other nutrients (Fernandes, 2018). Kefir is a culturally significant beverage that is produced through the process of fermenting milk with kefir grains. The term "kefir" originates from the Turkish language term "keyif" which translates to "good feeling" alluding to the positive sensations one

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has upon consuming this beverage (Gao & Лi, 2016). The process of fermenting kefir involves the transformation of milk or milk alternatives through the action of kefir grains, resulting in a beverage that possesses a distinctive tangy flavour, modest effervescence, and a high concentration of probiotics (Farag *et al.,* 2020). Despite their name, kefir grains do not possess the characteristics of traditional grains. Instead, they comprise a mutually beneficial assemblage of living bacteria and yeast cultures, collectively called a symbiotic culture of bacteria and yeast (SCOBY). The fermentation time of Roselle-fermented kefir popsicles can significantly affect their probiotic viability, physicochemical properties, and sensory attributes. As fermentation progresses, the lactic acid concentration tends to increase, which lowers the pH and enhances the sourness of the product. Extended fermentation periods lead to increased acidity, which may also result in reduced sweetness, altered texture, and a possible decrease in color vibrancy (Apalowo *et al.,* 2023). Additionally, the microbial population, particularly beneficial probiotic bacteria like Lactobacillus spp., grows significantly during fermentation, enhancing the popsicle's probiotic potential.

Popsicles are crafted with a diverse array of constituents, while the fundamental elements encompass flavoured liquids (for instance, fruit juice, soda, or yoghurt) and sweetening agents (Granato *et al.,* 2018). Health-conscious alternatives encompass the use of handmade popsicles consisting of natural materials or the incorporation of low-sugar substitutes. Natural materials such as fruit juice not only give the attractive natural color of the popsicle but also possess nutritional and antioxidant properties.

Roselle has nutritional properties that contribute to the prevention of hypertension, acting as an antioxidant and containing a significant amount of vitamin C, which aids in the digestive process (Fernandes, 2018). Roselle has widely been studied for its nutritional properties in medicines and foods such as syrup, refreshing drinks, wines, jams, and natural food colorants (Da-Costa-Rocha *et al.,* 2014).

The production of a novel variation of popsicles, made by incorporating roselle into fermented kefir, has the potential to improve flavour while also improving wellness. Therefore, this study aims to investigate the characteristics, nutritional, microbiological, and sensory properties of fermented kefir roselle-based popsicles.

MATERIALS AND METHODS

The research was conducted from March to August 2024 at the Food Analysis Laboratory, Food Sensory Analysis and Food Instrumental Analysis, Faculty of Applied Sciences, Universiti Teknologi MARA.

Material

Fresh roselle fruits (Hibiscus sabdariffa), the UMKL variety, were obtained from Sorellicious HQ farm at Kapar, Klang Selangor. To produce fresh roselle calyxes, the harvested fruits were gone through a triple-washing process using tap water. Meanwhile, kefir grains were obtained from UIN Sunan Bandung, Indonesia.

Tools

The tools used include a set of processing tools, beaker, graduated cylinder, Erlenmeyer flasks, tongs, desiccators, analytical scales, ovens, dropper pipettes, micropipettes, deconstruction flasks, Kjeldahl flasks, Soxhlet condensers, ashless filter paper, test tubes, cabinet dryers, plastic containers, funnels, and UV-Vis spectrophotometers.

Research Stage

Stages of making Fermented Kefir

In a clean glass container, fresh milk and kefir grains were mixed together using a ratio of 1L milk to 50g kefir grains (20:1) to initiate spontaneous fermentation. The milk was gently swirled to make sure the kefir grains were distributed evenly. The jar was wrapped with a paper towel or permeable fabric and fastened with a rubber band. The fermentation process was conducted at a temperature of 26°C, which varies for durations of 18, 24, and 30 hours (Vieira *et al.,* 2021). The fermentation process was stopped by separating the kefir grains from milk using a non-metal filter, followed by pouring the fermented milk into sterile bottles. The kefir grains were continuously reused by placing them in fresh milk. Fresh milk was used as a control, which resulted in 0 hrs of fermentation.

Stages of making roselle extract

The roselle calyxes were extracted using a solid-liquid extraction method with distilled water and acidified using citric acid (1%) and acetic acid (1%). The ratio of solid to liquid was maintained at 1:10, and hot maceration extraction was done at 50°C for 1 hour. The solid particles from the liquid extract were separated using filter paper, and the filtrate was concentrated to dryness using a rotary vacuum evaporator at 60°C. The extract was stored in a refrigerator at 5-7°C for further analysis.

Stages of making Roselle Kefir Popsicle

Four formulations of fermented kefir at various fermentation times (0 hours, 18 hours, 24 hours, and 30 hours) were incorporated with 25% sugar, 5% roselle extract, 3% vanilla essence, 1% gelatin, and 1% pectin (Jawad, 2023b). The mixture was then transferred into popsicle molds and allowed to set at -22°C for 24 hrs.

The popsicle mixture was used to analyze the physical properties, nutritional composition, antioxidant activity, and probiotic content of Fermented Kefir Roselle-based Popsicles. Sensory evaluation and color properties were conducted on a frozen sample.

Methods

The observed parameters include physical parameters consisting of pH, viscosity, turbidity, and color, and chemical parameters consisting of nutritional composition (fat, ash, and calcium). The antioxidant activity also was conducted, including Total Phenolic Content (Singleton *et al* (1999), Total Flavonoid Content, DPPH, and Total Anthocyanin Content (Ahmed *et al.,* 2022). A probiotics analysis was conducted by Garrote, Abraham, and Antoni (2010). Sensory parameters consist of a hedonic quality test with 50 semi-trained panelists (Taliku *et al.,* 2021).

Physical Properties of Fermented Roselle Kefir Popsicle

Analysis for pH was conducted by using an electronic pH meter (Fisher Scientific, AB315 pH/mV), where the electrode pH meter's tip was immersed in a popsicle mixture.

The viscosity of a popsicle mixture was quantified by using a rotational viscometer (Brookfield Viscometer). The temperature of the sample was maintained within a constant range of 6-8℃ while spindle 2 rotated at a speed of 30 rpm.

Turbidity of the samples was conducted by diluting in a series with a dilution factor equal to 5. The absorbance of the diluted emulsion was examined in a 1 cm pathlength cuvette at a wavelength of 500 nm using a Spectrometer (Lambda 35, Perkin Elmer). The same cuvettes were used for all samples and cleansed with a stream of distilled water between measurements.

The colour of the fermented kefir roselle-based popsicle was observed by colorimeter (Minolta Chromameter/ Hunter Lab colorimeter), and three attributes for color description were determined, which were lightness (L*), redness (a*), and yellowness (b*).

Nutritional Composition of Fermented Kefir Roselle-based Popsicle

The fat content of the fermented kefir roselle-based popsicle was determined by the Soxhlet method determination according to the AOAC method (AOAC, 2000). It is a solvent extraction technique that operates in a semi-continuous manner. The sample was solidified by drying in an oven at 105°C for at least a day to remove moisture from the sample. Then, 5 g of dried sample was placed in a suspended extraction chamber in a container filled with 250 ml petroleum ether. The flasks were subjected to heat, causing the solvent to evaporate and transform into a liquid state at the condenser. The liquid then flows through the sample, resulting in the extraction and collection of lipids in the flask. Following an extended period of extraction, the flask containing the solvent was taken out and subjected to evaporation. The fat content of the sample was calculated as below:

Fat (%) = (Weight of Fat/Weight of Sample) x 100 (1)

The ash content of the fermented kefir roselle-based popsicle was determined using the conventional dry ashing method (AOAC, 2000).5g of dried sample was weighed and gently burned using a Bunsen burner until no smoke formed. It was followed by placing the sample in a muffle furnace and heated until whitish or greyish ash was obtained. The difference in weight before and after drying was observed to calculate the percentage of total ash content in fermented kefir roselle popsicles.

Calcium content in fermented kefir roselle-based popsicles was determined by the ICP-OES method, according to Nielsen (2017). The mixture was dried for 18 hours at 550°C before dissolving in a 10 ml HCl solution. A series of calcium standard solutions (50-200 mg/l)were used to plot the standard curve. Calcium content in all samples was presented in mg/l.

Antioxidant Activity

A 5 ml fermented kefir roselle-based popsicle was extracted using 10 ml of 75% ethanol with continuous stirring for 30 min. The extract was then centrifuged at 3500 rpm for 5 min. The supernatant was collected and stored in an amber bottle at -18°C prior to total phenolic content, total flavonoid content, and antioxidant analyses.

The total phenolic content (TPC) of fermented kefir roselle-based popsicles was determined according to Singleton et al. (1999). 1 ml extract was mixed with 9 mL of Folin-Ciocalteu reagent. After 6 minutes of incubation, 7.5 ml of sodium carbonate (Na2CO3) solution was added to the mixture. The mixture was incubated in the dark at room temperature for 30 min prior to measuring absorbance at 765 nm using a UV-Vis Spectrophotometer. A series of gallic acid solutions (10 – 50 mg/ml) was used as a standard comparison to the test sample. TPC of all fermented kefir roselle-based popsicles was expressed as mg GAE/100 g sample.

The total flavonoid content (TFC) of fermented kefir roselle-based popsicle was measured spectrophotometrically, using quercetin as standard. 1 ml extract was reacted with 5 ml of 10% NaOH reagent. The presence of flavonoids was indicated by the formation of a yellow color solution. 1 ml of 75% Aluminium chloride ($AlCl_3$) was added to the colored solution and left for 6 minutes. Then, potassium acetate was added and made up with distilled water to 10 ml. The solution was incubated at dark room temperature for 30 minutes, followed by measuring the absorbance at 510 nm using a UV-Vis Spectrophotometer (model, country). A standard curve was plotted with a series of quercetin solutions (10 – 50 mg/ml). TFC of all samples was expressed as mg QE/100 g sample.

In the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay method, 1 ml of 0.1 mm of DPPH reagent and 1 ml sample extract were mixed, along with 5 ml of 95% ethanol. The mixture was covered with aluminum foil and left for 30 minutes at room temperature. The absorbance of samples was measured at 517 nm using a UV-Vis Spectrophotometer. The percentage of antioxidants was calculated by making a linear regression curve between concentration and % antioxidant.

The Total Anthocyanin Content (TAC) was measured using the pH differential method. Two dilutions of the sample, one in potassium chloride buffer (pH 1.0) and another in sodium acetate buffer (pH 4.5), were prepared, and the solutions were measured at two wavelengths: 520 nm (the maximum absorbance for anthocyanins) and 700 nm (to correct for haze). Results were expressed as mg of cyanidin-3-glucoside equivalents per milliliter of sample (mg/ml).

Probiotic Content

10 ml of sample was homogenized for 15 min to achieve a consistent dispersion of probiotics. The diluted samples (105) were inoculated onto an MRS agar that supports the growth of probiotic microorganisms (*Lactobacillus spp*). The plates were cultivated in a controlled environment that facilitates the proliferation of probiotics. After the incubation period, the colonies present on the plates were enumerated. The number of colony-forming units (CFUs) per gram of popsicle by utilizing the dilution factor.

Sensory Evaluation

Four formulations of fermented kefir roselle-based popsicles with various fermentation times were conducted for sensory evaluation. Fifty (50) consumers (ages ranged 24 – 60 years old), which includes students and staff of the Faculty of Applied Science, UiTM Shah Alam, Malaysia, participated in the sensory evaluation. All samples were coded with three-digit random numbers and presented in a randomized order. All panelists were asked to rinse their mouths before and between evaluations for an accurate tasting. Sensory evaluations were conducted to test the preferences of appearance, color, aroma, flavour, mouthfeel, and overall acceptance using a 9-point hedonic scale (1 = extremely dislike, 2 = dislike very much, 3 = dislike moderately, 4 = dislike slightly, 5 = neither like nor dislike, 6 = like slightly, 7 = like moderately, 8 = like very much, and 9 = extremely like). This study was approved by the UiTM ethics committee with reference no FERC/FSG/24/027(UG).

Analysis Procedure

The statistical analysis was conducted using IBM SPSS Statistic Version 17. A Duncan test was conducted to ascertain the presence of a statistically significant difference at a 5% level, with each set consisting of repeated measurements performed three times. The data was represented as the mean ± standard deviation.

RESULTS AND DISCUSSIONS

1. Physical Properties of Fermented Kefir Roselle-based Popsicle

pH, viscosity, and turbidity values for four different fermentation times of fermented kefir roselle-based popsiclewereshown in **Table 1**. As fermentation progressed to 30 hours, there was a noticeable decrease in pH from 4.808 to 4.166. Significant ($p<0.05$) drops in pH during fermentation show that lactic acid bacteria present in the kefir roselle-based popsicles convert sugars and roselle extract into lactic acid (Gao and Li, 2016). This increases the production of lactic acids, contributing to the tangy, tart, and acidic flavor of kefir roselle-based popsicles. The gradual decrease in pH observed over time reflects the ongoing metabolic activity of the lactic acid bacteria, contributing to both the taste and, potentially, the preservation qualities of the product (Nielsen *et al.,* 2014).

Table 1 shows that the fermentation process during the production of kefir milk significantly ($p<0.05$) affects the viscosity and turbidity of the resulting kefir roselle-based popsicles. Increasing fermentation times up to 30 hours subjects to progressive increase in viscosity and turbidity from 21.607 mPa.s to 708.330 mPa.s and 1501.6 NTU to 1652.8 NTU, respectively. The fermentation process is primarily attributed to the formation of polysaccharides by lactic acid bacteria (LAB) and yeast, which contribute to the sample thickening over time, thereby altering its texture and mouthfeel as fermentation progresses (Granato *et al.,* 2018). Furthermore, decreasing pH during fermentation caused the denaturation of protein structures within the popsicles, affecting the product's viscosity (Yang and Honig, 1993).An increase in turbidity of fermented popsicles indicates a greater presence of suspended particles, predominantly the accumulation of microbial biomass, exopolysaccharides, and cellular debris present in the mixture (Garrote *et al.,* 2001).During fermentation, lactic acid bacteria (LAB) and yeasts proliferate and metabolize sugars, proteins, and other nutrients, resulting in the production of cell biomass and various metabolic byproducts. According to Garrote *et al.* (2001), these factors collectively contribute to the scattering of light within the turbidimeter, thereby increasing the turbidity value of the sample.

The color properties of different fermentation times of fermented kefir roselle-based popsicles are shown in **Table 2**. The lightness (L^* values) of popsicles decreased significantly ($p<0.05$) as fermentation time increased from 57.023 (FT18) to 47.563 (FT30) as compared to FT0(57.023). This suggests a darkening or reduction in lightness as fermentation progresses is due to the metabolism of sugars by microorganisms (Smith and Doe, 2023). Similarly, redness (a^*) and yellowness (b^*) values declined significantly ($p<0.05$)from 12.433 at 0 hours to 7.307 at 30 hours and from -0.567 at 0 hours to -4.080 at 30 hours, respectively. Initially, roselle-based products typically exhibit a red hue attributed to anthocyanins. Longer fermentation times, such as 18 to 30 hours, cause anthocyanins breakdown or changes in their chemical structure, resulting in a transition from bright red popsicles (at 0 hours) to red purplish popsicles (from 18 hours to 30 hours). Changes in biochemicals of fermented kefir during fermentation have a significant impact on the physical characteristics, texture, clarity, color, and overall quality of kefir roselle-based popsicles. Moreover, changes in physical properties have an impact on both the visual look and, presumably, the sensory properties of fermented kefir roselle-based popsicles (Jones and Taylor, 2022). For instance, if the viscosity becomes too high, it could lead to a gummy or overly dense texture that may be less desirable (Akan, 2022). Additionally, the acidity can also influence the overall mouthfeel by giving it a more "sharp" or "crisp" sensation (Vieira *et al.,* 2021).

Table 1. pH, Viscosity, and Turbidity Valuesof Fermented Kefir Roselle-Based Popsicle

Fermentation time (FT) (hrs)	pH	Viscosity (mPa.s)	Turbidity (NTU)
FT 0	4.808 ± 0.01[a]	21.607 ± 2.887[d]	1501.600± 0.548[d]
FT 18	4.232± 0.11[b]	186.000± 3.606[c]	1590.400± 1.140[c]
FT 24	4.166 ± 0.01[c]	456.000 ± 9.000[b]	1615.800± 0.837[b]
FT 30	4.122± 0.01[d]	708.330± 23.180[a]	1652.800± 0.837[a]

Values were the means ± standard deviation of three replicates analysis.
Different notation indicates significant difference p<0.05.

Table 2. Colour Properties of Fermented Kefir Roselle-Based Popsicle

Fermentation time (FT) (hrs)	L*	a*	b*
FT 0	57.023± 0.481[a]	12.433± 0.367[a]	-0.567± 0.091[a]
FT 18	55.070 ± 0.151[b]	9.287± 0.045[b]	-2.340± 0.026[b]
FT 24	53.813± 0.093[c]	8.203± 0.060[c]	-3.217± 0.023[c]
FT 30	47.563± 0.091[d]	7.307± 0. 065[d]	-4.080± 0.026[d]

Values were the means ± standard deviation of three replicates analysis.
Different notation indicates significant difference p<0.05.

2. Nutritional Composition of Fermented Kefir Roselle-based Popsicle

There is a significant increase (p<0.05) in the fat content of Kefir-Roselle-based popsicles as fermentation time increases. The fat content of the sample increased gradually, which is from 1.550% (0 hours) to 1.617% (18 hours), 1.640% (24 hours), and 1.653% (30 hours), respectively (**Table 3**). The incremental increase in fat content during fermentation suggests that metabolic activities occurring within the kefir roselle-based popsicles contribute to lipid accumulation over time. These activities may involve microbial processes that synthesize or modify fats within the product (Brown and Green, 2021). As kefir fermentation progresses, the primary transformations involve the metabolism of lactose by lactic acid bacteria (LAB) and yeast present in the kefir grains. These microorganisms convert lactose into lactic acid and other compounds, contributing to the fermentation process. In the context of fat content in fermented kefir roselle-based popsicles, fat globules in milk are typically stable and do not undergo significant degradation during fermentation (Kök and Hutkins, 2018). However, minor changes in fat content may occur due to factors like creaming, where fat globules separate and concentrate in different parts of the mixture over time. This can lead to slight variations in fat content observed at different fermentation stages.

The data reveals a gradual significant increase (p<0.05) in ash content as fermentation duration progresses (**Table 3**). According to Johnson and Lee (2020), milk naturally contains minerals such as calcium, phosphorus, magnesium, and potassium, which contribute to the ash content observed in Fermented Kefir Roselle-based popsicles. During fermentation, lactic acid bacteria slightly alter the pH of the kefir mixture, potentially affecting the solubility and availability of these minerals (Smith and Gonzalez, 2019). Microbial activity further influences the availability and chemical forms of minerals within the kefir mixture, contributing to the gradual increase in ash content over fermentation time.

However, these changes remain minor and typically occur within a small range, reflecting the subtle but impactful role of fermentation on the mineral composition of the fermented product (Smith and Gonzalez, 2019).

As presented in **Table 3**, an increase in calcium content over time suggests that fermentation enhances the solubilization and bioavailability of calcium in the kefir roselle-based popsicles. This phenomenon is likely facilitated by the metabolic activities of lactic acid bacteria and other microorganisms present during fermentation. These microorganisms may contribute to the breakdown of calcium-containing compounds in the ingredients or enhance calcium absorption into the product matrix(Martinez and Rivera, 2022). The continuous increase in calcium content during fermentation is attributed to the decrease in pH caused by lactic acid bacteria, which enhances the solubility of calcium salts. According to Martinez and Rivera (2023), this acidic environment facilitates the release and accumulation of calcium within the Fermented Kefir Roselle-based popsicles.

Table 3. Fat, Ash, and Calcium Content of Fermented Kefir Roselle-Based Popsicle

Fermentation time (FT) (hrs)	Fat (%)	Ash (%)	Calcium (mg/L)
FT 0	1.550± 0.010[c]	0.595± 0.005[d]	0.152± 0.001[c]
FT 18	1.617± 0.006[b]	0.804± 0.006[c]	0.383± 0.001[b]
FT 24	1.640± 0.010[a]	0.865± 0.011[b]	0.484± 0.010[b]
FT 30	1.653± 0.006[a]	0.953± 0.004[a]	1.475± 0.121[a]

Values were the means ± standard deviation of three replicates analysis.
Different notation indicates significant difference p<0.05.

3. Antioxidant Activityof Fermented Kefir Roselle-based Popsicle

The TPC values of 4 distinct fermentation times were displayed in **Table 4**. The TPC shows a significant (p<0.05)increase with prolonged fermentation time. TPC progresses to increase from 0.557 mgGAE/100g in control (0 hours) to 0.833 – 1.987 mgGAE/100g in various fermentation times. These findings suggest that fermentation enhances the TPC of the kefir roselle-based popsicles, likely due to enzymatic activities and microbial transformations during fermentation. According to Perez and Kim (2018), lactic acid bacteria undergo enzymatic activities during fermentation, where they can modify and release phenolic compounds from their bound forms over time. Lactic acid bacteria in Fermented Kefir Roselle-based popsicles help to release and transform phenolic compounds found in ingredients (Perez and Kim, 2018), potentially increasing bioavailability and promoting the health characteristics of phenolic compounds in fermented foods.

Fermented Kefir Roselle-based popsicles showed a substantial increase in total flavonoid content (TFC) across different fermentation times (p<0.05) (**Table 4**). The increase in TFC in Fermented Kefir Roselle-based popsicles is attributed to flavonoid metabolism via enzymatic activity within kefir grains (Garrote *et al.,* 2001). Besides, the pH levels in popsicles have a crucial impact on the TFC content. Flavonoids contain multiple hydroxyl (OH) groups that are protonated at low pH levels (Chen & Wang, 2020). It shows that the decrement of pH levels over fermentation time (**Table 1**) potentially increases flavonoid concentration in popsicles (**Table 4**). However, prolonged fermentation under certain conditions may also lead to the degradation or loss of flavonoids, impacting their overall concentration in the final product. These biochemical processes highlight the complex interactions between microbial activity, pH dynamics, and the stability of flavonoids during fermentation of kefir roselle-based popsicles.

The antioxidant activity of fermented kefir roselle-based popsicles was measured by scavenging activity against DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals at IC_{50} values in milligrams per milliliter (mg/mL) as shown in **Table 4**. The IC50 value represents the concentration at which the popsicles inhibit 50% of DPPH radicals, serving as a quantitative measure of their antioxidant efficacy. Initially, at 0 hours of fermentation, the IC_{50} value is highest at 1.966 mg/ml, indicating a relatively lower antioxidant activity. As fermentation progresses, IC_{50} value decreases significantly (p<0.05), showing enhancement in antioxidant activity of fermented kefir roselle-based popsicles for 18 hours (1.881 mg/ml), 24 hours (1.726 mg/ml), and 30 hours (1.424 mg/ml). These findings suggest that lactobacillus fermentation aids in neutralizing free radicals by creating an acidic condition to facilitate the release of antioxidants from plant materials, hence enhancing their bioavailability (Zhao and Zhang, 2019). Furthermore, *Lactobacillus* has natural antioxidant activity, which can produce antioxidant compounds like exopolysaccharides and peptides during fermentation. These processes demonstrate that increasing fermentation time maximizes the antioxidant properties of Fermented Kefir Roselle-based popsicles, enhancing their ability to scavenge free radicals and potentially increasing their health-promoting benefits (Lee and Lee, 2022).

Anthocyanin content in kefir roselle-based popsicles increases significantly (p<0.05) with longer kefir fermentation time (**Table 4**). This suggests that fermentation enhances the extraction and accumulation of anthocyanin pigments from roselle into the popsicle mixture. Anthocyanin is well known for its antioxidant properties and contributes to the vibrant red color of roselle extract (Mazza and Miniati, 2021; Abdolmaleki *et al.,* 2023). Anthocyanins are stable at low pH levels, i.e., acidic conditions. As the fermentation process produces an acidic environment, it potentially breaks down the roselle cell walls and facilitates the release of anthocyanins bound within plant cells into the popsicle mixture (Gomez and Martinez, 2022).

Table 4.TPC, TFC, IC_{50}, and Anthocyanin Content of Fermented Kefir Roselle-Based Popsicle

Fermentation time (FT) (hrs)	TPC (mg GAE/100g)	TFC (mg QE/mL)	DPPH, IC_{50} (mg/ml)	Anthocyanin (mg/ml)
FT 0	0.557 ± 0.000[d]	0.274± 0.012[d]	1.966± 0.011[a]	0.015± 0.007[c]
FT 18	0.833± 0.00[c]	0.384± 0.012[c]	1.881± 0.017[b]	0.022± 0.001[c]
FT 24	1.343± 0.000[b]	0.505± 0.006[b]	1.726± 0.078[c]	0.044± 0.002[b]
FT 30	1.987± 0.000[a]	0.631± 0.004[a]	1.424± 0.008[d]	0.082± 0.004[a]

Values were the means ± standard deviation of three replicates analysis.
Different notation indicates significant differencep<0.05.

4. Probiotic Contentof Fermented Kefir Roselle-based Popsicle

Figure 1 shows the formation of Lactobacillus spp. after 5 days of incubation in all popsicle samples. Lactobacillus spp. The count was relatively low at the initial state (0 hours), which is 3.333 x 104 CFU/ml. There are significant(p<0.05) increases in *lactobacillus spp*. Count upon 18 hours (1.333 x 107 CFU/ml) and 24 hours (1.856 x 107 CFU/ml) fermentation, respectively (**Table 5**). This indicates a robust proliferation of Lactobacillus spp. in the fermented kefir roselle-based popsicles. This phase is characterized by an exponential increase in bacterial population fueled by available nutrients, particularly sugars derived from roselle, and optimal environmental conditions such as temperature and pH conducive to bacterial growth (Roberts and Johnson, 2021). As Lactobacillus bacteria proliferate, they metabolize these nutrients, producing lactic acid and other metabolic byproducts (Ganzle, 2015). Between 18 to 24 hours of fermentation, the Lactobacillus population reaches a relatively stable level, where the rate of growth balances with the rate of decline or bacterial death. This period typically marks the peak Lactobacillus population in the product. However, at 30 hours of fermentation of kefir, there was a noticeable significant decrease (p<0.05) in Lactobacillus spp. Count to 1.677 x 105 CFU/ml, indicating a potential stabilization or decline in microbial populations at this extended fermentation time. At this stage, the initial sugars and other nutrients from roselle may become depleted, limiting the capacity of Lactobacillus to continue multiplying. Additionally, within complex microbial environments like kefir, competition, and stress factors arise. Other microorganisms, such as yeasts or competing bacteria, may coexist and vie for nutrients or produce compounds that hinder *Lactobacillus* growth (Simova *et* *al.,* 2002). These dynamics illustrate the intricate microbial ecology involved in kefir fermentation, impacting the growth and viability of Lactobacillus populations over time.

Table 5. *Lactobacillus spp*. content in fermented kefir roselle-based popsicle

Fermentation time (FT) (hrs)	Total Viable Count (CFU/mL)
FT 0	$3.333 \times 10^4 \pm 57735.027^c$
FT 18	$1.333 \times 10^7 \pm 404145.188^b$
FT 24	$1.856 \times 10^7 \pm 611010.093^a$
FT 30	$1.677 \times 10^5 \pm 57735.027^c$

Values were the means ± standard deviation of three replicates analysis.
Different notation indicates significant difference p<0.05.

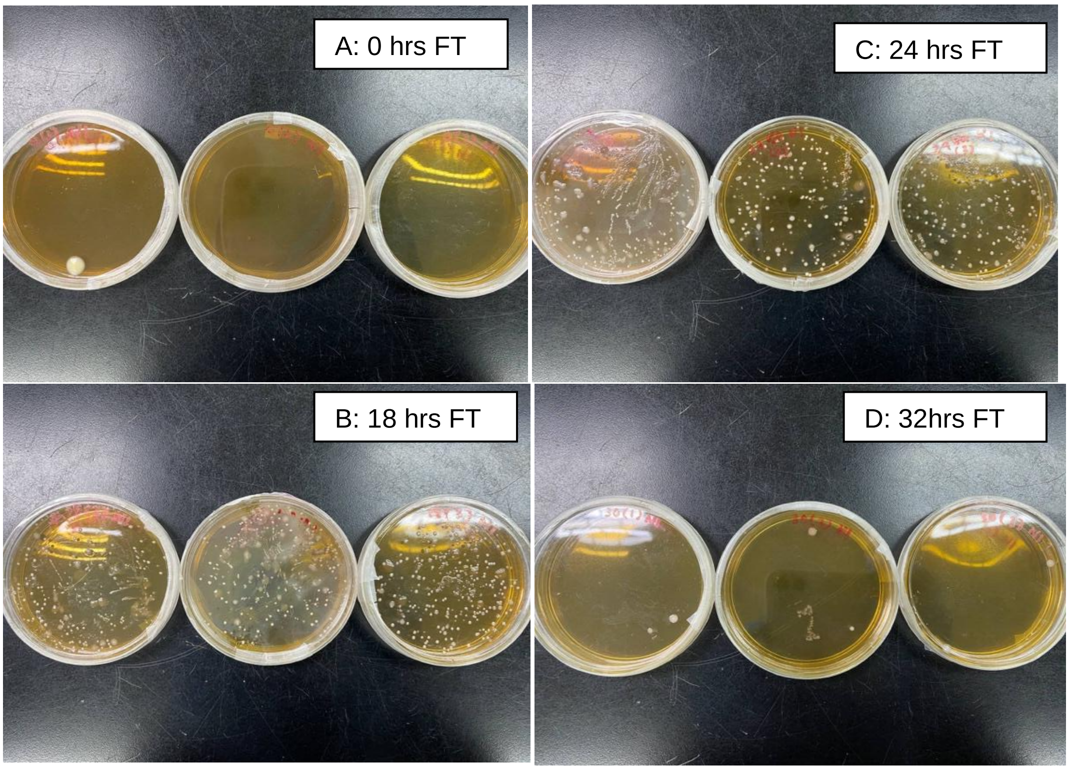

Figure 1. *Lactobacillus spp.* growth in 0,18, 24, and 32 hour fermented kefir roselle-based popsicle

5. Sensory Evaluation of Fermented Kefir Roselle-based Popsicle

Sensory acceptance of various Fermented Kefir Roselle-based Popsicles is presented in **Table 6**. There was no significant difference (p>0.05) in appearance acceptance across samples. Consumers moderately liked (with scores ranging from 6.200 to 7.070) the new innovation of natural and healthy formulated Fermented Kefir Roselle-based Popsicles. Fermented Kefir Roselle-based Popsicles at 18 hours of fermentation were observed to have an outstanding acceptance for their color, aroma, flavor, and mouthfeel attributes as compared to other samples.

Color perception peaks at popsicles of 18 hours fermentation time (8.070), indicating maximum visual appeal at this fermentation time. Furthermore, Fermented Kefir Roselle-based Popsicle at 18 hours was very much like for aroma and flavour, with scores of 7.730 and 7.870, respectively. According to Cadwallader and Singh (2009), flavour development in fermented products is closely tied to the metabolic activities of microorganisms. The scores of aroma and flavours (**Table 6**) show that at this fermentation stage (18 hours), the accumulation of pleasant volatile compounds and organic acids happens in developing a well-rounded and appealing flavour profile of fermented popsicles. Prolonging the fermentation time (>18 hours) causes the development of off-flavours (Dongmo *et al.,*

2016) due to the breakdown of desirable flavour compounds. This is supported by the notable dislike of the sample at 24 and 30 hours of fermentation by consumers.

In addition, consumers very much liked the mouthfeel of popsicles at 18-hour fermentation with a score of 7.730. At 18 hours of fermentation, there might be an optimal balance in the breakdown of proteins and polysaccharides, leading to a smoother and more pleasant mouthfeel (Alrosan *et al.,* 2024) of Fermented Kefir Roselle-based Popsicles. Excessive fermentation beyond this point might degrade these compounds further, resulting in a less desirable texture.

For the overall acceptance of Fermented Kefir Roselle-based Popsicles, consumers scored 5.230 to 6.470, neither like nor dislike to like moderately. As shown in **Table 6**, overall acceptance of popsicles at 18 hours was most preferred compared to popsicles at 30 hours. It can be proof that enhancing the sensory aspect of each attribute reflects the consumer's overall acceptance of popsicles at 18-hour fermentation (Costa *et al.,* 2017).

Sensory data explains the fermentation process influences the sensory acceptance of Fermented Kefir Roselle-based popsicles. This information is crucial for producers in optimizing fermentation conditions to ensure the products meet quality and palatability. Additionally, understanding these sensory dynamics helps in aligning product development with consumer preferences, thereby enhancing market competitiveness and customer satisfaction.

Table 6. Sensory evaluation of fermented kefir roselle-based popsicle

Attributes	**Fermented kefir roselle-based popsicle with different fermentation time**			
	0 hrs	**18 hrs**	**24 hrs**	**30 hrs**
Appearance	6.200 ± 2.058[a]	7.070± 1.530[a]	6.570± 1.870[a]	6.500± 1.81[a]
Colour	4.830± 1.724[b]	8.070± 0.868[a]	4.670± 1.788[b]	4.270± 2.196[b]
Mouthfeel	4.900± 1.583[b]	7.730± 0.785[a]	4.600± 1.993[b]	4.370± 1.921[b]
Aroma	5.470± 1.279[b]	7.730± 0.944[a]	4.500± 1.333[c]	3.330± 1.729[d]
Flavour	5.570± 1.278[b]	7.870± 0.900[a]	4.570± 1.455[c]	3.670±1.516[d]
Overall acceptance	6.070± 1.799[ab]	6.470± 2.460[a]	5.330± 2.324[ab]	5.230± 2.079[b]

Values were the means ± standard deviation of hedonic scoring (1 = extremely dislike, 2 = dislike very much, 3 = dislike moderately, 4 = dislike slightly, 5 = neither like nor dislike, 6 = like slightly, 7 = like moderately, 8 = like very much, and 9 = extremely like) from 50 panelists.
Different notation indicates significant difference $p<0.05$.

CONCLUSION

Fermented kefir roselle-based popsicles showed an increase in fat and calcium content with longer fermentation times, improving their nutritional profile. Antioxidant properties, measured by TPC, TFC, and IC50 values, also improved with extended fermentation. The process effectively enhanced the growth of probiotic strains, particularly *Lactobacillus spp*., adding potential health benefits. However, popsicles fermented for 30 hours, despite their high nutritional values, were less palatable due to over-fermentation, resulting in an unpleasant taste. In contrast, popsicles fermented for 18 hours were most preferred in sensory evaluations, offering a balanced and enjoyable sensory experience. This highlights the importance of balancing nutritional benefits with sensory quality to meet consumer preferences.

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