Understanding the effect of fermentation time on the physicochemical and microbiological characteristics of "*Popo*", a traditional Mexican beverage

Comprendiendo el efecto del tiempo de fermentación sobre las características fisicoquímicas y microbiológicas del "*Popo*", una bebida tradicional mexicana

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Abstract
The *Popo* is a traditional frothy beverage crafted with fermented rice, roasted cocoa beans, cinnamon, and green chupipe fruits. Despite its widespread consumption in southeastern Mexico, there are no studies providing information on the fermentation of its dough, combining all its components to classify it as a fermented product. Hence, the aim of this study was to assess the impact of fermentation time on the physicochemical and microbiological characteristics of *Popo*. The fermentation process of the *Popo* dough prepared at the laboratory level (T1) was conducted at 25 ± 2 °C for 120 h. The study revealed an initial yeast count of 4.35 ± 0.01 log CFU/g, with a significant decrease over time. In contrast, BAL showed an increase in the first 48 h of fermentation, reaching a value of 9.54 ± 0.04 log CFU/g. Fermentation inhibited the growth of coliform microorganisms, initially present at 2.10 ± 0.05 log CFU/g, thus making *Popo* dough a safe option for consumption. Upon concluding the fermentation process, significant changes were observed, including a decrease in pH to 3.9 ± 0.02, an increase in titratable acidity to 1.23 ± 0.03 %, and a moisture content of 39.67 ± 0.08 %. Therefore, it is recommended to subject *Popo* dough to a fermentation period of at least 48 h to enhance its microbiological quality.

Key Words: *Lactic acid bacteria, fermented beverage, microbial community, Popo*

Resumen
El *Popo* es una bebida espumosa tradicional elaborada con arroz fermentado, granos de cacao tostados, canela y frutos verdes de chupipe. Aunque se consume ampliamente en el sureste de México, no existen estudios que proporcionen información sobre la fermentación de sus masas, integrando todos sus componentes para identificarlo como un producto fermentado. Por lo tanto, el objetivo de este trabajo fue evaluar el efecto del tiempo de fermentación sobre las características fisicoquímicas y microbiológicas del *Popo*. El proceso de fermentación de la masa de *Popo* elaborada a nivel de laboratorio (T1) se realizó a 25 ± 2 °C durante 120 h. El estudio reveló un recuento inicial de levadura de 4.35 ± 0.01 log UFC/g, con una disminución significativa con el tiempo. En contraste, las BAL mostraron un aumento en las primeras 48 h de fermentación, alcanzando un valor de 9.54 ± 0.04 log UFC/g. La fermentación inhibió el crecimiento de microorganismos coliformes, inicialmente presentes en 2.10 ± 0.05 log UFC/g, lo que convirtió a la masa de *Popo* en una opción segura para el consumo. Al concluir el proceso de fermentación, se observaron cambios significativos, incluyendo una disminución del pH a 3.9 ± 0.02, un aumento en la acidez titulable del 1.23 ± 0.03 % y un contenido de humedad de 39.67 ± 0.08 %. Por lo tanto, se sugiere someter las masas de *Popo* a una fermentación de al menos 48 h para mejorar su calidad microbiológica.

Palabras claves: *Bacterias ácido lácticas, bebida fermentada, comunidad microbiana, Popo*

Introduction

Fermented foods represent a valuable source of cultural identity worldwide, gaining particular significance in countries like Mexico, where approximately 200 fermented products, including around 20 beverages dating back to pre-Hispanic times, have been identified (Robledo-Márquez et al., 2021). Notable examples of these beverages include Atole agrio, Tesgüino, Pozol, Atzokot, and *Popo*, prepared from cereals, fruits, and plants, consumed over the years (López-Sánchez et al., 2023; Rubio-Castillo et al., 2021; Castillo-Morales et al., 2005).

Popo is a non-alcoholic beverage originating from southeastern Mexico, obtained through a process involving the grinding of the following ingredients: roasted and husked cocoa beans (*Theobroma cacao L*.), cinnamon (*Cinnamomum*), green fruits of chupipe (*Gonolobus edulis*), and rice (*Oryza sativa*) previously soaked in water overnight (Hernández-Nolasco et al., 2023). For consumption, the resulting dense mass is dissolved in water, filtered to remove solid particles, and mixed with sugar and ice. Subsequently, it is vigorously shaken with a wooden grinder to generate abundant foam, which is then served in "jícaras" made from the “Jícaro” (*Crescentia cujete*) (Barros & Buenrostro, 2011).

During the rice soaking process, spontaneous fermentation occurs, promoting the growth of fermentative microorganisms, especially lactic acid bacteria (BAL) (Ghosh et al., 2014). BAL produce various antimicrobial compounds, including organic acids, creating a low pH environment that is detrimental to the development and survival of pathogenic and spoilage microorganisms (Mgomi et al., 2023). However, the synthesis of these compounds might not be effective enough to completely eradicate them from the final product, where the most adapted microorganisms determine the microbiota of traditionally fermented foods (Lhomme et al., 2016).

In a previous study, it was observed that traditionally produced *Popo* doughs exhibited the presence of total coliform microorganisms with a concentration exceeding 2 log CFU/g, surpassing the limit allowed by NOM-147-SSA1-1996 for safe consumption (Hernández-Nolasco et al., 2023). Generally, *Popo* production is carried out on a small scale or at home, employing traditional processing methods. Limited familiarity with good manufacturing practices and proper handling may lead to unhygienic production, involving the use of rudimentary equipment and a high dependence on manual labor.

To date, no studies have been conducted to provide information on the fermentation of *Popo* doughs, which involves the integration of all components to identify it as a fermented product. Therefore, the aim of this study is to evaluate the effect of fermentation time on the physicochemical and microbiological characteristics of *Popo*.

Materials and Methods

1. *Production of Popo Dough*

The ingredients, consisting of cocoa beans (*Theobroma cacao L*.), cinnamon (*Cinnamomum*), green chupipe fruits (*Gonolobus edulis*), and rice (*Oryza sativa*), were purchased from the local market in Acayucan, Veracruz, Mexico. All chemicals used were of analytical grade. The *Popo* dough, designated as treatment 1 (T1), was prepared according to a traditional procedure typical of the southeast Mexico, conducted at the laboratory level. The following ingredients were ground in a grain mill (SURTEK, model U1 MOGRA1, El Salto, Jalisco, Mexico): 1000 g of roasted and husked cocoa beans, 150 g of cinnamon, 400 g of seedless green chupipe fruits, and 3 kg of rice previously soaked for 12 h in potable water. The resulting dense dough was shaped into 300 g balls, which were wrapped in cling film and then incubated at 25 ± 2 °C for 120 h. Fermentation was monitored at 0, 24, 48, 72, 96, and 120 h for microbiological and physicochemical analyses, as detailed in sections 2 and 3. The production of T1 is presented in Figure 1.

***Figure 1**. Elaboration process of Popo dough*

2. *Bacterial Isolation*

To determine the quantity of viable bacterial cells present in T1, 10 g of each sample was taken and mixed with 90 mL of 0.85 % w/v saline solution. Subsequently, tenfold serial dilutions were performed. The diluted samples (1000 µL) were plate spread on tryptone glucose yeast extract agar (TGEA; BD, Bioxon, Cuautitlán Izcalli, EMX, Mexico), potato dextrose agar (PDA; BD) with the addition of 10 % w/v tartaric acid to adjust the pH to 3.5, violet red bile agar (VRBA; BD) adjusted to pH 7.4, and *Lactobacilli* MRS agar (MRS-VB; Difco, Sparks, MD, USA) (Table 1). The results were expressed as colony-forming units per gram of sample (CFU/g). The collected data were analyzed in terms of colony type and microbial population size (NOM-092-SSA1-1994, NOM-111-SSA1-1994, NOM-113-SSA1-1994, ISO 15214:1998; Jung et al., 2015).

***Table 1**. Specific medium used for viable cell isolation.*

Type of Medium	Colony	Incubation Conditions
TGEA[a]	Total mesophilic aerobic bacteria	35 ± 2 ºC, 48 h
PDA[b] (pH 3.5)	Yeasts and molds	25 °C ± 2 °C, 120 h
MRS[c]	Lactic Acid Bacteria (LAB)	35 °C ± 2 °C, 72 h
RVBA[d] (pH 7.4)	Total coliform microorganisms	35 °C ± 2 °C, 24 h

Note: aTryptone Glucose-yeast Extract Agar, bPotato Dextrose Agar, cLactobacilli MRS Agar, dRed Violet Bile Lactose Agar.

3. *Analysis of Physicochemical Properties*

3.1 *pH, titratable acidity, and moisture content*

Physicochemical analyses of the *Popo* samples fermented at 25 °C were conducted following the methodology proposed by AOAC, including pH measurement (AOAC 981.12), determination of titratable acidity (AOAC 942.15), and moisture content (MC) determination (AOAC 950.27).

3.2 *Determination of Water Activity and Color*

The measurement of water activity (a_w) was conducted using a water activity meter (Novasina LabMASTER, standard model, US) with internal temperature control at 25 °C after

prior calibration. Additionally, color parameters were determined using a Minolta colorimeter (Konica Minolta Sensing, Inc. CR-400, Osaka, Japan). The CIELAB system was employed to quantify the color of the samples. The total color difference (ΔE) was calculated using the following equation:

$$\Delta E=\sqrt{(L^*_f - L^*_i)^2+ (a^*_f - a^*_i)^2+(b^*_f - b^*_i)^2}$$

Where, L^*_f, a^*_f , and b^*_f are the values of the samples fermented over time, and L^*_i, a^*_i, and b^*_i are the values of the fresh samples.

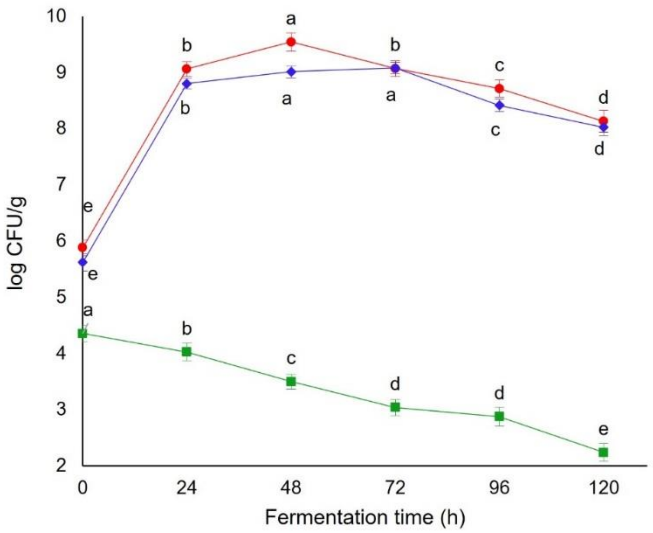
4. *Statistical Analysis*

The laboratory-prepared *Popo* dough (T1) underwent a 120 h fermentation period at a temperature of 25 ± 2 °C. Measurements were taken at six different time points: 0, 24, 48, 72, 96, and 120 h using a completely randomized design (CRD). Response variables included microbiological analyses (mesophilic aerobic bacteria, LAB, and yeast) and physicochemical analyses (pH, titratable acidity, MC, a_w, and total color difference (ΔE). The experiment was conducted in triplicate, and the results were expressed as mean values ± standard deviations (n = 3). Statistical analysis was performed using Minitab Inc., (State College, PA, USA). A one-way analysis of variance (ANOVA) was applied for each evaluated response variable, followed by the Tukey test for mean comparison, with a significance level of 5 % ($p<0.05$).

Results and Discussion

1. Bacterial Isolation

Traditional fermented foods are characterized by hosting lactic acid bacteria (LAB) and beneficial yeasts in their microbiota. These contribute to the consumer's health and enhance the quality, sensory attributes, and nutritional value of the foods (Aslam et al., 2020; Ilango & Antony, 2021). Figure 2 depicts the growth of the studied microbial groups throughout the fermentation process of the *Popo* dough prepared at the laboratory level (T1).



Note: abcd, different superscripts indicate statistically significant differences ($p<0.05$). Values are expressed as mean ± SD (n = 3).

Figure 2. *Fermentation kinetics of Popo dough (T1) at 25 °C: Growth profile of mesophilic aerobic bacteria (◆), lactic acid bacteria (●), and yeasts (■).*

The initial count of LAB was 5.88 ± 0.01 log CFU/g, a statistically significant exponential growth ($p<0.05$) was observed during the first 48 h of the fermentation process, reaching a maximum of 9.54 ± 0.04 log CFU/g. After 72 h, the population tends to decrease to 9.07 ± 0.00 log CFU/g. The count in the first 24 h of the study remained above the minimum recommended level for daily consumption in products with probiotic potential, which is 6 log

CFU/mL, as indicated by Enujiugha & Badejo (2017). Studies by Giri et al. (2018) and Miyagawa et al. (2016) showed slightly lower counts, with a maximum concentration of 8.98 and 7 log CFU/mL, respectively, in the case of Bhaati jaanr and Sochu/shochu rice-based beverages. The production of lactic acid triggers an increase in the activity of proteases, α-amylases, and glucoamylases in soaked rice, inducing the degradation of proteins and starch (Cheirsilp et al., 2023). This process provides additional substrate, ensuring greater growth of LAB (Axel et al., 2016).

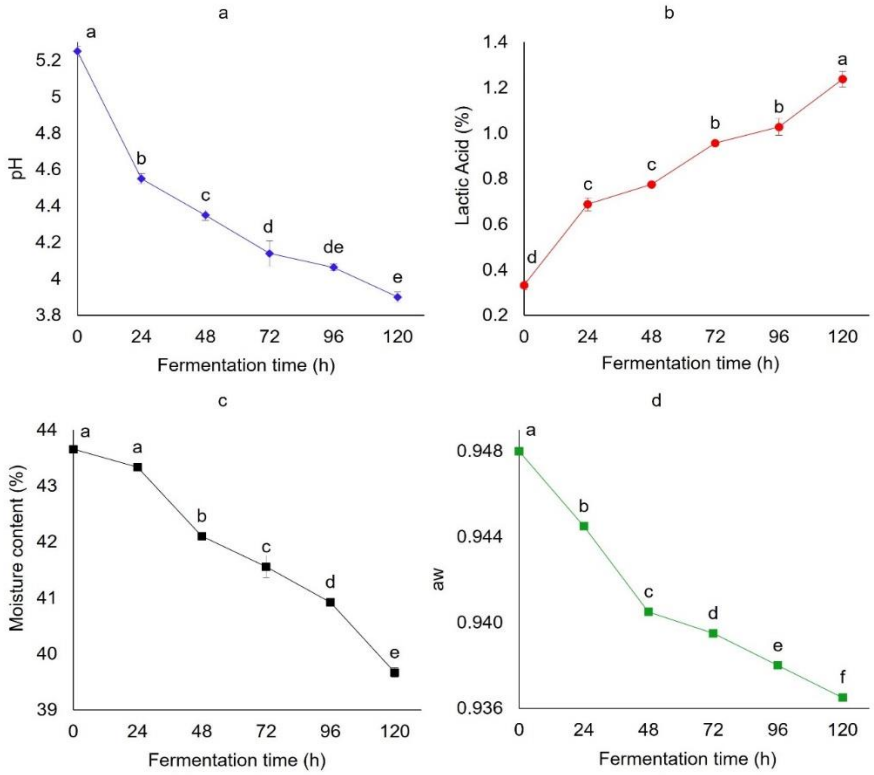
The initial concentration of aerobic mesophilic bacteria was 5.61 ± 0.01 log CFU/g. At 72 h, consistency in its growth was evident, reaching a maximum point at 9.07 ± 0.00 log CFU/g. In the beverage-making process, rice spontaneous and uncontrolled fermentation, driven by the native microbiota of the cereal (Mishra et al., 2022). Puerari et al. (2015) studied chicha, a rice beverage, where aerobic mesophilic bacteria showed an initial count close to that of LAB, reaching a maximum of 5.11 log CFU/mL. In another study, Ghosh et al. (2015) reported a higher concentration of 10.51 log CFU/g during the 48-72 h of fermentation of Haria, an ethnic fermented rice beverage.

In traditional fermented foods from various regions worldwide, yeasts are produced in significant amounts, ranging between 10^5 and 10^8 CFU/g (Rakmai et al., 2019). The initial yeast population was 4.35 ± 0.01 log CFU/g. However, over the 120-hour period, a significant decrease ($p<0.05$) in this population was observed, ultimately reaching 2.23 ± 0.05 log CFU/g. However, various studies have pointed out the limited presence of yeasts (Omemu et al., 2007; Sanni & Adesulu, 2013). The decrease in the yeast population can be attributed to various factors, such as variations in raw materials, the production process, the addition of water during milling, and the increase in acidity during fermentation, among others (Omemu et al., 2007; Teniola & Odunfa, 2001). This decrease could be a consequence of the anaerobic conditions present during the incubation of the *Popo* doughs (Ghosh et al., 2015). Lv et al. (2012) observed that the growth of LAB could influence the growth of yeasts. In the studies conducted by Pswarayi & Gänzle (2019) and Ghosh et al. (2015), yeast counts below 3 log CFU/g were identified in mahewu and Haria, cereal-based beverages. Finally, Piló et al. (2018) recorded counts of 5.8 log CFU/g in chicha, a traditional Andean sparkling beverage made from grains such as corn, rice, and oats.

The initial count of total coliform microorganisms was 2.10 ± 0.05 log CFU/g, subsequently decreasing to levels below the detection limit of 10 CFU/g in all samples throughout the fermentation process. The initial count could be linked to the microbiological quality of the soaked rice, which exhibited a count of 2.02 ± 0.09 log CFU/g (data not shown). This implies that the survival of these microorganisms might depend not only on the initial pH value (5.25) in the doughs but also on factors such as raw materials, production process, milling, and ambient temperature. Adinsi et al. (2017) reported the presence of coliforms ranging from 2.4 to 3.5 log CFU/g in Gowé samples, an African beverage made from sorghum and corn. The initial counts of LAB, aerobic mesophilic bacteria, yeasts, and total coliform microorganisms in T1 were similar to those obtained by Hernández-Nolasco et al., 2023, in traditionally prepared *Popo* doughs. The presence of fungi was not detected.

2. *Analysis of Physicochemical Properties*

The evaluation of physicochemical properties at different stages of fermentation provided an understanding of the process dynamics. Figure 3 displays the values obtained for the assessed physicochemical analyses: pH, titratable acidity, moisture content (MC), and water activity (a_w) during the fermentation process of T1 at analysis times of 0, 24, 48, 72, 96, and 120 h.



Note: abcd, different superscripts indicate statistically significant differences ($p<0.05$). Values are expressed as mean ± SD ($n = 3$).

Figure 3. *Physicochemical analysis kinetics in the fermentation of the Popo mass (T1) at 25 °C: pH (◆), titratable acidity (●), moisture content (■), and aw (■).*

An initial pH of 5.25 ± 0.02 and titratable acidity of 0.33 ± 0.01 % were recorded. As the fermentation time progressed, a significant decrease in pH ($p<0.05$) was observed, reaching 3.9 ± 0.02 (Figure 3,a), while titratable acidity significantly increased ($p<0.05$), reaching a value of 1.23 ± 0.03 % at 120 h (Figure 3,b). The decrease in pH is attributed to the concentration of antimicrobial substances and organic acids produced during the fermentation process, primarily by LAB. As part of their anaerobic fermentative metabolism, LAB generate lactic acid. This process leads to the acidification of the medium, creating unfavorable conditions that inhibit pathogenic, toxigenic, and spoilage organisms (Mgomi et al., 2023). It has been demonstrated that fermented beverages based on cocoa and rice, such as Pozol and Chicha, undergo a drastic decrease in their pH level, dropping from 7.3 to 4-3 (Robledo-Márquez et al., 2021; Silva et al., 2018).

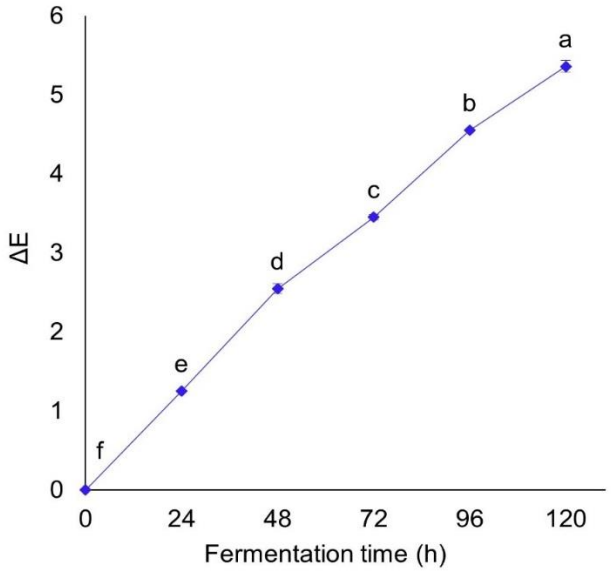
The titration of acids does not allow differentiation between different acidifying compounds; the percentage of titratable acidity is expressed in relation to the predominant known organic acid (Coelho et al., 2020). The presence of lactic acid and other organic acids can be attributed to the spontaneous fermentation that occurs during the soaking of rice, initiated by its native microbiota. LAB and yeasts produce various antimicrobial compounds, such as bacteriocins, hydrogen peroxide, ethanol, and organic acids (Puerari et al., 2015). Acidity significantly influences the sensory and nutritional characteristics of foods (Paredes et al., 2022). These results align with those reported by Ghosh et al. (2014), who studied the microbiota in Haria, a fermented rice-based beverage, demonstrating an increase in acidity from 0.01 % to 1.42 % at 96 h.

The MC was 43.65 ± 0.01 %, significantly decreasing ($p<0.05$) to a final value of 39.67 ± 0.08 % (Figure 3,c). However, these changes were deemed insignificant, as the use of cling film as wrapping material reduced the evaporation of water molecules and promoted the growth of LAB. Jiménez-Vera et al. (2010) observed a similar pattern of moisture loss

associated with fermentation time in balls of white pozol and cocoa wrapped in grilled banana leaves. The initial MC and a_w of T1 can be attributed to the soaking of rice. By subjecting rice grains to hydration, they absorb a certain amount of water until reaching equilibrium moisture content, due to the water diffusion mechanism (Balbinoti et al., 2018; Panda & Shrivastava, 2019).

The a_w is a fundamental parameter that determines the amount of water available in the food system, facilitating both microbial growth and biochemical and chemical reactions (Ostrowska-Ligęza & Lenart, 2015). In this context, the initial a_w in T1 was 0.94 ± 0.00, which was expected for a fresh food product. As the fermentation progressed, water at the molecular level gained greater mobility, causing a significant decrease ($p<0.05$) in a_w, ultimately reaching 0.93 ± 0.00 (Figure 3,d). The presence of elevated a_w values underscores the importance of maintaining rigorous quality and safety control. This is because most bacteria, molds, and yeasts require high levels of a_w (0.97 - 0.90) for their growth (Tapia et al., 2020).

The total color difference (ΔE) is a measure indicating the magnitude of color difference between two samples (test and control/reference) in the CIELAB three-dimensional color space (Patras et al., 2011). In Figure 4, it can be observed how the ΔE value increased significantly ($p<0.05$) from 1.24 ± 0.01 to 5.35 ± 0.07 during the fermentation process of T1. This indicates that it is possible to distinguish the color variation at 120 h compared to the initial time, as a ΔE > 5 is visually perceptible (Pathare et al., 2013).



Note: abcd, different superscripts indicate statistically significant differences ($p<0.05$). Values are expressed as mean ± SD (n = 3).

Figure 4. *Total color difference (ΔE) (—◆—) in the fermentation of the Popo mass (T1) at 25 °C.*

The changes in color in T1 could be primarily associated with the loss of MC during the fermentation process, incubation conditions, and packaging. Costa et al. (2017) indicated that color changes in a probiotic fermented beverage during storage could be related to the degradation of pigments and other constituents due to the metabolic activity of microorganisms involved in the fermentation process. The most significant total color difference ($p<0.05$) was observed in luminosity, which ranged from 55.21 ± 0.12 at the initial time to 50.02 ± 0.00 at the end of the fermentation process (data not shown), indicating that T1 gradually acquired a lighter appearance over time.

Conclusion

The fermentation of the *Popo* dough at 25 °C brought about significant changes in its physicochemical and microbiological characteristics as time progressed. The

community of mesophilic aerobic bacteria, yeasts, and LAB exhibited an efficient utilization of the available nutrients in the dough, highlighting the complex dynamics of the fermentation process. The study revealed an initial yeast count of 4.35 ± 0.01 log CFU/g, with a significant decrease over time. Meanwhile, at 48 h, a logarithmic phase for LAB exceeding 6 log CFU/g was recorded, significantly influencing the decrease in pH and the increase in titratable acidity throughout the process. Visually perceptible color changes were identified in the masses. Fermentation consistently demonstrated inhibition in the development of coliform microorganisms, transforming the *Popo* dough into a safe option for human consumption, in accordance with the standards established by NOM-147-SSA1-1996. While this study focused on a specific fermentation temperature, it is necessary to explore broader ranges of process variables to achieve a comprehensive understanding of their effects on the beverage's characteristics.

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