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Effect of Fermentation Duration on Physicochemical and Microbiological Properties of Tigernut (*Cyperus esculentus*) Milk

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Abstract

The study investigated the effect of the extent of fermentation time on the physicochemical and microbiological properties of tigernut milk using standard laboratory procedures. The unfermented (Control) sample was subjected to physicochemical and microbiological analysis, while the remaining samples were exposed at room temperature and allowed to undergo spontaneous fermentation for 12, 24, 36, and 48 hours, respectively, before analysis. The samples were analysed to determine the physicochemical properties, and the results were further analysed using ANOVA and ranged from pH (6.52a±0.30) of unfermented, (4.37b±0.42) of 12hrs fermented, (3.99b±0.04) of 24hrs fermented, (3.86b±0.26) of 36hrs fermented, (3.67b±0.32) of 48hrs of fermented., TTA(g\L) (0.20b±0.14) of unfermented, (0.45ab±0.07) of 12hrs of fermented (0.55ab±0.21)of 24hrs , (0.65ab±0.21) of 36hrs (0.85a±0.35) of 48hrs of fermented., Moisture (%) (90.41a±1.90) of unfermented, (89.58a±4.45) of 12hrs fermented, (88.68a±1.90)24hrs, (91.67a±3.79) of 36hrs (93.22a±4.74) of 48hrs, Total solid TS (%) (9.59a±1.90) of unfermented, (10.42a±4.45)12hrs of fermented,(11.33a±1.90) of 24hrs fermented, (7.84a±3.90) of 36hrs fermented, and (6.78a±4.74) of 48hrs fermented. The biochemical characterisation of the microorganisms in the juice samples fermented for 24 - 48 hours had increased significantly ($p < 0.05$) higher bacteria than the unfermented sample, and the sample fermented for 12 hours. The microbiological quality of the juice determined shows growths *of Staphylococcus aureus, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Penicillium* and *Aspergillus niger*. The findings conclude that these microorganisms can lead to food poisoning and reduce shelf life, leading to food spoilage. The presence of these microorganisms can be linked to improper hygiene practices during preparation or contamination from human handlers.

Keywords: Fermentation duration, Tigernut milk, Physicochemical, Microbiological

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1. Introduction

The perennial tigernut plant (*Cyperus esculentus L.*) produces firm, spherical tubers from scaly rhizomes near the base of the plant (Rojas-Sandoval & Acevedo-Rodríguez, 2022). Tigernut (also known as edible rush, yellow nut-grass, Zulu nut, chufa, water grass, and Earth almond) is a tuber rather than a nut (Hiko & Muktar, 2020). A thorough study on the benefits of using tiger nut tubers from the tiger nut plant, including how to make tiger nut drink, was researched by Maduka and Ire (2018) and Adesakin and Obiekezie (2020). However, a lot of people favour tiger nut drinks over commercially manufactured drinks since they are inexpensive, homemade, natural, and created using locally sourced ingredients. Maduka and Ire (2018) reveal that energy, fat, carbohydrates, glucose, fibre, and protein are all abundant in tiger nut drinks. Similarly, Yu *et al*. (2022) stated that tigernut protein has a significant biological value since it contains various critical amino acids. Nutrient-rich tiger nut milk is obtained from the nuts, and this milk has excellent nutritional value, but the degrading effects of several bacteria on it have made it difficult to produce in Nigeria (Wada, 2019; Agbo Tahir, 2018). This beverage should be drunk 2-4 hours after preparation because it has a relatively limited shelf life in tropical Africa and Nigeria under the current weather conditions. Nonetheless, pasteurisation and including ginger, garlic, and citric acid can increase the drink's shelf life (Maduka & Ire, 2018). The presence of bacteria that weaken milk and cause spoiling due to improper preparation, using contaminated raw materials, and using primitive utensils has hindered its production in Nigeria (Umaru *et al*., 2018). Therefore, it is essential to assess how fermentation duration influence the physicochemical and microbiological properties of tigernut milk, as well as its overall quality attributes.

2. Materials and Methods

Cyperus esculentus (Tiger-nut) were purchased at Tunga Market, Minna, Niger State, Nigeria. The materials were kept in clean containers before they were used.

2.1 Production of Tiger Nut Milk

The method described by Udeozor (2022) was used in the preparation of tiger nut milk. Fresh tiger nuts weighing one kilogram (1 kg) were manually sifted and cleaned to eliminate contaminants. The nuts were soaked for 12 hours in potable water at a nut: water ratio of 1:3. Three litres of water were added to the soaked nuts and ground into a slurry using a blender (Philips, Kenwood, UK). The slurry was filtered using a muslin cloth to obtain the milk. The extracted milk was homogenised, and the filtrate was collected into five sterile bottles, corked and pasteurised at 72℃ for 5 s. The samples were allowed to cool and kept in the Refrigerator at –18℃.The procedure is outlined in Figure 2.1.

2.1.1 Fermentation of Tiger Nut Milk

The control (Unfermented) sample of the tiger nut milk was immediately subjected to physiochemical and microbiological analysis, while the remaining samples were exposed at room temperature and allowed to undergo spontaneous fermentation for 12, 24, 36, and 48 hours, respectively, before analysis.

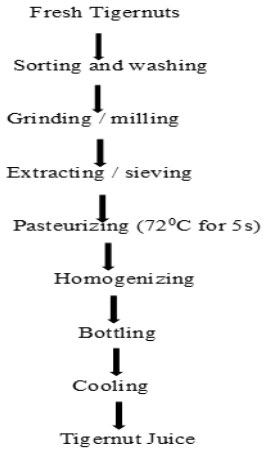


Figure 1: Flow chart for the production of tigernut milk (Udeozor, 2022).

2.2. Physicochemical Analysis

2.2.1 Determination of pH

The pH of 1 ml of the sample, which was diluted with 9 ml of distilled water, was determined using a pH meter (OHAUS). The electrode was inserted into the sample, and the readings displayed on the screen were recorded (AOAC, 2010).

2.2.2 Determination of TTA

The total titratable acidity (TTA) of the tigernut milk was determined according to the procedure outlined by AOAC (2010). 1 ml of the sample was diluted with 9 ml of distilled water to create a homogeneous solution. The sample was then titrated against 0.1 ml L^{-1} NOAH with phenolphthalein as an indicator to determine the amount of acid in the sample, and the result was expressed as % Lactic acid.

2.2.3 Determination of Moisture Content

The sample's moisture content was determined following the procedure outlined by AOAC (2010). First, the empty petri dish used to hold the sample was weighed (W1), and then 5 grams of it were weighed and placed in the petri dish (W2). The petri dish with the sample was then placed in a drying oven at 105℃ for 3 hours to remove the sample's moisture completely. After drying, the sample was removed from the oven, allowed to cool, and then weighed to obtain the weight of the dried sample (W3). The moisture content of the dried sample can be calculated as:

MC (%) = ×100

Where;

W1 = weight of empty petri dish

W2 = weight of sample + petri dish

W3 = weight of the dried sample.

2.2.4 Determination of Total Solid (TS)

The Total solid of the sample was determined by the difference after analyzing for moisture.

Mathematically;

$$TS\ (\%) = 100 - \left(\frac{W2-W3}{W2-W1} \times 100\right)$$

2 .3 Microbiological Analysis

Tunde-Akin and Souley's (2009) approach was used to determine the bacterial and fungi loads of the tiger-nut milk samples. Using peptone water, serial dilutions of the different tiger nut milk samples were created up to a 10-7 ratio. Each dilution's 0.1 ml was equally distributed over nutrient agar and incubated for 24 hours at 28℃. Following the incubation period, plates were inspected for distinct colonies, and the actual number of bacteria (total bacteria count) was calculated using the colony-forming unit per millilitre (CFU/ml) of the sample. Similar procedures were used for the total coliform count (TCC) and total fungal count (TFC) using MacConkey Agar and Sabouraud Dextrose Agar (SDA) media, respectively. Every instance involved duplicate plating and plate counts. After carefully examining each colony, separate colonies were removed, subcultured in a new, sterile medium, and then incubated for 24 hours at 37℃ to obtain pure cultures.

2.3.1Identification and Characterisation of Isolates

Purified isolates were characterised by gramme morphology and biochemical tests using Bergey's manual of determinative bacteriology (Holt *et al*., 2017), while fungal isolates were identified by cultural and morphological characteristics, as described by Gonzalez-Garcia *et al*. (2017).

2.3.2 Statistical Analysis

Data obtained were statistically analysed using analyses of variance by means of SPSS statistical software version 25.0. The difference between means was assessed using Duncan's multiple range tests. A significant difference was noted at $p<0.05$.

3. Results

3.1 Physico-chemical Properties of Tigernuts Juice Samples

The pH, TTA, moisture content and total solid of tigernut juice samples are shown in Table 1.

3.2 Microbiological Quality of Tigernuts Milk Samples

The total bacterial and fungal counts of the juice samples are shown in Table 2 while Table 3 outlines the biochemical characterization of the microorganisms present in the juice samples. Table 2 shows that samples fermented for 24-48 hours had significantly ($p<0.05$) higher bacteria than the unfermented sample and the sample fermented for 12 hours. The same trend was observed for the fungal count. Table 3 shows that *staphylococcus aureus, bacillus subtilis and Pseudomonas aeruginosa* were the organisms present in the juice samples. Table 4 shows the fungi identified in the fermented tiger nut juice samples after 24 hours. The two fungi present were *Penicillium and Aspergillus niger* which were detected by their morphological and colonial features.

Table 1: Physico-chemical Properties of Tigernuts Milk Samples

Parameters\Samples(hr)	Unfermented	12hrs fermented	24hrs fermented	36hrs fermented	48hrs fermented
pH	$6.52^{a}\pm0.30$	$4.37^{b}\pm0.42$	$3.99^{b}\pm0.04$	$3.86^{b}\pm0.26$	$3.67^{b}\pm0.32$
TTA(g\L)	$0.20^{b}\pm0.14$	$0.45^{ab}\pm0.07$	$0.55^{ab}\pm0.21$	$0.65^{ab}\pm0.21$	$0.85^{a}\pm0.35$
Moisture (%)	$90.41^{a}\pm1.90$	$89.58^{a}\pm4.45$	$88.68^{a}\pm1.90$	$91.67^{a}\pm3.79$	$93.22^{a}\pm4.74$
Total solid TS (%)	$9.59^{a}\pm1.90$	$10.42^{a}\pm4.45$	$11.33^{a}\pm1.90$	$7.84^{a}\pm3.90$	$6.78a^{a}\pm4.74$

Values are means of duplicate Values are means± standard deviation of duplicate determinations. Means in the same row with different superscripts are significantly different ($p<0.05$).

Table 2: Total Bacterial and Fungal counts of Tigernut Milk Samples

Parameters\Samples	Unfermented	12hrs fermented	24hrs fermented	36hrs fermented	48hrs fermented
Bacterial(cfu\ml)	$0.00^{b}\pm0.00$	$0.00^{b}\pm0.00$	$1.5x10^{3a}\pm7.07$	$1.0\times10^{3a}\pm0.00$	$1.0\times10^{3a}\pm0.00$
Fungi(cfu\ml)	$0.00^{a}\pm0.00$	$0.00^{a}\pm0.00$	$1.0\times10^{3a}\pm0.00$	$1.5\times10^{3a}\pm0.00$	$8.0\times10^{4a}\pm8.49$

Values are means ± standard deviation of duplicate determinations. Means in the same row with different superscripts are significantly different ($p<0.05$).

Table 3: Biochemical Characteristics of Bacterial Isolates from Tigernut Milk Samples

Sample	Unfermented	12hrs fermented	24hrs fermented	24hrs fermented	36hrs fermented	48hrs fermented
Shape	NG	NG	Cocci	Rod	Cocci	Rod
GR	NG	NG	+	+	+	-
CAT	NG	NG	+	+	+	+
COU	NG	NG	+	-	+	-
CIT	NG	NG	-	+	-	+
OXI	NG	NG	-	-	-	+
H_2S	NG	NG	-	-	-	-
MOL	NG	NG	-	+	-	+
MR	NG	NG	+	-	+	-
UR	NG	NG	-	-	-	-
IND	NG	NG	-	-	-	-
Organism Identified	No Organism	No Organism	*Staphylococcus aureus*	*Bacillus subtilis*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*

Keys: NG=No growth; GR= Gram staining; CAT=Catalase; COU=Coagulase; CIT=Citrate utilization OXI=Oxidase; H_2S=Hydrogen sulphide production; MOL=Motility; MR=Methyl red; UR=Urease IND=Indole

Table 4: Colonial Characterisation and Morphological Identification of Fungal Isolates from Tigernut Milk Samples

Samples	Colonial features	Morphology	Probable organism
Unfermented	No growth	No growth	No organism
12hrs fermented	No growth	No growth	No organism
24hrs fermented	Green and velvety	Colonies are smooth and ellipsoidal. Conidiophores are smooth and short. Mycelia are arranged irregularly with branches of various lengths.	*Penicillium*
36hrs fermented	Green and velvety	Colonies are smooth and ellipsoidal. Conidiophores are smooth and short. Mycelia are arranged irregularly with branches of various lengths.	*Penicillium*
48hrs fermented	Black colonies with white Edges	Conidia heads are large, globose, dark-brown and biseriate, conidia are globose and rough-walled. Conidiophores are smooth Walled	*Aspergillus niger*

4. Discussion

4.1 Physicochemical Properties of Tigernut Milk Samples

The observed decrease in pH and increase in TTA over the fermentation period indicate a rise in acidity due to the production of organic acids by fermenting microorganisms. Fermented foods often exhibit lower pH levels, which contribute to their safety and shelf life by inhibiting the growth of pathogenic organisms (Tamang *et al.*, 2016). Furthermore, a study by Dimidi *et al.* (2019) shows that increased acidity enhances flavours and acts as a natural preservative, reducing the need for artificial additives. In addition, Dimidi *et al*. (2019) stated that an increase in microbial counts, particularly bacteria and fungi, plays a role in the natural preservation of the milk by lowering the pH and producing antimicrobial compounds. These changes help prevent the growth of spoilage organisms and pathogens, which extends the product's shelf life. The moisture content remains relatively stable across different fermentation durations, with slight increases observed in later stages. This stability suggests that fermentation does not significantly alter the milks' water content. The variations in total solids, with an initial increase followed by a decrease, suggest changes in the concentration of soluble components. Studies indicate that later stages may reduce due to the consumption of solids by microorganisms, which can lead to a more refined and potentially more digestible product (El-Sayed, 2020).

4.2 Microbiological Quality of Tigernut Milk Samples

The bacterial counts indicate that no bacteria were detected in the unfermented and 12-hour fermented samples. This indicates that the initial stages of fermentation did not promote bacterial growth, likely due to insufficient duration for microbial colonisation and growth. Also, the bacterial count significantly increased at 24 hours, indicating that the environment had become favourable for bacterial growth. In addition, the bacterial counts stabilised at 1000 CFU/mL, indicating a balanced microbial environment or a competitive exclusion among bacterial species. This finding agrees with the study of El-Sayed (2020), which reveals that stability is crucial for consistent product quality and safety. The fungal counts are similar to the bacterial counts; no fungal presence was detected initially or after 12 hours of fermentation, suggesting that the conditions were not optimal for fungal growth. However, fungal growth became evident, with a mean count of 1000 CFU/mL, highlighting the role of fungi in the fermentation process, possibly contributing to flavour and texture development (24 hours fermented). Furthermore, fungal counts increased to 1500 CFU/mL, with variability indicating active fermentation and fungal metabolic activity (36 hours fermented). Furthermore, a large spike in fungal counts (mean 8000 CFU/mL) was observed, suggesting robust fungal activity (48 hours fermented). This result is consistent with Atuna *et al.* (2025), who reported that the high variability (standard deviation of 8485.28) may indicate diverse fungal species contributing to the fermentation process. The implication of *Staphylococcus aureus* in the tiger nut is that it produces heat-stable enterotoxins that can cause food poisoning even after pasteurisation or cooking (CDC, 2023). Health risks include the consumption of contaminated tiger nut milk, which can lead to staphylococcal food poisoning that causes symptoms like nausea, vomiting, diarrhoea, and abdominal cramps (CDC, 2023). *S. aureus* is often linked to improper hygiene practices during food preparation or contamination from human handlers, as this bacterium is commonly found on the skin and nose (CDC, 2023).

Bacillus subtilis is a food spoilage organism that can produce enzymes that degrade carbohydrates, proteins, and fats, leading to milk spoilage (Akinsemolu & Onyeaka, 2024). While generally not harmful to healthy individuals, some strains of *Bacillus* can produce toxins that cause gastrointestinal issues like nausea and vomiting, although this is rare (Akinsemolu & Onyeaka, 2024). *B. subtilis* forms endospores, making it resistant to heat treatment, which can complicate food preservation efforts (Akinsemolu & Onyeaka, 2024). *Pseudomonas aeruginosa* is also known for spoiling food products. It can break down proteins and lipids, resulting in unpleasant odours and tastes (WHO, 2024). These bacteria, particularly *S. aureus* and *P. aeruginosa*, indicate the potential for foodborne illness. Both *B. subtilis* and *P. aeruginosa* contribute to food spoilage, reducing the shelf life and quality of tiger nut milk (WHO, 2024). Therefore, contamination from S. aureus suggests inadequate sanitation and possible contamination from human handlers. *Penicillium* and *Aspergillus niger* are two types of fungi that can have implications for the safety and quality of tiger nut milk. The presence of *Penicillium* in the tiger nut sample after 24 hours can be a concern due to the potential production of mycotoxins, such as patulin and ochratoxin A. These mycotoxins have been associated with various health problems, including kidney and liver damage, immunosuppression, and carcinogenic effects (Pleadin *et al*., 2019). *Aspergillus niger* is a species of filamentous fungus that is commonly found in the environment and can also be associated with food products. *Aspergillus niger* is known to produce various enzymes, which can be beneficial for certain food processing applications. However, Goncalves *et al*. (2016) stated that some strains of *Aspergillus niger* can produce mycotoxins, such as ochratoxin A, which can be harmful to human health.

5. Conclusion

The study investigated the effect of fermentation duration on some quality aspects of tiger nut milk. We achieved the first objective by producing tiger nut milk and undergoing fermentation for 0, 12, 24, 36, and 48 hours. The physicochemical properties of the analysis, the observed decrease in pH and the increase in TTA over the fermented period indicate a rise in acidity due to the production of organic acids by fermenting microorganisms. The microbiological quality of the milk determined shows growths of the following microorganisms: *Staphylococcus aureus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Penicillium* and *Aspergillus niger*. The finding shows that these microorganisms can cause food poisoning and reduce shelf life, thereby leading to food spoilage. The presence of these microorganisms can be linked to improper hygiene practices during preparation or contamination from human handlers.

Conflicts of Interest
Authors declare that there is no conflict of interest.

Consent for Publication
All authors have given their consent for the manuscript to be submitted. There is no applicable data or material.

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