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*Full Length Research*

# Fermentation of tiger nut milk using *Lactobacillus plantarum*: An Implication for aflatoxin reduction

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**ABSTRACT:** Tiger nut (*Cyperus esculentus*) is a spherical rhizome crop that can be eaten raw, dried or processed. The study aims at examining the effects of fermentation on aflatoxins present in tiger nuts (*Cyperus esculentus* (L) milk using lactic acid bacteria. This study enumerated microorganisms in tiger nut milk during fermentation using standard microbiological methods. Extraction and quantification of aflatoxin were determined by HPLC analysis. All data obtained were statistically analysed using one-way analysis of variance (ANOVA). Unpasteurized big tiger nut milk had a total bacteria count of 6.40±0.57 CFU/mL, total Lactic Acid Bacteria (LAB) count of 0.80±0.07 CFU/mL and yeast count of 5.50±0.57 CFU/mL while pasteurized big tiger nut milk had a total bacteria count of 3.60±0.42 CFU/mL, total LAB count of 0.20±0.01 CFU/mL and yeast count of 0.30±0.03 CFU/mL. The Lactic acid bacteria isolated from the tiger nut drink were identified as lactococcus spp, *Lactobacillus plantarum* and *Lactobacillus fermentum*. Aflatoxin concentration of pasteurised big and small tiger nut milk fermented by *Lactobacillus plantarum* reduces as values range from 37.56 ±0.00 µg/kg at 0 h; 8.83 ±0.05 µg/kg at 24 h; 3.49 ±0.57 µg/kg at 48 h; 00.00 ±0.00 µg/kg at 72 h, and 96 h respectively. The study revealed that fermentation of tiger nut milk using *L. plantarum* significantly reduced the microbial loads and aflatoxins. Hence, the process of fermentation and pasteurisation improved the quality of tigernut milk and ensured stability and safety for consumers. I recommend that the tigernut milk should be kept in the freezer after 48-72 h of storage (room and fridge), which is sufficient for the reduction of the microbial load and aflatoxin in the tigernut milk.

**Keywords:** Aflatoxin, fermentation, lactic acid bacteria, mould, pasteurisation.

## INTRODUCTION

The cost of dairy milk and its products has led to the development of an alternative source of milk from plant materials in developing countries. Plant-based milks such as tiger nut milks act as a source of a suitable medium for probiotic bacteria, and are beneficial for people not consume dairy products (Mudgil, 2018). The consumption of tiger nut milk is becoming increasingly popular and taken by strict vegetarians and anyone with lactose intolerance, for it contains no lactose or fructose (Olagunju and Oyewumi, 2019). Tiger nut milk is a nourishing beverage but a suitable substrate of microorganisms such as *E. coli*, *S. aureus, Enterobacter*, Yeast and Moulds (Bibek, 2018). Despite its health benefits, tiger nut milk is prone to contamination by microbes and mycotoxins (Bibek, 2018; Shamsuddeen and Aminu, 2016). It can also be responsible for food-borne illnesses, which can be fatal if not handled well. Aflatoxins are hepatoxic and have been classified as Group 1 carcinogens by the International Agency for research on cancer (Koko *et al.,* 2024).

Fermentation has been used as a global method of food production and preservation for centuries. Lactic acid bacteria (LAB) are microbes applied in the development of plant-based fermented foods safely and effectively. The metabolic activity of food-fermenting LAB plays an

important role in the dietary value, sensory properties, shelf life and safety of products (Şanlier *et. al*., 2019).

This study reports the extensive effects of fermentation on aflatoxins present in tigernut (*Cyperus esculentus*) milk using *Lactobacillus plantarum*. Though there have been several reports on the potential of LAB fermentation for the reduction of mycotoxin levels in food products (Nduti, 2022), these studies mainly focus on staple foods and fermented dairy products, with limited attention to non-dairy plant-based beverages. This study provides a novel perspective by demonstrating that *L. plantarum* fermentation effectively reduces aflatoxin contamination in tiger nut milk. Unlike previous reports focusing on mycotoxin binding mechanisms, this study also examines the microbial load reduction during fermentation. The ability of *L. plantarum* to eliminate aflatoxins after 72 hours in tiger nut milk under controlled conditions is a novel finding, expanding the application of LAB-mediated mycotoxin reduction beyond conventional food matrices.

## MATERIALS AND METHODS

### Sample collection and preparation

Dried big and small tiger nuts were purchased from local markets in Abeokuta, Ogun State, Nigeria. The nuts were washed, soaked, blended with sterile distilled water and filtered to obtain tiger nut milk. Prior to the fermentation process, the extracted milk was pasteurised to destroy pathogenic microorganisms at 60°C for 30 mins.

### Enumeration, isolation and identification of microorganisms

Ten millilitres of tiger nut milk sample was homogenised with 90 ml of maximum recovery diluent (MRD, Oxoid) and serially diluted in the same diluents. Aliquots (0.1 ml) of appropriately diluted samples were spread plated on de Man Rogosa and Sharpe Agar (MRSA), Sabouraud Dextrose Agar (SDA) and Nutrient Agar (NA) plates. Plates were incubated anerobically at 30°C for 48h (LAB); aerobically at 25°C for 2-5 days (yeast and mould) and 37°C for 24 h (aerobic mesophilic bacteria) in triplicate. Colonial growths on the plates were enumerated, and the different colonies were described, isolated and assigned codes. The isolates were further purified by repeated streaking and subcultured on fresh MRS agar slant for storage at 4°C (Oluseun and Oluwatoyin, 2020). API 50 CHL system (Biomerieux® France) carbohydrate profiling in accordance with instructions stated by the manufacturer was used for further identification of LAB isolates.

### Preparation of starter cultures and fermentation of tiger nut milk

The *Lactobacillus plantarum* strain used as inoculum was prepared by transferring a loopful of an overnight culture from an MRS agar plate into 10 ml MRS broth and incubating at 35˚C for 24 hours in triplicate. 100 µl of the 24 hours old culture was transferred into 10 ml MRS broth and incubated at 35˚C for 16 hours (overnight). Subsequently, the cells were harvested by centrifugation at 3000 rpm for 10 mins; washed three times with 20 ml sterile distilled water and finally suspended in 10 ml of sterile distilled water, and these served as the isolate inoculum. Four flasks containing 500 ml of pasteurised and cooled rehydrated big and dried small tiger nut milk were inoculated with the washed LAB cells. Fermentation was allowed to occur in the flask at 37ºC for 24 hours, after which the tigernut milk was divided into two equal parts for storage at room temperature and in the refrigerator. Samples were withdrawn for all analysis at 0, 24, 48, 72 and 96 hours.

### Extraction and quantification of aflatoxin

According to method described by Manoochehri *et. al*. (2015) with modifications. About 2 g of the sample was weighed into 250 ml conical flask, 10 ml of purified water was added and shaken vigorously for 15 min. 5 ml of n-hexane was added to the mixture and continued the shaking for another 5 min in order to remove the fat. The n - hexane layer was separated from the aqueous layer. 5 ml of HPLC grade methanol and acetonitrile. 0.5 g of anhydrous sodium chloride was added and shaken for 10 minutes; the sample was later centrifuged for 5 min at 2000 RFP. The clear supernatant liquid was collected in a beaker, it was filtered through 0.45 mm Whattman filter paper. The filtrate was later cleaned up with C18 cartridges before injection for HPLC analysis. Agilent Technologies 1200 HPLC system was used with a ZORBAX Eclipse, C18 reversed phase column (150 × 4.6 mm), the flow rate was 0.8 ml/min. The mobile phase was a mixture of Purified water, HPLC grade Methanol and HPLC grade Acetonitrile in the ratio (50:40:10) v/v, and it was run on Isocratic mode. A Variable Wavelength detector (VWD) at a wavelength of 365 nm was used.

### Statistical analysis

Data obtained were subjected to statistical analyses using the Statistical Package for Social Sciences (SPSS) version 20.0 (IBM Corp., 2011). Mean values of unpasteurized and pasteurized Tiger nut milk were compared using One-Way Analysis of Variance (ANOVA). Mean values were compared by storage temperature and storage time using Multivariate Analysis of Variance (MANOVA). Results were presented as Mean±Standard Deviation. Post hoc test was done using the Student-Newman-Keuls (SNK). A probability value (p–value) less than 0.05 was considered to be statistically significant.

**Table 1.** Microbial count (x $10^7$ CFU/mL) in unpasteurized and pasteurized tiger nut milk (unfermented).

| Parameters | Total Bacteria count | Total LAB count | Yeast count |
|---|---|---|---|
| UBTM | 6.40±0.57[a] | 0.80±0.07[a] | 5.50±0.57[a] |
| PBTM | 3.60±0.42[b] | 0.20±0.01[b] | 0.30±0.03[b] |
| USTM | 5.50±0.57[a] | 0.30±0.03[b] | 1.40±0.42[a] |
| PSTM | 4.30±0.42[b] | 0.10±0.01[b] | 0.80±0.07[b] |

[abc]Mean values (±Standard deviation) in the same column having similar superscript are not significantly different (p>0.05). **Key:** UBTM - unpasteurized big tiger nut milk; PBTM - Pasteurized big tiger nut milk; USTM - Unpasteurized small tiger nut milk; and PSTM - Pasteurized small tiger nut milk.

**Table 2.** Biochemical characterization of lactic acid bacteria (LAB) isolates from spontaneously fermented tiger nut milk.

| Isolate code | Gram | Arabinose | Glucose | Lactose | Mannitol | Maltose | Cellulobiose | Raffinose | Ribose | Salicin | Rahmnose | Galactose | Sorbitol | Xylose | Trehalose | Catalase | Isolate |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | +R | + | + | + | + | + | + | + | + | + | - | - | + | + | + | - | *L. fermentum* |
| 2 | +C | + | + | - | + | + | + | + | - | + | - | + | + | - | + | - | *Lactococci* spp. |
| 3 | +C | + | + | - | + | + | + | + | - | + | - | + | + | - | + | - | *Lactococci* spp |
| 4 | +R | + | + | + | + | + | + | - | + | + | - | + | - | - | + | - | *L. plantarum* |

**Key:** +R - positive rods; +C - positive cocci; + - Positive; - - Negative; L – Lactobacillus.

## RESULTS

### Microbial count in unpasteurized and pasteurised unfermented tiger nut milk

Total bacteria count, total lab count, and yeast count of unpasteurized big tiger nut milk, pasteurised big tiger nut milk, unpasteurized small tiger nut milk, and pasteurised small tiger nut milk are shown in Table 1. Total bacterial count was highest in the unpasteurized big tiger nut milk. This was not significantly different from that of unpasteurized small tiger nut milk. Similarly, the total lab count was significantly ($p<0.05$) higher in the unpasteurized big tiger nut milk than in the pasteurised big tiger nut milk, the unpasteurized small tiger nut milk, and the pasteurised small tiger nut milk. The total yeast count was significantly lower in the pasteurised small tiger nut milk. Total yeast count recorded in the unpasteurized big tiger nut milk, pasteurised big tiger nut milk, and unpasteurized small tiger nut milk were not significantly ($p>0.05$) different.

### Biochemical characterisation of lactic acid bacteria (LAB) isolates from spontaneously fermented tiger nut milk

Two species (2) of genus Lactococci and two (2) species of Lactobacillus were successfully isolated from the spontaneously fermented tiger nut milk and identified based on relevant biochemical tests, as shown in Table 2. All the isolates were Gram-positive; the catalase test indicated that all isolates were non-catalase-producing bacteria. *Lactobacillus plantarum* fermented all the sugars except raffinose, xylose, sorbitol, and rhamnose, which indicates no colour change.

### Microbial count in pasteurised fermented big and small tiger nut milk

Table 3 shows the total bacterial, total LAB and yeast counts in pasteurised fermented big and small tiger nut milk at room temperature and refrigerator temperature. Total bacterial and total LAB counts were not recorded in the big and small tiger nut milk at 96 storage time at both room and refrigerator temperatures.

### Fermented big tiger nut milk

At room temperature, there was no significant difference in the mean total yeast count recorded in the big tiger nut milk stored for 0 hours and those stored for 72 hours. These were, however, significantly higher than those stored for 24 hours and 48 hours. Similarly, total bacterial count and total LAB count, total bacterial count and yeast count recorded in the big tiger nut milk were significantly highest

**Table 3.** Microbial count in fermented pasteurised big and small tiger nut milk (x $10^7$ CFU/mL).

| Storage | Time (hours) | Big yellow tiger nut milk | | | Small yellow tiger nut milk | | |
|---|---|---|---|---|---|---|---|
| | | Bacteria | LAB | Yeast | Bacteria | LAB | Yeast |
| Room temperature | 0 | 2.40±0.57$^{b}$ | 1.80±0.14$^{b}$ | 7.90±1.27$^{a}$ | 2.10±0.14$^{b}$ | 1.20±0.07$^{d}$ | 5.70±0.99$^{b}$ |
| | 24 | 1.10±0.03$^{b}$ | 3.60±0.42$^{a}$ | 2.10±0.14$^{b}$ | 1.00±0.14$^{b}$ | 6.80±0.14$^{c}$ | 1.51±0.08$^{d}$ |
| | 48 | 8.00±0.57$^{a}$ | 4.10±0.14$^{a}$ | 3.60±0.85$^{b}$ | 6.00±0.85$^{a}$ | 8.40±0.57$^{b}$ | 6.50±0.42$^{a}$ |
| | 72 | 0.00±0.00$^{c}$ | 2.20±0.14$^{b}$ | 7.40±0.57$^{a}$ | 2.00±0.28$^{b}$ | 9.10±0.14$^{a}$ | 4.00±0.42$^{c}$ |
| | 96 | 0.00±0.00$^{c}$ | 0.00±0.00$^{c}$ | 2.80±0.10$^{b}$ | 0.00±0.00$^{c}$ | 0.00±0.00$^{e}$ | 5.10±0.14$^{b}$ |
| Fridge temperature | 0 | 2.40±0.57$^{a}$ | 1.80±0.14$^{b}$ | 7.90±1.27$^{a}$ | 2.10±0.14$^{a}$ | 1.20±0.07$^{c}$ | 5.70±0.99$^{b}$ |
| | 24 | 1.40±0.28$^{b}$ | 2.40±0.42$^{b}$ | 1.40±0.21$^{c}$ | 1.60±0.42$^{b}$ | 5.40±0.42$^{b}$ | 8.00±0.85$^{a}$ |
| | 48 | 1.10±0.07$^{b}$ | 3.20±0.28$^{a}$ | 2.20±0.07$^{c}$ | 1.00±0.04$^{b}$ | 6.60±0.85$^{a}$ | 1.30±0.28$^{c}$ |
| | 72 | 0.00±0.00$^{c}$ | 1.80±0.07$^{b}$ | 5.00±0.28$^{b}$ | 0.00±0.00$^{c}$ | 7.20±0.28$^{a}$ | 6.00±0.57$^{b}$ |
| | 96 | 0.00±0.00$^{c}$ | 0.00±0.00$^{c}$ | 1.00±0.14$^{c}$ | 0.00±0.00$^{c}$ | 0.00±0.00$^{d}$ | 1.40±0.07$^{c}$ |
| Storage * Time | F value | 93.20 | 5.52 | 3.97 | 50.80 | 6.68 | 62.58 |
| | p value | 0.01* | 0.01* | 0.04* | 0.02* | 0.01* | 0.01* |

$^{abc}$Mean values (±Standard deviation) in the same column for big and small yellow tiger nut respectively having similar superscripts are not significantly different ($p > 0.05$); *Interaction significant at $p < 0.05$. **Key:** LAB – Lactic Acid Bacteria.

in the samples stored for 48 hours. At refrigerator temperature, the total bacterial count and yeast count of big tiger nut milk were significantly (p-value) higher in the samples stored for 0 hours. Also, the total LAB count recorded on big tiger nut milk was significantly (p-value) higher in the samples stored for 48 hours.

**Fermented small tiger nut milk**

At room temperature, the total bacterial count and total LAB count recorded in the big tiger nut milk and the total bacterial count and yeast count recorded in the small tiger nut milk were significantly high in the samples stored for 48 hours. On the other hand, the total LAB count of small tiger nut milk was significantly high at 72 hours of storage and lowest at 0 hours. At refrigerator temperature, the total bacterial count of small tiger nut milk was significantly highest in the samples stored for 0 hours. The total LAB count of small tiger nut milk was significantly highest in samples stored for 72 hours, which was the end of fermentation, and lowest in the samples stored for 0 hours. Also, the yeast count of small tiger nut milk was significantly high in samples stored for 24 hours. The size of the tiger nut and storage time showed significant statistical interaction ($p < 0.05$) with the total bacterial count, total LAB count and yeast count of the pasteurised fermented tiger nut milk at both room and refrigerator temperatures.

**Microbial count in pasteurized unfermented big and small tiger nut milk**

Table 4 presents the total bacterial, total lab, and yeast counts in pasteurised unfermented big and small tiger nut milk at room temperature and refrigerator temperature.

**Big tiger nut milk:** At room temperature, the total bacterial count of big tiger nut milk was significantly lower in the samples stored for 48 hours. Similarly, the total LAB count of the big tiger nut milk was highest (p-value) in the samples stored for 72 hours and least in those stored for 0 hours. Yeast count of the big tiger nut milk was, however, significantly higher (p-value) in those stored for 96 hours. At refrigerator temperature, the total bacterial count was significantly highest in the big tiger nut milk samples stored for 24 hours. On the other hand, total LAB count and yeast count of big tiger nut milk were significantly (p-value) higher in the samples stored for 72 hours.

**Small tiger nut milk:** At room temperature, the total bacterial count of small tiger nut milk was significantly lower in the samples stored for 48 hours. Also, the total LAB count of the small tiger nut milk was highest (p-value) in the samples stored for 72 hours and lowest in those stored for 0 hours. However, the yeast count of small tiger nut milk was significantly (p-value) higher in those stored for 24 hours. At refrigerator temperature, the total LAB count of small tiger nut milk was significantly (p-value) higher in the samples stored for 72 hours. Also, total bacterial count and yeast count were significantly (p-value) higher in the samples stored for 0 hours.

The size of the tiger nut and storage time showed a significant ($p < 0.05$) statistical interaction with the total LAB count and yeast count of the pasteurised unfermented tiger nut milk at room temperature. Also, the size of the tiger nut and storage time showed significant ($p < 0.05$) statistical interaction with the total bacterial count and

**Table 4.** Microbial count of pasteurised unfermented big and small tiger nut milk (x $10^7$ CFU/mL).

| Storage | Time (hours) | Big yellow tiger nut milk | | | Small yellow tiger nut milk | | |
|---|---|---|---|---|---|---|---|
| | | Bacteria | LAB | Yeast | Bacteria | LAB | Yeast |
| Room temperature | 0 | 3.60±0.42$^{a}$ | 2.00±0.14$^{b}$ | 1.20±0.07$^{c}$ | 4.30±0.42$^{a}$ | 1.00±0.03$^{c}$ | 8.00±0.71$^{b}$ |
| | 24 | 2.68±0.11$^{a}$ | 3.70±0.28$^{b}$ | 2.83±0.04$^{b}$ | 5.70±0.99$^{a}$ | 3.20±0.28$^{b}$ | 9.40±0.57$^{a}$ |
| | 48 | 1.32±0.03$^{b}$ | 7.60±0.85$^{a}$ | 1.35±0.07$^{c}$ | 3.40±0.57$^{b}$ | 4.30±0.42$^{b}$ | 1.40±0.07$^{d}$ |
| | 72 | 3.90±1.27$^{a}$ | 8.70±0.99$^{a}$ | 1.38±0.11$^{c}$ | 0.00±0.00$^{c}$ | 7.30±0.28$^{a}$ | 7.30±0.42$^{b}$ |
| | 96 | 0.00±0.00$^{c}$ | 6.70±0.28$^{a}$ | 3.30±0.42$^{a}$ | 0.00±0.00$^{c}$ | 5.00±0.35$^{b}$ | 5.70±0.28$^{c}$ |
| Fridge temperature | 0 | 3.60±0.42$^{b}$ | 2.00±0.14$^{c}$ | 1.20±0.07$^{c}$ | 4.30±0.42$^{a}$ | 1.00±0.03$^{c}$ | 8.00±0.71$^{a}$ |
| | 24 | 6.40±0.57$^{a}$ | 3.30±0.42$^{b}$ | 2.84±0.06$^{b}$ | 1.60±0.42$^{b}$ | 2.10±0.14$^{b}$ | 6.30±0.42$^{b}$ |
| | 48 | 4.80±1.13$^{b}$ | 3.60±0.85$^{b}$ | 7.20±0.28$^{a}$ | 1.30±0.28$^{b}$ | 2.40±0.14$^{b}$ | 4.00±0.42$^{c}$ |
| | 72 | 2.80±0.14$^{c}$ | 6.40±0.57$^{a}$ | 7.60±0.85$^{a}$ | 0.00±0.00$^{c}$ | 4.60±0.85$^{a}$ | 2.00±0.07$^{d}$ |
| | 96 | 1.00±0.08$^{d}$ | 1.80±0.14$^{c}$ | 2.40±0.28$^{b}$ | 0.00±0.00$^{c}$ | 1.40±0.21$^{c}$ | 4.00±0.28$^{c}$ |
| Storage * Time | F value | 12.48 | 14.77 | 111.46 | 17.62 | 15.28 | 44.46 |
| | p value | 0.01* | 0.01* | 0.01* | 0.01* | 0.01* | 0.01* |

$^{abc}$Mean values (±Standard deviation) in the same column for big and small yellow tiger nut respectively having similar superscript are not significantly different ($p > 0.05$); *Interaction significant at $p<0.05$. **Key:** LAB - Lactic Acid Bacteria.

**Table 5.** Morphological, biochemical and gram reaction of bacterial isolates from unfermented and fermented tiger nut milk.

| I/N | GR | shape | CA | CT | CO | Indole | Motility | Sugar Glucose | Fermentation Lactose | Sucrose | Identified organism |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | + | Cocci | + | + | + | - | - | AG | AG | AG | *Staphylococcus aureus* |
| 2 | + | Rod | + | + | - | - | + | + | + | + | *Bacillus subtilis* |
| 3 | - | Rod | + | + | - | - | + | AG | - | - | *Pseudomonas aeruginosa* |

**Key:** + - Positive, - - Negative, AG - Acid and Gas Production, I/N- Isolates number, GR- Gram's reaction, CA- catalase, CT- citrate, CO- coagulase.

**Table 6.** Cultural and morphological characteristics of mould isolates from tiger nut.

| Isolates code | Type of mycelium | Colour of mycelium | Shape of colony | Septation | Colour of spore | Type of reproduction | Suspected organism |
|---|---|---|---|---|---|---|---|
| 1 | Conidiospore | Grey | Circular | Septate | Yellowish green | Sexual with scospore | *Aspergillus flavus* |
| 2 | Conidiospore | Grey | Circular | Septate | Black | Sexual with scospore | *Aspergillus niger* |
| 3 | Conidiospore | Grey | Circular | Septate | Blue green | Sexual | *Penicillium* spp |

yeast count of the pasteurised unfermented tiger nut milk at fridge temperature. Table 5 shows the morphological, biochemical and gram reaction of bacterial isolates from unfermented and fermented tiger nut milk. The identified bacteria were *Staphylococcus aureus, Bacillus subtilis and Pseudomonas aeruginosa.* In Table 6, cultural and morphological characteristics of mould isolates on potato dextrose agar (PDA) from tiger nut were shown, and the identified organisms are *Aspergillus flavus, Aspergillus niger* and *Penicillium* spp. The morphological and biochemical characteristics of yeast isolated from unfermented and fermented tiger nut milk are shown in Table 7 as *Saccharomyces cerevisiae* and *Candida* spp.

**Aflatoxin level in unfermented, unpasteurized, pasteurised, and *lactobacillus plantarum* fermented big and small tiger nut milk**

Total aflatoxin concentration was significantly highest in unpasteurized big tiger nut milk, having 173.18 µg/kg and 42.22 µg/kg in unpasteurized small tiger nut milk. The aflatoxin concentration in the pasteurised tiger nut milk, for both big and small were 47.18 µg/kg and 34.28 µg/kg, respectively. Table 8 presents the total aflatoxin concentration of the *lactobacillus plantarum* fermented pasteurised big and small tiger nut milk at varying storage times. Aflatoxin concentration recorded in the big and

**Table 7.** Morphological and biochemical characteristics of yeast isolates from unfermented and fermented tiger nut milk

| Isolates code | Cultural characteristics | Reproduction | Sugar Fermentation | | | | | Identified organisms |
|---|---|---|---|---|---|---|---|---|
| | | | Glu | Lac | Mal | Suc | Fru | |
| 1 | Whitish, circular, smooth | Budding | A | - | - | A | A | *Saccharomyces cerevisiae* |
| 2 | Creamy, smooth, flat, circular, raised | Budding | A | A | AG | A | A | *Candida* spp |

**Keys:** A - Acid fermentation, AG - Acid and Gas production, Glu - glucose, Lac - lactose, Mal - maltose, Suc - sucrose, Fru – fructose.

**Table 8.** Total aflatoxin concentration of fermented pasteurised big and small tiger nut milk (µg/kg) by *Lactobacillus plantarum.*

| Storage | Time (hours) | Big | Small |
|---|---|---|---|
| Room temperature | 0 | 37.56±0.78$^{a}$ | 9.33±0.47$^{a}$ |
| | 24 | 5.67±0.08$^{b}$ | 3.57±0.10$^{b}$ |
| | 48 | 1.32±0.03$^{c}$ | 0.00±0.00$^{c}$ |
| | 72 | 0.00±0.00$^{d}$ | 0.00±0.00$^{c}$ |
| | 96 | 0.00±0.00$^{d}$ | 0.00±0.00$^{c}$ |
| Fridge temperature | 0 | 37.56±0.00$^{a}$ | 9.33±0.47$^{a}$ |
| | 24 | 8.83±0.05$^{b}$ | 3.72±0.03$^{b}$ |
| | 48 | 3.49±0.57$^{c}$ | 0.00±0.00$^{c}$ |
| | 72 | 0.00±0.00$^{d}$ | 0.00±0.00$^{c}$ |
| | 96 | 0.00±0.00$^{d}$ | 0.00±0.00$^{c}$ |
| Storage * Time | F value | 24.16 | 0.10 |
| | p value | 0.04* | 0.98 |

$^{abc}$Mean values (±Standard deviation) in the same column for big and small yellow tiger nut, respectively, having similar superscript are not significantly different (p>0.05); *Interaction significant at p<0.05. **Key:** Big - big yellow tiger nut milk, Small - small yellow tiger nut milk.

small yellow tiger nut milk stored under room temperature and those stored under refrigerator temperature followed the same trend. This was significantly (p-value) higher in the tiger nut milk stored for 0 hours, followed by those stored for 24 hours. Aflatoxins were not recorded in the big tiger nut stored for 72 hours and 96 hours. Similarly, aflatoxins were not recorded in the small tiger nut stored for 48 hours, 72 hour and 96 hours. Also, a significant (p = 0.001) statistical interaction was recorded between the tiger nut size and storage time on the aflatoxin levels of the tiger nut milk at room and refrigerator temperatures.

## DISCUSSION

The presence of *Bacillus subtilis* in the pasteurised and fermented tiger nut milk may be attributed to its thermoduric nature. It is a spore forming bacteria that is commonly found in soil, water (through soil water contamination) and on plants. The spores of *Bacillus subtilis* can survive harsh temperatures such as pasteurisation temperature (Ike *et al*., 2015), hence their survival in the pasteurised tiger nut milk sample. Other aerobic bacteria, which are *Staphylococcus aureus* and Pseudomonas spp, were also found in the beverage. *Staphylococcus aureus* is known as human skin normal flora, which could have been introduced by chance into the tiger nut milk. High moisture content typically allows microbial growth (Adesakin and Obiekezie, 2020).

Lactic acid bacteria (LAB) can dominate other bacteria involved in natural fermentation. They are an essential group of microorganisms that can secrete antimicrobial substances in foods, some of which may have antagonistic activities against food-borne pathogens and spoilage organisms (Zapaśnik *et al.,* 2022). According to Bintsis (2018), LAB are used to enhance food safety, preserve food quality, develop characteristic new flavours, and improve the nutritional qualities of foods.

Table 3 shows that as fermentation progresses, there was a steady increase in LAB count in each of the tiger nut milk drinks stored at room and refrigerator temperatures within 0 – 48 hours. However, between 72 - 96 hours, there was a reduction in lactic acid bacterial count in both the fermented big and small tiger nut-milk drink stored at room

and refrigerator temperatures, except for the uninoculated. The reduction in lactic acid bacterial count at 72 hours could be a result of fast-depleting nutrients in the tigernut-milk drink. Hence, the LAB might be at the stationary phase and probably as a result of stiffer antagonism for restricted fast-depleting nutrients (Maduka *et al*., 2017).

During the fermentation, *Saccharomyces cerevisae* and *Candida* spp were the yeast isolated. Researchers have reported that the presence of *Saccharomyces cerevisae* in the fermenting tiger nut milk could be due to its ability to tolerate acid, alcohol and grow at low temperatures. It could also be as a result of the substrate being a plant material with a carbohydrate source. The mould (*Aspergillus niger, Aspergillus flavus, Penicillium spp*.) isolated was from the sterile water used for rinsing the tiger nut, which is similar to the observation of Ike *et al.* (2017) during the microbial evaluation of tiger nuts.

The presence of aflatoxins in tiger nut and its beverage has been reported by some researchers (Sa'id *et al*., 2017). As nutritive and therapeutic as the tiger nut drink is, the presence of aflatoxin compounds, which are highly toxic, mutagenic and carcinogenic, can render it unacceptable and unhealthy. The reduction of aflatoxins from contaminated food is essential to improve the safety of such food. The use of biological agents like probiotic LAB is a vital approach. Microbial starters have been successfully used for fodder conservation and mycotoxin control in silage making (Ogunade *et al*., 2018), and studies carried out in Uganda, Tanzania and Ethiopia have suggested that using selected *Lactobacillus* strains as starters for fermentation reduced aflatoxin levels efficiently in milk and traditional maize-based fermented foods (Shigute and Washe, 2018; Wacoo *et al*., 2018).

As fermentation progressed, the aflatoxin content in the tiger nut milk was reducing, and in 72 hours of fermentation, which is the post-fermentation phase, aflatoxin content was below the analytical limit of detection **(**LOD**)**.

## Conclusion

The study revealed that fermentation of tiger nut milk using *L.plantarum* significantly reduced the microbial loads and the total reduction of aflatoxins (B1, B2, G1 and G2). The synergistic effects of fermentation and pasteurisation have the ability to improve the quality of the product. Hence, the use of tiger nut milk with *Lactobacillus plantarum* as a Nigerian fermented drink is novel, safe and beneficial to consumers.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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