

Microbiological assessment of tigernut milk as a potential probiotic product

Osho, M. B.* and Sobande, O. E.

Department of Biological Sciences, (Microbiology Unit) College of Natural & Applied Sciences,
McPherson University, Km 96, Lagos-Ibadan Expressway, Seriki Sotayo, P.M.B. 2094,
Abeokuta, Nigeria.

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Abstract

This study aimed at developing starter cultures as probiotic from spontaneous fermented tigernut milk (SFTM) by the natural flora. Fermented tiger nut milk (FTM) was produced from these varieties – small dried brown, big dried brown and fresh yellow tiger nuts. Microbiological analysis of SFTM and pasteurized fermented tiger nut milk (PFTM) were carried out using Nutrient agar, De Mann Rogosa and Sharp and Malt Extract Agar for isolation of aerobic bacteria, lactic acid bacteria and yeast respectively. The pH of SFTM and PFTM, and titratable acidity of the lactic acid produced were investigated. Lactic acid bacteria (LAB) *Lactococcus lactis* (LC) and two species of yeast *Pichia sp.* PM13 and *Pichia kudriazevii* CBS 245 were obtained from FTM at different fermentation periods. LAB were found to predominate the microflora of the FTM with their count ranging between 2.2×10^4 - 6.5×10^4 (SFTM) and 4.6×10^5 - 7.9×10^6 (PFTM) and pH ranged 4.3- 6.5. The quantity of lactic acid produced by the LAB isolate ranged between 0.46 g/l - 1.92 g/l and hydrogen peroxide between 0.16 g/l – 0.51 g/l. Thus, the propagation and selection of known starter cultures as a probiotic microbes might bring about uniformity of product.

Keywords: Tiger nut milk; Lactic acid bacteria, Spontaneous, Starter Culture; Probiotic Product

*Corresponding Author: osho_michael@rocketmail.com; +2348032698955addresses:

omowunmi.oluwole@gmail.com

Introduction

Tiger nut (*Cyperus esculenta*), popularly referred as "Ofio" in Yoruba, "Akiausa" Igbo and "Ayaya" Hausa in Nigeria has three varieties (brown, yellow and black) which can be eaten raw, roasted, dried, baked or be made into milk (Oladele and Aina, 2007). Ukwuru and Ogbodo, (2011) and Roselló-Soto et al. (2019) reported that four varieties of the milk product viz: pasteurized, natural, ultra-high temperature, and condensed tiger nut milk could be obtained when tigernut milk undergoes fermentation. *C. esculentus* is rich in energy content (starch, fat, sugar, and protein), minerals (mainly phosphorous and potassium), and vitamins E and C (Belewu and Belewu, 2007) thus making this

tuber also suitable for diabetics and for those intent on losing weight (Boerges et al., 2008). Starter culture micro-flora in raw milk and fermented milks belong to a family of bacteria collectively known as the Lactic Acid Bacteria (LAB) which occur naturally as indigenous organisms and are widely distributed in nature (Olokun et al., 2018). LAB have the ability to dominate other bacteria involved in natural fermentation of tigernut milk drink. They possess adhesional adaptation with ability to survive different environments such as diverse food matrices (Wakil et al., 2014). Tigernut can support the growth of lactic acid bacteria being slightly acidic pH 6.34. Maduka et al. (2017) reported that tigernut is a substrate that can sustain microbial

growth possibly due to the near neutral pH of tigernut tubers which favours the growth of many microorganisms. There is paucity of research on the effect of using spontaneous and/or pasteurized tigernut milk for LAB growth as a mean of appropriating lactic acid bacteria into tigernut milk drink to serve as a potential probiotic product and might not in some products such as yoghurt maintain adequate quantity of the probiotic bacteria. Hence, as an essential requirement, a labeled probiotic product must meet the number of probiotic bacteria (Irkin and Guldass, 2011). This study explored the efficiency of starter cultures isolated from the spontaneous fermentation for uniformity of product as a potential probiotic microorganisms.

Materials and Methods

Three different varieties of tiger nut - small brown tiger nut, big brown tiger nut, yellow tiger nut designated SBT, BBT and YTN respectively were purchased in Agege market, Agege Local Government Area, Lagos State, Nigeria and were packed properly in sterile polythene bags. De Mann Rogosa and Sharpe (MRS) (HiMedia Laboratories Pvt. Ltd), Nutrient agar (NA), Malt extract agar (MEA), Yeast extract, Hydrogen peroxide, Sodium hydroxide, Potassium permanganate, Tetraoxosulphate (VI) acid, phenolphthalein were of analar grade.

Production and Fermentation of Tigernut Milk

Wholesome tiger nuts were separated out to eliminate pebbles, stones, and dirt materials before rinsing in water to remove cling particles. The method of Ukwuru and Ogbodo (2011) was adopted for the preparation of the milk. The tuber were swilled in distilled water and soaked at 60 °C for 6 h to assuage the fiber. The tiger nut (200 g) was whisked by adding 500 ml of warm distilled water with sterile blender (Marlex, Electrolin). The mash was strained using a clean muslin cloth to remove shafts from the milk. The filtered milk was then transferred into conical flask and pasteurized in a water bath at 90 °C for 15 mins and cooled to a temperature of 43 °C. Spontaneous fermentation was conducted using the milk natural flora at 24 h and analysis such as pH, the microbial quality and chemical composition were also investigated at 0 h fermentation period.

Microbiological Determination of Spontaneous Fermented Tigernut Milk

De Mann Rogosa and Sharpe (MRS), Malt Extract Agar (MEA) and Nutrient agar (NA) were used for culturing of lactic acid bacteria, yeast and aerobic bacteria respectively. The media were prepared according to manufacturer's specifications and sterilized at 121 °C for 15 mins. Serial dilution was done using the tiger nut milk by taking 1 ml tiger nut milk into a test tube containing 9 ml distilled water and further transferred into other test tubes. Half (0.5) ml dilution factor (10^{-3}) was pour plated on each sterile molten agar, swirled and allowed to set. NA plates were aerobically incubated at 37 °C for 24 h and MRS agar was incubated anaerobically at 37 °C for 48 h and MEA at 30 °C for 48 h (all in triplicate). Colonies on the plates were counted, repeatedly streaked out to obtained pure cultures and maintained on agar slants at 4 °C. Total colony count was expressed in colony forming units per millilitre (cfu ml^{-1}) according to Harrigan and McCance (1990).

Characterization of Isolates

The isolates were identified on the basis of their cultural and morphological characteristics (Holt et al., 2000) and confirmed by using molecular method of characterization. Total genomic DNA from the bacteria was isolated according to the protocol of Zymo Research Bacterial DNA MiniPrep™ Instruction Manual and kit. Oligonucleotide primers were produced to amplify of the purified template genomic DNA. The forward primer 5'AGAGTTTGATCCTGGCTCAG 3' and the reverse primer, 5'AAGGAGGTGWTCARCCGCA 3' were obtained from Eurofins, UK. The DNA eluted from agarose gel was sequenced using AB 373a Strech (short gun) DNA sequencer according to the manufacturer's instruction. The comparison of the nucleotide sequences of the unique fragment with the sequences available in the GenBank database was carried out using the N C B I B L A S T program (<http://www.ncbi.nlm.nih.gov/blast>).

Preparation of Inoculums

The pure cultures of LAB and yeasts isolated from SFTM were used as potentials starters after their screening (AOAC, 2000). The LAB culture

was inoculated onto fresh MRS agar slants and anaerobically incubated at 30 °C for 48 h while the yeasts were inoculated on Malt Extract agar and incubated at 28 °C for 48 h. The cells were dispersed through shaken by adding 9 ml sterile distilled water to the media. Appropriate serial dilutions were obtained and number of cells calculated (Rodriguez-Tudela et al., 2003). Dilution factor of 10⁵cfu/ml was attained when viable count was checked on MRS agar. Inoculum size 3.1 × 10⁵ to 8.8 × 10⁵cfu/ml and 2.0 × 10⁵ to 7.2 × 10⁵cfu/ml as viable count for LAB and yeasts respectively were used for the preparation of starter fermented tiger nut milk.

Laboratory Preparation of Starter Fermented Tigernut Milk

Treatment of each tigernut milk variety mixtures were processed as described during and thereafter pasteurized and cooled. The mixtures were then inoculated with 1% washed cells of starter cultures and fermented at 45 °C for 18 h. Samples were intermittently taken to determine the quantity of lactic acid using titratable acidity and also the chemical analysis every 6 h.

Screening for Potential Starters

The starter cultures were screened for their potential to produce lactic acid and hydrogen peroxide using titratable acidity and determination of hydrogen peroxide method respectively (A.O.A.C., 2000).

Determination of Lactic Acid

The production of lactic acid was determined using titratable acidity method by titrating 10 ml homogenized tiger nut milk (SBT,

BBT and YTN) against 2.5 M Sodium hydroxide (NaOH) using phenolphthalein as indicator (AOAC, 2000). The acidity was calculated using the formula in Equation 1.

$$\text{Acidity} = \frac{\text{Volume of 0.1N NaOH used} * 0.2 * 6 \times 100}{10 * \text{volume of sample taken}} \dots \text{Eqn. 1}$$

Determination of Hydrogen Peroxide

Diluted H₂SO₄ (10 ml) was added to 12.5 ml of the fermented milk and was titrated against 0.1N Potassium permanganate according to A.O.A.C (2000). The strength was calculated using the formula in Equation 2.

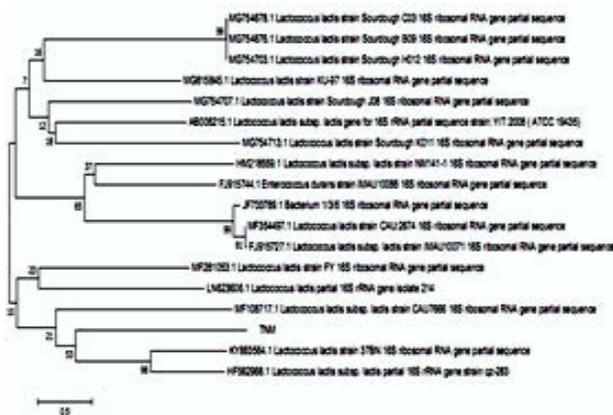
$$\text{Strength H}_2\text{O}_2 \% = \frac{N * V * \text{KMnO}_4}{0.25} \dots \text{Eqn. 2}$$

Statistical Analysis: Data were subjected to one-way analysis of variance (ANOVA) and means were compared using Turkey's test. Statistical significance was obtained at p<0.05.

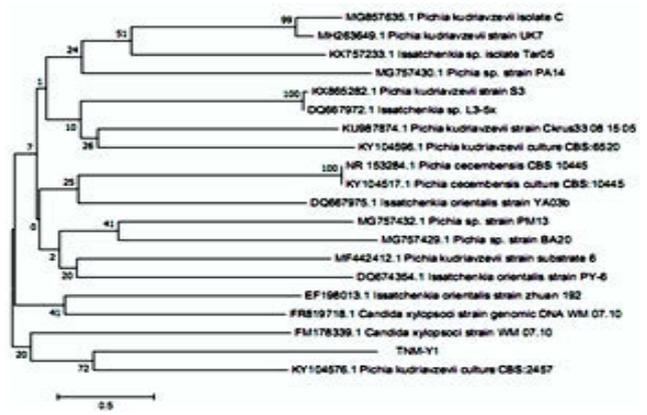
Results and Discussion

Characterization of Isolates

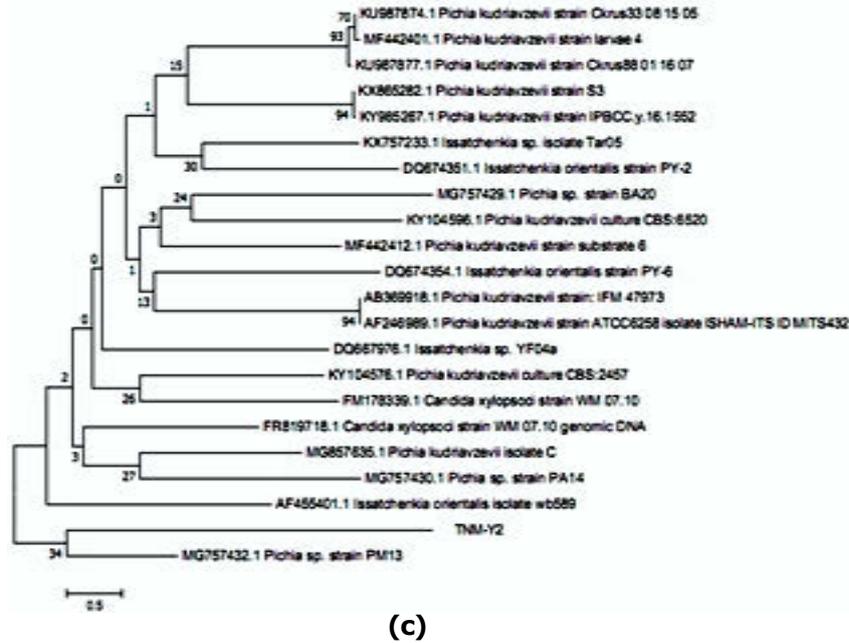
Based on the results of the cultural, morphological and molecular characterization, LAB and yeast species were identified. The isolate with NCBI Association number KY883564.1 was identified as *Lactococcus lactis*. Their maximum percentage identity was 96% and the NCBI Association number for yeasts are 37.19626813 and 23.87374498 identified as *Pichia kudriazevii* and *Pichia* sp. Their maximum percentage identity was 27% and 72%. Morphologically, the lactic acid bacteria present were cocci shape and Gram positive. The Phylogenetic trees in relation to other species are as shown in Fig.1 a, b and c respectively.



(a)



(b)



(c)

Fig. 1: Phylogenetic tree of (a) *Lactococcus lactis* strain TNM (b) *Pichia kudriavzevii* strain TNM-Y1; (c) *Pichia* sp. strain TNM-Y2

Microbiological Analysis of Fermented Tigernut Milk

Table 1(a) and 1(b) revealed the microbial count of spontaneous and pasteurized fermented tiger nut milk. The pasteurized milk had the highest microbial counts of all the isolates and samples as compared with the spontaneous milk. There was increase in the total aerobic count in all milk samples within 24 h of fermentation after which a decline was noticed. It ranges from 4.5×10^5 - 5.2×10^5 ; 3.4×10^5 - 5.9×10^5 ; and 5.2×10^5 - 6.4×10^5 for SBT, BBT and YTN respectively. Lactic acid bacteria had the highest among the isolates in all samples with YTN having 7.9×10^6 ; SBT - 7.2×10^6 and BBT - 7.1×10^5 at 6 h fermentation period. The LAB load in YTN and SBT met the requirement of minimum of biovalue (MBV) of probiotic product. Nyanzi and Jooste, (2012) recommended $6 \text{ Log } 10\text{cfu/ml}^{-1}$ per serving of probiotic microorganisms to ensure health deriving benefits from the product. This was contrary to the results of Maduka et al. (2017) that studied fermentation of tigernut by LAB and tigernut-milk drink fermentation by LAB as a potential probiotic product, though the study has demonstrated that inoculating LAB into

pasteurized tigernut resulted in higher LAB count than spontaneous tigernut but does not meet the MBV of probiotic product. Cogan et al., (1997) had high incidence of LAB species in fermented tiger nut milk from lactic acid bacteria of different fermented milk and dairy products. Moreover, as fermentation time increases there was an increase in total yeast count in all samples. According to Holzapfel (2000) that investigated the appropriate starter culture technologies for small scale fermentation reported that the substrate being a plant source might be responsible for the existence of yeast in the fermenting milk. However, the pasteurized fermented tiger nut milk recorded the highest microbial load of total LAB count especially in YTN 6.8×10^6 at 6 h and decreases to 4.8×10^6 while Total aerobic count of BBT had the lowest range from 2.8×10^4 and 3.8×10^4 . It has been reported by Nwachukwu et al. (2010) that there was an increase in total viable count in sterilized millet inoculated with *Lactobacillus plantarum*, *Lactobacillus cellobiosus* and *Pediococcus pentosaceus* during fermentation. LAB being the most predominant microorganisms during fermentation, an increase in LAB inoculum size obviously increased the LAB count.

Table 1(a): Microbial Count of Spontaneous Fermented Tigernut Milk (SFTM)

Sample	SBT				BBT				YTN			
	6	12	18	24	6	12	18	24	6	12	18	24
Total												
Aerobic Count	4.2 x10 ⁴	4.3 x10 ⁴	3.3 x10 ⁴	2.4 x10 ⁴	3.8 x10 ⁴	3.5 x10 ⁴	3.4 x10 ⁴	2.8 x10 ⁴	5.7 x10 ⁴	5.2 x10 ⁴	5.4 x10 ⁴	3.9 x10 ⁴
Total												
LAB Count	6.6 x10 ⁴	4.3 x10 ⁴	4.2 x10 ⁴	3.5 x10 ⁴	5.6 x10 ⁵	5.11 x10 ⁵	4.9 x10 ⁵	7.9 x10 ⁵	6.8 x 10 ⁶	6.1 x10 ⁶	5.5 x10 ⁶	4.8 x10 ⁶
Total												
Yeast Count	5.2 x10 ⁴	4.4 x10 ⁴	3.1 x10 ⁴	2.0 x10 ⁴	6.7 x10 ⁴	5.9 x10 ⁴	5.4 x10 ⁴	3.6 x10 ⁴	6.9 x10 ⁴	5.6 x10 ⁴	5.4 x10 ⁴	4.2 x10 ⁴
Values are means of triplicate determination												

Table 1(b): Microbial Count of Pasteurized Fermented Tigernut Milk (SFTM)

Sample	SBT				BBT				YTN			
	6	12	18	24	6	12	18	24	6	12	18	24
Total												
Aerobic Count	5.2 x10 ⁵	4.9 x10 ⁵	4.5 x10 ⁵	4.8 x10 ⁵	5.9 x10 ⁵	5.5 x10 ⁵	3.4 x10 ⁵	3.4 x10 ⁵	6.4 x10 ⁵	5.7 x10 ⁵	5.4 x10 ⁵	5.2 x10 ⁵
Total												
LAB Count	7.2 x10 ⁶	5.3 x10 ⁶	4.3 x10 ⁶	2.4 x10 ⁶	7.1 x10 ⁵	6.5 x10 ⁵	5.4 x10 ⁵	4.6 x10 ⁵	7.9 x10 ⁶	5.7 x10 ⁶	6.4 x10 ⁶	5.8 x10 ⁶
Total												
Yeast Count	4.8 x10 ⁵	4.3 x10 ⁵	3.1 x10 ⁵	2.0 x10 ⁵	6.4 x10 ⁵	5.5 x10 ⁵	4.4 x10 ⁵	3.6 x10 ⁵	7.4 x10 ⁵	4.5 x10 ⁵	6.4 x10 ⁵	6.2 x10 ⁵
Values are means of triplicate determination												

Production and Fermentation of Tiger Nut Milk

Table 2 revealed an increase in fermentation period as the pH of the fermented milk proportionally decreased. The highest pH 4.3 was observed in YTN milk at 18 h and 24 h and the least pH 6.5 was observed in SBT and BBT milk at 0 h for natural flora fermentation. The pH of starter developed fermented milk, using combined starters, as illustrated in Table 3 also showed that there was a decrease in pH as fermentation time increases. The lowest pH 3.7 was observed in *Lactococcus lactis*, *Pichia kudriazevii* of varieties of yellow at 18 h. The highest pH for variety big brown (6.5), small brown (6.5) and yellow (4.4) were all noted in the SFTM and was significantly different ($p > 0.05$). This was similar to the report of Makut et al. (2018) that the values for pH showed no significant difference ($p=.05$) as the values were so close between pH 4.0 - 4.4. Reed (1982) studied and noted that a good quality fermented

milk product should have a pH 4.15. This has been attributed to the fermentation process with other processing procedures such as cooking and roasting (Aletor 1993). Ndikom and Elutade (2016) in their study isolated LAB from tigernut tubers reported that it was slightly acidic (pH 6.34) can support the growth of lactic acid bacteria (Umerie et al., 1997). Maduka et al. (2017) also attested to the fact that decrease in pH could be as a result of fast LAB growth rate which break down the starch component during fermentation and ultimately resulted into an increase in quantity of lactic acid.

Screening for Potential Starters. The result of the production of the antimicrobial compounds as shown in Fig. 2 where the highest lactic acid measured using titratable acidity was observed at 24 h in LAB isolates. The highest value was produced by *Lactococcus lactis* (1.92g/mol) while the least was from *Pichia* sp. (0.36g/l).

The highest quantity of Hydrogen Peroxide produced was by *L. lactis* and *Pichia kudriazevii* culture. Fermentation was discovered to increase the titratable acidity and decrease the pH of starter fermented tiger nut milk. According to Wakil and Onilude (2011), the resulting acidification in fermentation of cereal weaning food fortified with cowpea was due to the dominance of lactic acid bacteria in the environment which degrades carbohydrates to simple sugars.

Laboratory Production of Starter Cultured Tiger Nut Milk

The result from production of lactic acid from starter culture pasteurized tiger nut milk

showed the highest quantity of lactic acid (Fig. 2) while the lowest lactic acid was measured in spontaneous tiger nut milk determination of the quantitative analysis of the lactic acid emanated an increase in incubation period followed by an increase in lactic acid content. *L. lactis* synthesized the highest quantity at 24 h. Wakil et al. (2014) recorded the highest value of lactic acid by *L. lactis* while the least was from *L. cremoris* at 6 h. All their isolates had an increase in production as incubation time increases. Spinnler and Corrieu, (1989) reported that the difference in acidity may be due to the peculiarity of the strain and the species. Badis et al., (2004) linked this difference to the acidification activity of strains and their capacity to use the substrate

Table 2: pH of Spontaneous fermented tigernut milk (SFTM)

Sample	pH				
	0 h	6 h	12 h	18 h	24 h
SBT	6.54±0.16 ^b	6.55±0.13 ^a	5.81±0.22 ^c	5.62±0.37 ^a	5.74±0.26 ^c
BBT	6.55±0.92 ^a	6.13±0.34 ^b	5.74±0.41 ^a	5.34±0.11 ^b	4.82±0.18 ^a
YTN	4.42±0.24 ^c	4.32±0.41 ^a	4.44±0.29 ^a	4.35±0.36 ^a	4.33±0.08 ^b

Value show means of duplicate analysis ± SD. Figures with different superscript across the row are significantly different (P < 0.05)

Table 3: pH of Starter developed fermented tiger nut milk

Sample/Time	SBT		BBT		YTN	
	0 h	18 h	0 h	18 h	0 h	18 h
LC	6.51±0.13 ^a	4.22±0.21 ^a	6.54±0.14 ^c	4.32±0.15 ^b	4.42±0.03 ^a	3.76±0.14 ^b
PK	6.52±0.11 ^c	4.22±0.16 ^c	6.53±0.15 ^a	4.35±0.13 ^c	4.45±0.08 ^a	3.84±0.12 ^c

Value show means of duplicate analysis ± SD. Figures with different superscript across the row are significantly different (P < 0.05).

Key: LC- *Lactococcus lactis*, PK- *Pichia kudriazevii*

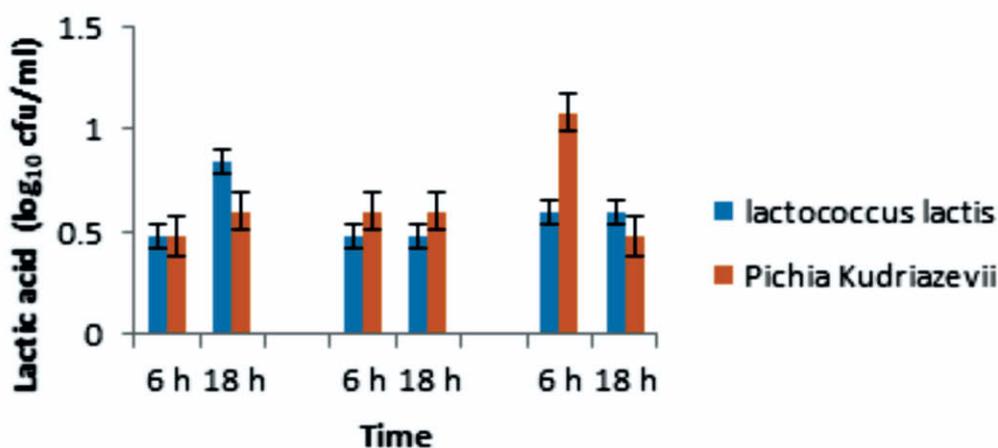


Fig 2: Lactic acid produced by the starter cultures - *Lactococcus lactis* and *Pichia kudriazevii* in a pasteurized fermented milk.

In conclusion, this study has demonstrated that the choice of microbial starters from indigenous origin for fermentation of tiger nut milk has greatly influenced its minimum biovalue (MBV) of probiotic parameter and generally regarded as safe (GRAS) which subsequently might decrease the hazard potency of its consumption especially as in the signified big brown tigernut and yellow tiger nut. The best quality milk was acquired from yellow variety milk pasteurized 90°C for 15 min and fermented with *Lactococcus lactis*, *Pichia kudriazevii* at 45°C for 18 h as juxtaposed to others.

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