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Microbial succession during the fermentation of blends of watermelon and tigernut

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Tigernut tubers (*Citrullus lanatus*) and watermelon fruits (*Cyperus esculentus*) are popularly consumed in Nigeria. This study investigated microbial succession occurring during the fermentation of tigernut and watermelon blends. The substrates were washed and ground into a homogenous pulp. The pulp was evenly divided into three sterile fermenters containing three litres of the blended substrates. The mixtures were allowed to ferment spontaneously (chance inoculation) for 5-days (primary fermentation). The mixtures were then sieved with a muslin cloth and transferred into a secondary fermenter, and fermented for 4 weeks. The fermented samples had an initial pH of 6.51 ± 0.00, 6.43 ± 0.00, 6.67 ± 0.00 which reduced towards the end of the fermentation to 3.15 ± 0.00, 3.16 ± 0.00, 3.22 ± 0.00 in the samples. The bacterial count ranged from 1.2 x 10^5 to 5.0 x 10^5, 3 x 10^4 x 4.0 x 10^5 3 x 10^4 to 6.8 x 10^5 CFU/mL for the samples. There was a significant reduction in sugar content after fermentation of the blends. At the early stages of fermentation, organisms from the genus *Clostridium, Bacillus, Lactobacillus, Corynebacterium, Gluconobacter, Pseudomonas, Streptococcus, Candida* and *Saccharomyces* were present within the first twenty-four hours of fermentation although *Lactobacillus plantarum, Bacillus species*, *Pseudomonas aeruginosa* and *Saccharomyces cerevisiae* were identified as completing the fermentation process. There was a variation in the total sugar content of the mix after fermentation. This study highlighted the microbial succession pattern in watermelon and tigernut fermentation.

Keywords: Fermentation, microbial succession, watermelon, tigernut.

INTRODUCTION

In the Arabian Peninsula, East Africa, Spain, and Asia, tigernuts (*Cyperus esculentus*), a perennial crop, are extensively grown. Chufa, golden nuts, earth almond, and ground almond are some of its other names, and it is a member of the *Cyperaceae* family (Abdelkader *et al.,* 2017). In Nigeria, there are three common varieties of this nut: brown, yellow, and black. The latter two are commonly sold on shelves in stores (Adenugba *et al.,* 2024). Among the two varieties, the yellow chufa is favored owing to its attractiveness, high milk yield, fleshier body, low fat and high protein content, and fewer anti-nutritional ingredients (Ponnampalam *et al.,* 2024).

In addition to being used to make flour, starch, cakes,and biscuits, this tuber is typically roasted, fried, baked, or consumed as a snack. These delicious tubers, which have a sweet nutty flavor, are typically steeped in water before eating. Because of its unique sweetness, it can also have an ice cream and cookie scent (Zhang and Sun, 2023). Tiger nuts have been dubbed "health" foods because, when consumed, they are thought to improve blood circulation, reduce the risk of colon cancer, and protect against thrombosis and heart disease (Gelberg, 2017). Tiger nuts can also be used to treat gas, diarrhea, dysentery, indigestion, and debility (Gelberg, 2017).

Compared to potatoes or sweet potatoes, tiger nut tubers contain about twice as much starch. Literature review reveals the isolation of a diverse group of microorganisms from tigernut. Obasi and Mani (2023) reported the presence of several microbial species, including *Bacillus subtilis*, *Staphylococcus aureus*, *Asper-*

Table 1. Consortia of prepared slurries.

Sample ID	Watermelon (%)	Tigernut	Total (%)
A	0.9L(30%)	2.1L(70%)	3L(100%)
B	2.1L(70%)	0.9L(30%)	3L(100%)
C	1.5L(50%)	1.5L(50%)	3L(100%)

gillus flavus, *A. niger*, *Fusarium solani*, *Saccharomyces cerevisiae*, *S. fubiligera*, and *Candida pseudotropicalis*, from tigernut exposed to ambient conditions.

In addition, it is used to make nougat, jam, beer, ice cream, and the local beverage kunnu. It is also utilized as a nutritional supplement and flavouring (Yu *et al.,* 2022). It has a large amount of high-quality oil that can be used naturally in salads or fried in them (Oke *et al.,* 2019). Tiger nut "milk" has been studied for use in lactose intolerant people's diets and as a milk substitute for fermented foods like yoghurt production.

Watermelon (*Citrullus lanatus*) is a fruit in the *Cucurbitaceae* family. Of all plant groups, the *Cucurbitaceae* family has one of the highest numbers and proportions of species that people eat. Cultivated for its delicious fruit, a special berry variety called botanically as a pepo, this large, spreading annual plant has yellow blossoms and rough, hairy leaves with pinnate lobes (Paris, 2015).

Watermelon fruit has a smooth, thick, deep green skin with vertical grey or light green stripes. Within the centre third of the crimson flesh of the fruit are tiny black seeds (Nadeem *et al.,* 2022). In Nigeria, watermelon can be fermented, combined, and eaten as a snack or appetizer, as well as in fruit cocktails, nectars, and juice. For instance, fruits like tiger nuts and watermelon are among the most vital nutrients for people since they are not only nourishing but also necessary for preserving health (Nadeem *et al.,* 2022).

Through fermentation of the fruit juice, watermelon has been employed in the creation of wine, similarly smoothies have been made using watermelon as substrate (Yusufu *et al.,* 2018). Adegunloye *et al.* (2020) reported the isolation *of Lactobacillus bulgaricus, Bacillus cereus, Streptococcus lactis, Staphylococcus aureus, Enterococcus* sp*, Micrococcus* sp*, Staphylococcus epidermidis,* and *Fusarium oxysporium, Mucor mucedo. Saccharomyces cerevisae, Aspergillus fumigatus, Aspergillus niger and Penicillium notatum* during the fermentation of water melon seeds.

Both fresh and processed fruits add essential nutrients such as vitamins, minerals, carbohydrates, and other nutrients to our diet. One of the main problems tropical countries like India face is the postharvest loss of fresh fruit. Due to inadequate post-harvest management and a lack of processing facilities, approximately 35 to 40% of horticulture production is squandered; therefore, fermentation is necessary to maintain these nutrients (Bhardwaj *et al.,* 2024).

The succession pattern and the relative influence of the various microbial genera to the fermentation process are not well understood, nor are the microorganisms involved in the natural fermentation process under rigorous control. Determining the microorganisms involved in the fermentation of these two fruit blends, as well as the microbial load at each stage of the fermentation process and the succession pattern of the related bacteria over a four-week period, are the objectives of this study.

MATERIALS AND METHODS

Sample collection and preparation

Samples of tigernut and watermelon were purchased from Oja Oba market Ado-Ekiti, Ekiti State and was transported in sterile ziplock bags to the laboratory for analysis. All the glass wares utilized were sterilized using dry heat (hot air oven) at 160^{O}C for one hour, while other materials were sterilized in an autoclave maintained at 121^{O}C for 15 min. Healthy and matured tigernut tubers were selected. A total of 1.4 kg w/v of the selected tigernuts were weighed out in sections, properly washed in two changes of clean water, and then steeped for 24 h before usage. The soaked tigernuts (1.79kg) were blended with 1000 mL of distilled water using a sterilized industrial blender (Qasa model). The purchased watermelons were sliced using a sterile knife with the seeds and rind (exocarp) carefully removed from the pulp (mesocarp), 2.60 kg of watermelon was then blended using an industrial blender (Qasa model) with 500 mL of water used during the blending process. The resulting slurries were divided between the sterilized fermenters after being evenly mixed with a stirrer that has been cleaned. Three litres of the prepared slurries were dispensed into sterile containers labelled A, B, and C as shown below in Table I.

Fermentation

The fermentation process was carried out in two phases: primary and secondary fermentation. The primary (natural) fermentation lasted for five (5) days and involved daily agitation. To begin, the samples were poured into sterilized plastic containers, covered with muslin cloth, and kept at room temperature (27°C). Aeration was achieved by agitating the slurries at 100 rpm each day throughout the five-day period.

After primary fermentation, the slurries were sieved to remove shafts and crushed fruit debris, following the method described by Awe *et al.* (2015). The resulting filtrates were then transferred into anaerobic fermentation jars and incubated at room temperature. This secondary fermentation phase was conducted over a period of four (4) weeks, with intermittent observation.

The pH of the fermentation liquor was determined using a pH meter. For microbial analysis, a ten-fold serial dilution was undertaken for the enumeration of microorganisms present by the pour plate method. Yeast enumeration was carried out using Yeast Extract Agar (YEA) supplemented with 50 mg/L chloramphenicol to inhibit commensal bacteria, while nutrient agar was utilized for enumeration of bacteria growth. After incubation, the plates were examined for colonies that appeared different in their cultural characteristics. These colonies were sub-cultured onto fresh media by the streaking method using a wire loop to obtain pure cultures. The identity of bacteria species obtained from the samples was confirmed by Gram staining and other biochemical tests using the methods of Fawole and Oso (2004). After incubation, the bacterial and yeast count was recorded using a colony counter. Few distinct hyphae of the yeast were picked with sterile needle on to a clean glass slide. A drop of Lactophenol cotton blue stain was added to the slide and allowed to react for two to three minutes. Excess stain was drained off and covered with a slip after which it was observed microscopically using ×10 and ×40 objectives for the arrangement of spores and characteristic shape. The microscopic features were then compared with standard pictorial mycology atlas (Alsohaili and Bani-Hasann, 2018).

Total titratable acidity

The total titratable acidity of the sample was determined as described by Ogu and Mgbebu (2011). Using phenolphthalein as indicator, 10 cm^3 of the sample was measured into a conical flask and titrated against 0.1 N solution of sodium hydroxide. The total titratable acidity was calculated as follows:

$$\frac{V1 \times N \times 75 \times 100}{1000 \times V}$$

Where; V_1 = Volume (cm^3) of NaOH, V = Volume (cm^3) of sample used, N = Normality of NaOH

Molecular Characterization

Yeast isolates were characterized by sequencing the Internal Transcribed Spacer (ITS) region of the nuclear DNA (rDNA). The universal primers ITS1 and ITS4 were used to amplify the ITS target region (White *et al.,* 1990). Genomic DNA was extracted from the samples using the Quick-DNA™ Fungal/Bacterial kit (Zymo Research, Catalogue No. D6005). The ITS target region was amplified using OneTaq® Quick-Load® 2X Master Mix (NEB, Catalogue No. M0486) with the primers presented in Table 1. The PCR products were run on a gel and cleaned up enzymatically using the EXOSAP method. The extracted fragments were sequenced in the forward and reverse direction using (Nimagen, BrilliantDye ™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050). The purified fragments were analyzed on the ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific) for each reaction for every sample, as listed in Section 1. DNASTAR was used to analyse the. ab1 files generated by the ABI 3500XL Genetic Analyzer and results were obtained by a BLAST search (NCBI).

Determination of total sugar

The total sugar content (brix) was determined using a refractometer. This was done by placing about 3 drops of the sample on top of the prism assembly and then closed with the daylight plate. The sample was then allowed to stand for approximately 30 seconds for it to adjust to the temperature of the refractometer. Then the result was taken by reading the calibrations of the refractometer through the eyepiece (Ohoke *et al.,* 2017).

Statistical analysis

All results presented are mean of triplicate readings.

RESULTS AND DISCUSSION

The fermenting consortia of samples resulted in significant pH variations. The pH at day 1 of steeping was 6.13 ± 0.00, 6.01 ± 0.00 and 6.24 ± 0.00 which reduced to 3.15, 3.22, and 3.16 for sample A, B and C, respectively after four weeks fermentation period as shown in Figure 1. Fleet (2013) attributed the decrease in the pH to the presence of lactic acid bacteria responsible for increased acidity during the fermentation of sugar substrates due to their proliferation in the substrates.

Total Titratable Acidity (TTA) also increased while the fermentation lasted as shown in Figure 1. This finding differs from the value (0.88%) reported by Ogodo *et al.* (2015) while fermenting a blend of watermelon, pawpaw and banana, (0.02 - 0.05%) recorded by Yabaya *et al.* (2016) in fermenting grape juice which were slightly lower. Although it was close to (0.97%) reported by Ohoke *et al.* (2017) in the fermentation of tigernuts and well below (2.23%, 2.16% and 2.08%) reported by Kantiyok *et al*. (2021) in fermenting pawpaw and watermelon blends. The decrease in pH and increase in TTA was attributed to the activities of the microorganisms which resulted in the production of organic acids from available sugar and nutrients present throughout the fermentation period for their growth and metabolism (Agbaje *et al.,* 2015).

The total sugar content of fermented blends decreased after fermentation to 34.47, 27.98 and 0.73 °Brix of the total sugar remaining after the secondary fermentation of the samples as shown in Figure 2. The decrease in the sugar content corroborated with earlier reports of Ogodo *et al.* (2015), Ohoke *et al*. (2017) and Kantiyok *et al.* (2021). The value of sugar present in sample E (0.73%) is similar to findings of Ogodo *et al.* (2015) who reported 0.54 - 0.94% of sugar present in the composition of fermented pawpaw, watermelon and banana juice. The value of sugar present in samples A and B is much higher than the values (1.25% and12.4-16.4%) reported by Ohoke *et al*. (2017) in the fermentation of tigernut, and Kantiyok *et al*. (2021) in the fermentation of consortium of watermelon and pawpaw. The reduction in the sugar content was expected as the fermenting microorganisms utilized the sugar present in the substrate.

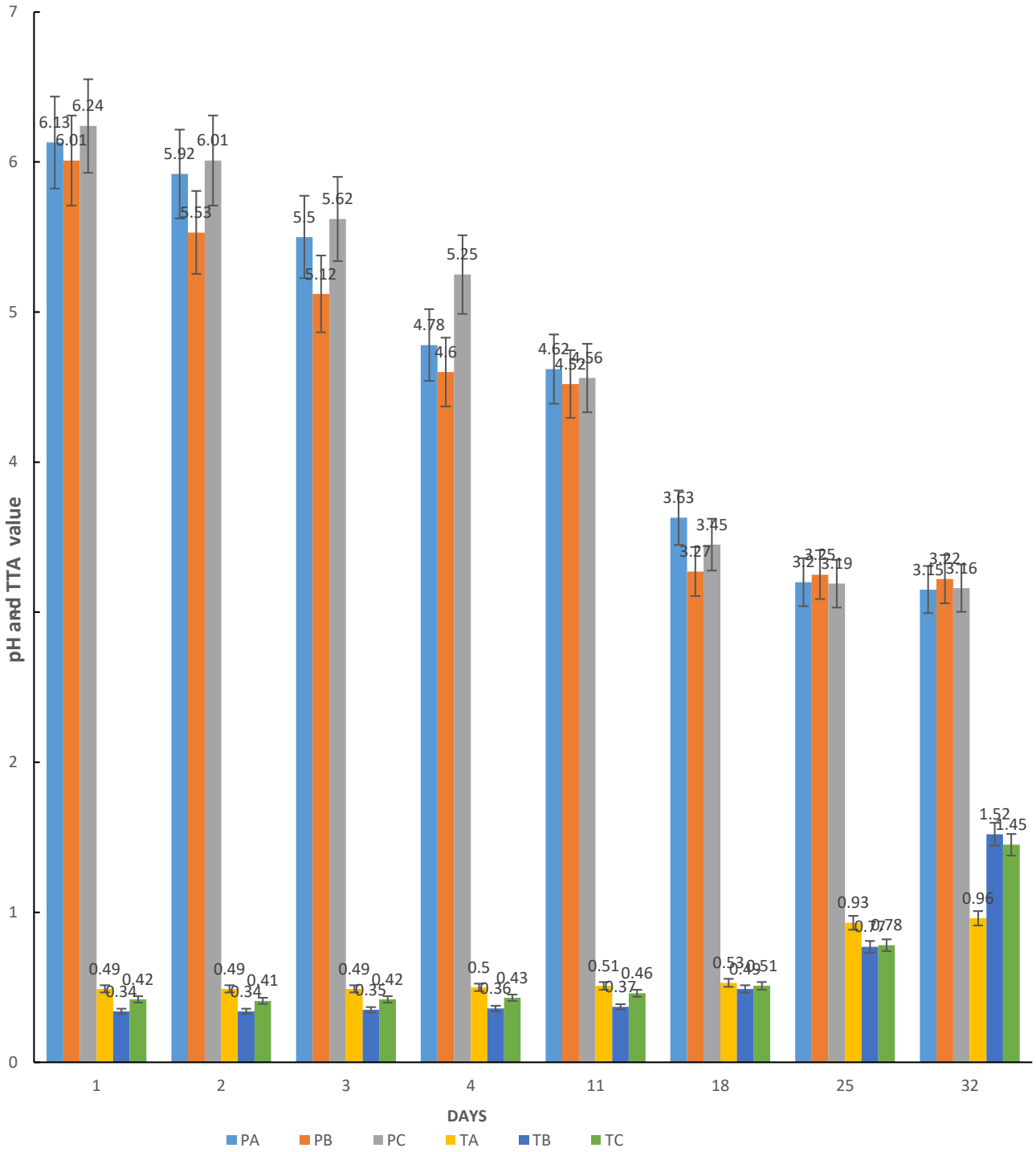

Figure 1. pH and total titratable acidity variation during four weeks of fermentation. **A** 30% watermelon + 70% tigernut, **B** 70% watermelon +30% tigernut, **C** 50% watermelon + 50% tigernut.

The bacteria count of the sample during primary fermentation showed a rapid decline in total bacteria count (TBC) after 48 h of fermentation, as shown in Figure 3. The increase in the TBC after the first 48 h of fermentation for sample C could be as result of the presence of sufficient nutrients that supported the growth of the microorganisms, which suggests that the microorganisms must have utilized the nutrients, thereby releasing some substances through extracellular activities (Ojokoh and Ojokoh, 2015), while the decrease in total bacteria count could be

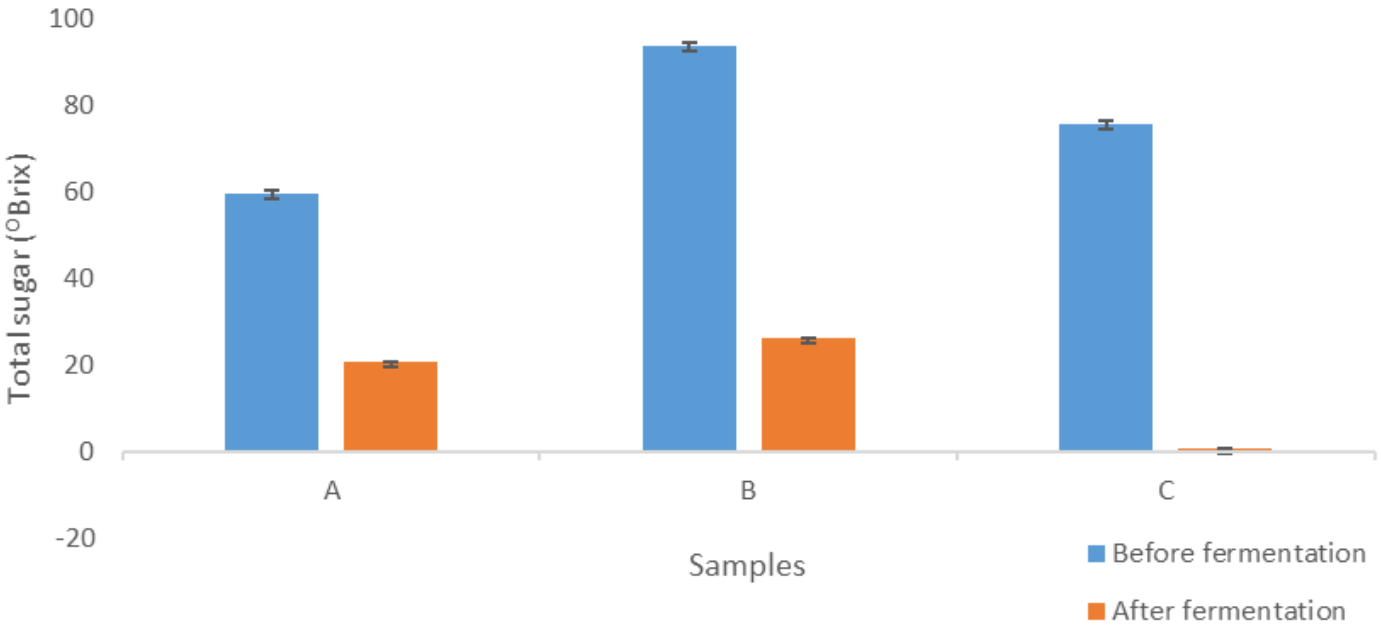

Figure 2. Total sugar after Fermentation **A:** 30% watermelon + 70% tigernut **B:** 70% watermelon +30% tigernut, **C:** 50% watermelon + 50% tigernut,

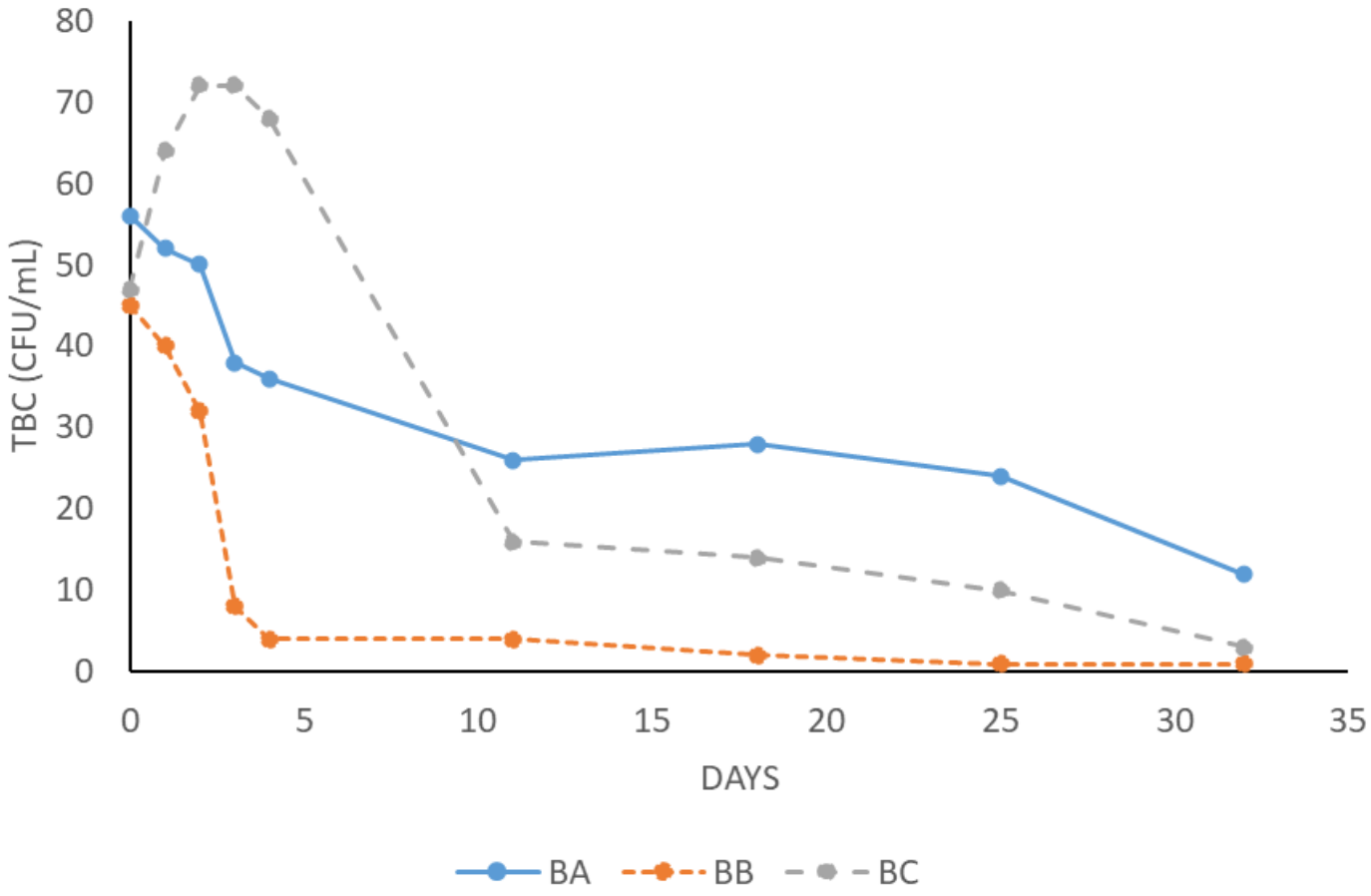

Figure 3. Variation in total bacteria count (TBC) for the fermenting Fruit Blend. **BA:** Bacteria count in 30% watermelon + 30% tigernut; **BB:** Bacteria count in 70% watermelon +30% tigernut; **BC:** Bacteria count in 50% watermelon + 50% tigernut.

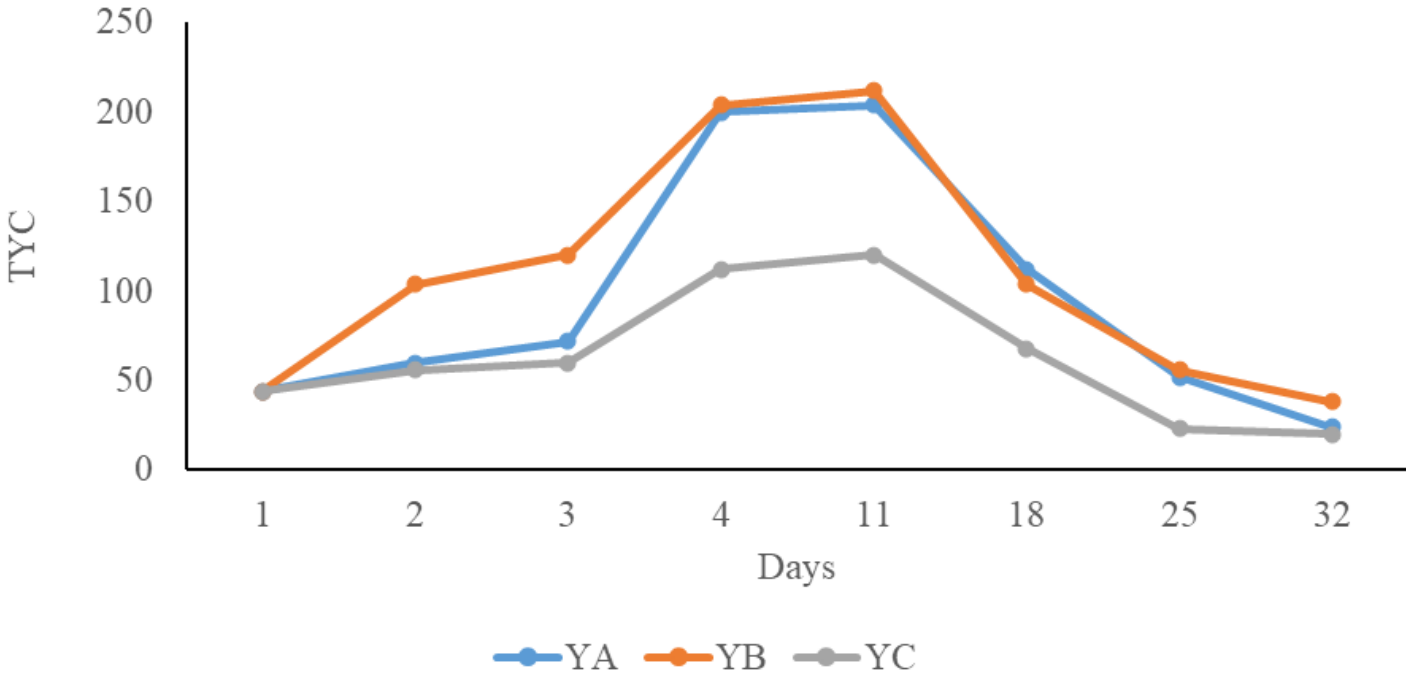

Figure 4. Variation in total yeast count (TYC) during the four weeks of fermentation. **A** 30% watermelon + 70% tigernut, **B** 70% watermelon +30% tigernut, **C** 50% watermelon + 50% tigernut.

attributed to the production of lactic acid by the Lactic Acid Bacteria (LAB), and lowered pH of the fermented product (Panda *et al.,* 2014).

The dominant microorganisms at 48 h incubation were *Bacillus* (Sample A, C) *Lactobacillus* (Sample A, B, C), *Pseudomonas* (Sample B) *Saccharomyces cerevisiae* (Sample A, B, C). All with differed ratios of the blends. This closely agrees with findings from other authors who reported the isolation of *Bacillus, Lactobacillus,* and *Saccharomyces* genus after 48 h incubation period (Oyekan and Oyetayo, 2021; Obasi and Mani, 2023).

A gradual increase in the yeast count also occurred, this is in close agreement with the findings of Awe and Nnadoze (2015) who stated that, the increase in total yeast count was expected owing to the daily aeration of the must thereby leading to cell propagation and multiplication as shown in Figure 4. Yeast play a prominent role in the production of alcoholic beverages, due to their ability to effectively utilize the available sugar and nutrient components, while, the decline in the number of yeast cells in the fermenting must during the secondary fermentation might be due to rapid utilization of the available sugar in the sample which lead to the fermentation of the must and increase in alcohol content thereby affecting the yeast growth rate (Awe and Nnadoze, 2015).

Amongst the microorganisms observed, *Bacillus* sp, *Lactobacillus plantarum*, *Pseudomonas aeruginosa* and *Saccharomyces cerevisiae* were the major microorganisms that concluded the fermentation of the samples as shown in Table 2 below with their corresponding Percentage Relatedness of the Gene Sequence of Isolates to Gene Sequence in NCBI GeneBank shown in Table 3. This is similar to reports by Ojokoh and Ojokoh (2015), Adisa and Enujiugha (2020), on the fermentation of sorghum and pumpkin blends and fermentation of Ogi respectively, they highlighted that, these organisms were responsible for the fermentation processes. Adisa and Enujiugha (2020) stated that, the occurrence of *Pseudomonas spp* in the raw samples / slurries could cause ropiness in the fermented product. The survival of some of these organisms could be as a result of the presence of essential nutrients that supported the growth of the microorganisms (Ojokoh and Ojokoh, 2015).

Conclusion

This study showed the microbial succession occurring during the fermentation of tigernut and watermelon blends. Findings showed the final microbiota of the fermented substrate was dominated by the genera *Bacillus, Lactobacillus plantarum* and *Saccharomyces cerevisiae.* Although the occurrence of *Clostridium, Streptococcus, Pseudomonas* and *candida* was noted.

There were significant physicochemical changes observed during fermentation, such as reduced pH resulting from the dominance of lactic acid bacteria. Also

Table 2. Occurrence of Microorganisms in the fermenting fruit blends.

Isolates	00hr			Day 1			Day 2			Day 3			Day 4			Day 11			Day 18			Day 25			Day 32		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Bacillus	+	-	+	-	-	-	+	-	+	-	+	-	-	-	-	-	+	-	+	-	-	+	+	-	+	+	-
Corynebacterium	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactobacillus	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Clostridium	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Streptococcus	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Candida	+	+	+	+	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Saccharomyces cerevisiae	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas	-	-	-	-	-	+	-	+	-	+	-	-	+	-	+	+	-	+	-	-	-	-	+	-	-	-	+
Gluconobacter	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-

A: 30% watermelon + 70% tigernut; B: 70% watermelon +30% tigernut; C: 50% watermelon + 50% tigernut

Table 3. Percentage Relatedness of the Gene Sequence of Isolates to Gene Sequence in NCBI Gene Bank.

Isolates	Accession number of close relatives from NCBI Gene Bank	Percentage relatedness (%)
Bacillus subtilis (BB1)	OP493507.1	96.32
Bacillus cereus (BB2)	MW911450.1	89.69
Bacillus sp (BB3)	GQ340496.1	84.81
Pseudomonas aeruginosa (P1)	MK530186.1	91.37
*Lactobacillus plantarum (*LB1)	CPO53571.1	96.65
Saccharomyces cerevisae (F1)	LC336452.1	98.38

during this study, blends containing equal ratios of the samples had lowered total sugar after fermentation. The associations of lactic acid bacteria (LAB) and yeast which can be used as starter cultures were found to be responsible for fermentation of these blends of watermelon and tigernuts.

Recommendation

Further studies should be carried out to understand the physiology of the fermenting organisms and the processes, this could be geared towards scaling up and subsequent commercialization upon satisfactory organoleptic analysis.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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