Effect of Different Preservatives on Microbial Load of *Zobo* Drink and Molecular Characterization of its Bacterial Flora

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Abstract: The potentials of various combinations of three preservation techniques: the use of chemical preservative (sodium benzoate), pasteurization and lime to preserve *zobo* drink at room temperature (28±2 ºC) were evaluated over six days. The samples were treated and stored for six days during which the proximate composition, physiochemical and microbiological properties of the *zobo* drink were determined daily. Isolates obtained were subjected to molecular characterization and identification. The proximate composition of *zobo* drink before treatment showed that it contains 92.52% moisture content, 0.56% ash content, 1.05% fat and oil, 2.16% protein, 0.17% fibre and 3.54% carbohydrate. The pH values ranged from 2.8 to 3.4, while the titratable acidity ranged from 0.2586 to 0.2816 %. There was increasing acidity of the zobo samples with storage (reduction in pH and increase in the titratable acidity). Bacterial counts ranged from 3.0×10^2 to 3.15×10^3 $cfuml^{-1}$ while fungal count ranged from 3.5×10^2 to 2.35×10^3 $cfuml^{-1}$. Six organisms comprising of four bacteria: *Bacillus cereus* ZB1 (MH566234), *Lactobacillus brevis* ZB2 (MH566235), *Staphylococcus aureus* ZB3 (MH559826), *Micrococcus luteus* and two fungi: *Aspergillus niger* and *Penicillium citrinum* were found in the *zobo* drink samples. Combining pasteurized with addition of preservatives was the most effective in reducing bacterial load followed by lime and sodium benzoate. This study revealed that the synergistic effect of pasteurization and lime, pasteurization and sodium benzoate introduction into *zobo* sample can be used to minimize bacterial load to acceptable limit for at least six days after production.
Key words: Zobo, Shelf-life, Bacterial, Fungal, Molecular characterization

INTRODUCTION

Zobo is a nutritional drink consumed by different classes of people irrespective of socio-economic status, sex and age in Nigeria especially in the Northern region and other neighboring African countries (Nwafor, 2012; Izah *et al.*, 2015). It is produced from the dried calyces of the roselle plant *Hibiscus sabdariffa* by boiling and filtration. The demand for *zobo* drink is due to its low prices, nutritional and medicinal properties (Ilondu and Iloh, 2007). *Zobo* drink has been shown to be a good source of natural carbohydrate, protein and vitamins C which constitutes the major reason for consuming soft drink and fruit juice (Ogiehor and Nwafor, 2004). Although, large scale production of zobo drink is limited because it deteriorates rapidly. *Bacillus, Streptococcus, Staphylococcus, Leuconostoc, Lactobacillus, Aspergillus, Penicillium, Geotrichum, Fusarium* and *Alternaria* have been associated with spoilage of *zobo* drink during storage (Ogiehor and Nwafor, 2004). Some of these spoilage microorganisms are associated with food poisoning, food intoxication, and death in severe cases (Omemu *et al.*, 2006). The proliferation of the associated microorganisms potentiates spoilage and the short shelf life associated with this relish beverage (Ogiehor and Nwafor, 2004). Considering the increasing acceptance of zobo drink and ready sources of vitamin C, the need to enhance and extend the shelf life by an effective means becomes imperative.

It is possible to improve the shelf life of *zobo* drink by slowing down the fermentation process, thus inhibiting discolouration of *zobo* drink and possibly permitting large-scale production and preservation for a longer period with maximum retention of nutritive values. Use of refrigeration for preservation is likely to increase cost of production because less than 50% of the about 170 million Nigeria are connected to the national grid and rely heavily on gasoline and diesel to power generators (Ohimain, 2014). In addition most *Zobo* producers are small-scale entrepreneurs. This study examined effect of different preservation techniques to increase the shelf life of *Zobo* drink.

MATERIALS AND METHODS

Source of *Hibiscus sabdariffa* calyces Sample

The dried calyces of *H. sabdariffa* were purchased from Oja Oba market in Ilorin and authenticated at the herbarium of Department of Plant Biology, University of Ilorin and assigned a voucher number UILH/601/1218.

Production of *zobo* drink

The dried calyces of *H. sabdariffa* were sorted, after which 25 grams was weighed and rinsed. The calyces were then boiled in 1 litre of sterile water for 40 minutes to enable extraction. The mixture was filtered with a clean muslin cloth and was allowed to cool before dispensing into various containers plastic bottles used for the analysis without addition of sugar and spices.

Determination of Proximate Composition of *zobo* drink

The proximate composition of the prepared *zobo* without preservative (moisture content, ash content, fat and oil content, crude protein content, fibre content and carbohydrate content) were determined at Department of Chemistry, University of Ilorin following the methods described by the Association of Official Analytical Chemists (AOAC, 2000).

Determination of Physicochemical Properties of *zobo* drink

pH determination

The pH of each group of containers with different treatment was determined for the period of storage starting from the day of production (day zero) using a pH meter (HI 96107). The pH of each of the treated samples and the control was measured twice and recorded.

Titrable acidity

Ten (10) ml of the *zobo* drink was measured into a 250 ml conical flask, and four drops of phenolphthalein indicator was added. This was titrated with 0.1N NaoH to golden brown point. The titer for the total acidity was expressed as percentage of citric acid (Umme *et al.,* 2001).

Treatment of *zobo* drink with preservatives

Two millimeters (2ml) of (0.02%) sodium benzoate was aseptically added to six containers containing 98ml of *zobo* drink. Another group of six containers containing 98ml of *zobo* drink were pasteurized at 60ºC for 1 hour (Maji *et al.,* 2011) and 2ml (0.02%) of sodium benzoate was aseptically added and stored at room temperature for the period of the experiment. Lime fruits used were first surface-sterilized with 70% ethanol and peeled using a pre-sterilized knife. The fruits were then halved and was squeezed aseptically (sterile gloves worn during preparation) into a sterile 100ml conical flask. Two millimeters of lime juice was added to six containers containing 98ml of *zobo* drink. Another set of six containers containing 98ml *zobo* drink were pasteurized at 60ºC for 1 hour and 2ml of lime juice was aseptically added and stored at room temperature for the period of the experiment while six containers with 100ml of *zobo* drink without any preservative or treatment served as the control.

Microbiological analysis of samples

Total viable heterotrophic bacteria and fungi were enumerated and isolated using standard pour plate technique and plated on nutrient agar and potato dextrose agar (Cheesbrough, 2002). Bacterial isolates were characterize using colonial and cellular morphologies as well as various biochemical tests. The bacteria were subjected to molecular characterization. Fungal identification was carried out using mycological atlas after microscopy (Beech *et al*., 1986; Kavanagh, 2005).

Bacteria Genomic DNA Extraction, Amplification, Sequencing and Blast

DNA was extracted from overnight culture of each isolates and amplified using the PCR technique. The amplicons were then subjected to electrophoresis and the DNA bands were visualized under Gel documentation System (Alpha Innotech).

Their sequences were generate from the ABI Prism 3130X1 genetic automated sequencer (Applied Biosystems) and were carefully edited, filtered and assembled for polymorphism detection using BioEdit software (Abarshi *et al.,* 2010). Sequence similarity was estimated by searching the homology in the Gene bank DNA database using BLAST. The sequence information was then imported into the MEGAS software programe for assembly and alignment. The 16SrDNA sequences of isolated bacterial strains were compared to sequences from typed strains held in Gene Bank.

Data analysis

The data was subjected to analysis of variance (ANOVA) and Duncan test was carried out using pre-packaged computer statistical software (SPSS 20.0).

RESULTS

Proximate Composition

The composition of untreated *zobo* drink had high moisture content (92.52 %), Ash (0.56 %), fat and oil (1.05 %), crude protein (2.16 %), fibre (0.17 %) and carbohydrate (3.54 %) (Table 1).

Physicochemical Parameters

Generally there was decrease in the pH values over the period of storage for all the samples analyzed. The pH of the treated *zobo* samples changed from an initial pH of 3.3 and ranged from 2.8 to 3.4 over a period of 6 days with control (untreated) having pH of 3.4 (Figure 1) while the titratable acidity (TTA) ranged from 0.25856 to 0.2816. The values of TTA increased with time irrespective of the preservative added as shown in Figure 2.

Microbiological Quality Assessment of Zobo Drinks

The population of microorganisms in the *zobo* drink over the storage period of 120 hours is shown in Table 2. Bacterial counts for control sample increased from 1.6 x 10^3 cfuml^{-1} to 3.15 x 10^3 cfuml^{-1}. Bacterial counts for *zobo* sample treated with sodium benzoate decreased from 1.85 x 10^3 cfuml^{-1} to 9.5 x 10^2 cfuml^{-1}. Bacterial count for *zobo* sample treated with lime decreased from 1.25 x 10^3 cfuml^{-1} to 3.5 x 10^2 cfuml^{-1}. For pasteurized sample treated with lime, bacterial counts ranged from 0 to 7.5 x 10^2 cfuml^{-1}. Bacterial count for pasteurized sample treated with sodium benzoate decreased from 1.1 x 10^3 cfuml^{-1} to 3.5 x 10^2 cfuml^{-1} at 120 hours.

Fungal count for control sample increased from 6.5 x 10^2 cfuml^{-1} to 2.35 x 10^3 cfuml. Fungal count for pasteurized sample, sample treated with lime and pasteurized *zobo* sample treated with lime increased from 3.5 x 10^2 cfuml^{-1} to 1.3 x 10^3 cfuml^{-1}, 6.0 x 10^2 cfuml^{-1} to 2.3 x 10^3 cfuml^{-1} and 4.0 x 10^2 cfuml^{-1} to 2.35 x 10^3 cfuml^{-1} at 120 hours respectively. There was no visible growth observed till 48 hours for samples treated with sodium benzoate and pasteurized sample treated with sodium benzoate (Table 3).

Six organisms were identified comprising of four bacteria and two fungi. The bacteria were identified as: *Bacillus cereus* ZB1 (MH566234), *Lactobacillus brevis* ZB2 (MH566235), *Staphylococcus aureus* ZB3 (MH559826) and *Micrococcus luteus* ZB4 (MH566236) based on molecular characterization (Table 4) The fungi were : *Aspergillus niger* and *Penicillium citrinum.* Figure 3 shows the Agarose gel Electrophoresis of the amplified DNA of the four bacterial isolates.

The distributions of fungal and bacterial isolates were shown in table 5 and 6 respectively. *Aspergillus niger* had the highest occurrence while *Lactobacillus brevis* MH566235 had the highest occurrence.

Table 1: Proximate composition of the untreated *zobo* drink

Parameters	% composition
Moisture content	92.52
Ash content	0.56
Fat and oil	1.05
Crude protein	2.16
Fibre	0.17
Carbohydrate content	3.54

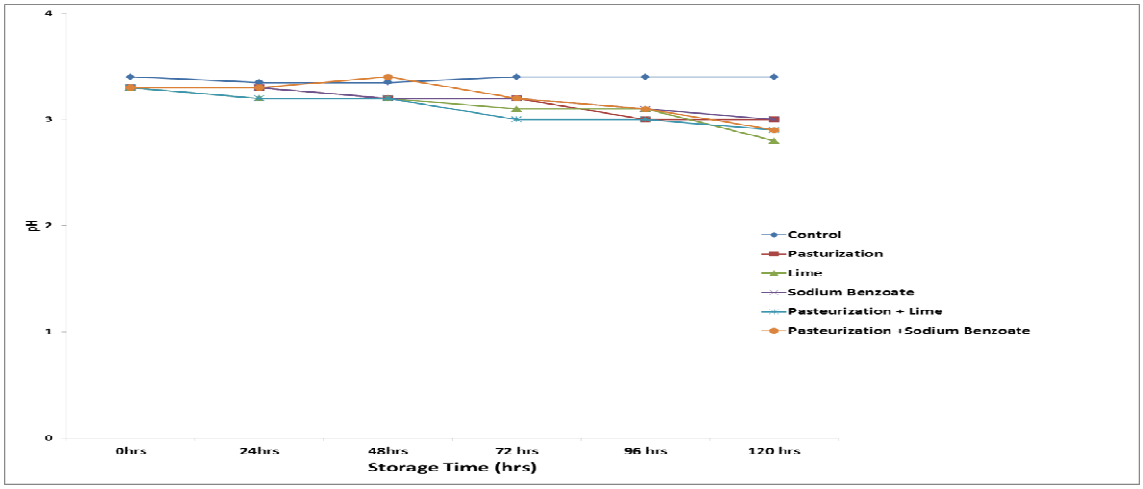

Figure 1: Changes in pH of *zobo* drinks during period of storage

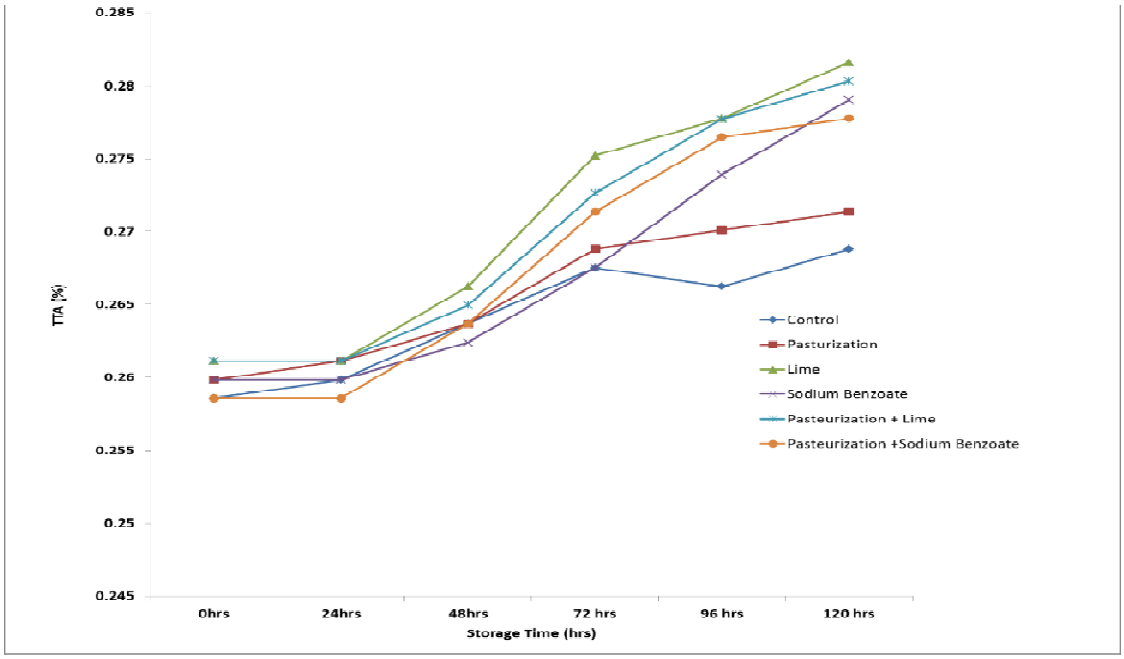

Figure 2: Changes in TTA of *zobo* drinks during period of storage

Table 2: Bacterial Count of *zobo* sample during storage period (cfu/ml)

SAMPLES	HOURS					
	0	24	48	72	96	120
Control	$1.6x10^{3c}$	$1.9x10^{3c}$	$2.2x10^{3c}$	$2.5x10^{3d}$	$2.9x10^{3d}$	$3.1x10^{3d}$
Pasteurization	NG	$8.0x10^{2a}$	$1.1x10^{3b}$	$1.6\ x10^{3c}$	$2.1x10^{3c}$	$2.6x10^{3c}$
Lime	$1.2\ x10^{3b}$	$9.0x10^{2a}$	$8.0x10^{2a}$	$7.0x10^{2b}$	$5.0x10^{2a}$	$3.5x10^{2a}$
Sodium benzoate	$1.5x10^{3c}$	$1.6\ x10^{3c}$	$1.8\ x10^{3c}$	$1.4\ x10^{3c}$	$1.1x10^{3b}$	$9.5x10^{2b}$
Pasteurization + Lime	NG	$7.5x10^{2a}$	$6.0x10^{2a}$	$4.0x10^{2a}$	$2.5x10^{2a}$	$2.0x10^{2a}$
Pasteurization + Sodium benzoate	$3.0\ x10^{2a}$	$1.1x10^{3b}$	$6.0x10^{2a}$	$5.0x10^{2a}$	$4.0x10^{2a}$	$3.5x10^{2a}$

Values are means of duplicate determinations. Values within a column with the same superscripts are not significantly different ($p<0.05$).
KEY: NG – No growth

Table 3: Fungal Count of *zobo* sample during storage period (cfu/ml)

SAMPLES	HOURS					
	0	24	48	72	96	120
Control	$6.5x10^{2c}$	$9.5x10^{2c}$	$1.4x10^{3b}$	$1.7x10^{3c}$	$2.1x10^{3b}$	$2.3x10^{3b}$
Pasteurization	$3.5x10^{2b}$	$5.5x10^{2b}$	$7.5x10^{2b}$	$9.0x10^{2b}$	$1.04x10^{3b}$	$1.3x10^{3a}$
Lime	$6.0x10^{2c}$	$1.0x10^{3c}$	$1.3x10^{3b}$	$1.7x10^{3c}$	$2.1x10^{3b}$	$2.3\ x\ 10^{3b}$
Sodium benzoate	NG	NG	NG	$4.0x10^{2a}$	$4.0x10^{2a}$	$4.0x10^{2a}$
Pasteurization + Lime	$4.0x10^{2b}$	$8.5x10^{2c}$	$1.2x10^{3c}$	$1.5\ x10^{3c}$	$1.9x10^{3c}$	$2.3x10^{3b}$
Pasteurization + Sodium benzoate	NG	NG	NG	$5.5x10^{2a}$	$5.5x10^{2a}$	$6.5x10^{2a}$

Values are means of duplicate determinations. Values within a column with the same superscripts are not significantly different ($p<0.05$).
KEY: NG – No growth

Table 4: Molecular identification of bacterial isolates

S/N	Isolate code	Reference from NCBI database	Accession numbers	Percentage % similarity
1	ZB1	*Bacillus cereus*	MH566234	100
2	ZB2	*Lactobacillus brevis*	MH566235	99
3	ZB3	*Staphylococcus aureus*	MH559826	100
4	ZB4	*Micrococcus luteus*	MH566236	100

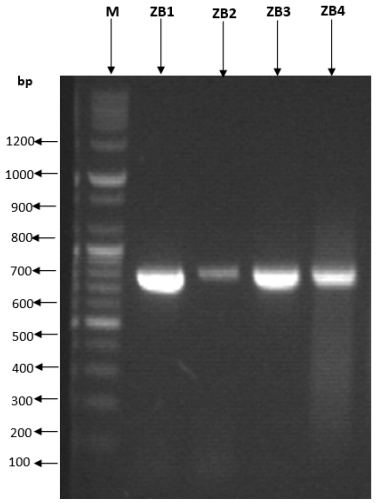

Figure 3: Gel electrophoresis of bacterial DNA.
Key: M: DNA ladder ZB1: *Bacillus cereus*, ZB2: *Lactobacillus brevis*, ZB3: *Staphylococcus aureus*, ZB4: *Micrococcus luteus*, **bp**: base pair

Table 5: Distribution of Fungal Isolates

ISOLATES	Control	Pasteurization	Lime	Sodium benzoate	Pasteurization + Lime	Pasteurization + Sodiun benzoate
Asperrgillus niger	+++	+++	+++	++	++	++
Penicillium citrinum	++	++	++	+	+	++

KEY: +++ ≥ 4 times, ++ ≥ 2times,< 4times, + 1time
NB: (Based on frequency of occurrence)

Bacillus cereus
Lactobacillus brevis
Staphylococcus aureus
Micrococcus luteus

Table 6: Distribution of Bacterial Isolates

ISOLATES	Control	Pasteurization	Lime	Sodium benzoate	Pasteurization + Lime	Pasteurization + Sodiun benzoate
Bacillus cereus	++	++	++	++	+	+
Lactobacillus brevis	+++	++	+++	++	++	++
Staphylococcus aureus	+	-	+	+	-	-
Micrococcus luteus	+	-	+	+	-	+

Key: +++ ≥4times
++ ≥2times, < 4times
\+ 1time
_ No appearance
NB: (Based on frequency of occurrence)

DISCUSSION

Proximate composition show that *zobo* drink contains high water content (92.52%) which is helpful in thirst-quenching. The low fat content (0.56%) of *zobo* drink makes it an ideal drink for obese people. The low protein composition obtained (2.16%) is similar to result obtained by Egbere *et al*. (2007) who investigated the effects of some preservation techniques on the quality and storage stability of *zobo* drink and reported that zobo drink has very low protein (0.046 – 0.463).

The pH values of treated *zobo* samples over the period of six days did not change significantly ($p>0.05$) with respect to preservation methods. This result is in agreement with Egbere *et al*. (2007) who reported *zobo* sample to be acidic over the period of storage irrespective of the additives added. Fasoyiro *et al.* (2005) also reported similar pH value when they worked on the chemical and storability of fruit-flavoured *H. sabdariffa* drink with the pH of the samples ranged from 2.19 to 3.32. The study also reported that the acidic nature of the samples was due to the acidic nature of the roselle calyx and that the roselle calyx is characterized as a highly acidic, rich in organic acids; oxalic, tartaric, malic and succinic acids. The titratable acidity (TTA) of *zobo* drink samples obtained ranged from 0.25856% to 0.2816%. Values increased with time irrespective of the preservatives added this may be due to acid producing ability of spoilage bacteria. Similar increase were observed in the work carried out by Fasoyiro *et al.* (2005) and Gbadegesin and Odunlade, (2016).

The increase in acidity accounts for the decrease in microbial population and could also enhance growth of acidophiles, the acidity can exert selective pressure on strains that make up the microbial population.

The zobo samples were found to undergo series of changes due to the presence of spoilage microorganisms; *Bacillus cereus*, *Staphylococcus aureus and Micrococcus luteus* in the drink. These changes include increase in total bacterial and fungi counts, titratable acidity and pH. All this were due to the growth and activities of the microorganisms present in the zobo drink samples. The bacterial and fungal count of the treated and untreated samples increased from the initial amount of 1.2 to 2.6 $x10^3$ $cfuml^{-1}$ and 1.6 to$3.16x10^3$ $cfuml^{-1}$ while for fungi $6.5x10^2$ $cfuml^{-1}$ to $2.3x10^3$ $cfuml^{-1}$ and $3.56x10^2$ $cfuml^{-1}$ to $2.3x10^3$ $cfuml^{-1}$ respectfully. For sample treated with lime, it was noticed that bacterial load was reduced with time hence the continuous decrease in population observed (Nwafor 2012). For *zobo* sample preserved with sodium benzoate, bacteria count started reducing after 48 hours from $1.85x10^3$ $cfuml^{-1}$ to $9.5x10^2$ $cfuml^{-1}$ at 120 hours. Braide *et al.* (2012) reported that sodium benzoate had no impact on microbial community of the sample but after days of application, a notable effect was observed most especially after 144 hours of application can be attributed to non-availability of utilizable sugar (sucrose) and other nutrient.. Pasteurized sample containing lime had no growth at 0 hour and fewer bacteria count was recorded during further storage. This might be due to synergistic effect of pasteurization at the initial day and presence of preservative during further storage. Pasteurized sample with sodium benzoate had growth at 0 hour.

The fungal counts were found to be consistently higher in all the *Zobo* samples. This may be attributed to the low pH and levels of titratable acidity which favours the growth of fungi over bacteria. In addition the treatment with sodium benzoate, pasteurization plus sodium benzoate had lower fungal count compared to other treatments. The two molds isolated were identified as *Aspergillus niger* and *Penicillium citrinum*. Molecular characterization of bacterial species showed that ZB1 has 100% similarity with *Bacillus cereus*, ZB2 has 99% similarity with *Lactobacillus brevis*, ZB3 has 100% similarity with *Staphylococcus aureus*, ZB4 has 99% similarity with *Micrococcus luteus* and ZB5 has 100% similarity with *Bacillus subtilis* after searching the homology in Gene bank DNA Database.

Zobo drink, raw or preserved supports the growth and proliferation of a wide variety of microorganisms. *Lactobacillus brevis* followed by *Bacillus cereus* were the predominant isolates. *Bacillus cereus* is a spore former that can withstand adverse effects of additives. Spores are extraordinarily resistant to environmental stress such as heat, ultraviolet radiation, chemical disinfectant and desiccation (Joanne *et al.*, 2016). *Staphylococcus aureus* and *Micrococcus luteus* have lesser occurrence in all samples. *Staphylococcus aureus* is a member of the normal flora of the body, frequently found in the nose, respiratory tract and on the skin. *Micrococcus luteus* is found in soil, dust, water and in human skin flora. It has also been isolated from foods such as milk. It is an atmospheric microorganism commonly found on environmental monitoring plates and it is one of the most common contaminants of lab cultures (Joanne *et al.*, 2016).

CONCLUSION

Result from the study showed that treatment of *zobo* drink with lime and sodium benzoate were effective in reducing the growth of microorganisms. A combination of pasteurization and lime, pasteurization and sodium benzoate are more effective in reducing microbial load of *zobo* drink. The microbial load obtained for lime treatment and the control are above the limit 10^3 for ready to drink soft drink while other treatment are within (FDA, 2013).

This might be due to the fact that the drink was produced under precautionary laboratory conditions. This implies that personal hygiene and Good Manufacturing Practice (GMP) should be employed during production, processing and packaging.

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