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Profile of resident microbes causing spoilage in "olewonyo", a locally produced non-alcoholic beverage in Kumasi, Ghana

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Abstract: Samples of *olewonyo* from two different localities together with a laboratory produced sample were analyzed for microbial changes, pH, titrable acidity and salt tolerance. High total viable counts (TVC) of 9.99 log cfu/ml, 9.23 log cfu/ml and 5.00 log cfu/ml were observed in the Aboabo, Anloga and Lab-simulated samples respectively at room temperature at day 3 (72hours). There was no significant differences in microbial load among the various samples among TVCs, lactic acid bacteria (LAB), lactic-acid cocci, and yeasts and moulds counts as the storage time increases ($p<0.001$). The pH of samples from Aboabo and Anloga showed a rapid decline from 6.55 and 6.60 to 4.55 and 4.30 respectively within 24-hour of storage. The changes in the Lab-produced sample however, showed a gradual decrease from 6.55 to 4.80 for the entire period of storage. There was no significant increase in titrable acidity of the laboratory sample ($p<0.02$) compared to the traditional samples. Of the 15 LAB isolates from the three sites, 40% were *Lactobacillus fermentatum*, 26.6% *Lactobacillus plantarum*, 20% *Lactobacillus acidophilus* and 13.4% *Lactobacillus brevis*. In the API gallaries, the dominant species were able to ferment ribose, galactose, D-glucose, D-fructose and mannitol. The present study shows that *Leuconostoc mesenteroides, Lactobacillus plantarum, L. acidophilus, L. brevis, L. fermentatum, Corynebacterium, Saccharomyces cerevisiae, Aspergillus flavus and A. parasiticus* may be involved or associated in the spoilage of *olewonyo*.

Keywords: olewonyo, titrable acidity, lab-simulated, microbial load, spoilage.

I. INTRODUCTION

'Olewonyo' or corn wine is a traditional non-alcoholic everage prepared from maize (*Zea mays*). It is dark-brown in colour with sweet taste and is consumed locally in Ghana and Togo. Different ethnic groups have different names. In Ghana, the Akans call it '*Olewonyo*', the Ewes call it '*Aliha*' whilst the Gas refer to it as '*Asaana*'.

Many such traditional beverage products are home to a complex microbial ecosystem, which is responsible for the broad diversity of tastes, aromas, and textures that are associated with them [1]. Many bacteria make a positive contribution to the organoleptic qualities of both fermented and non-fermented beverages, while others may have adverse effects or may even constitute a health risk. Deak and Beuchat [2] reported that, the beneficial activities of yeasts and microorganisms in general are of great economic significance and they have been used for millennia in the production of fermented foods and alcoholic beverages.

It is again reported that, lactic acid bacteria (LAB) enhance cheese flavor and diversity [3]. These LAB are characterized by a succession of largely undefined microbial communities on their surface and have a strong impact on the appearance, odor, flavor and texture development of respective products [4]. There is a growing interest in the biodiversity and ecology of microorganisms associated with different foods [5]. This is due to the realization that they can interact with themselves and other species in different ecosystems and that the outcome of these interactions may affect the role(s) that microorganisms have in foods.

Traditionally, '*olewonyo*' preparation is a batch process carried out on a small scale, once or twice a week. The grains are consistently sprinkled with water and spread on fiber sacks and allowed to germinate. The germinated grains are left in the sun for 2 days to dry. The malted grains are then coarse grounded and mixed with water and boiled for an hour till it is cooked. The cooked drink is left overnight to allow particles to settle. The supernatant is then decanted and brown sugar is added to the creamy supernatant which gives the drink its characteristic brown colour and the sweet taste.

Olewonyo, like many other traditional foods and beverages are prepared in small scale in the village homes. Their quality therefore depends on the skills of the households, as inherited over the years. The drink and its production add value and significance to the Ghanaian culture and economy. It is served chilled in the villages as soft drinks during festive occasions and now, as a source of income to some low income women who produce them and go round the big markets in the cities to sell them in very big calabashes and pots.

While there have been several studies on the microbial composition of these [6-8], little is known on the diversity of yeasts and other microorganisms associated with these foods. The mode of production makes this non-alcoholic beverage prone to microbial contaminations, hence, its short shelf-life of one to maximum three days.

In view of the slight differences in methodology on the skills of the producers in the preparation and the environmental conditions in its production which affects

its shelf-life, there is the need to conduct a detailed study to isolate and characterize the resident microbial profile in 'olewonyo' and determine their role in the spoilage of the drink.

II. MATERIALS AND METHODS

A. Sample collection

Traditional *olewonyo* samples were obtained from two different local markets in Aboabo and Anloga which are all suburbs of Kumasi, the regional capital of Ashanti, Ghana. A third sample was prepared in the laboratory under aseptic conditions. The raw materials (maize) used in preparation of the drink were bought at a local market in the study areas and divided into three batches. Two of the batches were used for traditional preparation, while the third batch was used for the laboratory study at the Microbiology Laboratory of the Department of Theoretical and Applied Biology, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.

For the traditional processing, the materials were given to six randomly selected local women experienced in preparation of *olewonyo* (three in each of the localities). They were requested to prepare the product using the traditional method. Households with women experienced in the art of *olewonyo* preparation were selected and the numbers randomized by the calculator random method.

The samples from the women were collected in sterile sample containers, cooled to 4°C and transported to the laboratory in a cool box containing freeze packs to maintain them at the temperature between collection and analysis. The samples were again divided into two batches and stored at room temperature (25°C) and refrigeration temperature (4°C). The sample from the laboratory-simulated production were also maintained at 4°C and 25°C prior to the microbiological analysis.

B. Analytical methods

During the preparation of *olewonyo* from the traditional sites and the laboratory, samples were taken at 24-hour intervals for Total Viable Counts (TVCs), Lactic acid bacteria (LAB – *lactococci* and *lactobacillus*), yeasts and moulds counts and total coliforms. On the other hand, samples were taken at 12-hour intervals for the analysis of pH and titratable acidity (TA). The pH was measured using a pH meter (Orion Model 420A). Titratable acidity was determined according to AOAC methods (AOAC, 1984) [9]. A sample of 10 ml was titrated with 0.1 N NaOH using phenolphthalein as an indicator. The titratable acidity was calculated as percent lactic acid.

C. Microbiological analysis

The methods and procedures used were as described by Harrigan and McCance, [10], FDA, bacteriological analytical manuals (FDA, [11] and ALPHA, compendium of methods for microbiological examination of foods (APHA, 1992). Total viable counts (TVC) were determined using the pour plate method of Harrigan and McCance [10]. Decimal dilutions were made with 0.1 percent bacteriological peptone. One millilitre of the 10^{-8} to 10^{-14} dilutions was used to prepare pour plates using plate count agar (PCA). The plates were incubated at 30°C for 48 hrs. The colonies on plates with between 30-300 colonies were counted.

LAB counts were determined using MRS (deMan Rogosa Sharpe) and M17 agar incubated anaerobically at 30°C for 3 days. Isolates of Lactic acid bacteria (LAB) were tentatively identified by determining their pattern of carbohydrate fermentation using the API 50 CH (BioMérieux, Marcy-l'Etoile, France) and comparing them to the API database.

The counts of yeasts and moulds were determined using potato dextrose agar (PDA), acidified with 10% tartaric acid to pH 3.5 by incubating at 30°C for 3-5 days. Coliform numbers were determined using violet red bile glucose agar incubated at 37°C for 48 hrs.

D. Salt Tolerance Test and Growth at different pH

Salt tolerance test was done using MRS (Oxoid CM359) containing 6.5% and 18% (w/v) NaCl with incubation period of 4 days at 15°C and 45 °C. pH was adjusted to 4.4 and 9.6. Growth were observed in MRS broth.

E. Statistical analysis of the data

The data collected was entered in MS Access and MS Excel solver as data base for optimization. The data was analyzed using Genstat 5 Release 3.2 statistical package and Statistical Package for the Social Sciences (SPSS 12.0 for Windows). The data validity and interpretation was monitored to ensure quality control of the results.

III. RESULTS

A. Microbial content and changes of *olewonyo* during storage at room temperature (25°C).

There was a general increase in microbial numbers in samples of *olewonyo* stored at room temperature with increasing time of storage.

Table 1 shows the microbial counts in *olewonyo* during storage. This increase was different for all the samples from Aboabo, Anloga and the laboratory-produced. High total viable counts (TVC) of 9.99 log cfu/ml, 9.23 log cfu/ml and 5.00 log cfu/ml were observed in the Aboabo, Anloga and Lab-simulated samples respectively at room temperature at day 3 (72hours) with the lactic acid bacteria (LAB) being the predominant microbes. There was no significant differences in microbial load among the various samples between TVCs, LAB, lactic-acid cocci, and yeasts and moulds counts of the traditional and laboratory-simulated products as the storage time increases ($p<0.001$).

The results showed that the microbial contents in *olewonyo* produced in the laboratory, were almost comparable to those of the traditional products. The TVCs were high and continue to increase steadily throughout the storage period. The lactococci showed a fairly constant trend during the storage period with values ranging from 3.45 -8.44 $\log_{10}$ CFU/ml. The yeast and moulds counts were relatively constant.

In all cases, the laboratory-simulated samples recorded the least number of microorganisms during the storage period.

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TABLE 1: Mean microbial counts in traditionally and laboratory-simulated *olewonyo* under different storage temperature.

	Time (hrs)	Microbial Count (log_{10} CFU/ml)					
		Aboabo		Anloga		Laboratory-simulated	
Microorganism(s)		4C	25°C	4°C	25°C	4°C	25°C
Total Viable Count	0	2.41	2.41	2.71	2.71	1.82	1.82
	24	2.61	4.29	2.83	5.42	1.94	2.28
	48	2.91	7.89	4.01	7.17	2.00	4.62
	72	3.22	9.99	4.62	9.23	2.96	5.00
Lactic acid bacteria	0	2.10	2.10	1.98	1.98	1.00	1.00
	24	2.61	4.41	2.11	4.23	1.72	1.89
	48	3.91	8.20	2.90	8.40	1.75	2.41
	72	5.51	9.41	4.23	9.12	1.91	2.80
Lactic acid cocci	0	1.80	1.80	1.74	1.74	1.14	1.14
	24	1.94	3.91	1.88	2.61	1.80	2.61
	48	3.31	7.22	2.61	6.88	2.22	3.01
	72	4.88	8.42	4.12	8.44	2.71	3.45
Yeast and moulds	0	1.10	1.10	1.20	1.20	0.41	0.41
	24	1.21	1.40	1.92	1.54	0.48	0.61
	48	1.41	2.00	1.95	2.22	0.61	1.01
	72	1.86	2.80	1.99	2.76	0.91	1.22

Values are means of 3 replicate

B. Microbial load and changes of *olewonyo* during storage at room temperature (4°C)

Although there was an increase in the microbial load in samples of *olewonyo* from all the samples stored at refrigeration temperature (4°C), they were not as the rate of increase in samples stored at room temperature. The rate followed the same pattern as that of room temperature with total viable counts of 3.22 log cfu/ml, 4.62 log cfu/ml and 2.96 log cfu/ml for Aboabo, Anloga and Lab-produced samples respectively at day 3 (Table 1).

Under this temperature, the predominant microbes were the LAB and lactic acid cocci. Unlike storage at 25°C where Aboabo recorded the highest total viable counts followed by Anloga in the traditional sites, the situation was different at 4°C. Anloga and Aboabo recorded 4.62 log cfu/ml and 3.22 log cfu/ml respectively at day 3 (Table 1). There was no significant difference in microbial load among the various samples ($p<0.011$) between TVCs, LAB, lactic-acid cocci, and yeasts and moulds counts between the traditional and laboratory produced products as the storage time increases. The lactococci showed a fairly constant trend during the storage period with values ranging from 2.71 -4.88 log_{10} CFU/ml. The yeast and moulds counts were relatively constant

C. Biochemical changes during *olewonyo* storage.

The pH, titratable acidity changes and microbial profile were monitored during the storage of *olewonyo* at a 12-hour interval for 60 hours at 4° C . The changes in pH and titratable acidity during storage of *olewonyo* are shown in Figures 1 and 2 respectively. The mean pH of samples from Aboabo and Anloga showed a rapid decline from an initial value of 6.55 and 6.60 to 4.55 and 4.30 respectively within 24-hour of storage. This was followed by a steady decline from a pH of 4.55 and 4.30 to 2.80 and 2.65 in the next 36 hours of storage.

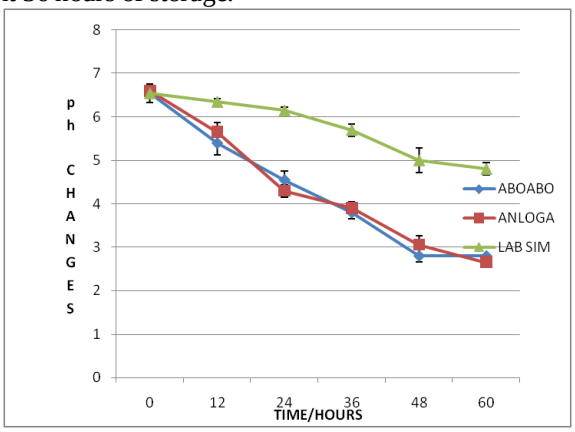

Figure 1: Mean changes in pH during storage of *olewonyo* at 4°C

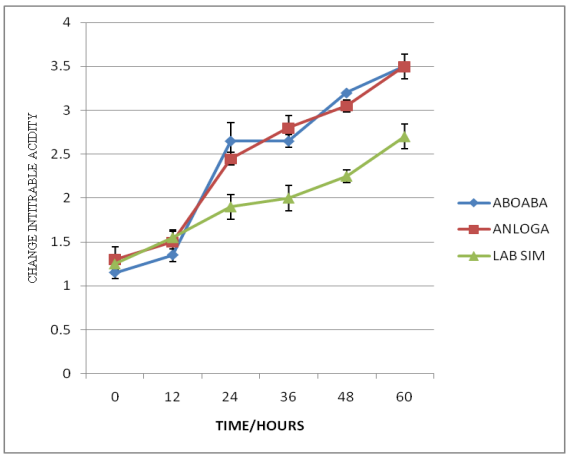

Figure 2: Changes in titratable acidity during storage of *olewonyo* at 4°C

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The changes in the mean pH of the Lab-produced sample however, showed a gradual decrease from 6.55 to 4.80 for the entire period of storage. The titratable acidity of samples from Aboabo and Anloga increased from a value of 1.15 to 3.50 and 1.30 to 3.50 respectively during storage of *olewonyo* (Figure 2).

Although the laboratory-simulated sample also showed increase in titratable acidity, it was not significant ($p<0.02$) as those from the two traditional samples.

D. Biochemical characteristics of lactic acid bacteria isolates in olewonyo using API-50 CH

Out of the 15 lactic acid bacteria isolates from *olewonyo* from the three sites (five from each site, Table 2), 40% were *Lactobacillus fermentatum*, 26.6% *Lactobacillus plantarum*, 20% *Lactobacillus acidophilus* and 13.4% *Lactobacillus brevis.*

The dominant species were able to grow at 45°C but not at 15 °C. They also grew in 6.5% NaCl and at pH 4.4 and 9.6 (Table 2).

Lactobacillus plantarum on the other hand showed growth at 15 °C but not at 45 °C. They also showed growth in both pHs 4.4 and 9.6. In the API gallaries, they were able to ferment ribose, galactose, D-glucose, D-fructose and mannitol. Two other species identified based on their biochemical characteristics and pattern of carbohydrate fermentation were *Lactobacillus acidophilus* and *Lactobacillus brevis.*

E. Microorganisms isolated from *olewonyo* samples from the different sites stored at different temperatures.

The laboratory-simulated samples had the lowest number of different species of microorganisms at 4° C and 25° C respectively (Table 3). Aboabo had the highest isolates of 9 at both 4° C and 25° C while Anloga had 7 and 9 isolates at 4°C and 25°C respectively. The most frequent species are *Lactobacillus plantarum* and *L. fermentatum.* The laboratory-simulated samples did not record *L. mesenteroides*, *L. brevis*, *E. coli* and *Aspergillus flavus* even though they were found in the Aboabo and Anloga samples (Table 3)

TABLE 2: Biochemical characteristics of lactic acid bacteria isolates in *olewonyo* samples using API-50 CH from the sample sites

	Sample Sites														
	Aboabo					**Anloga**					**Laboratory-simulated**				
Test	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Tetrad formulation	-	-	-	-		-	-	-	-	-	-	-	-	-	-
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Anaerobic growth	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CO_2 from glucose	+	-	+	+	+	-	+	+	-	+	-	-	-	+	-
Nutrient broth, pH 4.4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nutrient broth pH 9.6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth in 6.5% NaCl	+	-	+	+	+	+	+	+	-	+	+	-	+	+	
Growth in 18% NaCl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 15°C	+	+	-	-	-	+	-	+	-		+	-	+	-	-
Growth at 45°C	-	+	+	+		+	-	+	-	+	+	-	+	+	
Species identified	*LB*	*LP*	*LF*	*LF*	*LF*	*LA*	*LB*	*LF*	*LP*	*LF*	*LP*	*LA*	*LP*	*LF*	*LA*

LA - *Lactobacillus acidophilus* ; LB - *Lactobacillus brevis;* LF – *Lactobacillus fermentatum* ; LP – *Lactobacillus plantarum*

Table 3: Microbial species isolated from *olewonyo* samples from different sites and stored at different storage temperatures (4°C and 25°C)

	Sample Sites					
	Aboabo		**Anloga**		**Lab-sim.**	
	Storage Temperature (°C)					
	4	**25**	**4**	**25**	**4**	**25**
Leuconostoc mesenteroides	+	+	+	+	-	-
Lactobacillus plantarum	+	+	+	+	+	+
Lactobacillus acidophilus	-	-	+	+	+	+
Lactobacillus brevis	+	+	+	+	-	-
Lactobacillus fermentatum	+	+	+	+	+	+
Corynebacterium	+	+	-	+	-	-
Saccharomyces cerevisiae	+	+	+	+	-	+
Escherichia coli	+	+	-	+	-	-
Aspergillus flavus	+	+	+	-	-	-
Aspergillus parasiticus	+	+	-	+	-	-
Total	**9**	**9**	**8**	**9**	**5**	**6**

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IV. DISCUSSION

A. Microbial load and Biochemical changes during storage at 4 °C and 25 °C.

High microbial load was observed in *olewonyo* with initial counts of between 1.82-2.71 log 10 CFU/ml at both storage temperatures. The relatively high levels of reducing sugars and sucrose are possibly the reason why there were high counts of LAB. The initial counts increased to high levels of 9.41 and 9.12 log 10 CFU/ml for Aboabo and Anloga respectively during the storage period. These figures are similar to microbial counts reported in the Zimbabwean *mangisi* [12]. It has also been reported that in the preparation of *kirario* (a Kenyan fermented porridge of the Merus), a microbial load of more than 9.3-9.5 log 10 CFU/ml were recorded [13]. This is very similar to the results in this study. The low pH of 2.8, 2.6 and 4.8 for Aboabo, Anloga and the Laboratory simulated samples respectively in *olewonyo* is also in agreement with other cereal based fermented beverages with pH values in the range of 3.0 - 4.8. It is reported that most species of LAB and acetic acid bacteria (AAB) grow at pH 3.6-3.8, and some even at pH 3.0. The recorded low pH can be attributed to the high lactic acid bacteria (LAB) counts observed in the product. In previous studies, it has been found that the lactococci group of LAB decreases during fermentation as a result of the decrease in pH. . LAB are known to be able to tolerate environments with low pH. Lawlor et al. [14] reports that LAB typically enter breweries from raw materials and juice ingredients. It is also reported that the lactococci group, especially the Leuconostoc, is less resistant to low pH than the Lactobacillus.

The gradual decrease in pH values and increased titrable acidity may be as a result of microbial activity on the carbohydrates to produce organic acids such as lactic acid in the olewonyo from all the sample sites. Namugumya and Muyanja [15] reported that a decreased pH during the fermentation process could accelerate LAB growth.

B. Microorganisms isolated from *olewonyo* samples from different sites at different temperatures.

The raw materials and the preparation method used are likely to influence the type of microorganisms involved in the spoilage of olewonyo. The presence of these ten microorganisms other than yeast in *olewonyo* (Table 3) could lead to faster deterioration of the *olewonyo* as they are permanently and consistently present in the samples.

Lactobacillus plantarum, L. fermentatum and *Saccharomyces cerevisiae* were isolated in samples from all the sites and at the two different temperatures. Sawadogo, et al. [16] in their studies on *pito* production (a locally brewed alcoholic beverage from millet in northern Ghana) reported of *L. fermentatum* as the dominant species during acidification of the product. The presence of *L. brevis*, *L. acidophilus* and Corynebacterium species has also been reported in other traditional beers such as *chibuku* and *tchapalo* [17]. Faparusi et al. [18] found the presence of contaminants in the various stages of *burukutu* liquor production (a locally made alcoholic beverage from millet) and indicated that most of the bacteria survived at low acidity. This observation had been confirmed by other researchers [19-20]. *S. cerevisiae* which appeared in all the sites could be the predominant fermenter while most of the other microorganisms were there as opportunistic and contaminants from the environment. Studies have shown that the microoraganisms involved in the natural fermentation of cereals are essentially the microflora of the raw materials and equipment [21-22].

The presence of *E. coli* in the Aboabo and Anloga sample sites indicate a very low level of hygienic conditions of the environment and the individuals involved in the production since the laboratory produced samples did not record *E. coli*. In Nigeria, Kolawale et al.[19] attributed the presence of *E. coli* to improper sanitary condition during processing of *brukutu* from water supplies, unsterilized utensils and contaminations by flies.

C. Spoilage microbes in *olewonyo*

A range of microbes can be associated with *olewonyo* production, but only a few may be able to cause spoilage. As microbes differ in their growth requirements, different beverages support different spoilage microorganisms [14-23]. Lactic and acetic acid bacteria are the most common spoilage bacteria found in soft drinks. Their ability to tolerate environments with low pH is essential for growth in soft drinks. The presence of *Leuconostoc mesenteroides*, *Lactobacillus brevis, L. plantarum*, and *L. acidophilus* in this study may be responsible for the spoilage. Microbiological spoilage leads to deterioration of the sensory quality and typically appears as off-flavours, odours and visual changes in the product. In addition to direct spoilage mediated by viable cells [24], carry-over of microbial metabolites from raw materials can lead to indirect spoilage. LAB ferment sugars predominantly to lactate. Depending on the species and growth conditions, sugar catabolism can also lead to formation of ethanol, acetate, formate or succinate [25]. Some strains produce diacetyl, which tastes and smells buttery, and is an unwanted metabolite in soft drinks.

Yeasts are considered as the primary spoilage microbes in carbonated products mainly due to their ability to withstand high carbonation levels. Most species grow in the pH range 1.5–8.5 [26] and have their growth optimum in the pH range 3.0–6.0 [14]. Reports indicate that *Saccharomyces cerevisiae* is the most frequent spoiler of lemonades and fruit juices [27]. It is also reported that brewer´s yeasts which are ubiquitous contaminants in a brewery environment may also cause spoilage.

V. CONCLUSION

The present study shows that *Leuconostoc mesenteroides, Lactobacillus plantarum, L. acidophilus, L. brevis, L. fermentatum, Corynebacterium, Saccharomyces cerevisiae, Aspergillus flavus and A. parasiticus* are involved or associated in the spoilage of *olewonyo*. The level of bacteria yeast diversity was found to correlate with the chemical characteristics of *olewonyo* in terms of moisture content, salt content, and pH. The storage and the environmental conditions under which it is prepared can greatly contribute in prolonging the shelf-life of *olewonyo*

beyond the maximum three days. The lab-simulated samples recorded the least microbial load as well as only four (4) of the possible spoilage organisms isolated from the Aboabo and Anloga samples.

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