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Bacterial and Fungal Communities Associated with the Production of A Nigerian Fermented Beverage, "Otika"

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Abstract

"Otika" is a Nigerian indigenous alcoholic beverage produced from sorghum. The present work investigates the microbial community and exhibition of mutualism or antagonistic interaction during the production of 'Otika'. The microbes were isolated, enumerated and identified by pour plate, streak, morphological and biochemical characterization methods. Microbial interactions between the isolates identified were investigated by Agar well assay technique. Total bacterial, fungal, lactic acid bacterial and enterobacteriaceae counts, respectively, increased from $1.6\times 10^6 \pm 0.33$ cfu//ml, $3.4 \times 10^5 \pm 0.10$ cfu/ml, $3.0 \times 10^6 \pm 0.0$ cfu/ml and $1.5\times 10^6 \pm 0.15$ cfu/ml to $4.6 \times 10^7 \pm 0.30$ cfu/ml, $4.5 \times 10^6 \pm 0.10$ cfu/ml, $9.0 \times 10^7 \pm 0.05$ cfu/ml and $3.7 \times 10^7 \pm 0.2$ cfu/ml on the sorghum grains and at early stages of fermentation. Later, bacterial load decreased steadily along the fermentation period while enterobacteriaceae decreased until it was undetectable. *Bacillus* species, *Staphylococcus aureus*, *Enterobacter cloacae*, *Escherichia coli*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Pediococcus acidilactici*, *Enterococcus faecalis*, *Leuconostoc mesenteroides*, *Saccharomyces cerevisiae*, *Saccharomyces* species, *Candida krusei*, *Candida tropicalis*, *Aspergillus* species and *Penicilium italicum* were identified. Microbial occurrence through the production stage ranged from 33.3% each for *E. cloacae*, *A. fumigatus* and *Penicilium italicum* to 100% each for *L. plantarum*, *S. cerevisiae* and *C. tropicalis.* Yeasts and lactic acid bacteria exhibited positive interaction. There were no antagonistic interactions that existed among *L. plantarum*, *L. fermentum*, *Leuconostoc mesenteroides* and *S. cerevisiae* whereas both were antagonistic against other bacteria. The present study sheds more light on the populations and types of bacteria and fungi with their associations that characterized the production of "Otika" which will be useful information for production of consistent quality "Otika".

Key Words: "Otika" a Nigerian beverage, Microbial communities, Microbial interactions, Sorghum.

1. Introduction

Indigenous traditional beverage plays a vital role in the daily social, economic, nutritional and cultural aspects of people's life especially in developing countries (Kadjogbé *et al*., 2015; Fowoyo and Ogunbanwo, 2016). "Otika" is brownish-opaque, sweet with slightly sour taste (Ogunbanwo and Ogunsanya, 2012). It is an indigenous alcoholic beverage produced originally from sorghum grains through traditional process involving indigenous fermentation technology (Achi, 2005; Ogunbawo and Ogunsanya, 2012). "Otika" is commonly made in the South-Western Region of Nigeria "Otika" is used for various traditions including hospitality, friendliness and as part of the etiquette of most families. "Otika" also serves to seal harmonious relationships between individuals (Solange *et al*., 2014).

Fermentation is the process whereby chemical transformations of organic substances are broken down into simpler compounds by the actions of enzymes (Rina and Sonali, 2016). It has many advantageous attributes, which include improving nutritional value and safety of foods against pathogens over non-fermented foods (Adebayo *et al.*, 2014). Fermentation contributes to the reduction of some secondary metabolites, such as tannins and polyphenols in addition to enhancing the taste, aroma, shelf life, texture, nutritional value and other attractive properties of foods (Nzigamasabo and Nimpagaritse, 2009; Embashu, 2014; Stephanie *et al*., 2015). Fermented foods form about 25% of the foods consumed worldwide (Adebayo *et al.*, 2014).

Wide spectrum of microorganisms is involved in the production processes of fermented foods but a few types usually determine the quality of the end products. In order to access the types of microbes involved in determining the quality of "Otika", the present work was designed to investigate the microbial flora and exhibition of mutualism and antagonistic interaction during the production of "Otika." Therefore, isolation, characterization and identification of the microorganisms involved in the production with a prospective selection of starter cultures that are adapted to "Otika" production would be important to support the

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technical process and obtain a predictable end product with desired quality.

2. Materials and Methods

Traditional Preparation of "Otika"

Sorghum grains and water were mixed in ratio 1:3 (w/v) and left for two days at 25±2°C to steep. Malting followed in moistened fresh banana leaves for 3 days at 25±2°C. The germinated grains were dried at ambient temperature of 25±2°C for 2 days and milled using grinding instrument. Wort was extracted from the milled malted sorghum grains by cooking (100°C) for 7 hours, allowed to cool, poured into earthenware pots and left to naturally ferment for 3 days at 25±2°C (Ogunbanwo and Ogunsanya, 2012).

Sample Collection of "Otika"

Sample of traditionally fermented "Otika" were obtained at each step of production (Sorghum to the 120hrs of fermentation) from local home-made "Otika" producer (This production site is a major location where other retailers patronize from) in 'Iseyin', Oyo State, Nigeria. It was collected three different times from the period of March to May, 2012. These samples were put in sterilized bottles and transported to the Department of Microbiology's Laboratory, Federal University of Technology Akure for microbial analysis.

Microbial Analysis

Enumeration and isolation of bacteria, moulds, yeasts, Lactic Acid Bacteria (LAB) and Enterobacteriaceae members at each stage of the "Otika" preparation were conducted by pour plating using Nutrient Agar (NA) for bacteria, Potato Dextrose Agar (PDA) for moulds, Malt Extract Agar (MEA) (supplemented with streptomycin sulphate) for yeasts, Man Rugosa Sharpe Agar (MRS) at pH 5.5 for LAB anaerobically and Eosin Methylene Blue (EMB) agar for the members of Enterobacteriaceae. Incubation was carried out with bacteria for 24 hr and LAB for 48 hr at 37°C and with fungi at 27°C for 48 hr. Colonies and spore forming units formed on the media were counted and subcultured. The Bacteria isolates were examined using microscopy, Gram staining, sugar fermentation test, biochemical tests, such as urease test, catalase test, citrate utilization test and indole test according to the methods of Fawole and Oso (2007) and Brenner *et al*. (2005) while fungal identification was done using the fungi conventional identification method. Each fungal isolate was microscopically examined by putting a drop of lactophenol-in-cotton blue on a clean glass slide. A sterile inoculating loop was used to transfer a small piece of the mycelium into the lactophenol. The mycelium was spread out carefully with the sterile needle, covered with cover slip and examined firstly with the low-power objective lens, then with the high-power objective lens of the light microscope for vegetative and reproductive bodies. The fungi were identified based on the morphologic characteristics of their mycelia and spores according to Deak and Beuchat (1994) and Sanni *et al*. (1994).

Test for Selected Positive and Negative Microbial Interactions between the Isolates

Mutualism/commensalism and antagonism were the respective positive and negative interactions determined between the microbial isolates. Agar Well Assay method with slight modification was employed to determine the exhibition of the mutualistic or commensalistic and antagonistic associations among the isolated microbes during the production of the "Otika". Muller Hillton Agar (MHA) was prepared and poured in Petri dishes. Cultures of microorganism were swabbed uniformly on the individual plates using sterile cotton swab. Well was bored using a sterile cork borer of 5 mm diameter and with micropipette, 1 mL of each test isolate was transferred into each well and incubated for 24 hours at 37°C (Benkerroum et al., 2004). The agar was examined for zones of inhibition which were measured in millimetres. Creation of inhibitory zone indicated antagonism and absence of zone of inhibition signified mutualism or commensalism. Every laboratory experiment was carried out aseptically.

Analysis of Data

Experiment was carried out in triplicate. Numerical data obtained were subjected to Analysis of Variance (ANOVA) and means were separated with Duncan's New Multiple Range Test at 95% confidence level using SPSS 16.0 version.

3. Results

Types, Occurrence and Population of Microbial Isolates during "Otika" Production

Based on the cultural, microscopic and biochemical characteristics, twelve and nine different species of bacteria and fungi were isolated during the preparation of "Otika," respectively (Table 1). Among the bacteria, two were *Bacillus* species; six were species of Lactic Acid Bacteria (LAB), three species belong to the family of Enterobacteriaceae and one species was in the genus *Staphylococcus*. Among the fungal isolates, one and three species belong to *Penicillum* and *Aspergillus*, respectively. Two species of yeasts were separately identified as *Saccharomyces* and *Candida*.

All bacteria and fungi except Enterobacter cloacae, Lactobacillus brevis and Leuconostoc mesenteroides were isolated on the sorghum grains. Bacillus subtilis, B. cereus, Staphylococcus aureus, Escherichia coli, Lactobacillus plantarum, Lactobacillus fermentum, Pediococcus acidilactici, Aspergillus flavus and all the yeasts were present throughout the steeping, malting and milling and at 24^{th} hour of fermentation, but Aspergillus flavus was absent at 24^{th} hour of fermentation (Table 1).

More of the isolated microorganisms disappeared towards the end (120 hours) of the fermentation. Reoccurrence of these microorganisms was observed for only *Bacillus subtilis* and *E. faecalis* at 96 hours and 120 hours of fermentation, respectively. *Lactobacillus plantarum*, *Saccharomyces cerevisiae* and *Candida tropicalis* showed 100% occurrence during the "Otika" production while isolates with the lowest level of occurrence (30%) were *Enterobacter cloacae*, *A. fumigatus* and *P. italicum*.

Table 1. The types and occurrence of bacteria and fungi isolated during the production of "Otika"

Microorganisms	Sorghum Grains	Steeping	Malting	Milling Boiling	Fermentation (Hr)					Number of time microbe Occurred (%)	
					24	48	72	96	120		
Bacillus subtilis	+	+	+	+	+	-	-	+	+	70	
Bacillus cereus	+	+	+	+	+	-	-	-	-	50	
Enterococcus spp	+	+	+	-	-	-	-	-	+	40	
Staphylococcus aureus	+	+	+	+	+	-	-	-	-	50	
Escherichia coli	+	+	+	+	+	+	-	-	-	60	
Enterobacter clocae	-	+	-	+	+	-	-	-	-	30	
Listeria monocytogenes	+	+	-	+	+	-	-	-	-	40	
Klebsiella spp	+	+	+	+	-	-	-	-	-	40	
Lactobacillus plantarum	+	+	+	+	+	+	+	+	+	100	
Lactobacillus fermentum	+	+	+	+	+	+	+	+	-	80	
Lactobacillus brevis	-	+	-	+	+	+	+	-	-	50	
Leuconostoc mesenteroides	-	+	+	+	+	+	+	-	-	60	
Pediococcus acidilactici	+	+	+	+	+	+	-	-	-	60	
Aspergillus flavus	+	+	+	+	-	-	-	-	-	40	
Aspergillus niger	+	+	+	-	+	+	-	-	-	50	
Aspergillus fumigatus	+	+	+	-	-	-	-	-	-	30	
Penicillium italicum	+	+	+	-	-	-	-	-	-	30	
Sacharomyces cerevisiae	+	+	+	+	+	+	+	+	+	100	
Saccharomyces species	+	+	+	+	+	+	+	-	-	70	
Candida krusei	+	+	+	+	+	+	+	-	-	70	
Candida tropicalis	+	+	+	+	+	+	+	+	+	100	

Legend: + = Present, - = Absent

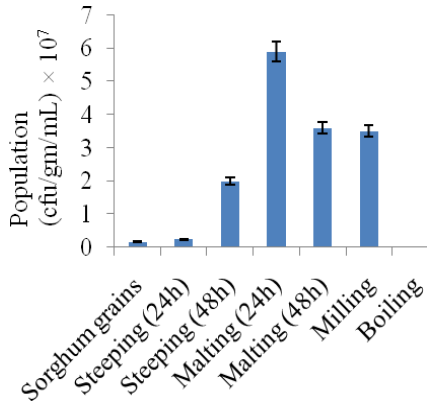

Figure 1a. Pre-fermentation total bacterial load

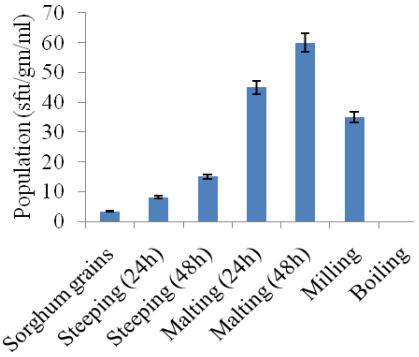

Figure 1b. Total bacterial load during fermentation of malted sorghum grains.

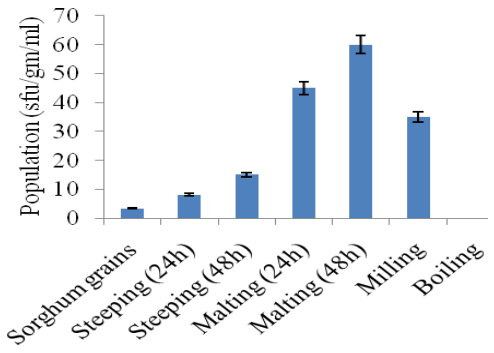

Figure 2a. Pre-fermentation fungal load

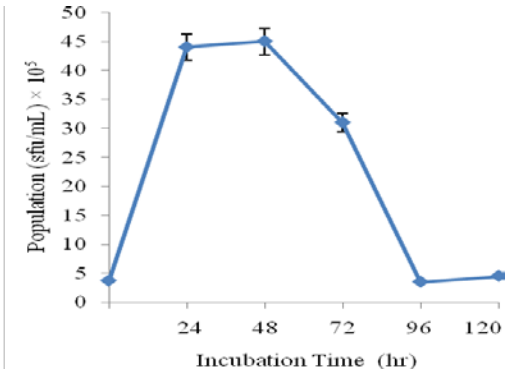

Figure 2b. Fungal load during the fermentationof malted sorghum grains

The total viable bacterial count increased significantly from 1.6 x 10^6 ± 0.33 cfu/gm/ml on the sorghum grains sample to 5.9 × 10^7 ± 0.05 cfu/ml during malting and later decreased to 2.5×10^7± 0.03 cfu/gm/ml after boiling. During fermentation, the population increased to 4.6 ×10^7 ± 0.33 cfu/gm/ml at 24 hr and decreased afterwards to 2.4 × 10^6 ± 0.05 cfu/gm/ml at 120 hr of fermentation (Figures 1a and b). Fungal population increased from 3.4 × 10^5 ± 0.05 cfu/gm/ml on the sorghum grains to 6.0 × 10^6 ± 0.05 cfu/ml after milling and declined to 0.0 sfu/ml during boiling (Figures 2a and b). During fermentation, the fungal load increased sharply after 0 hr at 3.7 ×10^5 ±0.03 cfu/ml to 48 hr at 4.5 ×10^6 ± 0.11 cfu/ml but dropped to 3.5 ×10^5 ± 0.11 cfu/ml at 96 hr of fermentation and again increased slightly (4.5 × 10^5 ± 0.11 cfu/ml) at 120 hr. Similar trend of change was recorded for the total viable lactic acid bacteria (LAB) population (Figures 3a and 3b), which increased significantly (<0.05) from 3.0 × 10^6 ± 0.0 cfu/gm/ml on sorghum grains to 3.2 × 10^7 ± 0.08 cfu/ml at 24 hr of malting. Then after, there was a reduction in the LAB population to 0 cfu/ml during boiling which increased to 9.0 × 10^7 ± 0.05 cfu/ml at 48 hr of fermentation and gradually decreased to 2.3 × 10^6± 0.0 cfu/ml at the end (120 h) of the fermentation. The total viable enterobacteriaceae load of the sorghum grains increased significantly from 1.5 × 10^6 ± 0.15 cfu/ml to 2.3 × 10^7 ± 0.08 cfu/ml during steeping (Fig. 4a). During the period of fermentation, there was an increase in this population to 4.2 × 10^6 ± cfu/ml at 0 hr which decreased to 3.7 × 10^7 ± 0.2 cfu/ml at 24 hr of fermentation (Fig. 4b). Then after, it decreased gradually and at 120 hr of fermentation the bacteria were undetectable.

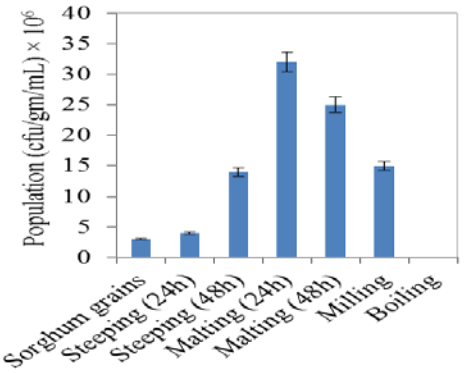

Figure 3a. Pre-fermentation lactic acid bacteria load

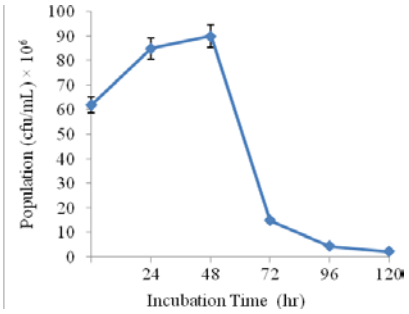

Figure 3b. Lactic acid bacteria load during the fermentation of malted sorghum grains

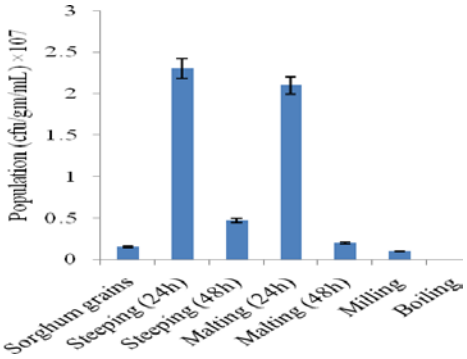

Figure 4a. Pre-fermentation enterobacteriaceae load

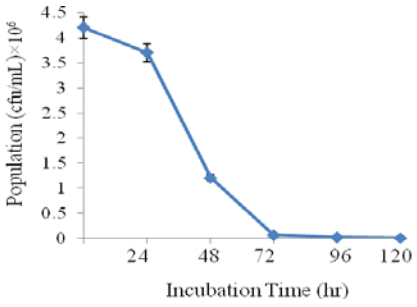

Figure 4b. .Enterobacteriaceae load during the fermentation of malted sorghum grains

Mutualism/Commensalism and Antagonism between the Lactic Acid Bacteria, Yeasts and Other Bacteria Isolated

Table 2 shows the interactions which existed between the bacteria, yeasts and Lactic Acid Bacteria (LAB). Lactic acid bacteria inhibited the growth of other bacteria but not its own members. The interaction between the yeasts and the LAB was positive as zone of inhibition was not created when they were co-cultured. Yeasts also showed a negative relationship against non-LAB bacteria by showing the zone of inhibition.

Table 2. Interaction between Lactic acid bacteria, yeast isolates and some of the bacteria isolated during "Otika" production

Microorganisms (LAB/Yeasts)	*Escherichia coli*	*Pediococcus acidilactici*	*Listeria monocytogenes*	*Lactobacillus fermentum*	*Bacillus subtilis*	*Staphylococcus aureus*	*Lactobacillus plantarum*	*Enterbacter faecalis*	*Enterococcus cloacae*	*Leuconostoc mesenteroides*	*Bacillus cereus*
Leuconostoc mesenteroides	+	-	+	-	+	+	-	+	+	-	+
Lactobacillus Fermentum	+	-	+	-	+	+	-	+	+	-	+
Lactobacillus Plantarum	+	-	+	-	+	+	-	+	+	-	+
Pediococcus acidilactici	+	-	+	-	+	+	-	+	+	-	+
Saccharomyces Cerevisiae	+	-	+	-	+	+	-	+	+	-	+
Candida tropicalis	+	-	+	-	+	+	-	+	+	-	+

Legend: LAB= Lactic Acid Bacteria

\+ = Zone of inhibition formed (Antagonism)

\- = Lack of zone of inhibition (Mutualism/Commensalism)

4. Discussion

Various species of microorganisms were isolated during the production of "Otika" which revealed the arrays of microorganisms. They exhibited cultural, cellular and biochemical properties similar to those described by Deak and Beuchat (1994) and Sanni *et al*. (1994) and Bergey's Manual of Systemic Bacteriology (Brenner *et al*., 2005); hence their probable names were as presented in Table 1. The presence of *Bacillus subtilis* at the beginning of fermentation of the milled malted grains suggests that the microorganisms were not completely killed during boiling; some could have also re-grown through their spores. *Bacillus subtilis* and *B. cereus* are spore formers and these spores help them to be resistant against heat that is, they can survive extreme temperatures. This was also confirmed by Boboye (2007) who worked on bacterial changes in sorghum larger beer where *Bacillus* spp. was isolated at the beginning of fermentation. *Lactobacillus plantarum* and two of the yeasts (*Saccharomyces cerevisiae* and *Candida tropicalis*) isolated were found throughout the production stages of "Otika". These *Lactobacillus* species and yeasts were reported to be predominant during cereal fermentation (Avicor *et al*., 2015; Kadjogbé *et al*., 2015). These data therefore mean that *L.* spp. and the yeasts play considerable roles in the fermentation of malted sorghum grains for "Otika" production. These roles might be similar to those played during cereal fermentation. The disappearance of some microorganisms during the production of the "Otika" may be attributed to the increased acidity and the lowered pH of the fermenting malted grains. Acid and lowered pH below 4 or 3 restrict the growth and survival of spoilage organisms and some pathogenic microorganisms, such as *Shigella*, *Salmonella* and *Escherichia coli* (Muyanja *et al*., 2003; Chelule *et al*. 2010; Nyanzi and Jooste, 2012; Rina and Sonali, 2016).

The increase in bacterial load at the initial stages before fermentation was a result of their dominancy due to favourable conditions. The studies carried out by some scientists showed these microorganisms are dominant fermenting microorganisms in fermented foods (Akinleye *et al*., 2014).

The increase in microbial loads before boiling indicates that the raw, steeped and malted grains contained appropriate nutrients for the microbes to utilize and multiply. This favourable condition could have resulted by the dominancy of bacteria at the initial stages before fermentation. Similarly, the fermenting malted, milled sorghum grains must have provided the microorganisms with sufficient and appropriate nutrients that caused the increase in their populations. Générose *et al*. (2016) who worked on "gowe", a fermented sorghum beer, reported that the volatile compounds observed during the primary fermentation stage supported an increase in the lactic acid bacteria and yeast counts. These compounds include alcohols, aldehydes, esters, hydrocarbon, furan, phenol, piperidine and acids; some of which were identified from 0 h of fermentation and may have been initiated during the stages preceding fermentation which are steeping and malting of sorghum kernels.

The later decrease in the loads of other bacteria besides LAB, after 24 hr of fermentation could be due to increased population of the LAB that must have produced acid causing reduction in pH (acidity) which seems to be detrimental to the mesophilic bacteria. A similar result has been reported by Babatunde and Oladejo (2014) and Teshome (2015) who posited that LAB produce many organic acids, such as lactic, acetic and propionic acids produced during fermentation as end products which provide an acidic environment unfavourable for the growth of many pathogenic and spoilage microorganisms. Acidic medium favoured yeast growth which underlies yeasts multiplication observed in the present study.

The mutual association that existed between yeasts and lactic acid bacteria has been noted in several cereal foods (Omemu *et al*., 2007; Omemu, 2011; Ogunbanwo *et al*., 2013). Enterobacteriaceae members are common on fermenting plant materials and have also been found in the natural fermentation of cereal products; thus their high load obtained before fermentation in the present work could be due to their possible presence on the sorghum grains from the farm where they were harvested. The bacterial isolates might have originated from the plants, utensils and vessels used previously during handling, malting, milling and fermentation. These data are similar to the report of Ogunbanwo *et al*. (2003). The presence of the microbes except *Aspergillus flavus* on the sorghum grains and during the fermentation implies that they are important for the production of "Otika". The differences observed in the level of occurrence of the fungi and bacteria mean that these microorganisms performed different

functions as they were associated with various stages of the "Otika" production. The presence of moulds at the initial stage of fermentation of cereal for "Ogi" production and the subsequent elimination was also reported previously Omemu *et al*. (2007). Fungi play vital roles at initial phase of fermentation mostly in saccharification of the substrates (Thapa and Tamang, 2004; Adebayo *et al*., 2014).

All LAB and yeasts evaluated in the present study showed antagonistic interaction to other isolated bacteria. This means that these microbes might have produced inhibitory metabolites. This conforms to the report of De Martinis *et al.* (2001) and Ogunbanwo *et al.* (2003) who reported that the antimicrobial compounds produced by LAB enable them to exert strong antagonistic activity against food contaminating microorganisms. None of the LAB isolates inhibited any of the yeast cultures. The relationship between yeasts and the entire LAB could therefore be mutualism or commensalism. Several authors have reported similar coexistence and positive interactions between yeasts and lactic acid bacteria in different African fermented foods (Omemu *et al*., 2007; Gulitz *et al*., 2013). The positive interaction between yeasts and LAB could have caused co-metabolism between them. Thus, the two groups of microbes appeared to have adapted to the food systems in the sorghum particularly the non-fermentable such as starch. This enabled them to adequately utilize substances in the fermenting sorghum thereby resulting to increased populations as observed in the figures. The stimulatory effect of yeasts on lactic acid bacteria during fermentation has been attributed to the provision of some compounds such as soluble nitrogenous compounds, B-vitamins, CO_2, pyruvate, propionate, acetate and succinate (Stadie *et al*., 2013).

5. Conclusion

The present study has provided useful information on the types of microbial communities and their associations that characterized the production of "Otika". *Lactobacillus plantarum*, *Saccharomyces cerevisiae* and *Candida tropicalis* were found to be predominant in the production of "Otika" and they have the ability to inhibit pathogenic microorganisms. There was re-occurrence of spoilage microorganisms after 72 hr which mean it start deteriorating after 72 hr which will not be safe for consumers. The present study also established the antimicrobial interaction between microorganisms which is either positive or negative. The information from this work would assist in the production of consistent quality of "Otika" beverage.

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No conflict of interest

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