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Dominant Strains of Microorganisms Involved in the Spontaneous Fermentation of *Obiolor*, a Nigerian Cereal Beverage

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Abstract

The study was conducted to determine the microflora of *obiolor* beverage produced from the natural fermentation process of sorghum and millet. One hundred and fourty four microorganisms were isolated and characterized using phenotypic and biochemical methods. Results revealed that yeast count increased from 4.33±0.06 to 5.50±0.01 log cfu/ml from 6 h -24 h fermentation time. Growth of lactic acid bacteria (LAB) was not observed at the 6th hour, however at the 18th and 24th hour LAB count ranged from 8.17±0.00 to 8.25±0.061 respectively. Population of aerobic mesophilc count was on the rise from 7.05±0.22 cfu/ml to 8.23±0.02 cfu/ml from the start to the end of fermentation period. The frequencies of dominance of LAB were *Lactobacillus delbrueckii* (44.29%), *Lactobacillus leichmanii* (31.43%), *Lactobacillus fermentum* (12.86%), *Lactobacillus plantarum* (10%) and *Lactobacillus bulgaricus* (1.43%). Yeast strains were identified as *Candida sojae* (66%), *Saccharomyces cerevisiae* (26%), *Kluyveromyces lactic* (6%) and *Saccharomyces bisporus* (2%). Aerobic mesophilic bacteria as well as indicator organisms were also present in the microbial population such as *Bacillus subtilis, Bacillus megaterium*, *Bacillus mycoides, Staphylococcus saprophyticus, klebsiella oxytoca* and *Enterobacter cloacae.* Results have shown that lactic acid bacteria and yeast were mainly responsible for the fermentation of *obiolor* and could serve as possible starter culture(s) for *obiolor* production.

Keywords:

Obiolor, Lactic acid bacteria, Yeast, Sorghum, Millet, Beverage

Introduction

Fermented beverages obtained from certain microbial based conversion of food raw materials have been staple drinks for millennia (Marco *et al*., 2017). It has therefore been considered important as part of the human diet for reasons of generally improved shelf-life, palatability, safety and nutritional quality (Marco *et al*., 2017). Beverages are consumed globally and can be alcoholic or non-alcoholic. In Africa, fermented beverages made from cereals are popular (Marsh *et al*., 2014). The raw materials used are maize, barley, millet, oats, rye, wheat, rice and sorghum. Examples of cereal based beverages are *bushera* from Uganda, *gowe´* from Benin, *oti-oka* from Nigeria, *tchapalo* from cote d'ivoire, *umqombothi* from South Africa, e.t.c. (Solange *et al*., 2014). In Nigeria, a non-alcoholic beverage known as *Obiolor* is produced from fermented sorghum and millet malts. As a consequence, it is a sweet-tasting thin gruel consumed by the Igala tribe and is extremely associated with good health. The fermentation process of *obiolor* depends on the vital information acquired by observation and experiences passed on from generation to generation (Chelule *et al*., 2010). Fermentation procedure is dependent on the natural action of microorganisms for production of metabolites which can hold back the increase and continued existence of unwanted microflora in food products (Ross *et al*., 2002). Fermentation may be a helpful method for reducing bacterial contamination of food and could also help to reduce the prevalence of diarrheal diseases (Mensah *et al*., 1990). It is the most economical system in which microorganisms play an essential role in food production and preservation. Lactic acid bacteria and yeast are the leading organisms that ferment cereals (Suman *et al*., 2015). The fundamental fermentation process involves the enzymatic activities of lactic acid bacteria, yeasts and moulds (Mensah, 1997). The principal function of these microorganisms in food fermentation is to convert carbohydrates to desired metabolites such as alcohol, lactic acid, acetic acid or carbon dioxide (Ajanaku *et al*., 2012). Lactic acid bacteria are popular owing to their reputed abilities to improve gastrointestinal health while yeast stimulate the growth of lactic acid bacteria, inhibit production of mycotoxin, degrade cyanogenic glucosides and produce tissue degrading enzyme e.g. pectinase and cellulose (Saijirahu, 2008). Studies on the natural fermentation of *obiolor* is on the rise, however, not much has been done on the diversity of microflora responsible for its fermentation. Hence, the need to characterize the principal microflora which could be a basis for selection of microorganisms with relevant technological traits for controlled fermentation of *obiolor* beverage.

Materials and Methods

Sample Collection

Sorghum and Millet grains used in this study was purchased at a retail market in Abeokuta, Nigeria, transported in a polythene bag to the laboratory in the Department of Microbiology, Federal University of Agriculture, Abeokuta and stored at room temperature in a dry place.

Spontaneous Fermentation of *Obiolor*

The germinated grains were wet milled with sweet potatoes and prepared into slurry by mixing in water. The slurry was then mixed in boiled water (ratio 1:4 v/v) and stirred. The mash was cooled, filtered and the residue discarded, while the filtrate was concentrated by boiling for 30 mins with constant stirring. The resulting gruel was cooled rapidly and allowed to spontaneously ferment for 24 h at ambient temperature (Bonno, 2011).

Enumeration of Isolated Microorganisms

10 ml of sample was homogenized with 90 ml of maximum recovery diluent (MRD, Oxoid) and serially diluted in the same diluents. Aliquots (0.1 ml) of appropriately diluted samples were spread plated on de Man Rogosa and Sharpe agar (MRSA), sabouraud dextrose agar (SDA) and nutrient agar (NA) plates. Plates were incubated anerobically at 30°C for 48 h for LAB and aerobically for 25°C for 2-5 days for yeast and 37°C for 24 h for aerobic mesophilic bacteria. Colonial growth on the plates were enumerated and the different colonies were described, isolated and assigned codes. The isolates were further purified by repeated streaking and subcultured on fresh MRS agar slant for storage (Harrigan and McCance, 1998).

Phenotypic Characterization and Identification of Isolated Microorganisms

Characteristics of the isolated strains such as catalase test, Gram staining and morphology were studied. Strains which were catalase negative and Gram positive were preliminary identified as LAB. Further identification of LAB and other mesophiles were performed by using the following tests: growth at different temperatures (15 and 45°C), different pH (3.9 and 9.6) as well as the ability to grow in different concentrations of NaCl (4.0 and 6.5%) in MRS broth; gas production from glucose determined in MRS broth containing inverted Durham tube; hydrolysis of arginine tested on MRS – Arginine broth, citrate utilization; and production of acetoin from glucose which was determined by using the

Voges- Proskauer test. The fermentation of carbohydrates was done in modified MRS broth (in which meat extract and glucose were omitted) containing phenol red (0.04 gL^{-1}) as a pH indicator which was supplemented with 1% of the following carbohydrates; xylose, galactose, sorbitol, mannitol, maltose, melibiose, ribose, trehalose, salicin, lactose, raffinose, cellobiose, sucrose and mannose (Harrigan and McCance, 1998). Yeast isolates were identified by determining their pattern of fermentation of various carbohydrates: glucose, maltose, lactose, galactose, sucrose and fructose; as well as their ability to utilize ethanol (Harrigan and McCance, 1998). All aerobic mesophiles were also characterized, and tentatively identified using phenotypic methods (Harrigan and McCance, 1976).

Statistical Analysis

Analysis of variance (ANOVA) was used to determine statistically significant differences among treatments using SPSS statistical software, version 17.

Results and Discussion

Population of Microorganisms Involved in Fermentation of *Obiolor*

Figure 1 shows the counts of isolates on MRSA, SDA and Nutrient Agar. There was no growth of LAB at 6 h fermentation of *Obiolor*. However, there was a rapid growth (8.17±0.00 Cfu/ml) at 18 h fermentation which increased significantly till the end of fermentation (8.25±0.01 Cfu/ml). This is not surprising as LAB have been reported to be the main microorganisms implicated in the fermentation of cereal based beverages like *kunu-zaki* and *burukutu* (Okoronkwo, 2014). Their growth is favoured by the acidic pH of the medium. Lactic acid bacteria usually play very vital roles in the production of varieties of traditional cereal based fermented foods and beverages, including *ogi* (Odunfa, 1985), *kenkey* (Halm *et al*., 1993); *togwa* (Mugula *et al*., 2003), etc. Different works have observed diverse counts of these microorganisms in different fermented foods depending on the duration of the fermentation technique, initial number of microflora present, nature of substrate for the fermentation, and even occasionally the ambient temperature of the local area where the fermentation process is going on (Owusu-Kwarteng *et al*., 2010). The population of yeast during the fermentation of *obiolor* showed a significant rise in the number of yeast throughout fermentation period. Yeast count was observed to be 4.33±0.06 cfu/ml at the start of fermentation which increased to 5.50±0.01 cfu/ml at the end of the fermentation process. The number of yeast rose with increase in fermentation time and this results agrees with Muhammet *et al.* (2017) who reported that yeast count in sourdough samples ranged from 3.78 to 6.28 log Cfu/g. Similar observation was revealed in the work of Wakil and Daodu (2011) in which there was an early increase in yeast count in the first 48 h after which there was a decline at the 72nd h during the fermentation for *ogi* production. Interactions between yeasts and lactic acid bacteria during the production of fermented foods are therefore observed to involve a 'symbiotic' association due to a mutual growth stimulation based on their amino acids and carbohydrate metabolisms (Martinez-Anaya *et al*., 1990). The aerobic mesophilic count at 6 h of fermentation was observed to be 7.05±0.22 cfu/ml which increased significantly to 8.14±0.00 cfu/ml and 8.23±0.02 cfu/ml at 18 and 24 h respectively. The rise in bacteria load of *Obiolor* may be due to available nutrient from the composite sorghum and millet grains.

Morphological and Biochemical Characterization

A total of seventy LAB colonies were isolated from *Obiolor*. They were circular in shape, creamy in colour and opaque. They had convex elevations, smooth surfaces and entire edges. They were all Gram positive, catalase negative bacilli which were non-nitrate reducers. 50 yeast were isolated during the fermentation of *Obiol*or and 27 aerobic mesophiles were isolated, of which 5 were cocci while the others were rod-shaped. The biochemical characteristics exhibited by the isolates (Table 1-3) were compared with those of standard strains for their identification ((Harrigan and McCance, 1998).

Percentage Occurrence of Isolated Microorganisms during Fermentation of *obiolor*

Figure 2 shows the percentage of occurrence of the lactic acid bacteria involved in the fermentation of *Obiolor*. They were *L. delbrueckii 44.29%, L. leichmannii 31.43%, L. fermentum 12.86%, L. plantarum 10%, and L. bulgaricus 1.43%.* This established previous findings which have revealed its dominance in many natural lactic fermentation of non-alcoholic based cereal foods and beverages (Afolabi and Akintokun, 2008; Suman *et al*., 2015). *L. leichmanni* was observed to be the second dominant LAB species as revealed by physiological characterization. *L. fermentum* found in this study has been reported to play an important role in the fermentation of *Masa Agria*, a traditional maize fermented product (Vanegas-Gamboa *et al*., 2009). It was also reported to be involved in the production procedure of *Togwa*, a popular fermented beverage in Tanzania (Mugula *et al*., 2003). The function of *L. fermentum* in aroma formation has also been described for fermented maize dough (Annan *et al*., 2003). Properties exhibited by *L. fermentum* may be helpful in the finishing product through the production of flavour which is a vital quality attribute of *Fura* (Owusu-kwarteng *et al*., 2015). *L. plantarum* has been reported in a number of cereal based food or beverages such as *Togwa* (Mugula *et al*., 2003), *Fura* (Olasupo *et al*., 1997), *Ogi* (Blandino *et al*., 2003) and *Obiolor* as in the case of this study amongst several others. *L. bulgaricus* is mostly involved in the fermentation of *Tarhana*, a wheat fermented

product (Economidou and Steinkraus, 1993). The percentage occurrence of yeast is shown in Figure 3. The most predominant yeast was *Candida sojae* (66%) followed by Saccharomyces cerevisiae (26%), *Kluyveromyces lactis* (6%) and *Saccharomyces bisporus* (2%). *C. sojae* (Y_{14}) have been reported by Nakase *et al*. (1994) to be isolated from an extraction process of water-soluble substances of defatted soybean flakes. This *Candida* isolates have also been shown to be involved in xylose consumption and xylitol production which could be of interest and relevance to the food industry as a sweetener (Borelli *et al*., 2016). Saccharomyces species was also found during the fermentation of *Obiolor*. They are *Saccharomyces cerevisiae* and *Saccharomyces bisporus*. *Saccharomyces* species in general have been observed to stimulate the growth of other microorganisms, including lactic acid bacteria, by providing necessary metabolites such as pyruvate, amino acids and vitamins. On the other hand, they had been reported to utilize certain bacterial metabolites as carbon sources (Gadaga *et al*., 2001). *Kluyveromyces lactis* was also recognized to be present in the course of fermentation of sugary Kefir grains (Magalhães-Guedes *et al*., 2018).

Yeasts and lactic acid bacteria are implicated in the fermentation of a wide variety of traditional food and beverage fermentation. While yeasts are known to facilitate alcoholic fermentations, lactic acid bacteria produce lactic acid as a part or major by-product during the fermentation of carbohydrates (Olaoluwa, 2013). Isolated aerobic mesophilic bacteria (Figure 4) involved in *Obiolor fermentation* are *Bacillus subtilis* (51%), *Bacillus mycoides* (11%)*, Staphylococcus saprophyticus* (11%) and *klebsiella oxytoca* (11%). *Bacillus megaterium* (7%) was also present during fermentation with the least dominating bacteria known as *Staphylococcus epidymidis* and *Enterobacter cloacae* having a percentage occurrence of 3%. The presence of aerobic mesophilic bacterial encountered during the study does not mean they were involved in the fermentation process. However, they may have been introduced due to the unhygienic practices associated with the process.

Conclusion

The study showed that diverse strains of lactic acid bacteria and yeasts were responsible for the fermentation of *obiolor*. The most frequently lactobacillus species observed during the fermentation was *L. delbrueckii* while the least frequent lactobacillus was *L. bulgaricus.* Yeast isolated were *Candida sojae, Saccharomyces cerevisiae*, *Kluyveromyces lactis* and *Saccharomyces bisporus*. The technological properties of these microorganisms could be further investigated and developed for application in small scale production of *obiolor* with enhanced health benefits.

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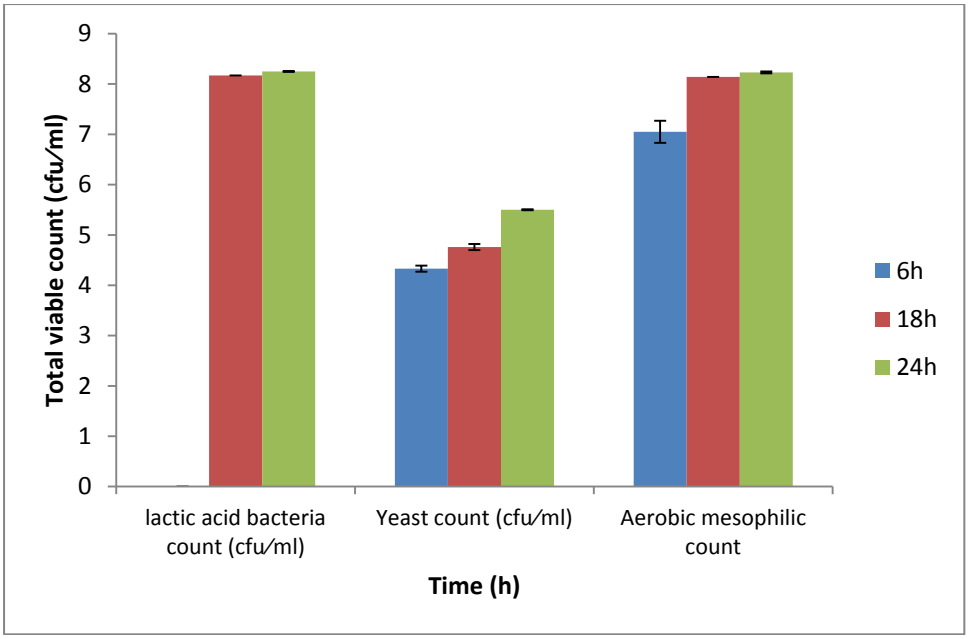


Figure 1: Total Viable Counts (log cfu/ml) during Natural Fermentation of *Obiolor*

Table 1: Morphological and Biochemical Characterization of Lactic Acid Bacteria Isolated from *Obiolor* Beverage

Number of Strains	31	22	7	9	1
Gram reaction /Morphology	GPR	GPR	GPR	GPR	GPR
Catalase	-	-	-	-	-
MR	-	-	-	-	+
VP	-	-	+	-	-
Arginine	-	+	-	+	+
MRS_{gas}	-	-	-	+	-
pH 3.9	-	-	+	-	-
pH 9.6	+	-	+	+	-
NaCl 4.0%	-	-	+	+	-
NaCl 6.5%	-	-	+	+	-
15°C	-	-	+	-	-
45°C	+	+	+	+	+
Xylose	-	+	+	-	-
Galactose	+	+	+	+	+
Mannitol	+	+	+	+	-
Maltose	-	+	+	+	-
Lactose	-	-	+	+	+
Cellobiose	-	+	+	+	-
Sucrose	+	+	+	+	-
Identification	*L.delbrueckii*	*L. leichmanii*	*L. plantarum*	*L. fermentum*	*L. bulgaricus*

Key: GPR – Gram positive rod + = Positive

L – Lactobacillus - = Negative

Table 2: Morphological and Biochemical Characterization of Yeast Isolated from *Obiolor* Beverage

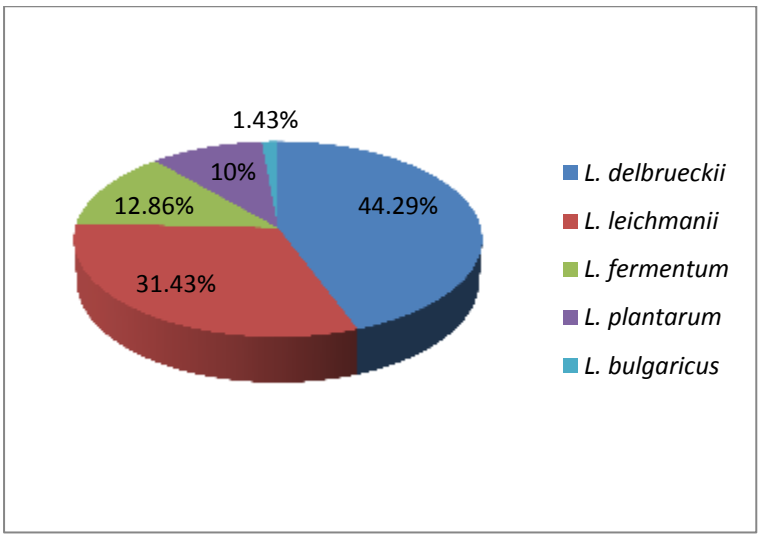
Number of Strains	33	13	3	1
Glucose	+	+	+	+
Maltose	-	+	+	+
Lactose	-	-	+	-
Galactose	+	+	+	+
Sucrose	+	+	+	+
Fructose	+	+	+	-
Utilization of Ethanol	+	+	+	-
Identification	*Candida sojae*	*Saccharomyces cerevisiae*	*Kluyveromyces lactis*	*Saccharomyces bisporus*

+ = Positive - = Negative

Table 3: Morphological and Biochemical Characterization of Aerobic Mesophilic Bacteria Isolated from *Obiolor* Beverage

Number of Strains	14	1	3	2	3	3	1
Gram reaction /Morphology	GPR	GPC	GPC	GPR	GPR	GNR	GNR
Catalase	-	-	+	+	-	+	+
Motility	+	+	-	-	-	+	-
Indole	-	-	-	-	-	+	-
Citrate	+	-	-	+	-	+	+
Oxidase	-	-	-	-	-	-	-
H_2S	-	+	-	-	-	-	-
MR	-	-	+	+	+	-	-
VP	+	+	-	+	+	+	+
Urease	-	+	+	+	-	-	-
Nitrate Reduction	+	+	-	-	+	+	+
Glucose	+	+	+	+	+	+	+
Lactose	+	+	+	+	-	+	+
Mannitol	+	-	+	+	-	+	+
Maltose	+	+	+	+	-	+	+
Sucrose	+	+	+	+	+	-	+
Identification	*B. subtilis*	*S. epidymidis*	*S. saprophyticus*	*B. megaterium*	*B. mycoides*	*K. oxytoca*	*E. cloaca*

Key: GPR – Gram positive rod; + = Positive; B - Bacillus
GPC – Gram positive cocci; - = Negative; S - Staphylococcus
GNR – Gran negative rod; E - Enterobacter; K - Klebsiella



Key: L - Lactobacillus

Figure 2: Percentage Occurrence of Lactic Acid Bacteria Isolated during the Natural Fermentation of *Obiolor*

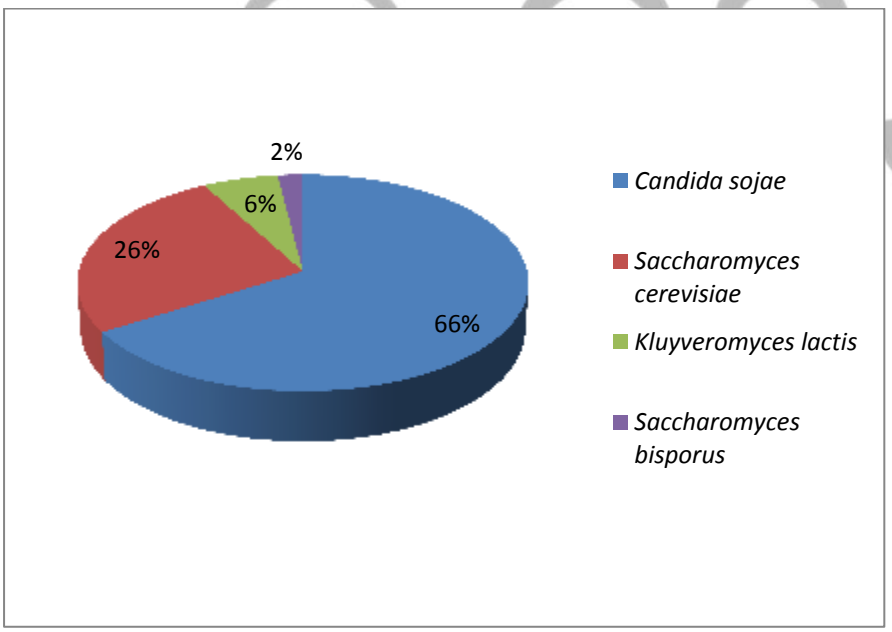


Figure 3: Percentage Occurrence of Yeast Isolated during the Natural Fermentation of *Obiolor*

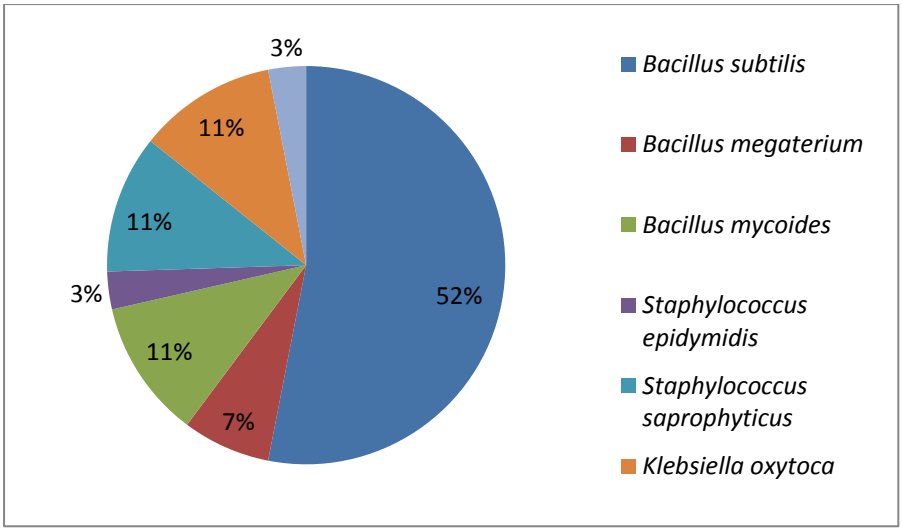


Figure 4: Percentage Occurrence of Aerobic Mesophilic Bacteria Isolated during the Natural Fermentation of *Obiolor*