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Microorganisms Associated with the Production of a Nigerian Fermented Beverage, 'Agadagidi'

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Authors' contributions

This work was carried out with the collaboration of all authors. Authors OO and BB designed the study and performed the statistical analysis. Author OO performed the laboratory analysis and wrote the first draft of the manuscript. Authors BB and AF assisted in experiment implementation and corrected the first draft. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The microbial types, occurrence, loads and interactions were studied during the production of a Nigerian fermented beverage, 'Agadagidi', from overripe plantains.
Place and Duration of Study: Federal University of Technology Akure, Ondo State, Nigeria. March- July, 2012.
Methodology: Isolation, enumeration and identification of bacteria and fungi were carried out by using standard pour plate, morphological, biochemical and physiological characterization methods. Antagonistic and mutualistic interactions among the microorganisms were investigated using agar well diffusion method.
Results: *Bacillus subtilis*, *B. megaterium*, *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus* species, *Lactobacillus plantarum*, *Pediococcus acidilactici*, *L. fermentum*, *Leuconostoc mesenteroides*, *Aspergillus flavus*, *A. niger*, *A. fumigatus*, *Penicilium notatum*, *Trichoderma viridea* *Saccharomyces cerevisiae*, *Candida utilis* and *Geotrichum* species were identified during the production of the beverage. The loads of total bacteria (TB), Lactic Acid Bacteria (LAB), enterobacteriaceae and fungi of the uncrushed plantain pulp were $2.6 \times 10^7 \pm 0.05$ cfu/ml, 6.7×10^6

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± 0.05 cfu/ml, 3.8 × 10^5± 0.05 cfu/ml and 2.0 × 10^7 ± 0.05 cfu/ml. At 0 hour of fermentation, the loads of total bacteria, fungi and enterobacteriaceae increased. Then after, the total bacteria, enterobacteriaceae and fungi counts decreased to 2.0×10^5 ± 0.11 cfu/ml, 1.3 × 10^5± 0.11 cfu/ml and 1.03 × 10^5 ± 0.05 cfu/ml respectively. In contrast, the LAB cell number increased to 8.6 × 10^7 ± 0.1cfu/ml at the end (48 hours) of fermentation. The level of the microbial occurrence was 25 to 100% with *B. subtilis*, *L. plantarum*, *L. mesenteroides*, *S. cerevisiae* and *C. utilis* occurring as the highest. *B. megaterium*, *E*. spp., *A. niger* and *T. viridea* occurred least. There was positive co-existence between Yeast and LAB. The yeasts and LAB exhibited antagonism against other bacteria.

Conclusion: The data obtained in this work has shown some functional microflora and their relationship during the production of "Agadagidi". This information can contribute to a better understanding of the "Agadagidi" production process for a consistent quality beverage.

Keywords: Microbial community; "Agadagidi"; fermented beverage; microbial loads; antagonism.

1. INTRODUCTION

Plantain (*Musa sapientum* var. Paradisiacal Linn) is one of the most important staple food crops for millions of people both in developed and developing countries. It is one of the foods commonly consumed in the West Africa sub-region, Northern America, Mexico and the Caribbean. In Nigeria, its consumption cuts across the indigenous groups and the numerous socio-economic classes because of the ease of preparation and consumption [1]. In West and central Africa, more than 10 million tons are produced annually and are traded locally [2,3].

''Agadagidi,'' a cloudy effervescent sweet-sour taste typical African traditional alcoholic beverage is made from overripe bananas and plantains through fermentation. It is common in south-western part of Nigeria [4].

The techniques used in the production of wines from tropical fruits are similar to those of grape wine production which include pressing out the juice, fermenting, maturing and bottling [5]. Fermentation leads to changes in appearance of food which is characterized by different reactions of microorganisms [6]. Food fermentation represents one of the oldest known uses of biotechnology. Fermented foods and beverages forms a significant proportion of all diets worldwide; they are typically about one-third of the foods consumed globally. Fermentation of foods covering a wide range of microbial and enzymatic processing of foods and ingredients is used to achieve desirable characteristics such as prolonged shelf-life, improved safety, attractive flavour, nutritional enrichment and promotion of health [7,8]. The fermentation of overripe plantain to produce ''Agadagidi'' is a waste prevention processing of plantain. Plantain is a perishable crop which has much less value when it is overripe; hence it is used for wine production [9-11].

At present, there is no adequate information on the spectrum of microorganisms and microbial interaction associated with the production of overripe plantain pulp to yield 'Agadagidi'.i.e. from the raw material to the finished product. Microbial information on the production of "Agadagidi" will contribute to the development of starter cultures with predictable characteristics, for use in small-scale and commercial production of stable and consistent quality 'Agadagidi'. Therefore, this research was proposed to reveal the microbial community and confirmation of mutualism or commensalism and antagonistic interaction during the production of ''Agadagidi''.

2. MATERIALS AND METHODS

2.1 Traditional Preparation of ''Agadagidi'' Sample

The production of ''Agadagidi'' was done in the laboratory based on the local or indigenous method. The plantains used were bought from the king's market, Akure, Ondo State. The Overripe plantain pulps peels were washed in tap water to remove debris and dirt. The plantain was peeled and the pulps were crushed in portable tap water at a ratio of 1:5 (w/v) in a sterilized container, covered and left to ferment for 2 days at ambient temperature of 27±2°C. Samples were withdrawn from peeled, uncrushed pulps and at 0 h (This is immediately after water is added into the crushed plantain pulp), 24 h and 48 h of fermentation. The fermented liquid was filtered with a sterilized muslin cloth to remove the plantain mashes. The liquid then served as "Agadagidi".

2.2 Enumeration and Isolation of Microorganisms

Serial dilutions (1:10 v/v) were made with the samples collected from peeled, uncrushed pulps and at 0 h, 24 h and 48 h of fermentation and pour plated on nutrient agar for mesophilic bacteria, Man Rogosa Sharpe agar at pH 5.5 for lactic acid bacteria (LAB), Malt Extract Agar (supplemented with streptomycin sulphate) for yeasts, Eosin Methylene Blue for the isolation of the member enterobacteriaceae. Incubation was carried out at 37°C for total bacteria and LAB and 27°C for fungi. Colonies and spore forming units formed on the media were counted and subcultured. The Bacteria isolate were observed using microscopy, Gram staining, sugar fermentation test, biochemical tests such as urease test, catalase test, citrate utilization test and indole test While Fungal identification was done using the fungi conventional identification method according to the methods of [12-15].

2.3 Determination of Positive and Negative Microbial Interactions between the Isolates

Mutualism/commensalism and antagonism between the microbial isolates were determined using Muller Hilton agar and Agar Well Assay method with slight modification as described by [16].” Pre-poured Muller Hillton agar (MHA) in separate Petri dishes containing various bacterial and yeast’s cells were bored using a sterile cork borer of 5 mm diameter and 1 mL of each test isolate was transferred into each well, incubated for 24 hours at 37°C. 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 no inhibition.

2.4 Statistical Analysis

Data obtained were subjected to Analysis of Variance (ANOVA) and separation of means was done with Duncan’s New Multiple Range Test at 95% confidence level using SPSS 16.0 version.

3. RESULTS

The microbial composition of ‘Agadagidi’ is made up of nine bacteria and eight fungi. The bacteria consist of two species of *Bacillus* and *Lactobacillus*, one species of *Staphylococcus*, *Enterococcus*, *Escherichia coli*, and *Leuconostoc*. *Pediococcus acidilactici* was also isolated. Among the fungi, three species of *Aspergillus*, one species of *Trichoderma* and *Penicilium* were the moulds isolated. *Geotrichum*, *Saccharomyces* and *Candida utilis* were the yeasts obtained (Table 1).

Table 1 also revealed the types of microbes associated with the ‘‘Agadagidi’’ production. *Bacillus subtilis*, *Leuconostoc mesenteroides*, *Lactobacillus plantarum*, *Saccharomyces cerevisiae* and *Candida utilis* had the highest (100%) occurrence throughout the stages of production. *Bacillus megaterium*, *Enterococcus* spp., A. *niger* and *T. viridea* occurred lesser (25%) than other microorganisms.

During the production of “Agadagidi”, the load of bacteria, fungi and the members of enterobacteriaceae followed the same trend. Their populations increased at 24 hr and decreased at 48 hours of fermentation respectively (Figs. 1, 2 and 3). The pattern of lactic acid bacteria load differs with decrease load at 24 hours and increase at 48 hours (Fig. 4).

The total bacterial load of the overripe plantain pulp was $2.6 \times 10^7 \pm 0.05$ cfu/ml which increased before fermentation i.e. after adding water to the overripe plantain pulp (0 hr) to $5.2 \times 10^7 \pm 0.05$ cfu/ml (Fig. 1). This load decreased significantly (<0.05) to $2.0 \times 10^5 \pm 0.11$ cfu/ml at 48 hours of fermentation. Fungal load of $2.0 \times 10^7 \pm 0.05$ cfu/ml was obtained on the ripe plantain pulp which increased and decreased at 0 hr and 24 hr of fermentation to $2.2 \times 10^7 \pm 0.05$ cfu/ml and $4.3 \times 10^5 \pm 0.05$ cfu/ml respectively (Fig. 2). There was a continuous decrease till the end (48 hr) of fermentation, having a load of $1.03 \times 10^5 \pm 0.05$ cfu/ml. Fig. 3 shows enterobacteriaceae load during the preparation of “Agadagidi.” The load was 2.8×10^7 cfu/ml at 0 hr of fermentation. There was a drastic reduction in the enterobacteriaceae load to $1.3 \times 10^5 \pm 0.11$ cfu/ml at 48 hr of fermentation.

Lactic acid bacteria had a load of $6.7 \times 10^6 \pm 0.05$ cfu/ml on the overripe plantain pulp (Fig. 4). The load reduced after 24 hr of fermentation to $4.2 \times 10^5 \pm 0.05$ cfu/ml and thenafter increased to $8.6 \times 10^7 \pm 0.1$ cfu/ml at 48 hr.

Table 1. The occurrence of bacteria and fungi during the production of "Agadagidi"

Microorganism	Plantain pulp (Uncrushed)	Fermentation: (hr) 0	24	48	Level of occurrence (%)
Bacillus subtilis	+	+	+	+	100
Bacillus megaterium	+	-	-	-	25
Staphylococcus aureus	+	+	-	-	50
Escherichia coli	+	+	-	-	50
Enterococcus species	-	+	-	-	25
Lactobacillus plantarum	+	+	+	+	100
Lactobacillus fermentum	+	+	+	-	75
Leuconostoc mesenteroides	+	+	+	+	100
Pediococcus acidilactici	-	+	+	+	75
Aspergillus flavus	+	+	-	-	50
Aspergillus niger	+	+	-	-	50
Aspergillus fumigatus	+	+	-	-	50
Penicillium notatum	+	+	-	-	50
Trichoderma viridea	+	-	-	-	25
Sacharomyces cerevisiae	+	+	+	+	100
Candida utilis	+	+	+	+	100
Geotrichum species	+	+	+	-	75

Legend: + Present - Absent

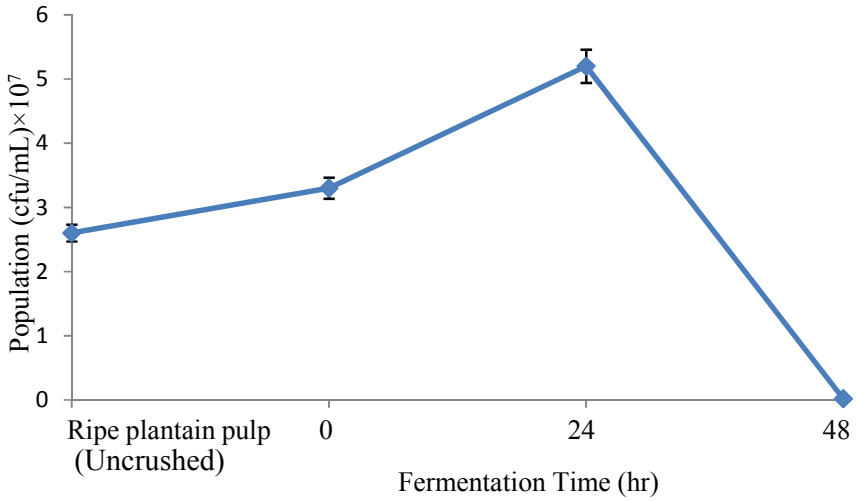

Fig. 1. Total bacterial load during production of "Agadagidi"

Each value represents the mean value (log Cfu/mL), standard deviation (SD) from three trials and standard error

Table 2 shows the interactions which occurred among the microorganisms *in vitro*. Lactic acid bacteria (LAB) and yeasts inhibited the growth of other bacteria which were all spoilage and pathogenic microorganisms. The interaction between the yeasts and the lactic acid bacteria were positive as zone of inhibition was not created when they were co-cultured.

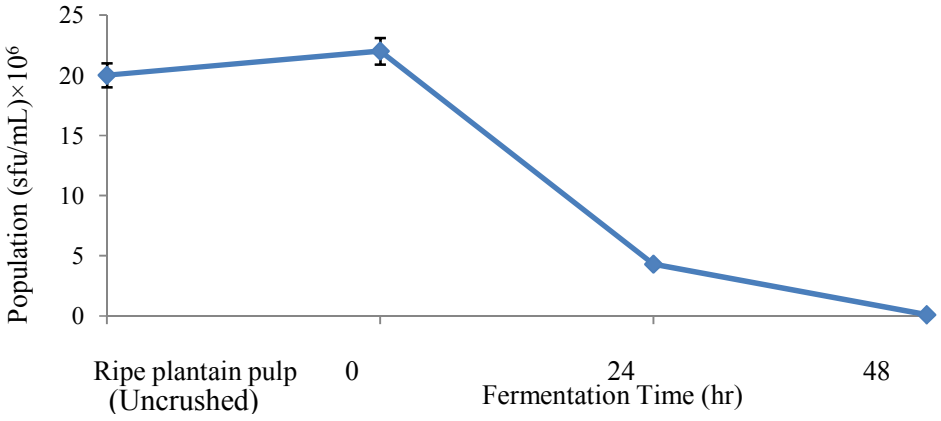

Fig. 2. Fungal load during the production of "Agadagidi"

Each value represents the mean value (log Cfu/mL), standard deviation (SD) from three trials and standard error.

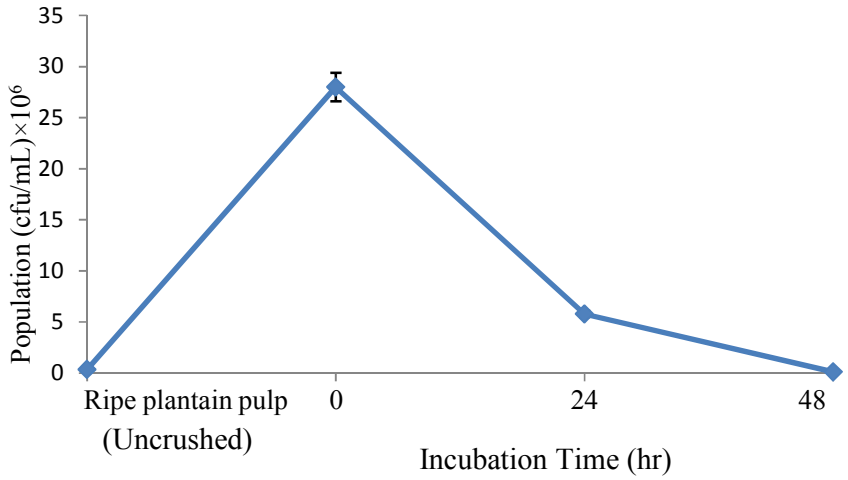

Fig. 3. Enterobacteriaceae load during the production of "Agadagidi"

Each value represents the mean value (log Cfu/mL), standard deviation (SD) from three trials and standard error.

4. DISCUSSION

Many species of microorganisms were isolated during the production of "Agadagidi." The production stages comprises of both pre-fermentation and during fermentation. The cultural and biochemical properties exhibited by the microorganisms were similar to those described by [12-14] which in turn gave the names of the microorganisms in Table 1. The 100% occurrence of *Leuconostoc mesenteroides*, *L. plantarum* and *Bacillus subtilis* isolated could be as result of their dominancy and the ability to withstand acidic condition associated with the fermentation stages. *Leuconostoc mesenteroides* and *L. plantarum* are heterofermentative, hence they were highly resistant to acid. [17] confirmed that their dominancy is determined by the sensitivities of microorganisms to the acidic conditions that develop during the fermentation. The presence of *B. subtilis* during the production of "Agadagidi" may be due to contamination through their endospores from dust, air and peels. Bacilli are spore forming bacteria, able to withstand harsh conditions which are widely distributed in nature and in many cases with a pH as low as 3.9 [18,19]

Table 2. Interaction between Lactic acid bacteria, yeasts and some of the bacteria isolated during the production of "Agadagidi"

Microorganism (LAB and yeast)	*Enterococcus* spp.	*Lactobacillus plantarum*	*Bacillus subtilis*	*Lactobacillus Fermentum*	*Escherichia coli*	*Staphylococcus aureus*	*Bacillus megaterium*	*Leuconostoc mesenteroides*	*Saccharomyces cerevisiae*	*Candida utilis*
Leuconostoc Mesenteroides	+	-	+	-	+	+	+	-	-	-
Lactobacillus fermentum	+	-	+	-	+	+	+	-	-	-
Lactobacillus plantarum	+	-	+	-	+	+	+	-	-	-
Lactococcus Lactis	+	-	+	-	+	+	+	-	-	-
Saccharomyces cerevisiae	+	-	+	-	+	+	+	-	-	-
Candida utilis	+	-	+	-	+	+	+	-	-	-

Legend: + = Positive interaction (No Zone of inhibition found)
- = Negative interaction (zone of inhibition)

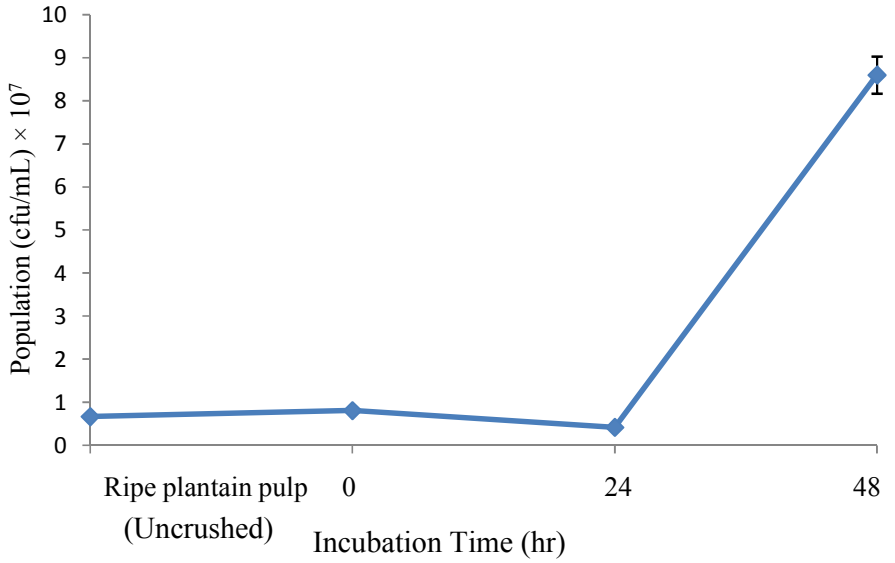

Fig. 4. Lactic acid bacteria load during the production of "Agadagidi"
Each value represents the mean value (log Cfu/mL), standard deviation (SD) from three trials and standard error.

The appearance of the two yeasts, *Saccharomyces cerevisiae* and *Candida utilis* throughout the stages of the production of 'Agadagidi' can be attributed to the environment and the fruits itself. These yeasts are naturally associated with ripened fruits and they are known to be responsible for alcoholic fermentation [20-21]. This findings is similar to the report of [22] who identified yeasts species in "Tchapalo" production and observed highest frequency with *S. cerevisiae* (87.36%), followed by *Candida tropicalis* (5.45%) and *Meyerozyma caribbica* (2.71%). The presence of moulds on the uncrushed ripened plantain pulp, after adding water may be as a result of improper handling of the ripe plantain. The peel possibly contained moulds which might have been transferred the plantain pulp. The elimination of moulds stated during fermentation may be as result of high loads and activities of LAB during fermentation. Bacteria have been shown to suppress the growth of mould during fermentation [23].

[24] who worked on yeasts and moulds associated with "Ogi" similarly observed moulds on the surface of raw maize grains for "Ogi" production. The increase in the load of bacteria, fungi and enterobacteriaceae in mashed overripe plantain pulp (0 hr fermentation) may be as a result of the potable tap water added. Portable water passes through pipes that may not have been treated or sanitized for a long period of time and as a result microorganisms present in the pipes might have increased the microbial loads. [25,26] confirmed the presence of coliform bacteria in "Kunun zaki" where tap water was the source of water used. The decrease in total bacterial and fungal loads after 24 hr of fermentation may be as a result of the increase in population of the LAB that must have formed acid thereby reducing pH (acidity) which seems to be detrimental to the other bacteria. This data agrees with the work of [27,28] who explained that LAB produce many organic acids such as lactic, acetic and propionic acids as end products during fermentation which provide an acidic environment unfavourable for the growth of many pathogenic and spoilage microorganisms.

The increment LAB population after 24 hr may be due to their ability to proliferate in the presence of the metabolic product synthesized by the yeasts. This observation is similar to that of [29] who worked on "Akamu". They observed that LAB occurred early in the fermentation, increasing rapidly from 1.6×10^7 cfu/g to 7.1×10^8 cfu/g after 72 hr. The drop in population of enterobacteriaceae during fermentation was as a result of the low pH and acid production by some of the bacteria which could destroy the cells of these microbes. Similar observation was obtained in the [30] work, who reported decrease in the population of enterobacteriaceae as the pH dropped was due to the effect of lactic acid bacteria present in 'bushera' and 'kirario'

LAB and yeasts evaluated in this study showed antagonistic interaction against other isolated bacteria, which mean that these microbes might have produced inhibitory metabolites. This agree with the report of [31,32] who documented that the ability of antimicrobial compounds produced by LAB enabled them to exert strong antagonistic activity against food contaminating microorganisms. None of the LAB isolates inhibited any of the yeast cultures. The association between the yeasts and LAB may therefore be mutualism. Several authors have reported similar co-existence and positive interactions between yeasts and lactic acid bacteria in different African fermented foods [24, 33].

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 [34]. It has also been shown that yeasts multiplication is associated with an increase in acid formation in fermented products.

5. CONCLUSION

This study has provided valuable information on the types of microbial communities and their interactions during the production of "Agadagidi". *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, *B. subtilis*, *S. cerevisiae* and *C. utilis* were found to be predominant in the production of the beverage. This research also established positive and negative interactions between the isolated microorganisms. This will contribute to the development of strains with predictable characteristics for use in small and large scale production.

6. RECOMMENDATION

It is therefore recommended that there should be proper handling of the overripe plantain pulp and tap water should also be boiled before use so as to reduce or eliminate undesirable microorganisms.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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