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Characterization and Identification of Fungi in the Sorrel Beverage (Zobo) Hawked in Ifite Awka, Anambra State, Nigeria

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Abstract: Sorrel beverage (Zobo) is a nutritive, medicinal and refreshing drink made from the dried calyces of Hibiscus sabdariffa. The fungal assessment of the Zobo samples purchased from ten hawkers in Ifite Awka, Nigeria as well as the control sample were carried out using standard methods. The pH values of the samples were also determined. The average fungal counts of the hawked samples ranged between 110 and 210 cfu/ml while the pH ranged between 2.1 and 2.6. No fungus was isolated from the control sample prepared under aseptic conditions in the laboratory while it's pH was 2.2. The fungi were identified as *Aspergillus niger* (23.77%), *Rhizopus stolonifer* (19.48%), *Fusarium oxysporum* (8.57%), *Penicillium expansum* (17.14%), and *Saccharomyces cerevisiae* (31.04%). *Aspergillus niger, Rhizopus stolonifer, Fusarium oxysporum, Penicillium expansum* and *Saccharomyces cerevisiae* were present in 70%, 60%, 30%, 50% and 90% of the samples respectively. The yeast *Saccharomyces cerevisiae* was the predominant fungus isolated from the samples. The presence of most of these fungi in significant numbers in the product is a public health problem. Effective preservation methods should be used while adequate quality control during production, packaging and storage as well as good personal and environmental hygiene should be encouraged. These measures will minimize the entry and proliferation of fungi and the health risk associated with them.

Keywords: Fungi, Characterization, Identification, Sorrel Beverage, Ifite Awka, Nigeria

1. Introduction

Roselle (Hibiscus sabdariffa L.) is an edible leafy vegetable that is believed to originate from East Africa [1]. It is locally called Zoborodo (Hausa), Isapa (Yoruba) in Nigeria and Sorrel in English [2]. Many parts of the roselle including the seeds, leaves, fruits, calyces, shoots and roots are used in various foods. Fleshy calyces of roselle are used fresh for making wine, juice, jam, jelly, syrup, gelatin, pudding, cakes, ice cream and flavours and are also dried and brewed into tea among others [3].

Various medicinal uses of infusions of leaves or calyces have been reported such as being a diuretic, cholerectic, anti-hypertensive, anti-helminthic and antimicrobial and decreasing viscosity of the blood, stimulation of intestinal peristalsis and for the treatment of the after effect of drunkenness [4-6]. The calyces have been found to be rich in natural carbohydrates, proteins, vitamin C and other antioxidants [7] and minerals [8]. The consumption of Zobo is reported to play an active role in bone and teeth formation [8].

Oboh and Elusiyan [9] found the glycemic index of Zobo drink to be 33 ± 3 which consequently made it possibly suitable for the maintenance of normal blood sugar, weight reduction and athletics. Sorrel beverage (Zobo) is a refreshing beverage prepared by boiling and filtering the red succulent calyx of Hibiscus sabdariffa with sugar and other spices such as ginger added to produce the local nutritious drink with a unique colour, flavour, aroma and taste [10, 11].

The proximate composition of the beverage is 88.88% water, 0.046% protein, 0.16% fat, 0.16% crude fiber, 0.21% ash and 10.64% carbohydrate [12]. Zobo drink is highly

affordable and very popular with acceptability cutting across many ethnic groups and socio-economic classes in Nigeria and other African countries especially among the youths who see it as an alternative source of cheap and relaxing non-alcoholic drink in social gathering [10].

The non-alcoholic nature of the drink has made it to be readily consumed by both Christians and Muslims as a substitute for alcoholic drinks [13]. The drink is commonly hawked in market places, schools, churches, hospitals, motor parks and other public places and is usually packaged in polythene bags and recycled plastic containers. Zobo drink is produced by many people especially the poor in the society. It is usually produced and packaged under poor hygienic environments thereby making its microbiological quality questionable.

The presence of pathogenic organisms such as fungi in significant numbers in the drink can pose a serious danger to the health of the consumers of the product since they are known to produce mycotoxins thus in this work, the fungi in the Sorrel beverage (zobo) hawked in Ifite Awka, Anambra State, Nigeria, were characterized and identified. It is hoped that this work will educate the producers and vendors of the product on the need for adequate hygiene during the production and distribution of zobo beverage.

2. Materials and Methods

2.1. Samples Collection

Samples of Sorrel (Zobo) drinks were purchased from ten hawkers in Ifite Awka and labeled 1-10. The samples were in used plastic bottles. They were taken to the Microbiology laboratory of Nnamdi Azikiwe University Awka, Nigeria in ice packs for analysis. Fresh calyces of roselle were purchased from Eke Awka market and also taken to the laboratory in sterile polythene bag to prepare the control sample for comparative analysis. The study was carried out in October, 2017.

2.2. Media Preparation

All media used were prepared according to the manufacturers instructions and sterilized in the autoclave at 121°C for fifteen minutes.

2.3. Preparation of the Control Sample

The calyces were washed and boiled in hot water for thirty minutes. The extract was sieved under aseptic conditions, sweetened with granulated sugar, allowed to cool and packaged in a sterile plastic container.

2.4. Determination of the pH of the Samples

The pH of the samples was determined using the pH meter (JENWAY, Model 320) which had been standardized with pH buffers. The standardized pH meter was inserted into each of the samples contained in a beaker and the pH values were read and recorded when the meter readings stabilized.

2.5. Fungal Isolation

The spread plate method was used. 0.1 milliliter of the sample was introduced into a sterile Petri dish containing already prepared and sterilized Sabouraud dextrose agar (SDA) and spread uniformly with a sterile glass rod. The plate also had chloramphenicol at a concentration of 0.05mg/ml to inhibit bacterial growth. The plate was inverted and incubated at 28°C for three days. The experiment was carried out in duplicates. The fungal colonies that developed after incubation were determined and expressed as colony forming units per ml. They were purified by repeated subculturing on fresh sterile SDA plates and stored on sterile SDA slants for characterization and identification.

2.6. Characterization and Identification of the Fungi

The fungi were characterized and identified on the basis of their colonial, microscopic and biochemical characteristics. Lactophenol cotton blue staining and slide culture test were carried out to characterize the molds while germ tube test, sugar fermentation test, motility test, urease test, Gram staining and nitrate reduction test were used to characterize the yeasts. The tests were carried out using the methods described by Cheesbrough [14]. The fungi were identified following the description given by Oyeleke and Manga [15].

2.6.1. Lactophenol Cotton Blue Staining

A drop of lactophenol cotton blue solution was placed on a clean grease-free glass slide. A small portion of the test mold was introduced into the solution and spread properly on the slide using a straight wire. A cover slip was gently placed on the slide and the excess stain was removed using a filter paper. The slide was mounted and viewed under the X10 and X40 objective lens of the microscope.

2.6.2. Slide Culture Test

Sterile Sabouraud dextrose agar was inoculated with a test mold in a Petri dish. A cover slip was inserted beside the mold and the dish was thereafter incubated in an inverted position at 28°C for 7days. The cover slip was removed and placed on a slide containing a drop of lactophenol cotton blue stain and examined under X10 and X40 objective lens of the microscope for the presence of fungal features.

2.6.3. Germ Tube Test

A colony of the yeast was picked with an inoculating loop and emulsified in 1.0ml of human serum. The emulsion was incubated at 37°C for 4hours and examined under the microscope for the presence of narrow protrusions growing out from the cells.

2.6.4. Sugar Fermentation Test

The test yeast was inoculated into a test tube containing sterile 1% solution of different sugars, peptone water, bromothymol blue indicator and a Durham's tube which was inserted into the test tube to detect gas production. The test tube was incubated at 37°C for 48hours and observed for acid and gas production. Colour change from bluish-green to

yellow indicated acid production while a void in the concavity at the top of the Durham's tube indicated gas production. The sugars used were glucose, sucrose and lactose.

2.6.5. Motility Test

The test yeast was stab-inoculated into sterile semi-solid nutrient agar with a straight wire and incubated at 37°C for 24hours. Growth away from the line of stab indicated a positive result.

2.6.6. Urease Test

A portion of the test yeast was transferred to a test tube slant of Christensen agar which contains urea. The tube was incubated at 28°C and examined daily for four days. A change in the amber color of the medium to pink or red indicated urease production due to the formation of ammonia.

2.6.7. Gram Staining

A smear of the yeast isolate was made on a clean slide and allowed to dry. It was heat-fixed by passing it thrice through the Bunsen flame. The smear was thereafter flooded with crystal violet solution and left for one minute before rinsing with water. Lugol's iodine solution was added and the slide was allowed to stand for one minute and rinsed with water. The smear was next decolourized with acetone and left for ten seconds. The slide was then rinsed with water, counter-stained with safranin and left for a minute before being rinsed with water and allowed to dry in air. The slide was thereafter viewed under the oil immersion lens of the microscope.

2.6.8. Nitrate Reduction Test

The test yeast was inoculated into nitrate broth and incubated at 30°C for 96 hours. 0.1ml of a mixture of sulfanilic acid and alpha-naphthylamine was thereafter added to the nitrate broth culture and observed for colour change. The development of a red colour indicated a positive reaction while it's absence indicated a negative reaction.

3. Results

The average fungal counts and pH of the hawked and control Sorrel (Zobo) samples are presented in Table 1. The fungal counts of the hawked samples ranged between 110cfu/ml and 210cfu/ml while their pH values were between 2.1 and 2.6. Sample 8 had the highest fungal count of 210cfu/ml and pH value of 2.6 while sample 10 had the least fungal count of 110cfu/ml and pH value of 2.1. However, the fungal count of the control sample was 0cfu/ml and it's pH was 2.2.

Table 1. *Average fungal counts and pH of the hawked and Control Sorrel (Zobo) samples.*

Samples	Average fungal counts (cfu/ml)	pH
1	180	2.4
2	160	2.4
3	120	2.2
4	140	2.3
5	150	2.1
6	130	2.2
7	190	2.5
8	210	2.6
9	150	2.3
10	110	2.1
Control	0	2.2

The colonial, microscopic and biochemical characteristics of the fungi isolated from the hawked Sorrel (Zobo) samples are shown in Table 2. The fungi were identified as *Aspergillus niger, Rhizopus stolonifer, Fusarium oxysporum, Penicillium expansum and Saccharomyces cerevisiae.*

Table 2. *Colonial, microscopic and biochemical characteristics of the fungi isolated from the hawked sorrel (Zobo) samples.*

S/n	Colonial characteristics	Microscopic and biochemical characteristics	Identity
1.	Colonies were circular, opaque, smooth and flat with small black spots on short white hyphae.	Hyphae were septate. The conidia were arranged like mop head.	*Aspergillus niger*
2.	Colonies were white, cottony and covered the entire culture plate.	Hyphae were non-septate and branched. Sporangiospores were enclosed in a sporangium.	*Rhizopus stolonifer*
3.	Colonies were yellow, fast growing and cottony.	Hyphae were septate. Conidia were sickle-shaped.	*Fusarium oxysporum*
4.	Colonies were white at first but later became blue-green with white margin.	Hyphae were septate. Conidia were attached to sterigma and were borne in brush-like formation.	*Penicillium expansum*
5.	Colonies were flat, moist, circular, creamy and smooth with waxy surface.	Gram positive, spherical and budding cells were seen. The cells were motile, positive to glucose, sucrose, lactose and urease but were negative to germ tube test and did not reduce nitrate.	*Saccharomyces cerevisiae*

The distribution of the fungi in the hawked sorrel (Zobo) samples is shown in Table 3. The mold *Aspergillus niger* was detected in seven of the samples analysed, *Rhizopus stolonifer* in six, *Fusarium oxysporum* in three, *Penicillium expansum* in five and *Saccharomyces cerevisiae* in nine of the samples.

Table 3. *Distribution of the fungi in the hawked sorrel (Zobo) samples.*

Samples	Aspergillus niger	Rhizopus stolonifer	Fusarium oxysporum	Penicillium expansum	Saccharomyces cerevisiae
1.	+	+	+	-	+
2.	+	+	-	-	+
3.	+	+	-	+	+
4.	-	-	-	+	+
5.	-	+	-	+	-

Samples	Aspergillus niger	Rhizopus stolonifer	Fusarium oxysporum	Penicillium expansum	Saccharomyces cerevisiae
6.	-	+	-	-	+
7.	+	-	+	+	+
8.	+	+	-	-	+
9.	+	-	-	+	+
10.	+	-	+	-	+

+ = Present
= Absent

The number of hawked sorrel (Zobo) samples with the fungal isolates is presented in Table 4. Seventy percent of the samples had the mold *Aspergillus niger,* 60% had *Rhizopus stolonifer,* 30% had *Fusarium oxysporum,* 50% had *Penicillium expansum* while 90% of the samples had the yeast *Saccharomyces cerevisiae.*

Table 4. *Number of hawked sorrel (Zobo) samples with the fungal isolates.*

Isolates	Number of samples with the isolates	% Occurrence
Aspergillus niger	7	70%
Rhizopus stolonifer	6	60%
Fusarium oxysporum	3	30%
Penicillium expansum	5	50%
Saccharomyces cerevisiae	9	90%

The frequency of isolation of the fungi in the hawked sorrel (Zobo) samples is shown in Table 5. The frequency of isolation of *Aspergillus niger* was 23.77%, Rhizopus stolonifer, 19.48%, *Fusarium oxysporum,* 8.57%, *Penicillium expansum,* 17.14% while that of *Saccharomyces cerevisiae* was 31.04%. The yeast *Saccharomyces cerevisiae* was isolated most frequently while *Fusarium oxysporum* had the lowest frequency of isolation.

Table 5. *Frequency of isolation of the fungi in the hawked sorrel (Zobo) samples.*

Fungi	Number isolated	Frequency of occurrence
Aspergillus niger	366	23.77
Rhizopus stolonifer	300	19.48
Fusarium oxysporum	132	8.57
Penicillium expansum	264	17.14
Saccharomyces cerevisiae	478	31.04

4. Discussion

The assessment of the hawked sorrel beverage (Zobo) samples showed that they were contaminated with fungi. The samples were acidic while the average fungal counts were high (Table 1). Wong et al [7] reported that Zobo drink has high acidity because it is rich in organic acids such as oxalic, tartaric, malic and succinic acids. The high acidity of the samples in this work probably supported the growth of the organisms since fungi thrive better in acidic environments. Fungi were however not isolated from the control sample though it's pH was also acidic (2.2), probably because of the observance of good personal and environmental hygiene during the preparation of the drink.

Onuorah et al [16] in their work on the bacteriological quality of locally produced Sorrel beverage (Zobo) vended in Awka campus of Nnamdi Azikiwe University Awka, Nigeria reported a pH range of 2.2 – 2.4 for the samples they analysed. Adesokan et al [17] however reported a pH range of 3.94 – 7.67 for the Zobo drinks they examined, while Omemu et al [18] reported a pH range of 2.67 – 2.77 for the Zobo juice they analysed. The variation in the pH values of the Zobo samples examined may be attributed to the concentration of the extract used and volume of the water used in diluting the product.

Zobo drink, raw or preserved supports the growth and proliferation of a wide range of organisms including fungi. The fungi isolated from the Zobo samples in this study were *Aspergillus niger, Rhizopus stolonifer, Fusarium oxysporum, Penicillium expansum* and *Saccharomyces cerevisiae* (Table 2). These fungi may come from the calyces, air, unsterile packaging materials, dilution water, soil, the handlers and the additives used and may be in the beverage as a result of poor hygienic practices and inadequate heat treatment during production. The contamination may also have occurred during the packaging, storage and hawking of the beverage. Braide et al [19] in their work on the perspectives in the hurdle techniques in the preservation of a non alcoholic beverage, Zobo, reported the presence of *Saccharomyces cerevisiae* and *Rhizopus stolonifer* in the product.

Nwafor [20] studied the growth inhibitions of three fungal isolates from locally-prepared Zobo drinks using sorbic and benzoic acids. The isolates were *Penicillium citrinum, Rhizopus stolonifer* and *Aspergillus niger*. Ogiehor and Nwafor [21] also reported that *Aspergillus, Penicillium*, and *Fusarium* have been associated with Zobo beverage during storage. Omemu et al [18] reported *Aspergillus niger, Aspergillus flavus, Rhizopus oligosporus, Penicillium citrinum* and *Saccharomyces cerevisiae* as some of the microflora of Hibiscus sabdariffa (roselle) and the resulting Zobo juice while Amusa et al [13] isolated *Aspergillus niger, Aspergillus flavus, Aspergillus tamarii, Penicillium oxalicum, Fusarium oxysporum* and *Rhizopus spp* from hawked sorrel drinks (Soborodo) consumed in Nigeria.

Osuntogun and Aboaba [22] isolated *Penicillium sp, Aspergillus sp* and *Saccharomyces sp* from some non-alcoholic beverages including Zobo drinks while Doughari et

al [23] studied the effect of some chemical preservatives on the shelf life of Sobo drink and reported the presence of *Aspergillus niger, Aspergillus flavus* and *Aspergillus fumigatus* in the drink. Nwafor and Ikenebomeh [24] also isolated *Aspergillus niger* and *Penicillium citrinum* in Zobo drink during storage at 30 ± 2°C.

Ilondu and Iloh [1] isolated *Aspergillus niger, Aspergillus flavus* and an unidentified yeast from Zobo and studied their inhibition using hurdle technique while Egbere et al [12] studied the effects of some preservation techniques on the quality and storage stability of Zobo and isolated *Saccharomyces sp and Aspergillus niger* from the drink. In addition, Adebayo-tayo and Samuel [2] isolated *Aspergillus flavus, Aspergillus terreus*, *Aspergillus glaucus, Aspergillus niger, Penicillum expansum, Fusarium oxysporum, Rhizopus sp* and *Saccharomyces cerevisiae* from dried Hibiscus sabdariffa calyces in Uyo, Nigeria. Ayandele [25] carried out the microbiological analyses of Zobo drinks sold within LAUTECH campus Ogbomosho, Oyo State, Nigeria and isolated *Aspergillus niger*, *Rhizopus sp* and *Saccharomyces cerevisiae*.

Majority (90%) of the samples analysed contained the yeast *Saccharomyces cerevisiae* while 30% of the samples had *Fusarium oxysporum* (Tables 3 and 4). Yeasts are the most significant group of microorganisms associated with the spoilage of Zobo drinks. Zobo drink when left for a long time at room temperature usually becomes sour as a result of fermentation due to microbial action especially the yeasts. The fermentation may lead to loss of taste, nutritional value, turbidity, increased rate of browning and production of noxious smell.

Saccharomyces cerevisiae (31.04%) was the dominant fungus isolated from the samples while the mold *Fusarium oxysporum* had the least frequency of isolation (Table 5). This could be attributed to the fact that yeasts thrive better in acidic environments than the molds. The low pH level of the samples provided a conducive environment for the proliferation of the yeast, which is also a fermentative organism. Yeasts also grow better in super-rich substrates and their small size usually results in high surface/volume ratio that favours their rapid acquisition of essential nutrients.

Most of these fungi have been reported to be pathogenic to humans [26] when present in significant numbers. Ogiehor and Nwafor [21] reported that the proliferation of *Aspergillus, Penicillum* and *Fusarium* can potentiate spoilage and the short shelf life of 1-4 days associated with Zobo beverage. *Aspergillus niger* is an asexual, filamentous, saprophytic fungus that can be isolated from many habitats such as soil, plant debris, rotting fruits, indoor air environments and stored grains. The fungus produces several toxins such as Malformin C and Ochratoxin A that are harmful to man. *Aspergillus niger* has been associated with Otomycosis, an infection of the ear that can be painful [27], cutaneous infections [28] and pulmonary diseases. The deleterious role of *Aspergillus niger* in low pH and high sugar foods has been reported [29].

Rhizopus stolonifer is a filamentous saprophytic fungus that is associated with a variety of substrates such as vegetables, mature fruits, jellies, bread, leather, peanuts and syrups [30]. It is an opportunistic agent of human zygomycosis and can be fatal. *Rhizopus stolonifer* infection may be a complication of diabetic ketoacidosis [31]. Ekrakene and Igeleke [32] reported that *Rhizopus* add significantly to food spoilage and food infection through the production of toxins. *Penicillium expansum* is present in soils and plant debris from both tropical and antarctic regions but tends to dominant spoilage in temperate regions [2]. Many strains are important spoilage organisms and some produce potent mycotoxins such as patulin, Ochratoxin A, Citinin and Penitrem A [2]. *Penicillium expansum* is pathogenic especially to immuno compromised individuals such as HIV patients and it is used as a marker in endemic areas.

Fusarium oxysporum is a ubiquitous soil fungus that is pathogenic to humans and animals. It's mycotoxins cause the diseases fungal keratitis, Onchomycosis and Hyalohyphomycosis [33]. Clinical manifestations of the diseases caused by *Fusarium oxysporum* are more in immuno compromised individuals especially those with cutaneous and subcutaneous infections, inflammations, arthritis or dialysis [33]. However, *Saccharomyces cerevisiae* is well known in the baking, brewing and wine making industries and is also used as a probiotic in humans.

The nutrient value as well as the acceptability of Sorrel beverage (Zobo) by the public necessitate that this important drink must not contain fungi in the type and number that are deleterious to health. All the hawked samples analysed contained fungi in excess of the number recommended by regulatory bodies, therefore good hygienic practices during the processing, packaging and distribution must be practiced. Effective preservation methods such as the use of food grade chemicals and pasteurization should be employed.

5. Conclusion

Zobo drink though nutritious and healthy to human beings could serve as a vehicle for the transmission of fungi with serious health implications. The non-detection of fungi in the control sample after incubation indicated that there is a relationship between strict hygienic processing and packaging of the drink and its level of fungal contamination. The relevant regulatory bodies such as the Standards Organization of Nigeria, National Agency for Food, Drug Administration and Control, Consumer Protection Agency and the Ministries of Health, Water Resources and Environment should sensitize and educate the citizens particularly those engaged in the commercialization of Zobo drink on the standard operating procedure to be adopted during the preparation, packaging, storage and distribution of the drink to ensure minimal contamination by fungi and improved quality of the beverage.

Conflict of Interests

There is no conflict of interests.

Author's Contribution

Onuorah Samuel designed, gathered the literature, carried out the laboratory work and put down the results and discussion while Odibo Frederick supervised the work. Both authors read and approved the final manuscript.

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