Quest Journals
Journal of Research in Pharmaceutical Science
Volume 10 ~ Issue 12 (2024) pp: 01-06
ISSN(Online) : 2347-2995
www.questjournals.org

Research Paper

Isolation And Identification Of Bacteria Associated With Non-Alcoholic Local Drinks Sold In Azare, Bauchi State.

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Abstract
The aim of this research is to isolate and identify bacteria associated with the non-alcoholic drinks samples.The physiochemical properties such as the pH, moisture, fat and protein were all determined using standard methods. The total bacteria count was obtained using serial dilution and pour plate method. Streak plate method was employed to obtain a pure culture. The bacterial isolates were characterized and identified by observation of colonial and morphological characteristics, Gram reactions and biochemical tests. The pH of the Zobo drinks samples ranged from 2.6-2.8, while that of Kunun-zaki drinks samples ranged from 4.10-4.40. The bacteria count of Zobo drinks samples ranged from 13.00 ± 0.57 × 10^2 CFU/mL to 16.00 ± 2.00 × 10^2 CFU/mL while that of Kunun-zaki drinks ranged from 2.50 ± 4.61 × 10^4CFU/mL to 2.90 ± 5.00 × 10^4 CFU/mL. Four bacterial isolates were obtained from Kunun-zaki drinks samples which were Escherichia coli, Staphylococcus aureus, Proteus vulgaris, and Staphylococcus epidermidis, while three bacterial isolates were obtained from Zobo drinks samples which were Escherichia coli, Staphylococcus aureus and Proteus vulgaris. It was concluded that some of the Kunun-zaki and Zobo drinks samples were contaminated and also contained different pathogenic microorganisms which can serve as sources of infection to human. Therefore, proper hygienic and sanitary measures need to be enforced during processing and packaging of these local non-alcoholic drinks.

Key words: *Isolation, Bacteria, Zobo, pH, contamination, microorganisms*

***R**eceived 07 Dec., 2024; **R**evised 15 Dec., 2024; **A**ccepted 19 Dec., 2024 © The author(s) 2024.*
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I. INRODUCTION

In every society, drinks of indigenous origins are produced in different ways and served sometimes at occasions [1];[2]. Some of these drinks are prepared by fermentation, which is a widely practiced ancient technology and these fermented foods are essential part of diet in all regions of the world [3]. Non-alcoholic beverages such as Zoborodo (Zobo) drink and Kunun-Zaki drink of recent are among the popular traditional food drinks which are very important in the dietary pattern of people in the Northern part of Nigeria [4]. The non-alcoholic nature of Zobo drink and Kunun-Zaki drink makes them to be readily consumed by Christians and Muslims alike as a substitute for alcoholic ones [3].

Kunun-Zaki is a non-alcoholic beverage produced by fermentation of several cereals such as millet, sorghum, maize and rice [5]. Kunun zaki (in Hausa) is a traditional fermented non-alcoholic beverage consumed by a large population of the people in Northern Nigeria [6]. Preparation protocol varies among people and can generally be produced from either the following substrates; millet (*Pennisetumtyphoideum*), maize (*Zea mays*) sorghum (*Sorghum typhoideum* or *Sorghum bicolar)*. Spices such as ginger, black pepper, red pepper, cloves and sugar are commonly added as flavor to enhance acceptability and as a taste improver [5];[7].

Zobo is an indigenous drink obtained from the infusion of dry calyces of Roselle plant (*Hibiscus sabdariffa*). The drink enjoys patronage mostly in the Northern Part of Nigeria, where it has become a

household name since it is affordable among the low income earners. Other people use it during social occasions such as birthdays, marriage, thanksgiving, weddings and naming ceremonies as refreshing drink.The water used is often obtained from the local water sources often boreholes and in some cases well water is used for preparation. It is these conditions that result in the microbial contamination of the product, coupled with contamination that may result from handling ([8];[9]. Some of its nutritive elements are various amino acids, proteins, carbohydrate, vitamins, and fats among others [10];[5];[3].

Nutrients of a food play an important role in the kinds and number of microorganisms that grow on it because of the great organic compound in food and the numerous kinds of microorganisms that can decompose them. Many chemical changes are possible and many kinds of products are yielded. However, like any other non-alcoholic locally, brewed drinks, the presence of microbial contaminant is not always underestimated due to the unhygienic condition of preparation, hence, microbial isolates reportedly associated with these drinks include, *Lactobacillus* species, *Leuconostoc*species, *Bacillus* species, *Staphylococcus* species, *Candida* species and *Saccharomyces* species. Others include *Salmonella*, *Enterobacter*species and *Clostridium* species [4].

Food spoilage is a major concern throughout the world. It can occur at any point in the food production process, growth, harvesting, transport, storage or final preparation. Spoilage can also occur if foods are not stored properly. When fungi grow in foods, especially cereals and grains, they produce important diseases causing chemicals, including aflatoxins and fumonisins (carcinogens) to contaminate the food. Microorganisms usually grow rapidly and can make an attractive and appealing food becomes sour, off-flavour and poisonous. This leads to visible changes which include changes in color, change in texture and change in smell [11].

Kunun-Zaki and Zobo are liable to microbial spoilage if not adequately stored and could act as an important medium for the transmission of pathogenic microorganisms. Many organisms can use the carbohydrate content for their fermentation processes producing undesirable changes in them. The sugar used as a sweetening agent could also contribute to these changes. The microorganisms can be in dormant or semi-metabolic changes, but others are of public health significance since the microbes may be potential pathogens or can produce toxins in food, which can cause illness to consumers [8];[12].

Millets are one of the cereals asides the major wheat, rice, and maize. Millets are major food sources for millions of people, especially those who live in hot, dry areas of the world. They are grown mostly in marginal areas under agricultural conditions in which major cereals fail to give substantial yields [13]. Millets are classified with maize, sorghum, and Coix (Job's tears) in the grass sub-family Panicoideae [14]. Millets are important foods in many underdeveloped countries because of their ability to grow under adverse weather conditions like limited rainfall.

The economic and religious situation in Nigeria has made the Zobo drink gain wide acceptance in different occasions. It's used as refreshment, entertainment in parties or as appetizers before the main dish is served and it is also sold in market to various consumers [15];[16]. The Zobo drink is a red liquid drink and taste like fruit punch, served as a fair source of vitamin A, riboflavin, niacin, calcium and iron, and is low in sugar content. This drink also contains anthocyanins and Vitamins C, among others and it is used in curing minor stomach ailments, sore throat and strengthening the heart among other uses[17];[18]. Zobo drink is extracted from the dried reddish purple calyces of the plant Hibiscus sabdriffa [19].The juice drink which is usually obtained by extraction of the calyx of Hibiscus contains about 1% solid. The drink contains some microorganisms which can cause food spoilage [20].

At present, the production processes is neither mechanized nor standardized. Consequently, the shelf life of the drink is less than two days. Furthermore, the mode of packaging or dispensing of the juice in nylon or astic container before retailing, that is taken as Zoboi.e the largely unregulated nature of the trade, and poor hygienic practices as well as lack of running water, toilet, proper storage and waste disposal facilities at preparation and services point has resulted in poor unsanitary conditions exposure to potential contaminants and an increased risk to public health [20];[21].

Consequently, street drinks and foods safety has remained a major public health concern globally, and more importantly in Nigeria were the regulation of this critical sector is virtually non-existent or inadequate, making street foods and drinks hazardous source of nutrition [22].

Some of the risks involved in consuming local beverages is the presence microbial contaminations like *Proteus species*, *Staphylococcus aureus*and *Bacillus species* in the samples mayattributed to handling during production. *Staphylococcus aureus* is a normal flora of the body and mucos membrane a common etiological agent of *Septic arthritis* [23]. The organisms can pass onto the food during harvesting, processing or even storage. The consumer is at risk of acquiring food borne disease. *Staphylococcus aureus*is the major cause of Staphylococcal food poisoning. The poisoning is characterized by diarrhea and vomiting [24];[25].

II. MATERIALS METHODS

Sample Collection

Four samples of Kunun Zaki and Zobo drinks samples were collected by purchasing randomly from four different locations in Azare. Samples of Kunuzaki and Zobo drinks were purchased and properly labelled in sterile plastic containers. The samples were brought to laboratory in ice pack for microbiological analysis respectively.

Determining the Percent Fat in Whole Kunun-zaki and Zobo Drinks Samples

A clean dry empty 100mL beaker was weighed and the mass was recorded. 5mL of the drink sample was measured using graduated cylinder and poured into the beaker and the mass of the beaker and drink sample were recorded. The mass of the drink was recorded, then 20mL of water was added to the beaker. The drink samples were poured into a test tube and 25mL of methylene chloride was added to the drink sample. The test tubes were agitated for 30 seconds, the contents of the test tube was allowed to separate into layers. A pasture pipette was used to remove the drink sample layer leaving behind the methylene chloride/ fat layer. The sample was put back into the 100mL beaker andthe mass was determined by weighing [3].

Determining the % Protein in Whole Kunun-zaki and Zobo Drinks Samples

One drop of concentrated acetic acid was added to the fat content. The beaker was swirl for 30 seconds and it was allowed to sit for a few minutes. A filter paper was used to filter out the liquid from the precipitate. Once the liquid has been separated from the precipitate, the filter paper was spread with the solid on to a watch glass and it was placed on a water bath then weighed [10].

Determination of pH of the Drinks Samples

The pH of the various samples were determined using sterile probes of the pH meter.

Preparation of Nutrient Agar

7 g of Nutrient Agar powder was measured using a weighing balance, and then poured into a conical flask containing 250 ml of distilled water. The mixture was then boiled and sterilized by autoclaving at 121ºC for 15 minutes [12].

Preparation of MacConkey Agar

13 g of MacConkey agar powder was measured using a weighing balance and poured into a conical flask containing 250 ml of distilled water. It was then mixed thoroughly. The mixture in the conical flask was then sterilized by autoclaving at 121 ºC for 15 minutes. It was allowed to cool at 47 ºC and poured into Petri dishes [6].

Preparation of Mannitol Salt Agar

27 g of Mannitol salt agar powder was measured using a weighing balance and poured into a conical flask containing 250 ml of distilled water. It was then mixed thoroughly. The mixture in the conical flask was then sterilized by autoclaving at 121 ºC for 15 minutes. It was allowed to cool at 47 ºC and poured into Petri dishes [7].

Isolation of Bacterial Isolate

Samples were serially diluted aseptically using 1ml of kunuZaki and Zobo samples with 9ml of sterile distilled water to reduce the microbial load. After dilution, about 0.1ml of appropriate dilution was used to inoculate Nutrient agar (NA) plates in triplicates for isolation of bacteria. The culture plates for isolation of bacteria were incubated at 37 °C for 24 hours for enumeration of colonies. The mean triplicate results on NA were then enumerated for total bacterial count, coliform and recorded as colony forming unit per millimeter (CFU/ml) of the samples [20].

Purification of Microbial

Isolated colonies were sub-cultured on Mannitol salt agar (MSA) and MacConkey agar (MAC) and incubated at 37 °C for 24 hours to obtain pure isolates of bacteria. After incubation, pure bacterial isolates were stored on NA and prepared in Bijou bottles respectively. The stock cultures were then preserved in a refrigerator at 4 °C until used for further microbiological analyses [23].

Characterization and Identification of Bacterial Isolates.

Bacterial isolates were characterized and identified by observation of colonial, and morphological characteristics, Gram reaction and biochemical tests. The various biochemical test that were carried out for identification were; catalase, coagulase, indole and oxidase test [12].

Catalase Test

Hydrogen peroxide was poured into the bottle and the organism was emulsified in it with the sterile wire loop. It produced bubbles of oxygen, the organism was catalase positive [16].

Coagulase Test

A drop of normal saline was dropped on a clean slide using inoculating wire loop, the loop was then flamed and allowed to cool before being used and was added to the normal saline which was used to make a smear that gives a creamy coloration. A drop of plasma was then added to it and mixed properly to observe agglutination. Agglutination was observed [5].

Oxidase Test

A few drops of oxidase reagent (1% tetra methyl p-phenylene-diamicdihydrochloride) were dropped on a filter paper, a clean slide was used to pick colonies of the organism to be tested and smear on a paper. A positive test was indicated [3].

Indole Test

Peptone water was pipetted into the epidoff tube with the help of a micropipette, the organism to be tested was emulsified in the medium, and incubated overnight. After incubation a drop of Kovacs reagent was added. If there is a pinkish ring in the solution was observed [19].

III. RESULTS AND DISCUSSION

Physiochemical Parameters of Zobo and Kunun Zaki Drink Samples

According to Table. 1. The physiochemical parameters of the samples were presented in table 1. Zobo sample from three sample sites has the highest pH value of 2.6 and Kunun- zaki from two sample sites has the highest pH value of 4.1. Zobo sample from sample sites one, two and three has a moisture percentage of 55.9%, 60.6% and 58.6%, and that of Kunun-zaki samples were 74.5%, 72.4% and 64.9%, the crude protein percentage of Zobo samples from sample sites one two and three were; 1.14, 1.16 and 1.23 and that of Kunun-zaki were 6.1, 7.8 and 7.4. The fat percentage of Zobo samples from sample sites one, two and three were; 11.60, 12.50 and 12.30, while that of Kunun-zaki samples were 18.20, 17.70 and 15.70.

Bacteria Count

The bacteria count of the samples were presented in Table 2. Zobo from sample site two with dilution 10^2 had the highest bacteria count of 1.6×10 CFU/mL and all the values of the bacteria count of the Zobo samples from sample sites one, two and three of dilution 10^2were 1.3×10 CFU/mL, 1.6×10 CFU/mL and 1.5×10 CFU/mL and Kunun-zaki from sample site three of dilution 10^2 had the highest bacteria count of 2.9×10^3 CFU/mL and all the values of the bacteria count of the Kunun-zaki samples from sample site one, two and three of dilution 10^3 were 2.5×10^3 CFU/mL, 2.6×10^3 cfu/ml and 2.9×10^3 CFU/mL.

Table 1: Physiochemical Parameters of Zobo and Kunun-zaki drinks Samples.

	Zobo Samples			Kunun-zaki Samples		
Parameters	**Sample Site 1**	**Sample Site 2**	**Sample Site 3**	**Sample Site 1**	**Sample Site 2**	**Sample Site 3**
pH	2.70	2.60	2.80	4.10	4.40	4.30
Moisture %	38.0	42.20	39.0	74.5	72.4	64.9
Crude Protein %	1.14	1.16	1.23	6.1	7.8	7.4
Crude Fat %	11.60	12.50	12.30	18.2	17.7	15.7

Table 2:The bacteria count (cfu/mL) of Zobo and Kunun-zaki drinks Samples.

	Zobo Samples		Kunun-zaki samples
Location	Total bacteria count (cfu/mL) 10^2	Location	Total bacteria count (cfu/mL) 10^4
Sample site 1	13.00±0.57	Sample site 1	25.33±4.61
Sample site 2	16.00±2.00	Sample site 2	26.33±4.04
Sample site 3	15.00±2.64	Sample site 3	29.00±5.00

Key: Sample site 1- Azare main marketSample site 2- Coline bus stationSample site 3- Bamako round about.

Morphological Characteristics

Isolate A1 and B3 of zobo and kunun-zaki sample had a creamy, irregular, crenated and flat cultural characteristics which was Rod, Gram negative, catalase positive, coagulase negative, indole negative and oxidase negative and the probable organism was identified as *Escherichia coli*. Isolate A2 and B2 of zobo and kunun-zaki sample had a circular, entire, yellowish and flat cultural characteristics which wasCoccus, Gram positive, catalase positive, coagulase positive, indole negative and oxidase negative and the probable organism was identified as *Staphylococcus aureus*. (Table 3).

Table 3:Cultural Characteristics, Gram Reaction and Biochemical test of the drink samples.

Isolate	Cultural characteristics	Cell Morphology	Gram Reaction	Catalase	Coagulase	Indole	Oxidase	Probable Organisms
A1 and B3	Creamy, irregular, entire, Flat	Rod	_	+	_	+	_	*Escherichia Coli*
A2 and B2	Circular, entire, yellowish, flat	Coccus	+	+	+	_	_	*Staphylococcus Aureus*
A3 and B1	Circular, entire, yellowish, convex	Rod	_	+	_	+	_	*Proteus Vulgaris*
B4	Circular, entire, yellowish, flat	Coccus	+	+	_	_	_	*Staphylococcus Epidermidis*

Key: + = Positive, - = Negative. Isolates A stands for Zobo samples while B isolates stands for Kunun-zaki samples.

Kunun-zaki and Zobo drinks were two non-alcoholic drinks prepared and consumed in large quantities in Azare Town .This drinks were widely accepted by the community and is being produced in large quantities as substitutes and complements to carbonated drinks.Kunun-zaki and Zobo drinks are contaminated with bacteria which may be potentially pathogenic to human beings. The occurrence of *S. aureus, S. pyogenes, Proteus*sp, *E. coli* in Kunun-zaki and Zobo drinks was considered detrimental to the health of the consumers. The results obtained from this study was similar to the one reported byOyewo*et al*.,[16]who isolated *S. aureus, Proteus* sp, *Streptococcus* sp, *Bacillus* sp, *E. coli* from Hawked Kunun-zaki and Zobo. Therefore, there is need for high degree of sanitation during the processing of these beverages. The occurrence of *E. coli* in kunun-zaki and Zobo is an indication of faecal and environmental contamination and a signal for the presence of other enteric pathogens. Therefore, their presence may be linked to faecal, environmental and human contaminations which may occur probably due to the use of water during production. *E. coli* generally is confined to human intestines, however, in a debilitated or immunosuppressed host or when the bacteria is introduced to the other tissues even in a healthy person (Center for Disease Control and Prevention, 2005). *S. aureus* can pass onto the food during harvesting, processing or even storage. The consumer is at risk of acquiring food borne disease. *Staphylococcus aureus*is the major cause of Staphylococcal food poisoning. The poisoning is characterized by diarrhea and vomiting [22].

IV. CONCLUSION

The microbial content of all the hawked marketed Kunuand Zobo drinks was higher and contaminated with microorganisms which may be potentially pathogenic to human beings. There is therefore need to maintain adequate hygienic conditions during processing, preparation and storage of these beverages to eliminate these microbial contaminants and improve the quality of the final product.

CONFLICT OF INTEREST

The authors declared that conflict of interest do not exist

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