Asian Journal of Research in Biology

Volume 7, Issue 1, Page 109-121, 2024; Article no.AJRIB.12651

Evaluation of Microbial Contents of Bottled Zobob Beverages Sold in Port Harcourt Metropolis

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

Article Information

DOI: https://doi.org/10.56557/ajrib/2024/v7i142

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://prh.ikprress.org/review-history/12651

Original Research Article

Received: 26/10/2024
Accepted: 30/12/2024
Published: 31/12/2024

ABSTRACT

This study investigated the microbial contents of bottled Zobo drinks in Port Harcourt Metropolis. A total of 3 samples were collected from mile 1 market, "Big Tree" and Ignatius Ajuru University of Education, Port Harcourt main gate. Each sample was carefully stored in a cooler with ice packs to prevent any change in microbial quality during transportation to the Biology laboratory, for microbial analysis, to assess the microbial and fungal quality of bottled Zobo drink, using nutrient agar culture medium, a sterile sampling technique was employed to collect drink samples. Using a sterile pipette, drops of the diluted samples were spread onto nutrient agar plates and incubated at 37°C for 24 to 168 hours to allow microbial and fungal growth. The results of heterotrophic bacteria on nutrient agar media showed a mean count range of 1.32 x 106 to 4.56 x 106 cfu/lm in the Zobo drink, while heterotrophic fungal mean count range of 2.4 x 105 cfu/ml to 3.5 x 105 cfu/ml. The study recovered seven bacteria genera namely: Bacillus subtilis, Klebsiella, Pseudomonas

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Cite as: *Faith, Nnaa, Lelebubari, and Lebari Barine Gboeloh. 2024. "Evaluation of Microbial Contents of Bottled Zobob Beverages Sold in Port Harcourt Metropolis". Asian Journal of Research in Biology 7 (1):109-21. https://doi.org/10.56557/ajrib/2024/v7i142.*

aeruginosa, Lactobacillus acidophilus, Streptococcus lactis Escherichia coli and Staphylococcus sp. Lactobacillus sp. and Bacillus sp. had an occurrence of 25% each, Staphylococcus sp. had 18% while Streptococcus lactic, Pseudomonas sp, Escherichia coli, and Klebsiella sp. had 8% occurrence respectively in the Zobo drink. Four fungi species were recovered, namely: Aspergillus niger, Candida sp., Saccharomyces sp. and Rhizopus stolonifera. The study identified a high prevalence of microbes in Zobo drinks sold in Port Harcourt Metropolis. Hence, it recommends that consumers and retailers of Zobo drink should be vigilant and observant of the source of products and producers to maintain absolute cleanliness.

Keywords: Microbial contents; bottled zobo drinks; Port Harcourt metropolis.

1. INTRODUCTION

The significance of natural fruit drinks lies in their ability to provide a refreshing and nutritious alternative to artificially flavoured beverages. Nigeria has a rich cultural tradition of using natural ingredients to make delicious drinks, such as zobo, which is believed to have originated from traditional medicine. *Hibiscus sabdariffa*, the primary ingredient of Zobo, has been used in African traditional medicine for centuries to treat conditions such as high blood pressure, heart disease, and even diabetes [1]. Over time, Zobo evolved from being a traditional medicinal drink to a popular refreshment, appreciated for its tangy and sweet flavour. Zobo drink, also known as hibiscus tea or "sorrel" in the Caribbean, is a popular West African beverage made from the dried and crushed calyces of the Hibiscus sabdariffa plant. It has been traditionally prepared in Nigeria and other West African countries for centuries, offering a refreshing, tart, and slightly sweet taste [2].

In Port Harcourt Metropolis, Zobo drink has gained popularity as a healthy, non-alcoholic alternative to sugary beverages. Zobo drink, also known as hibiscus tea or sour tea, is a popular beverage in Nigeria, particularly in the South-South region. It is traditionally prepared from dried and crushed calyces of Hibiscus sabdariffa, which is rich in antioxidants and minerals [3]. Recently, there has been an increase in the commercial production and sale of bottled Zobo drinks, with the rise of small and medium-scale beverage companies [4].

In recent times, bottled Zobo drinks have become increasingly popular in Nigeria, particularly in urban centers like Port Harcourt. According to a study by Ajirotutu and Olukayode in Dadson [5], the demand for bottled Zobo drinks has risen significantly due to their convenience, portability, and perceived health benefits. This trend is partly driven by consumer demand for healthier, natural alternatives to carbonated soft drinks. These small and medium-scale companies have contributed to the increased availability of bottled Zobo drinks in both urban and rural areas, making the beverage more accessible to a broader range of consumers. The rise of bottled Zobo drinks also reflects the entrepreneurial spirit of many Nigerians, who recognize the potential of traditional drinks to appeal to modern consumer tastes and preferences. While bottled Zobo drinks offer a convenient and tasty alternative to other beverages, they can also pose potential safety concerns.

According to Ayinla et al. in Alanamu [6]. The processing, packaging, and storage conditions of bottled Zobo drinks can contribute to the growth of microorganisms, leading to microbial contamination.

Microbial contents, in this context, refer to the presence and distribution of microorganisms (bacteria, fungi, viruses, etc.) in the “Zobo” drink samples. Microbial contamination can occur during various stages of production, from harvesting and processing to storage and distribution. This contamination can cause food poisoning, especially when the drink is consumed after its expiration date or has been exposed to extreme temperatures. Poor handling during transportation and distribution can also lead to contamination, resulting in compromised product quality and safety. Health risks associated with microbial contamination in beverages can be addressed through various measures.

According to Marathas and Martino in Aadil [7], effective microbial control measures, such as Hazard Analysis Critical Control Points (HACCP), have become essential in the food and beverage industry to ensure product safety, maintain public health, and prevent costly product recalls. By implementing effective

microbial control measures, companies can safeguard consumer health, maintain their reputation, and avoid costly legal and regulatory issues. This highlights the importance of adhering to strict quality standards in the production and distribution of bottled Zobo drinks.

Despite the growing popularity of bottled Zobo drinks in Nigeria, there remains a significant knowledge gap regarding their microbial quality and safety. This research gap is exacerbated by the limited availability of comprehensive studies on bottled Zobo drinks sold in Port Harcourt Metropolis, which poses a potential health risk to consumers. According to Mibro et al. in Nwaiwu et al. [8], few studies have been conducted to assess the microbiological quality of Zobo drinks in Nigeria. This dearth of information highlights the pressing need for scientific investigation into the microbial contents of bottled Zobo drinks sold in Port Harcourt Metropolis. By conducting such research, scientists can help establish baseline microbial quality standards for these products, providing a foundation for further improvements in food safety and quality control measures.

1.1 Statement of the Problem

The relationship between microbial contents and bottled Zobo drinks in Port Harcourt Metropolis is crucial due to the potential health risks associated with microbial contamination. Zobo drinks, which are made from the calyx of the hibiscus flower (*Hibiscus sabdariffa*), are popular beverages known for their refreshing taste and purported health benefits. However, improper handling, processing, or storage of bottled Zobo drinks can lead to microbial contamination, resulting in the presence of harmful microorganisms such as bacteria, molds, and yeast. Microbial contamination in bottled Zobo drinks can occur at various stages, including during preparation, bottling, transportation, and storage. Factors such as inadequate sanitation practices, unhygienic production environments, and prolonged storage periods can contribute to microbial growth and proliferation in the beverage. Additionally, the use of contaminated water, equipment, or ingredients during the production process can introduce harmful microorganisms into the final product.

The problem of the study lies in the potential health hazards posed by microbial contamination in bottled Zobo drinks sold in Port Harcourt Metropolis. The widespread consumption of bottled Zobo drinks in local markets poses a significant risk to public health due to limited scientific research examining their microbial quality and safety. Despite their popularity, these beverages are often prepared and stored under unsanitary conditions, making them a breeding ground for harmful microorganisms. A staggering 25% of bottled Zobo drinks sampled from local markets in Nigeria contained E. coli, a common indicator of fecal contamination Adesulu-in Ndukwe et al. [9]. Furthermore, World Health Organization (WHO) research revealed that 50% of street-vended Zobo drinks in Ghana contained Salmonella, a pathogen that can cause food poisoning World Health Organization in Nwaiwu et al. [8]. The lack of adequate microbial quality control measures puts consumers at risk of foodborne illnesses, including diarrhea, vomiting, and gastrointestinal infections. A survey of 100 bottled Zobo drinks sold in markets in Lagos, Nigeria, found that 30% had microbial counts exceeding acceptable limits, indicating poor hygiene practices during preparation and storage Ogunjobi & Ogunjobi in Wood et al. [10].

This is particularly concerning, as the Centers for Disease Control and Prevention (CDC) reports that foodborne illnesses affect approximately 1 in 10 people worldwide yearly (Centers for Disease.

(Control and Prevention, 2022). Moreover, a study by Afoakwa and Agyemang Afoakwa and Agyemang (2020), found that 70% of foodborne outbreaks in Africa were linked to contaminated beverages, including Zobo drinks. Therefore, the study aims to investigate the microbial contents of bottled Zobo drinks in Port Harcourt Metropolis to assess their safety and identify potential microbial hazards. Examining the prevalence and types of microorganisms present in these beverages, provides valuable insights into the microbial quality of bottled Zobo drinks and informs regulatory measures to ensure consumer protection and public health.

1.2 Aim and Objectives of the Study

The study aims to investigate the microbial contents of bottled Zobo drinks in Port Harcourt Metropolis. Specifically, the objectives are to:

- Determine the prevalence of microorganisms in bottled Zobo drinks sold in Port Harcourt Metropolis.
- Identify the microorganisms isolated in bottled Zobo drinks sold in Port Harcourt Metropolis.

1.3 Conceptual Review

1.3.1 Microbial contamination in zobo drink

Zobo drink, a popular beverage in Nigeria, has a rich history that dates back to the pre-colonial era. Its origins can be traced back to the Hausa people of Northern Nigeria, who traditionally consumed a drink made from the flowers of the Hibiscus sabdariffa plant, commonly known as roselle or bissap [11]. This plant, native to West Africa, has been used for centuries for its medicinal and culinary purposes. The traditional recipe for the Zobo drink involves boiling the roselle flowers in water, sweetening the mixture with sugar or honey, and flavouring it with spices like ginger, cloves, and cinnamon. The resulting drink is a sweet, tangy, and refreshing beverage that has become an integral part of Nigerian culture. Over time, the Zobo drink has evolved, and modern recipes may include additional ingredients like fruit juice, herbs, and preservatives.

Osueke and Ehirim [12], further added that Zobo drink is a rich source of vitamins, minerals, and antioxidants, making it a nutritious and healthy beverage option. The drink is also low in calories and sugar content, making it a popular choice for health-conscious individuals. The authors also noted that Zobo drink is comparable in quality and taste to other soft drinks but with the added benefit of being a natural and herbal beverage. Overall, Zobo drink is a unique and refreshing beverage that offers a range of health benefits and culinary uses. Adelekan et al. [13] also asserted that the Zobo drink is a versatile beverage that can be enhanced with various fruits to improve its flavour and nutritional value. Adelekan et al. further developed a fruit-enhanced Zobo drink by adding different fruits such as pineapple, orange, and mango to the traditional recipe. The resulting drink was not only refreshing and flavorful but also rich in vitamins, minerals, and antioxidants.

1.4 Factors Influencing Contamination

Microbial contamination in Zobo drink is a significant concern that can pose health risks to consumers. Several factors contribute to contamination, including:

Poor hygiene practices: During preparation and storage, poor hygiene practices can introduce microorganisms into the drink. This includes inadequate handwashing, unclean equipment, and unsanitary environments [11]. Poor hygiene practices during the preparation and storage of Zobo drink can lead to the introduction of microorganisms into the drink, making it unsafe for consumption. One of the main causes of poor hygiene practices is inadequate handwashing.

Inadequate pasteurization: Inadequate pasteurization is a significant risk factor for microbial contamination in Zobo drinks. Pasteurization is a critical step in the production process that involves heating the drink to a high temperature to kill harmful microorganisms. However, if the pasteurization process is not done correctly, some microorganisms may survive and contaminate the drink [14]. Inadequate pasteurization can occur due to various reasons such as inadequate temperature, insufficient holding time, or faulty equipment. This can lead to the survival of microorganisms like *E. coli, Salmonella, and Listeria*, which can cause serious foodborne illnesses.

Contamination from raw materials: Contamination from raw materials is a significant risk factor for microbial contamination in Zobo drinks. Raw materials such as water, sugar, and fruit can harbor microorganisms like bacteria, viruses, and fungi. If these raw materials are not properly cleaned, sanitized, and handled, they can introduce microorganisms into the drink For example, untreated water can contain harmful bacteria like E. coli, while unclean fruit can carry fungal spores.

Unhygienic handling: Unhygienic handling is a critical factor in microbial contamination of Zobo drinks. Handling the drink with unclean hands or utensils can introduce microorganisms into the drink, making it unsafe for consumption [15]: Food handlers may not wash their hands frequently or thoroughly enough, leading to the transfer of microorganisms from their hands to the drink. Additionally, using unclean utensils or equipment can also contaminate the drink.

Storage conditions: Improper storage conditions can significantly contribute to microbial growth in Zobo drinks. High temperatures, humidity, and exposure to light can create an environment conducive to microbial growth, leading to contamination and spoilage FDA in Caly, 2020 [16]. For example, temperatures between 40°F and 140°F (4°C and 60°C) can support the growth of harmful microorganisms like Staphylococcus aureus and Escherichia coli EcoLab in Ramírez-Gómez [17].

Fig. 1. Zobo Drink-Making Items in Nigerian
Source: https://www.google.com/search?sca

Equipment Cleaning and Sanitizing: Inadequate cleaning and sanitizing of equipment is a significant risk factor for microbial contamination in Zobo drinks. Residual microorganisms can survive on equipment surfaces if not properly cleaned and sanitized, and can subsequently contaminate the drink. Osueke et al. [12] emphasized the importance of proper equipment cleaning and sanitizing in preventing microbial contamination in Zobo drinks.

Water Quality: Using contaminated water in Zobo drink production is a significant risk factor for microbial contamination. Water is an essential ingredient in Zobo drink production, and if it is contaminated with microorganisms, it can introduce ha (harmful bacteria, viruses, and fungi into the drink [18].

Ingredient Quality: Using low-quality ingredients that are contaminated with microorganisms is a significant risk factor for microbial contamination in Zobo drinks. Ingredients like fruit, sugar, and spices can harbour harmful microorganisms like bacteria, viruses, and fungi. If these ingredients are not properly cleaned, sanitized, and handled, they can introduce microorganisms into the drink. Contaminated ingredients can lead to the growth of microorganisms in the drink, making it unsafe for consumption.

Production environment: An unclean production environment is a significant risk factor for microbial contamination in Zobo drinks. A production environment that is not properly cleaned, sanitized, and maintained can harbour microorganisms like bacteria, viruses, and fungi [11]. These microorganisms can then contaminate the drink during production, making it unsafe for consumption. Unclean equipment, utensils, and surfaces can also spread microorganisms throughout the production environment, increasing the risk of contamination.

Lack of Quality Control Measures: Failure to implement quality control measures is a significant risk factor for microbial contamination in Zobo drinks. Quality control measures, such as testing for microorganisms, are essential to ensure the safety and quality of the drink [14]. Without these measures, microorganisms can go undetected, leading to contamination and potential harm to consumers. Regular testing for microorganisms, such as bacteria, yeast, and mould, can help identify potential contamination sources and prevent their growth.

1.5 Types of Microorganisms in Bottled Zobo Drinks Sold

According to a recent study by Ovutor et al. [19] and Ire, Ire et al. [20] bottled Zobo drinks sold in Port Harcourt Metropolis have been found to contain various types of microorganisms, including [21]:

***Escherichia coli* (*E. coli*):** commonly referred to as E. coli, is a type of bacteria that is ubiquitous in our environment. It can be found in the gastrointestinal tract of animals and humans alike and is typically harmless. However, certain strains of *E. coli* can be detrimental to human health, causing food poisoning and other serious illnesses. The bacteria can contaminate food and water through various means, including fecal matter, untreated wastewater, contaminated surfaces, and infected animals.

***Staphylococcus aureus*:** commonly referred to as S. aureus, is a type of bacteria that is notoriously known for its ability to cause a wide

range of illnesses, from mild to severe. This versatile pathogen can infect various parts of the body, including the skin, respiratory tract, and even the bloodstream. S. aureus is particularly notorious for its ability to cause skin infections, such as boils, abscesses, and cellulitis.

***Bacillus cereus*:** is a type of bacteria that is commonly found in soil, water, and the gastrointestinal tract of animals. While it is generally harmless, *B. cereus* can produce toxins that can cause food poisoning in humans. When ingested, *B. cereus* can cause a range of symptoms, including nausea, vomiting, diarrhoea, and stomach cramps. In severe cases, it can lead to more serious conditions, such as gastrointestinal inflammation and even kidney failure.

***Aspergillus flavus*:** is a type of fungus that is commonly found in soil, decaying organic matter, and even in the air we breathe. While it is generally harmless, *A. flavus* can produce toxic compounds called aflatoxins, which can have devastating effects on human health. When ingested, aflatoxins can cause a range of symptoms, including nausea, vomiting, diarrhea, and abdominal pain. Prolonged exposure to these toxins can lead to more serious conditions, such as liver damage, kidney failure, and even cancer.

***Candida albicans*:** is a type of fungus that is commonly found on the skin and in various bodily cavities. While it is typically harmless, *C. albicans* can cause infections when it overgrows, leading to a condition known as candidiasis. When *C. albicans* infects the body, it can cause a range of symptoms, including itching, burning, and redness. In women, it can cause vaginal yeast infections, characterized by thick, white discharge and discomfort during urination and sex.

2. MATERIALS AND METHODOLOGY

2.1 Study Area

The study will focus on the bottled Zobo drinks sold in various markets and locations within Port Harcourt Metropolis, a vibrant city in Rivers State, Nigeria. This metropolis is home to numerous markets, including the bustling Iwofe Market, Wimpey Market, Mile 1 & 3 Market, and Creek Road Market, where locals and visitors alike can find a variety of goods, including food and drinks. In addition to markets, the study will also examine bottled Zobo drinks sold by street food vendors, supermarkets, retail shops, and other locations where these drinks are readily available.

2.2 Isolation of Bacteria

Sample collection: A total of 3 samples were collected from various markets and locations. These locations will include the bustling Mile 1 market, where 1 sample will be collected, and Creek Road Market, where 1 sample was obtained. Additionally, 1 sample was collected from street food vendors, who are an integral part of the city's food landscape.

2.3 Sample Preparation/ Preparation of Stock Solution

The sample was homogenized by mixing 10ml in 90ml of sterile distilled water and 1ml from the stock was used for serial dilution.

Serial dilution: Each sample was serially diluted in six test tubes (10'1 to 10'6) using.

Sterile distilled water as diluents.

- Using a sterile wait pipette, 9ml of the diluents (water) was dispensed into the sterile test tube.
- The test tube was plugged with cotton wool, put together in a big beaker, and covered with aluminum foil.
- Then autoclaved at 121^0 C for 15 minutes as 15PSI.
- The sterile test tubes were then arranged in the test tube rack & labelled correctly indicating the dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4},10^{-5}, 10^{-6}) and each sample code.
- 1 ml or 1g of homogenously mixed samples was transferred using a sterile pipette into the first test tube (10^{-1}) containing 9 ml of normal saline. This gives 1 in 10 dilutions (10^{-1}) for a tenfold serial dilution.
- Test tube one (10^{-1}) was agitated to mix and then 1ml was transferred from it to test tube two (10^{-2}). This process was repeated until the desired dilution was reached.

Media preparation: The following media were used for the isolation of the various bacteria: Nutrient agar (bacteria), *Salmonella shigella* agar (*Salmonelle),* Mannitol salt agar

(*Staphylococcus sp),* EMB agar (gram-negative enteric bacilli). All media were prepared according to the manufacturer's instructions.

Nutrient agar (NA): Nutrient Agar (NA) was prepared by suspending 28g in 1000 ml distilled water in a conical flask. The conical flask was corked with cotton wool, boiled to dissolve and autoclaved at 121^0C, 15mins and 15psi. It was allowed to cool to around 45-50^0C and the poured into sterile petri dishes and allowed to solidify. It was then dried in hot air oven before it was inoculated with 0.1ml of the aliquot.

Eosin methylene agar (EMB): It was prepared suspending 36g of EMB powder in 1000ml distilled water into a conical flask. The conical flask was corked with cotton wool, boiled to dissolve and autoclaved at 121^0C, 15mins and 15psi. It was allowed to cool to around 45-50^0 C and then poured into sterile petri dishes and allow to solidify. It was then dried in hot air oven before it was inoculated with 0.1ml of the aliquot.

***Salmonella shigella agar* (SSA):** It was prepared by suspending 38g of salmonella agar (according to manufactures standard) in 1000ml distilled water into a sterile conical flask. The conical flask was corked with cotton wool, boiled to dissolve and autoclaved at 121^0 C, 15mins and 15psi. It was allowed to cool to around 45-50^0 C and then poured into a sterile petri dishes and allowed to solidify. It was then dried in a hot air oven before it was inoculated with 0.1ml of the aliquot.

Mannitol Salt Agar (MSA): It was prepared by suspending 111g of salmonella agar (according to the manufacturer's standard) in 1000ml distilled water in a sterile conical flask. The conical flask was corked with cotton wool, boiled to dissolve, and autoclaved at 121^0 C, 15mins and 15psi. It was allowed to cool to around 45-50^0 C and then poured into sterile petri dishes and allow to solidify. It was then dried in hot air oven before it was inoculated with 0.1ml of the aliquot.

Isolation and sub-culture:

Isolation: The spread plate method was used to isolate the bacteria present. 0.1ml of 10'3 serially diluted samples were pipetted onto the agar and spread with a bent glass rod. The plates were incubated at 37^0 C for 24 hours.

Sub-culture: After 24 hours, discrete colonies were sub-cultured on fresh agar plates using streaking technique for the development of pure isolates which were stored on agar slants for biochemical tests.

Enumeration of bacteria count:

The colonies that developed on the nutrient agar plates were counted and used to determine the total bacterial count of the samples (cfu/ml). The count within range of 30-300 is accepted over a standard size petri dish.

$$Colony\ forming\ unit\ = \frac{Number\ of\ colonies}{Dilution\ x\ volume\ plated}$$

Isolation of Fungi:

Media Preparation

SDA: Sabouraud dextrose agar (SDA) was prepared according to manufacturer's standard (65g in 1000ml), boiled to dissolve and autoclaved at 121^0 C, 15mins and 15psi. It was allowed to cool and 25mg of tetracycline was added to the media to suppress growth of bacteria.

Culture and sub-culture:

Isolation:

Method 1: The spread plate method was used to isolate the bacteria present 0.1ml of 103 serially diluted samples were pipetted onto the agar and spread with a bent glass rod. The plates were incubated at 37^0 C for 5-7 days.

Method 2: The spoilt part of the sample was sliced thinly using a scalpel and placed on a solidified agar plate. The plates were incubated at 37^0 C for 5-7 days.

Sub-culture: After one week, Discrete colonies were sub-cultured on fresh agar plates using the streaking technique for the development of pure isolates which were stored on agar slants for biochemical tests.

Identification of bacteria isolates:

Colonial Morphology: A colony of the isolate was picked and streaked on the freshly prepared agar and incubated at 37^0C for 24 hours. After incubation, morphological features; shape, size, colour, edge, texture and elevation of the colony of the isolate observed visually with hand lens.

Cell morphology: Gram-stained slides were mounted on the microscope to observe the cell morphology.

Biochemical Tests: The following biochemical tests were carried out to aid in the identification of the isolates:

Catalase Tests: This test was used to determine the ability of an organism to break down hydrogen peroxide (H_2O_2) into oxygen and water. Only organisms that have the enzyme catalase can catalyze the reaction. The presence of the enzyme in a bacterial cell is evident when a small inoculum was introduced into a 3% hydrogen peroxide solution and rapid production of effervescence occurs. The absence of catalase is evident by a lack of or weak production of effervescence.

Citrate Test: The citrate test was used to determine the ability of an organism to utilize sodium citrate as its sole source of carbon and inorganic ammonium salt as its only source of nitrogen. Bacteria that can grow on this medium turn the bromothymol blue indicator from green to blue. Simmon citrate agar was prepared in a capped tube according to manufacturer's instruction. A sterile wire loop was used to pick a loopful of the test organism and streaked on slant surface. The tube was incubated at 37^0C for 48h. Change in colour from green to blue was indicative of positive result while no change in colour was indicative of a negative result.

Indole Test: This test was used to determine the ability of an organism to split the amino acid tryptophan to form pyruvic acid, ammonia, and indole using the enzyme tryptophanase.

A loopful of the test organism was inoculated into a sterile peptone water medium and incubated at 37^0C for 48h. Thereafter, 0.3-0.5ml of Kovac's reagent was added using a Pasteur's Pipette. The appearance of a red ring layer on medium was indicative of the position indole test while the development of a yellow ring was indicative of a negative result.

Methyl Red/Voges Proskauer (MR/VP) TEST MR VP: This test was used to determine the ability of an organism to produce and maintain stable acid end products from glucose fermentation and to determine the ability of some organisms to produce neutral end products such as acetyl-methyl carbinol or acetone from glucose fermentation. The MR/VP broth medium is used for this test. A loopful of test organisms is inoculated into 10ml sterile MR/VP broth medium prepared according to the manufacturer's instructions. The tube was incubated at 35-37^0C for 48h. After incubation, the broth culture was shared into two equal parts (5ml). one part was used for the methyl red test and the other part for the Voges Proskauer test.

To the part for MR, 5-6 drops of methyl red reagent was added and to the part for VP, 0.6ml (6 drops) of 5% a-naphthol and 0.2ml (2 drops) of 40%KOH reagent were added. Development of bright red colouration is indicative of positive MR/VP tests

Motility Test: This test was used to determine if an organism is motile. An organism must possess flagella (a locomotory organelle) to the motile. Semi-solid nutrient agar was used for this test. Half strength of the medium was prepared following manufacturer's direction. A young (fresh) colony was picked with a sterile straight wire and inoculated by stabbing into the medium. Thereafter, the tube incubated at 37^0C for 24-48 h. Growth (in diffuse form) from line of stab into the medium was indicative of positive result, whereas growth only along the line of stab was indicative of a negative result.

Oxidase Test: This test was used to determine the presence of bacterial cytochrome oxidase using the oxidation of the substrate tetramethyl-p-phenylenediamine dihydrochloride to indolphenol.

A filter paper soaked with 1% tetramethyl-p-phenylenediamine dihydrochloride was used to perform the test. A platinum wire loop was used to pick a small portion of the test organism which was then rubbed on the soaked filter paper. Observation for 10min for the development of purple colouration on the smeared portion is indicative of a positive result for the oxidase test while no change in colour of the smeared portion was indicative of a negative oxidase test.

Triple Sugar Iron Agar (TSIA) test: This test was used to determine whether the bacterial isolates could ferment glucose and lactose or sucrose and form hydrogen sulfide (H_2S), gas and acids.

The triple sugar iron agar (TSIA) was used for this test. The slant was prepared according to manufacturer's recommendation. To perform this

test, a straight sterile inoculation needle was used to pick the organism which was then inoculated by first stabbing the centre of the medium (not to the bottom of the tube) and streaking the surface of the agar slant. Thereafter, the tubes were incubated at 35-37^{0}C for 24-48h. Change in colour of medium from pink to yellow indicated acid production resulting from glucose fermentation. When in addition to glucose, lactose and/or sucrose are fermented; the large amount of fermentation products formed on the slant will neutralize the alkaline amines and render the slant acidic (yellow). Black colouration indicated production of hydrogen sulfide (H_2S) and cracks in the medium or complete upward shift of the agar slant from bottom of the tube indicates positive gas production.

Sugar Fermentation: This test was used to determine the ability of an organism to ferment a sugar to produce acid and gas. Peptone broth (1%) incorporated with 1% sugar was used for the test. Bromocresol purple (0.3%) indicator was added to the sugar medium with Durham's tube added in the tube in inverted (upside down) position. After sterilization, a loopful of test tubes were then incubated at 35-37^{0}C for 24-48 h. Change in colour from purple to yellow and gas production (collected in the inverted Durham's tube) constitute positive sugar fermentation test. Production of acid resulted in a change in colour of the medium from purple to yellow while acid production is detected in the inverted Durham's tube.

Method of Data Analysis: The data was analyzed using statistical software, such as SPSS or R, to ensure accurate and reliable results. The results will be presented in tables, figures, and graphs to facilitate easy understanding and interpretation.

3. RESULTS

3.1 Enumeration of the Bacteria and Fungi Load in the Zobo Drink

The result in Table 1 shows the bacterial and fungal load in the Zobo drink samples. Heterotrophic bacteria on nutrient agar media showed a mean count range of 1.32 x 10^6 to 4.56 x 10^6cfu/lm present in the zobo drink while a heterotrophic mean count range of 2.4 x 10^5 cfu/ml to 3.5 x 10^5 cfu/ml were noted/reported.

3.2 Prevalence of Bacteria in the Zobo Drink Sample

Table 2 showed the percentage frequency of occurrence of the bacteria isolates recovered from the Zobo drink.

The bacteria, *Lactobacillus* sp. and *Bacillus* sp. were noted in the zobo drink with a percentage occurrence of 25 each, while *Streptococcus lactic Pseudomonas* sp*, Escherichia coli* and *Klebsiella* sp. had a 8 percentage occurrence each in the zobo drink. Consequently, *Staphylococcus sp.* had a 18 percentage occurrence.

Table 1. Mean Load of Bacterial and Fungal Load in the Zobo Drink

Zobo Drink Sample	Total Heterotrophic Bacterial Count (CFU/ml)	Total Heterotrophic Fungal Count (CFU/ml)
1	1.32 x10^6	3.1 x10^5
2	3.60 x10^6	3.5 x10^5
3	4.56 x10^6	2.4 x10^5

Note, THF= Total Heterotrophic Fungi, FC= Fungal Counts, CFU= Coliform forming unit

Table 2. Prevalence of Bacteria in the Zobo Drink Sample

Bacteria	Frequency of Occurrence	Percentage Occurrence
Lactobacillus sp	3	25
Streptococcus lactic	1	8
Bacillus sp	3	25
Pseudomonas sp	1	8
Staphylococus sp	2	18
Escherichia coli	1	8
Klebsiella sp.	1	8

Table 3. Biochemical Characterization of Bacterial Isolates from the Zobo Drink

S/N	Catalase	Oxidase	Citrate	Indole	Spore	Methyl Red	Urease	Gas	VP	Glucose	Lactose	Sucrose	Mannitol	Suspected Bacteria
1	+	+	+		+				+	+	+	+	+	*Bacillus substilis*
	+		+			+	+		+	+	+	+	+	*Staphylococcus* sp.
2	+			+		+		+		+	+			*Escherichia coli*
3	+		+				+	+	+	+	+	+	+	*Klebsiella* sp
	+	+							+	+				*Pseudomonas aeriginosa*
										+	+	+	+	*Lactobacillus acidophilus*
										+	+	+	+	*Streptococcus lactis*

Key: VP= Voges Proskauer, + = Positive, - = Negative

3.3 Characterization of Bacterial Isolates

Table 3 showed the biochemical reactions of some bacterial isolates recovered from the zobo drink sample observed in the study. The study recovered seven bacteria genera namely: *Bacillus subtilis, Klebsiella, Pseudomonas aeruginosa, Lactobacillus acidophilus, Streptococcus lactis*, *Escherichia coli* and *Staphylococcus* sp.

Biochemical reaction characterized the bacterial isolates, whereby *Bacillus substilis* showed positive reaction to catalase, oxidase, citrate, spore formation, glucose production, lactose, sucrose and mannitol fementation however negative reaction was noted for indole reaction, urease production, gas and voges prauskeraur in their respective reactions.

For Staphylococcus sp which was identified with positive reaction to catalase, citrate, urease and methyl red, glucose production, lactose, sucrose and mannitol fermentation however negative reaction was noted for oxidase, indole, spore formation and, gas released and voges prauskeraur in their respective reactions.

Escherichia coli showed positive reaction to catalase, indole, methyl red, gas production, glucose and lactose however oxidase, spore fomma, glucose production, lactose, sucrose and mannitol fermentation however negative reaction was noted for indole reaction, urease production, gas and voges prauskeraur in their respective reactions.

3.4 Frequency of Fugal Occurrence

Table 4 showed the percentage frequency of occurrence of the fungi isolates recovered from the Zobo drink.

The fungi, *Aspergilus niger*. were noted in the zobo drink with a percentage occurance of 22, and the following isolates *Rhizopus* sp*, Candida* sp and *Sacchromyces* sp. at a percentage occurance of 12, 33 and 33 respectively.

3.5 Colonial/ Macroscopic Characterization of the Isolated Fungi

Table 5 showed the characterization of the fungi isolates recovered from the zobo drink. colonial classification of the fungal isolates as seen in the chart below, showed the appearance of the fungal in terms of color, size, growth rate and texture. The fungi *Aspergillus niger* were noted dark brown colour with a large size, rough walled and cottony texture.

Candida sp. were observed with a milky large colony, a shiny textured appearance, thus showing an ovoid shape. The fungi *Sacchromyces* sp. had a white creamy colour, that is large and the yeast cell showing budding. *Rhizopus stolonifer* the fungi appeared whitish in colour with a small sized colony that is densely cotton-like. Four fungi species were recovered, namely: *Aspergillus niger, Candida* sp., *Sacchromyces* sp. and *Rhizopus stolonifera.*

Table 4. Frequency of Fugal Occurrence

Fungal	Frequency of occurrence	Percentage occurrence
Aspergilus niger	2	22
Rhizopus sp	1	12
Candida sp	3	33
Sacchromyces sp	3	33

Table 5. Macroscopic Characterization of the Fungi Isolate

Isolates	Structural description				Identification
	Colour	Size	Growth rate	Texture	
2	Dark brown	Small	Slow	Rough walled	*Aspergillus niger*
3	Blue-green	Small, ovoid shape	Fast	Shiny	*Candida sp*
4	Cream/white	Large	Slow	Shiny	*Sacccharomyces* sp.
5	White	Small and smooth walled	Fast	Dense cottony	*Rhizopus stolonifer*

4. CONCLUSION

The study identified a high prevalence of microbes in Zobo drinks. It therefore noted the incidence of lactobacillus sp*, Streptococcus lactis, Candida sp., Saccharomyces* sp*. Bacills* sp*., Pseudomonas* sp*. Staphylococcus* sp *Escherichia coli, Klebsiella* sp., *Aspergillus niger,* and *Rhizopus* sp*.* in the Zobo drink could constitute a public health hazard, where the drink can result in outbreaks of diseases, gastroenteritis, and diarrhea.

5. RECOMMENDATION

The study advises that consumers and retailers of zobo drink be vigilant and observant of the root source of products and therefore encourage producers to maintain absolute cleanliness and keep to standard hygiene practices.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

We the author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during the writing or editing of this manuscript.

COMPETING INTERESTS

The authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
https://prh.ikprress.org/review-history/12651