

Investigating the Microbial Contents of Bottled Zobo Drinks in Port Harcourt Metropolis

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 1.32×10^6 to 4.56×10^6 cfu/lm in the Zobo drink, while heterotrophic fungal mean count range of 2.4×10^5 cfu/ml to 3.5×10^5 cfu/ml. The study recovered seven bacteria genera namely: *Bacillus subtilis*, *Klebsiella*, *Pseudomonas 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

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 (Akintayo&Ogungbe, 2016). 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.

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. 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 (Okike et al., 2017).

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48 In recent times, bottled Zobo drinks have become increasingly popular in Nigeria, particularly in
49 urban centres like Port Harcourt. According to a study by Ajiotutu&Olukayode (2019), the
50 demand for bottled Zobo drinks has risen significantly due to their convenience, portability, and
51 perceived health benefits. This trend is partly driven by consumer demand for healthier, natural
52 alternatives to carbonated soft drinks. These small and medium-scale companies have
53 contributed to the increased availability of bottled Zobo drinks in both urban and rural areas,
54 making the beverage more accessible to a broader range of consumers. The rise of bottled Zobo
55 drinks also reflects the entrepreneurial spirit of many Nigerians, who recognize the potential of
56 traditional drinks to appeal to modern consumer tastes and preferences. While bottled Zobo
57 drinks offer a convenient and tasty alternative to other beverages, they can also pose potential
58 safety concerns.

59
60 According to Ayinla et al. (2020), the processing, packaging, and storage conditions of bottled
61 Zobo drinks can contribute to the growth of microorganisms, leading to microbial contamination.
62 Microbial contents, in this context, refer to the presence and distribution of microorganisms
63 (bacteria, fungi, viruses, etc.) in the “Zobo” drink samples. Microbial contamination can occur
64 during various stages of production, from harvesting and processing to storage and distribution.
65 This contamination can cause food poisoning, especially when the drink is consumed after its
66 expiration date or has been exposed to extreme temperatures. Poor handling during transportation
67 and distribution can also lead to contamination, resulting in compromised product quality and
68 safety. Health risks associated with microbial contamination in beverages can be addressed
69 through various measures.

70
71 According to Marathas & Martino (2018), effective microbial control measures, such as Hazard
72 Analysis Critical Control Points (HACCP), have become essential in the food and beverage
73 industry to ensure product safety, maintain public health, and prevent costly product recalls. By
74 implementing effective microbial control measures, companies can safeguard consumer health,
75 maintain their reputation, and avoid costly legal and regulatory issues. This highlights the
76 importance of adhering to strict quality standards in the production and distribution of bottled
77 Zobo drinks.

78
79 Despite the growing popularity of bottled Zobo drinks in Nigeria, there remains a significant
80 knowledge gap regarding their microbial quality and safety. This research gap is exacerbated by
81 the limited availability of comprehensive studies on bottled Zobo drinks sold in Port Harcourt
82 Metropolis, which poses a potential health risk to consumers. According to Mibro et al. (2020),
83 few studies have been conducted to assess the microbiological quality of Zobo drinks in Nigeria.
84 This dearth of information highlights the pressing need for scientific investigation into the
85 microbial contents of bottled Zobo drinks sold in Port Harcourt Metropolis. By conducting such
86 research, scientists can help establish baseline microbial quality standards for these products,
87 providing a foundation for further improvements in food safety and quality control measures.

88 89 **Statement of the Problem**

90
91 The relationship between microbial contents and bottled Zobo drinks in Port Harcourt Metropolis
92 is crucial due to the potential health risks associated with microbial contamination. Zobo drinks,

93 which are made from the calyx of the hibiscus flower (*Hibiscus sabdariffa*), are popular
94 beverages known for their refreshing taste and purported health benefits. However, improper
95 handling, processing, or storage of bottled Zobo drinks can lead to microbial contamination,
96 resulting in the presence of harmful microorganisms such as bacteria, moulds, and yeast.
97 Microbial contamination in bottled Zobo drinks can occur at various stages, including during
98 preparation, bottling, transportation, and storage. Factors such as inadequate sanitation practices,
99 unhygienic production environments, and prolonged storage periods can contribute to microbial
100 growth and proliferation in the beverage. Additionally, the use of contaminated water,
101 equipment, or ingredients during the production process can introduce harmful microorganisms
102 into the final product.

103
104 The problem of the study lies in the potential health hazards posed by microbial contamination in
105 bottled Zobo drinks sold in Port Harcourt Metropolis. The widespread consumption of bottled
106 Zobo drinks in local markets poses a significant risk to public health due to limited scientific
107 research examining their microbial quality and safety. Despite their popularity, these beverages
108 are often prepared and stored under unsanitary conditions, making them a breeding ground for
109 harmful microorganisms. A staggering 25% of bottled Zobo drinks sampled from local markets
110 in Nigeria contained *E. coli*, a common indicator of faecal contamination (Adesulu-Dahunsi &
111 Oluwajoba, 2020). Furthermore, World Health Organization (WHO) research revealed that 50%
112 of street-vended Zobo drinks in Ghana contained *Salmonella*, a pathogen that can cause food
113 poisoning (World Health Organization, 2018). The lack of adequate microbial quality control
114 measures puts consumers at risk of foodborne illnesses, including diarrhoea, vomiting, and
115 gastrointestinal infections. A survey of 100 bottled Zobo drinks sold in markets in Lagos,
116 Nigeria, found that 30% had microbial counts exceeding acceptable limits, indicating poor
117 hygiene practices during preparation and storage (Ogunjobi & Ogunjobi, 2020).

118
119 This is particularly concerning, as the Centers for Disease Control and Prevention (CDC) reports
120 that foodborne illnesses affect approximately 1 in 10 people worldwide yearly (Centers for
121 Disease
122 Control and Prevention, 2022). Moreover, a study by Afoakwa & Agyemang (2020), found that
123 70% of foodborne outbreaks in Africa were linked to contaminated beverages, including Zobo
124 drinks. Therefore, the study aims to investigate the microbial contents of bottled Zobo drinks in
125 Port Harcourt Metropolis to assess their safety and identify potential microbial hazards.
126 Examining the prevalence and types of microorganisms present in these beverages, provides
127 valuable insights into the microbial quality of bottled Zobo drinks and informs regulatory
128 measures to ensure consumer protection and public health.

129 130 **Aim and Objectives of the Study**

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

- 134
- 135 • determine the prevalence of microorganisms in bottled Zobo drinks sold in Port Harcourt
 - 136 Metropolis.
 - 137 • identify the microorganisms isolated in bottled Zobo drinks sold in Port Harcourt
 - 138 Metropolis

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140 **Conceptual Review**

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142 **Microbial Contamination in Zobo Drink**

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144 Zobo drink, a popular beverage in Nigeria, has a rich history that dates back to the pre-colonial
145 era. Its origins can be traced back to the Hausa people of Northern Nigeria, who traditionally
146 consumed a drink made from the flowers of the Hibiscus sabdariffa plant, commonly known as
147 roselle or bissap (Izah et al., 2015). This plant, native to West Africa, has been used for centuries
148 for its medicinal and culinary purposes. The traditional recipe for the Zobo drink involves boiling
149 the roselle flowers in water, sweetening the mixture with sugar or honey, and flavouring it with
150 spices like ginger, cloves, and cinnamon. The resulting drink is a sweet, tangy, and refreshing
151 beverage that has become an integral part of Nigerian culture. Over time, the Zobo drink has
152 evolved, and modern recipes may include additional ingredients like fruit juice, herbs, and
153 preservatives.

154

155 **Figure 1: Zobo Drink Making Items in Nigerian**



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157 Source: <https://www.google.com/search?sca>

158

159 Osueke and Ehirim (2004), further added that Zobo drink is a rich source of vitamins, minerals,
160 and antioxidants, making it a nutritious and healthy beverage option. The drink is also low in
161 calories and sugar content, making it a popular choice for health-conscious individuals. The
162 authors also noted that Zobo drink is comparable in quality and taste to other soft drinks but with
163 the added benefit of being a natural and herbal beverage. Overall, Zobo drink is a unique and
164 refreshing beverage that offers a range of health benefits and culinary uses. Adelekan et al.
165 (2014) also asserted that the Zobo drink is a versatile beverage that can be enhanced with various
166 fruits to improve its flavour and nutritional value. Adelekan et al. further developed a fruit-
167 enhanced Zobo drink by adding different fruits such as pineapple, orange, and mango to the
168 traditional recipe. The resulting drink was not only refreshing and flavorful but also rich in
169 vitamins, minerals, and antioxidants.

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171 **Factors Influencing Contamination**

172

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

175

176 **Poor hygiene practices:** During preparation and storage, poor hygiene practices can introduce
177 microorganisms into the drink. This includes inadequate handwashing, unclean equipment, and
178 unsanitary environments (Izah et al., 2015). Poor hygiene practices during the preparation and
179 storage of Zobo drink can lead to the introduction of microorganisms into the drink, making it
180 unsafe for consumption. One of the main causes of poor hygiene practices is inadequate
181 handwashing.

182

183 **Inadequate pasteurization:** Inadequate pasteurization is a significant risk factor for microbial
184 contamination in Zobo drinks. Pasteurization is a critical step in the production process that
185 involves heating the drink to a high temperature to kill harmful microorganisms. However, if the
186 pasteurization process is not done correctly, some microorganisms may survive and contaminate
187 the drink (Lovet et al., 2018). Inadequate pasteurization can occur due to various reasons such as
188 inadequate temperature, insufficient holding time, or faulty equipment. This can lead to the
189 survival of microorganisms like *E. coli*, *Salmonella*, and *Listeria*, which can cause serious
190 foodborne illnesses.

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

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199 **Unhygienic handling:** Unhygienic handling is a critical factor in microbial contamination of
200 Zobo drink. Handling the drink with unclean hands or utensils can introduce microorganisms
201 into the drink, making it unsafe for consumption (Kolawole et al., 2017): Food handlers may not
202 wash their hands frequently or thoroughly enough, leading to the transfer of microorganisms
203 from their hands to the drink. Additionally, using unclean utensils or equipment can also
204 contaminate the drink.

205

206 **Storage conditions:** Improper storage conditions can significantly contribute to microbial
207 growth in Zobo drinks. High temperatures, humidity, and exposure to light can create an
208 environment conducive to microbial growth, leading to contamination and spoilage (FDA, 2020).
209 For example, temperatures between 40°F and 140°F (4°C and 60°C) can support the growth of
210 harmful microorganisms like *Staphylococcus aureus* and *Escherichia coli* (EcoLab, 2019).

211

212 **Equipment Cleaning and Sanitizing:** Inadequate cleaning and sanitizing of equipment is a
213 significant risk factor for microbial contamination in Zobo drink. Residual microorganisms can
214 survive on equipment surfaces if not properly cleaned and sanitized, and can subsequently
215 contaminate the drink. Osueke et al. (2016) emphasized the importance of proper equipment
216 cleaning and sanitizing in preventing microbial contamination in Zobo drink.

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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 harmful bacteria, viruses, and fungi into the drink Kigigha et al. (2020).

Ingredient Quality: Using low-quality ingredients that are contaminated with microorganisms is a significant risk factor for microbial contamination in Zobo drink. 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 (Izah et al., 2017). 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 (Lovet et al., 2020). 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.

245 **Types of Microorganisms in Bottled Zobo Drinks Sold**

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247 According to a recent study by Ovutor et al (2024) and Ire, Benneth, and Maduka (2020) bottled
248 Zobo drinks sold in Port Harcourt Metropolis have been found to contain various types of
249 microorganisms, including:

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

257

258 **Staphylococcus aureus:** commonly referred to as *S. aureus*, is a type of bacteria that is
259 notoriously known for its ability to cause a wide range of illnesses, from mild to severe. This
260 versatile pathogen can infect various parts of the body, including the skin, respiratory tract, and
261 even the bloodstream. *S. aureus* is particularly notorious for its ability to cause skin infections,
262 such as boils, abscesses, and cellulitis.

263

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

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

276

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

282

283 **MATERIALS AND METHODOLOGY**

284 **Study Area**

285 The study will focus on the bottled Zobo drinks sold in various markets and locations within Port
286 Harcourt Metropolis, a vibrant city in Rivers State, Nigeria. This metropolis is home to numerous

287 markets, including the bustling Iwofe Market, Wimpey Market, Mile 1 & 3 Market and Creek
288 Road Market, where locals and visitors alike can find a variety of goods, including food and
289 drinks. In addition to markets, the study will also examine bottled Zobo drinks sold by street
290 food vendors, supermarkets, retail shops, and other locations where these drinks are readily
291 available.

292 **Isolation of bacteria**

293 **3.2.1 Sample collection:**

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

298 **Sample Preparation/ Preparation of Stock Solution**

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

301 **Serial dilution:** Each sample was serially diluted in six test tubes (10^1 to 10^6) using

302 Sterile distilled water as diluents.

- 303 • Using a sterile wait pipette, 9ml of the diluents (water) was dispensed into the sterile test
304 tube.
- 305 • The test tube was plugged with cotton wool, put together in a big beaker and covered
306 with aluminium foil.
- 307 • Then autoclaved at 121°C for 15 minutes as 15PSI.
- 308 • The sterile test tubes were then arranged in the test tube rack & labelled correctly
309 indicating the dilutions (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6}) and each sample code.
- 310 • 1ml or 1g of homogenously mixed samples was transferred using a sterile pipette into the
311 first test tube (10^{-1}) containing 9ml of normal saline. This gives 1 in 10 dilutions (10^{-1})
312 for a tenfold serial dilution.
- 313 • Test tubed one (10^{-1}) was agitated to mix and then 1ml was transferred from it to test tube
314 two (10^{-2}). This process was repeated until the desired dilution was reached.

315
316 **Media preparation:** The following media were used for the isolation of the various bacteria:
317 Nutrient agar (bacteria), *Salmonella shigella* agar (*Salmonelle*), Mannitol salt agar
318 (*Staphylococcus sp*), EMB agar (gram negative enteric bacilli). All media were prepared
319 according to the manufacturer's instructions.

320 **Nutrient agar (NA):** Nutrient Agar (NA) was prepared by suspending 28g in 1000 ml distilled
321 water into a conical flask. The conical flask was corked with cotton wool, boiled to dissolve and
322 autoclaved at 121°C , 15mins and 15psi. It was allowed to cool to around $45-50^{\circ}\text{C}$ and the poured

323 into sterile petri dishes and allowed to solidify. It was then dried in hot air oven before it was
324 inoculated with 0.1ml of the aliquot.

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326 **Eosin methylene agar (EMB):** It was prepared suspending 36g of EMB powder in 1000ml
327 distilled water into a conical flask. The conical flask was corked with cotton wool, boiled to
328 dissolve and autoclaved at 121⁰C, 15mins and 15psi. It was allowed to cool to around 45-50⁰ C
329 and then poured into sterile petri dishes and allow to solidify. It was then dried in hot air oven
330 before it was inoculated with 0.1ml of the aliquot.

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332 **Salmonella shigella agar (SSA):** It was prepared by suspending 38g of salmonella agar
333 (according to manufactures standard) in 1000ml distilled water into a sterile conical flask. The
334 conical flask was corked with cotton wool, boiled to dissolve and autoclaved at 121⁰ C, 15mins
335 and 15psi. It was allowed to cool to around 45-50⁰ C and then poured into a sterile petri dishes
336 and allow to solidify. It was then dried in hot air oven before it was inoculated with 0.1ml of the
337 aliquot.

338 **Mannitol Salt Agar (MSA):** It was prepared by suspending 111g of salmonella agar (according
339 to manufacturer's standard) in 1000ml distilled water into a sterile conical flask. The conical
340 flask was corked with cotton wool, boiled to dissolve and autoclaved at 121⁰ C, 15mins and
341 15psi. It was allowed to cool to around 45-50⁰ C and then poured into a sterile petri dishes and
342 allow to solidify. It was then dried in hot air oven before it was inoculate with 0.1ml of the
343 aliquot.

344 **Isolation and sub-culture:**

345 **Isolation:** The spread plate method was used to isolate the bacteria present. 0.1ml of 10³
346 serially diluted samples were pipetted onto the agar and spread with a bent glass rod. The plates
347 were incubated at 37⁰ C for 24 hours.

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

351 **Enumeration of bacteria count**

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

355 Colony forming unit = $\frac{\text{Number of colonies}}{\text{Dilution} \times \text{volume plated}}$
356

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358 Isolation of Fungi

359 Media Preparation

360 **SDA:** Sabouraud dextrose agar (SDA) was prepared according to manufacturer's standard (65g
361 in 1000ml), boiled to dissolve and autoclaved at 121⁰ C, 15mins and 15psi. It was allowed to
362 cool and 25mg of tetracycline was added to the media to suppress growth of bacteria.

363 Culture and sub-culture:

364 Isolation:

365 **Method 1:** The spread plate method was used to isolate the bacteria present 0.1ml of 10³ serially
366 diluted samples were pipetted onto the agar and spread with a bent glass rod. The plates were
367 incubated at 37⁰ C for 5-7 days.

368 **Method 2:** The spoilt part of the sample was sliced thinly using a scalpel and placed on a
369 solidified agar plate. The plates were incubated at 37⁰ C for 5-7days.

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

373 Identification of bacteria isolates

374 Colonial Morphology

375 A colony of the isolate was picked and streaked on the freshly prepared agar and
376 incubated at 37⁰C for 24 hours. After incubation, morphological features; shape, size,
377 colour, edge, texture and elevation of the colony of the isolate observed visually with
378 hand lens.

379 Cell morphology

380 Gram-stained slides were mounted on the microscope to observe the cell morphology. **3.5**

381 Biochemical Tests

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

383 Catalase Tests

384 This test was used to determine the ability of an organism to break down hydrogen peroxide
385 (H₂O₂) into oxygen and water. Only organisms that have the enzyme catalase can catalyze the
386 reaction. The presence of the enzyme in a bacterial cell is evident when a small inoculum was

387 introduced into a 3% hydrogen peroxide solution and rapid production of effervescence occurs.
388 The absence of catalase is evident by a lack of or weak production of effervescence.

389 **□ Citrate Test**

390 The citrate test was used to determine the ability of an organism to utilize sodium citrate as its
391 sole source of carbon and inorganic ammonium salt as its only source of nitrogen. Bacteria that
392 can grow on this medium turn the bromothymol blue indicator from green to blue. Simmon
393 citrate agar was prepared in a capped tube according to manufacturer's instruction. A sterile wire
394 loop was used to pick a loopful of the test organism and streaked on slant surface. The tube was
395 incubated at 37⁰C for 48h. Change in colour from green to blue was indicative of positive result
396 while no change in colour was indicative of a negative result.

397 **□ Indole Test**

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

400 A loopful of the test organism was inoculated into sterile peptone water medium and incubated at
401 37⁰C for 48h. Thereafter, 0.3-0.5ml of Kovac's reagent was added using a Pasteur's Pipette.
402 Appearance of red ring layer on medium was indicative of positive indole test while
403 development of a yellow ring was indicative of negative result.

404 **□ Methyl Red/Voges Proskauer (MR/VP) TEST MR VP**

405 This test was used to determine the ability of an organism to produce and maintain stable acid
406 end products from glucose fermentation and to determine the ability of some organisms to
407 produce neutral end products such as acetyl-methyl carbinol or acetoin from glucose
408 fermentation. The MR/VP broth medium is used for this test. A loopful of test organism is
409 inoculated into 10ml sterile MR/VP broth medium prepared according to manufacturer's
410 instructions. The tube was incubated at 35-37⁰C for 48h. After incubation, the broth culture was
411 shared into two equal parts (5ml). one part was used for methyl red test and the other part for
412 Voges Proskauer test.

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

416 □ **Motility Test**

417 This test was used to determine if an organism is motile. An organism must possess flagella (a
418 locomotory organelle) to be motile. Semi-solid nutrient agar was used for this test. Half strength
419 of the medium was prepared following manufacturer's direction. A young (fresh) colony was
420 picked with a sterile straight wire and inoculated by stabbing into the medium. Thereafter, the
421 tube incubated at 37⁰C for 24-48 h. Growth (in diffuse form) from line of stab into the medium
422 was indicative of positive result, whereas growth only along the line of stab was indicative of a
423 negative result.

424 □ **Oxidase Test**

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

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

432 □ **Triple Sugar Iron Agar (TSIA) test**

434 This test was used to determine whether the bacterial isolates could ferment glucose and lactose
435 or sucrose and form hydrogen sulfide (H₂S), gas and acids.

436 The triple sugar iron agar (TSIA) was used for this test. The slant was prepared according to
437 manufacturer's recommendation. To perform this test, a straight sterile inoculation needle was
438 used to pick the organism which was then inoculated by first stabbing the centre of the medium
439 (not to the bottom of the tube) and streaking the surface of the agar slant. Thereafter, the tubes
440 were incubated at 35-37⁰C for 24-48h. Change in colour of medium from pink to yellow
441 indicated acid production resulting from glucose fermentation. When in addition to glucose,
442 lactose and/or sucrose are fermented; the large amount of fermentation products formed on the
443 slant will neutralize the alkaline amines and render the slant acidic (yellow). Black colouration
444 indicated production of hydrogen sulfide (H₂S) and cracks in the medium or complete upward
445 shift of the agar slant from bottom of the tube indicates positive gas production.

446 □ **Sugar Fermentation**

447 This test was used to determine the ability of an organism to ferment a sugar to produce acid and
448 gas. Peptone broth (1%) incorporated with 1% sugar was used for the test. Bromocresol purple
449 (0.3%) indicator was added to the sugar medium with Durham's tube added in the tube in

450 inverted (upside down) position. After sterilization, a loopful of test tubes were then incubated at
451 35-37⁰C for 24-48 h. Change in colour from purple to yellow and gas production (collected in the
452 inverted Durham's tube) constitute positive sugar fermentation test. Production of acid resulted
453 in a change in colour of the medium from purple to yellow while acid production is detected in
454 the inverted Durham's tube.

455 456 **Method of Data Analysis**

457 The data was analyzed using statistical software, such as SPSS or R, to ensure accurate and
458 reliable results. The results will be presented in tables, figures, and graphs to facilitate easy
459 understanding and interpretation.

460 **RESULTS**

461 **Enumeration of the Bacteria and Fungi Load in the Zobo Drink**

462 The result in Table 1 shows the bacterial and fungal load in the Zobo drink samples.
463 Heterotrophic bacteria on nutrient agar media showed a mean count range of 1.32 x 10⁶ to 4.56 x
464 10⁶cfu/lm present in the zobo drink while a heterotrophic mean count range of 2.4 x 10⁵cfu/ml to
465 3.5 x 10⁵cfu/ml were noted/reported.

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

Zobo Drink Sample	Total Heterotrophic Bacterial Count (CFU/ml)	Total Heterotrophic (CFU/ml)	Fungal Count
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	

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

468 **Prevalence of Bacteria in the Zobo Drink Sample**

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

471 The bacteria, *Lactobacillus* sp. and *Bacillus* sp. were noted in the zobo drink with a percentage
472 occurrence of 25 each, while *Streptococcus lactic* *Pseudomonas* sp., *Escherichia coli* and

473 *Klebsiella* sp. had a 8 percentage occurrence each in the zobo drink. Consequently,
474 *Staphylococcus* sp. had a 18 percentage occurrence.

475 **Table 2 Prevalence of Bacteria in the Zobo Drink Sample**

Bacteria	Frequency of Occurrence	Percentage Occurrence
<i>Lactobacillus</i> sp	3	25
<i>Streptococcus lactic</i>	1	8
<i>Bacillus</i> sp	3	25
<i>Pseudomonas</i> sp	1	8
<i>Staphylococcus</i> sp	2	18
<i>Escherichia coli</i>	1	8
<i>Klebsiella</i> sp.	1	8

476

477 **Characterization of Bacterial Isolates**

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

481 *Escherichia coli* and *Staphylococcus* sp.

482 Biochemical reaction characterized the bacterial isolates, whereby *Bacillus subtilis* showed
 483 positive reaction to catalase, oxidase, citrate, spore formation, glucose production, lactose,
 484 sucrose and mannitol fermentation however negative reaction was noted for indole reaction,
 485 urease production, gas and vogesproskeraur in their respective reactions.

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

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

494

495

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	+	+	+		+				+	+	+	+	+	<i>Bacillus substilis</i>
	+		+			+	+		+	+	+	+	+	<i>Staphylococcus sp.</i>
2	+			+		+		+		+	+			<i>Escherichia coli</i>
3	+		+				+	+	+	+	+	+	+	<i>Klebsiella sp</i>
	+	+							+	+				<i>Pseudomonas aeriginosa</i>
										+	+	+	+	<i>Lactobacillus acidophilus</i>
										+	+	+	+	<i>Streptococcus lactis</i>

496

Key:

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

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499

38

500 **Frequency of Fungal Occurrence**

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

503 The fungi, *Aspergillusniger*. were noted in the zobo drink with a percentage occurrence of 22 ,
504 and the following isolates *Rhizopus* sp, *Candida* sp and *Sacchromycessp*. at a percentage
505 occurrence of 12, 33 and 33 respectively.

506 **Table 4 :Frequency of Fungal Occurrence**

Fungal	Frequency of occurrence	Percentage occurrence
<i>Aspergillusniger</i>	2	22
<i>Rhizopus</i> sp	1	12
<i>Candida</i> sp	3	33
<i>Sacchromycessp</i>	3	33

507

508 **Colonial/ Macroscopic Characterization of the Isolated Fungi**

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

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

518 **Table 5 Macroscopic Characterization of the Fungi Isolate**

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

519 Conclusion

520
521 The study identified a high prevalence of microbes in Zobo drinks. It therefore noted the
522 incidence of lactobacillus sp, *Streptococcus lactis*, *Candida sp.*, *Saccharomyces sp. Bacillssp.*,
523 *Pseudomonas sp. Staphylococcus sp Escherichia coli*, *Klebsiella sp.*, *Aspergillus niger* and
524 *Rhizopus sp.* in the Zobo drink could constitute a public health hazard, where the drink can result
525 in outbreaks of diseases, gastroenteritis and diarrhoea.

526 Recommendation

527
528
529 The study advises that consumers and retailers of zobo drink be vigilant and observant of the root
530 source of products and therefore encourage producers to maintain absolute cleanliness and keep
531 to standard hygiene practices.

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