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PREVALENCE OF MICROBES IN EXPIRED CANNED BEVERAGES SPOTTED IN SOME SUPERMARKETS IN PORT HARCOURT

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Abstract: With the continuous presence of expired can beverages in the market space, the determination of microbial prevalence following increased cases of fungal diseases have become paramount. One hundred (100) canned beverage samples (expired and non-expired) were spotted and analyzed for microbial density, heavy metal concentration and proximate analysis using standard microbiological procedure. The result showed no pseudomomad counts. However, growth was observed for Staphylococcal, Acetobacter, and heterotrophic bacteria/fungi. Heterotrophic bacteria reported 20 x 10^2 and 7 x 10^2 CFU/ml for expired and non-expired beverage respectively. Similarly, Staphylococcal counts of 1 x 10^2 and 0 x 10^2 CFU/ml were noted for expired and non-expired beverage respectively. Acetobacter reported counts of 6 x 10^2 CFU/ml for expired beverage while non-expired beverage showed 5 x 10^2 CFU/ml counts. Heterotrophic fungi were noted with 10 x 10^2 CFU/ml counts for expired beverage and non-expired beverage showed 4 x 10^2 CFU/ml counts. Statistically, the microbial counts obtained were not significant in both expired and non-expired. A total of 6 microbes, 3 fungal and 3 bacteria genera were obtained. The fungal genera and percentage prevalence were: *Penicillin* sp (35%), *Mucor* sp. (20%) and *Aspergillus* sp. (45%). Similarly, the bacteria and percentage prevalence were: *Acetobacter aceti* (29%), *Proteus* sp. (43%) and *Staphylococcus aureus* (29%). The heavy metal and proximate compositions of the beverage samples showed cadmium was not detected. Consequently, the metals (zinc, magnesium, calcium, iron) and proximate (moisture, crude protein, crude lipid, ash, fiber and carbohydrate) compositions were significantly not different in the samples. Thus, the study identified high prevalence of *Proteus* sp. and *Aspergillus* sp. in expired beverage samples that is significant, and have the capacity to disease outbreak following consumers' negligence on expiry date. The study therefore advice consumers to be vigilant and observant on purchase as *Proteus* sp. and *Aspergillus* sp. have been reported with high prevalence.
Keywords: Canned beverages; Expired; Microbes; Port Harcourt; Supermarkets

1 INTRODUCTION

The report of expired canned beverages in some market space specifically supermarket have become worrisome [1]. Microbes have been associated with food product were they produce large dose of toxins, biofilms, foam, sediments and off-flavored taste [2]. Microbes digest food substrates and in the process proliferate to large doses. High prevalence of microbes in beer have been reportedly identified [3]. Hence, the prevalence of microbes has become necessary due to the increased or rising cases of fungal disease which has heat nearly a billion people [4]. Beverages provide essential nutrients that the body needs. It is consumed mostly to hydrate the body, hence maintain the fluid level of the body. Expired product could be toxic to consumers and death may arise [5]. Juice, flavoured drinks, water and many more beverages have been associated with expiration [6]. The harmful effect of the consumption of expired food consumables includes development of diarrhea, digestive issue, nausea, all of which are caused by pathogenic microbes. Prior, to consumption, Beverages or foods are processed in can packaged material to prevent the proliferation of microorganisms [1]. Consequently, when expired, the food additives are inactive. Additives such as hop extract whose function is antifungal becomes functionless and thus the fungal breeds to heavy density [7]. Additionally, the expired state is associated with changed water activity and acidic levels amongst many other factors. The water activity allows the proliferation of microbes as in the isolation of *Acetobacter aceti, Acetoacter cerevisiae, Acetobacter fabarum* etc[7]. Thus, microbial increase due to acidic levels may influence microbial proliferation. [6], were some microbes specifically lactic acid bacteria and *lactobacillus* are commonly associated with soured items/food materials [8]. Following all these challenges, the proximate and heavy metal compositions of the drink are considered factors to be investigated as they could have imparted on microbial load with respect to the package in cans made of steel [9]. The significance of the study therefore serves as an early warning sign to consumers and hence informed her of the prevalence of microbes with respect to associated infections in the public space.

2 MATERIALS AND METHODS

2.1 Study Area/ Study Design

The study area, Oroekpo, Rumuepirikom Community of Port Harcourt, Nigeria is associated with the sale of expired canned beverage. The area is a densely populated with shops identified in strategic points along the street. Hence, a probability exists of identifying an expired product in every ten sales points.

2.1.1 Beverage sample purchase/collection

One hundred (100) canned beverage samples (expired and non-expired) were purchased/collected from sales point within the study area. The beverage sample were clearly identified expired from the printed on the can and then transferred to the Biology Laboratory of Ignatius Ajuru University of Education, Port Harcourt.

2.1.2 Preparation of the beverage samples

The beverage samples were serially diluted, to a dilution factor of 10^{-2} by a process, were one (1) mill of the sample was transferred into a nine (9) mill normal saline to get a 10^1 dilution, and thereafter, one (1) mill again from the 10^1 dilution transferred to another nine (9) mill normal saline to get a 10^2 dilution needed for the study. The 10-2dilution is hereby adopted to recover the least microbial growth within the range of 30 and 300 coliform forming unit (CFU) on freshly prepared media [10].

2.1.3 Media preparation

Nutrient agar, a media for heterotrophic bacteria growth, sabouraud dextrose agar (for isolation and growth of fungi), mannitol salt agar (for isolation of *Staphylococcus* spp.), centrimede agar (for growth of *Pseudomonas* spp), and glucose-yeast extract-calcium carbonate (GYC) agar (for growth of *Acetobacter* spp.) were weighed, allowed to dissolved in a measured distilled water in a conical flask, cocked properly and autoclaved according to the manufacturer instructions. The agar at the end of autoclaving were allowed to cool before dispensing into a sterile petri dish for sample inoculation exercise [11].

2.2 Microbial Load in the Beverage Samples (Expired and Non-Expired)

The isolation of viable microbial cells in the samples involved the use of spread plate technique, were a glass spreader was used to spread 0.1ml inoculum of 10^2 dilutions around the freshly prepared media, thereafter the media incubated at a temperature of 350C for 24 hours' bacteria cell growth and incubation at 25oC for 5 day fungi growth. The number of colonies/growth recovered were counted and reported as colony forming unit per mill (cfu/ml) [12].

2.2.1 Colonial and morphological characterization of the isolates

Colonial characterization of the isolates involved macroscopic description of the colony appearance for the bacterial and fungal isolates. Features such as; the color, shape, size, elevation and opacity of the colonies were observed in details. Morphological features involved determination of the motility of the isolate and gram staining procedure were necessary [13].

2.2.2 Motility test

This test determined if the isolates recovered were with flagella (a moveable structure). In carrying out the test, a semi solid nutrient media (in a tube) was prepared and with the aid of a sterile straight needle used to pick a colony and inoculate on the medium by stabbing. The tube was then incubated at 370C for 24 - 48 hrs. Growth in diffused form, from the line of stab into the medium indicated a positive result (presence of flagella), whereas growth only along the line of stab indicated a negative result (absence of flagella) [13].

2.2.3 Gram staining

The Gram stain screening was carried out to identify the isolates response to stains. This involved heat fix of the isolate on a glass slide followed by application of crystal violet, iodine, ethanol and safranin independently at different time interval with a rinse of water each time accordingly, the slides after all processes were viewed under a microscope. A view of pink indicated a Gram negative isolate while purple indicated Gram positive isolate [13].

2.2.4 Biochemical properties of the bacteria isolates

The isolates were further identified and classed, from where the isolates were inoculated into several biochemical test reagents. The mannitol salt test, coagulase test, indole test, capsule formation test, catalase test, lactose test and Urea test were all employed [14].

2.2.5 Mannitol fermentation test

The test involved preparation of a broth, a liquid media, by adding, 1% of mannitol, peptone agar and litmus red indicator reagent to constitute the broth. Sterilization of the broth followed before the test isolate was inoculated into it. The inoculated broth, now culture was then incubated at 370C for 18 - 24 hours. Change in color of the culture from red to yellow indicated mannitol fermentation [14].

***2.2.6* Coagulase test**

The test involved introducing a loopful of the isolate into a clean glass slide followed by the addition of a rabbit plasma and the component stirred. After 20 seconds, clump appearance indicated a coagulase positive reaction while a non-coagulase reaction was indicated by no clump formed [14].

2.2.7 *Indole test*

The indole test employed the use of a peptone reagent from which the medium was prepared. A loop-full of the test organism was inoculated into sterile peptone water medium and incubated at 370C for 48 hours. Thereafter, 0.3 – 0.5 ml of

Kovac's reagent was added using a Pasteur's pipette. A red ring layer, observed on the medium indicated a positive indole test while a yellow ring color indicated a negative result [14].

2.2.8 ***Capsule stain***

The capsule stain involved the introduction of some drops of crystal violet onto a clean slide, followed by the introduction of the test organism onto crystal violet slide. Both components were stirred and thereafter viewed under a light microscope. A light blue appearance signified the test cell possess capsule while an absence of a light blue appearance indicated an absence of capsule [14].

2.2.9 Catalase test

The test involved the transfer of a loop-full of the isolate into a clean glass slide. Thereafter, Hydrogen Peroxide was introduced onto the slide and the components stirred to observe the breakdown Hydrogen Peroxide into Oxygen and Water. Rapid production of effervescence indicated the enzyme catalase while the absence of catalase was indicative of weak effervescence [14].

2.2.10 **Lactose test**

The test involved preparation of lactose broth which is composed of 1% lactose, peptone agar and a litmus red indicator reagent. A Durham's tube was placed in an inverted position inside a test tube. thereafter, a loopful of the isolate was then inoculated into the broth and incubated at 370C for 18 - 24 hours. Change in color of the broth culture from red to yellow and a gas inside the Durham's tube indicated a positive lactose whereas a reverse is indicative of negative reaction [14].

2.2.11 **Urease test**

The urease test, was carried out with the aid of the urease agar media. The media was prepared, dispensed into a test tube and sterilized. Thereafter the isolate was introduced into the medium. A change in color from pink to yellow indicates the isolate could utilize the urease while [14].

2.3 Determination of Trace Element in the Samples

The metals (Cadmium (Cd), Zinc (Zn), Magnesium(Mg), Calcium (Ca) and Iron(Fe)) were analyzed for; to determine their composition before and after expiry of the beverage samples. Samples were analyzed using atomic absorption spectrophotometer (ASS) of UNICAM 919 model. Where the beverage which appears to be in suspension was placed in a flask and digested by heating for 20 minutes in the presence of acid. The flask and its content was then cooled for 30 minutes, filtered to get a filtrate, which was then transferred into a 50ml volumetric flask. Deionized water was added to the filtrate to make 50ml volume. And the volume injected one after the other into the Atomic Absorption Spectrometry device and allowed to run, the heavy metals were separated in their scale and identified accordingly. The results from the atomic absorption spectrometry machine was then expressed in mg/kg [15].

2.4 Determination of Proximate Composition in the Samples

The proximate analysis of the beverage was done to determine the major constituent present in the beverage before and after expiration. The analysis determines the safety of the beverage with respect to crude protein, moisture, lipids, total ash, crude fibre and carbohydrate contents in the beverage before and after expiration etc. all major constituent of the beverages were determined with the spectrophotometric method [14].

2.5 Statistical Analysis

The data recovered were analyzed using statistical analysis system wherein the mean/average of the microbial load and also percentage occurrence of the isolates were determined.

3 RESULTS

3.1 Enumeration of Microbial Population on the Culture Media from Beverages (Expired and Non- Expired)

The result in Table 1 showed the microbial load in expired and non-expired drink samples. Centrimede media showed no counts for both expired and non- expired beverage samples. However, growth was observed in mannitol salt media, GYC medium, SDA medium and Nutrient medium. Nutrient agar media showed counts of 20 x 102 and 7 x 102 CFU/ml for expired and non-expired beverage respectively. Similarly, counts of 1 x 102 and 0 x 102 CFU/ml were noted for expired and non-expired beverage respectively on mannitol salt medium. GYC medium showed 6 x 102 CFU/ml count for expired beverage and non-expired beverage showed 5 x 102 CFU/ml. SDA media noted 10 x 102 CFU/ml counts for expired beverage and non-expired beverage showed 4 x 102 CFU/ml counts. Statistically, the fungi and heterotrophic fungal counts obtained were in significant in both expired and non-expired.

Table 1 Mean Counts of Microbes in Culture Media from the Beverages

Media	Expired (CFU/ml)	Non-Expired (CFU/ml)	p= 0.05
Centrimed	0	0	p>0.05

Mannitol Salt	1 x 10^2	0 x 10^2	P>0.05
GYC Medium	6 x 10^2	5 x 10^2	p>0.05
SDA	10 x 10^2	4 x 10^2	P<0.05
Nutrient	20 x 10^2	7 x 10^2	P<0.05

Note, CFU= Coliform Forming Unit

3.2 Colonial Characterization of the Bacteria Isolates Recovered

Table 2 showed the colonial appearance of the isolates on the media plates. The nutrient agar plate showed fishy smell, colorless colonies, opaque colonies while the GYC media showed colorless colonies that were smooth, round and small. The mannitol salt media plates showed golden yellowed colonies.

Table 2 Colonial Characterization of the Bacteria Isolates Recovered

Media Plates	Color	Opacity	Edge	Elevation	Surface	Shape	Size
Nutrient Media	Golden Yellow	Opaque	Entire	Convex	Smooth	Round	Small
GYC Media	Colorless	Opaque	Round	Convex	Smooth	Round	Small
Mannitol Salt Media	Greyish White	Opaque	Round	Low Convex	Smooth	Round	Large

3.3 Colonial/ Macroscopic Characterization of the Isolated Fungi

Macroscopic characterization of the fungi isolates as recovered showed in Table 3 denoted that three fungi were recovered from both beverage samples. The fungi *Mucor* sp. were noted whitish in colour with a large size and cottony texture. *Penicillium* sp. were observed with a tint of greenish-blue appearance and a powdery textured surface. The fungi *Aspergillus* sp. appeared with shades of yellow-green in color with a small sized colony.

Table 3 Macroscopic Characterization of the Fungi Isolates Recovered

Isolates	Structural description				Identification
	Colour	Size/shape	Growth rate	Texture	
1	Whitish	Large/Circular	Slow	Cottony/woolly	*Mucor* sp.
2	Blue-green	Small/Circular	Fast	Powdery	*Penicillium* sp.
3	Yellow	Small	Rapid	Powdery	*Aspergillus sp.*

3.4 Biochemical Characterization of Bacterial Isolates

Table 4 showed the biochemical features of three bacteria genera isolated from the sample. *Staphylococcus aureus* was identified with a positive Gram reaction while *Acetobacter aceti* and *Proteus*. were identified with a positive Gram reaction. In further characterization of the bacteria, Acetobacter aceti was noted positive for motility and catalase while indole, motility and urease were positive for *Proteus* sp. A Gram positive reaction was reported for *Staphylococcus aureus*. The isolate, *Staphylococcus aureus* was further identified by a positive coagulase, mannitol and catalase test.

Table 4 Biochemical Characterization of Bacterial Isolates

Mann	Moti	Coa	Indo	Cap	Cata	Urea	Lac	Gram Stain	Bacteria
	+	-	-	-	+	-	+	-	*Acetobacter aceti*
+	-	+	-	-	+	-	+	+	*Staphylococcus aureus*
-	+	-	+	-	-	+	-	-	*Proteus* sp.

Keys: Mann= Mannitol Fermentation, **Moti**= Motility, **Coa**= Coagulase, **Indo**=Indole, **Cap**=capsule formation, **Cata**=Catalase, **Lac**=Lactose

3.5 Frequency of Bacteria Prevalence

Table 5 showed the percentage frequency of occurrence of the bacterial isolates recovered from the expired and non-expired beverage. The bacteria, *Staphylococcus aureus* were noted in expired drink with a percentage frequency of 29 and in non-expired beverage with a percentage frequency of 14. The bacterial isolates *Acetobacter aceti* and *Proteus* sp. had 29 and 43

percentage frequencies respectively for expired beverages while non-expired had 29 and 57 percentages for *Acetobacter aceti* and *Proteus* sp. respectively.

Table 5 Bacterial Prevalence Across the Beverages

Media	Frequency of Isolates in Non- Expired Beverage	% Frequency	Frequency of Isolates in Expired Beverages	% Frequency
Staphylococcus sp.	1	14	2	29
Acetobacter aceti	2	29	2	29
Proteus sp.	4	57	3	43

3.6 Frequency of Fungal Prevalence

Table 6 showed The percentage frequency of occurrence of the fungal isolates recovered from the expired and non-expired beverage. The fungal, *Mucor* sp. were noted in expired drink with a percentage frequency of 20 and in non-expired beverage with a percentage frequency of 21. The fungal isolates *Penicillium* sp and *Aspergillus* sp. had 35 and 45 percentage frequencies respectively for expired beverages while non-expired had 29 and 50 percentages for *Penicillium* sp. and *Aspergillus* sp. respectively.

Table 6 Fungal Prevalence Across the Beverages

Media	Frequency (Non- Expired)	% Frequency	Frequency (Expired)	% Frequency
Mucor sp	3	21	4	20
Penicilium sp.	4	29	7	35
Aspergillus sp.	7	50	9	45

3.7 Heavy Metal and Proximate Composition of Expired and Non Expired Beverages

Table 7 showed the heavy metal and proximate composition of expired and non-expired beverage samples. cadmium was not detected in either of the test samples. Consequently, the metals (zinc, magnesium, calcium, iron) and proximate (moisture, crude protein, crude lipid, ash, fiber and carbohydrate) composition in the beverage samples were significantly not different in both expired and non-expired beverages.

Table 7 Heavy Metal and Proximate Composition of Expired and Non Expired Beverages

Parameters	Units	Expired	Non-Expired	Permissible limit
CD	Mg/L	ND	ND	0.003
Zn	Mg/L	3.19	3.56	5 mg/L
Mg	Mg/L	14.73	14.73	50 mg/L
Ca	Mg/L	59	54	50mg/L
Fe	Mg/L	0.5	0.4	0.3mg/L
Moisture	g/100Ml	0.68	0.61	0.5 g/100Ml
Crude Protein	g/100Ml	0.59	0.55	0.5 g/100Ml
Crude lipid	g/100Ml	0.1	0.1	0.5 g/100mL
Total ash	g/100Ml	526	521	500 g/100mL
Crude Fibre	g/100Ml	0.65	0.61	0.5 g/100mL
Carbohydrate	g/100Ml	12.8	12.8	10 g/100Ml

Key: CD= Cadmium, Zn= Zinc, Mg=Magnesium, Ca=Calcium, Fe = iron

4 DISCUSSION

The increased fungal count in expired canned beverage over the non-expired may be attributed to the extreme physiochemical condition [16] which the expired drink portrays. Fungi in an extreme physiochemical condition is noted to proliferate than normal hence the expired beverage provides this favorable condition. Similarly, with respect to the increased heterotrophic bacteria load in canned food were expiration was indicated [17]. The canned beverages are of low acid content and hence could encourage the growth of spore-forming bacteria [18], however, *Clostridium* sp., a spore former was not investigated. Canned food of low acid (pH>4.5) guarantees acceptable levels of bacteria unlike high acid (pH<4.5) [18]. The bacteria namely, *Staphylococcus aureus, Acetobacter aceti* and *Proteus* sp. as identified in this study is not in accordance [19] with studies on Kunu drink, a Nigerian local beverage. The isolation and identification of *Proteus* sp. in this study with high prevalence, have also been noted high [20]. Although, it is reported that, the bacteria in the Kunu drink was attributed to poor hygiene and preparation practices. Consequently, canned beverages can be associated with indirect

contamination if not properly or adequately cleaned or sterilized before use [21]. With reference to the fungal recovered in the present study, yeast was totally not recovered. Fungi such as *Saccharomyces* sp. plays a crucial role in the brewing industry [20]. As important as *Saccharomyces* spp. are, the yeast was not recovered in both expired and non-expired beverages. Basically, the presence of *Saccharomyces* sp. indicates fermentation property [22]. The fungi, *Saccharomyces* sp. is able to carry out fermentation, an anaerobic process in which sugars are converted into alcohol and carbon dioxide [23]. The yeast unrecoverable status in the beverages were questionable, whereas, high prevalence of *Aspergillus* sp was noted/recovered. The high prevalence of *Aspergillus* sp. may be due to the sugar content of the beverage, wherein the fungi are able to withstand competition from other indigenous microorganisms which resulted to higher growth rate [24]. The proximate and metal concentrations before and after the drink expired had no significant influence on the microbial prevalence and population. Hence, the microbes may have considered the permissible condition at which the metal and proximate concentrations were established, before and after expiry [25]. Although, the presence of Cadmium would have changed the population of microbes, consequently, Cadmium was absent before and after expiry. Cadmium is reported to inhibit microbial population even at less concentration. Basically, cadmium has been reported to have negative effect on human health, its presence would have not been tolerated [26].

5 CONCLUSION

The study identified high prevalence of *Proteus* sp and *Aspergillus* sp. in the expired beverage samples. The prevalence of these microbes is significant as they have the capacity to cause outbreak of disease following the inability of consumers to check expiry date before purchase. It is also noted the incidence of *Mucor* sp. *Staphylococcus aureus*, *Acetobacter aceti* and *Penicillium* sp. could constitutes public health hazard.

6 RECOMMENDATION

The study advice that consumers to be vigilant and observant on purchase as *Proteus* sp. and *Aspergillus* sp. have been reported with high prevalence.

COMPETING INTERESTS

The authors have no relevant financial or non-financial interests to disclose.

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