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Antibiogram of bacteria and fungi associated with surfaces of canned beverages within a tertiary institution in port Harcourt, Rivers State

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Abstract
Beverage cans are exposed to various environments during production, storage and shipping during which it may be contaminated with microorganism. This study assesses the presence of microorganisms on the surface of canned beverages sold in a tertiary Institution in Port Harcourt, Rivers State. Swab samples were aseptically collected from the surfaces of canned beverages at five different retail outlets within the institution. Organisms were enumerated and identified using standard microbiological techniques. Kirby Bauer disc method was used for antibiotic sensitivity. Mean Total heterotrophic bacterial counts ranged from 1.03±0.02 to 3.8±0.14 CFU/cm^2 while mean total fungal counts ranged from 1.2±0.14 to 7.5±0.7 CFU/cm^2. Ten (10) Bacteria isolates identified and their percentage occurrence were; *Pseudomonas* sp. 1(10%), *Bacillus* sp. 2(20%), *Enterobacter* sp. 1(10%). *Staphylococcus* sp. 2(20%), *Enterococcus* sp. 1(10%), *Micrococcus* sp. 1(10%), *Streptococcus* sp. 2(20%), while seven (7) fungal isolates were; *Candida albicans* 2(18.2%), *Trichoderma* sp. 1(9.1%), *Penicillium* sp. 3(27.3%), *Mucor* sp. 3(18.2%), *Aspergillus* sp. 3(27.3%). Percentage occurrence from bacterial isolates showed that *Streptococcus* sp., *Staphylococcus* sp. and *Bacillus* sp. were the most dominant bacterial specie isolated while *Asergillus* sp. and *Penicillium* sp. were the predominant fungi isolated in all samples. Sensitivity results showed that *Bacillus* sp. was more susceptible to Levofloxacin, Gentamycin, Ciprofloxacin, Septrin and showed more resistance to Ampiclox, Streptomycin and Amoxil. *Staphylococcus* sp. was more susceptible to Levofloxacin, Chloramphenicol, Septrin, Streptomycin and showed more resistance to Gentamycin, Amoxil and Ampiclox. *Enterococcus* was susceptible to Ampiclox, Erythromycin, Gentamycin and resistant to Levofloxacin, Ciprofloxacin and Septrin. *Pseudomonas* sp. was more susceptible to Augmentin, Ciproflox, Streptomycin, Nalidixic acid and was resistant to Ampicilin, Tarivid and Gentamycin. The presence of these antibiotic resistant organisms on surfaces of canned beverages poses public health risks. Washing of canned drinks surfaces is likely to minimize the occurrence these microbes from the can surfaces and improved hygiene and handling by vendors is recommended. Usage of straws instead of drinking directly from the cans is also recommended and could minimize potential public health outbreaks.

Keywords: Canned beverages, microorganisms, antibiotic resistance, tertiary institution

Introduction
Beverages such as canned and bottled energy drinks, alcoholic beverages, juice and water are currently gaining popularity in the food industry. They are usually processed and transported to consumers under different handling conditions and could be consumed by many with little or no regard to the sentry condition of the orifice or opening of the can (Adem and Muktar 2020) [1].

Beverages cans are widely made for convenience and they are exposed to various environments during production, storage and shipping which may lead to the can being contaminated with a wide range of microorganisms thus when drinking from a can, an individual's mouth comes in direct contact with the can lid (Orifice) allowing possible transfer of microorganisms.

Canned beverages have one of the highest levels of contamination for a variety of surfaces humans are exposed to on a daily basis (Jiang *et al.*, 2002) [9]. According to (Cunningham *et al.*, 2011) [5], even though the risks of having dirty cans are quite different from food cans, there is a general abhorrence to drinking out of a can with a visibly dirty top, many people use a perfunctory to wipe of cans with paper products rather than rinsing or washing with soap and water (Michaels *et al.*, 2003) [11].

Despite the cleanliness of the surface of canned drink it cannot guarantee the absence of microbes. Adequate washing of any canned drink irrespective of whether it is from refrigerator or not will reduce or eliminate these microbes from the canned surfaces.

Moreover, studies have shown that certain bacterial and fungal species of public health concern can survive refrigeration temperature (Gündüz *et al.*, 2019) [8] and microorganisms present on the surface of beverage cans, may not cause diseases directly, but could be an opportunistic pathogen once ingested into the human body and may cause serious diseases such as respiratory and urinary tract infections, and tuberculosis among others (Gündüz *et al.*, 2019) [8]. This research is aimed at determining the bacterial and fungal isolates associated with surface of canned beverages sold in a tertiary institution, in Port Harcourt.

Materials and Methods

Description of Study Area

The study was carried out in a tertiary institution, in Port Harcourt City. Rivers State, Nigeria. The samples were obtained from five different locations by rotating a moist swap stick on the orifices and surfaces of the different beverage cans.

Sample Collection

Beverage can samples were bought from different locations within the tertiary institution, labelled and transported immediately in an ice pack to the Microbiology Laboratory in the institution, for microbiological analysis. Samples were collected by swabbing the surfaces and orifices of the beverage cans

Sample Preparation

The two-fold serial dilution was adopted. In this method, a sterile swab was moistened with normal saline and used to swab the surfaces and orifices of the beverage cans and transferred into test tubes containing sterile 2ml diluent (Normal saline) which gave an initial diluent of 1:2ml. Subsequent dilutions were carried out by transferring 1ml from the initial dilution to other test tubes containing 1ml sterile diluent. This was repeated until a dilution of $1:2^{-4}$ was reached (Ogbonna *et al*., 2022) [12].

Enumeration of Bacteria and Fungi

The total heterotrophic bacterial, and fungal load on the different cans were enumerated using standard plate count (Prescott *et al.*, 2011) [13]. After the serial dilutions, aliquot of 2^{-3} and 2^{-4} dilutions were transferred in duplicates into prepared Nutrient agar and Mannitol Salt agar, aliquot of 2^{-2} and 2^{-4} were transferred in duplicate on prepared Sabouraud Dextrose agar which had been fortified with tetracyclin antibiotics for the inhibition of bacterial growth (Ogbonna *et al*., 2022) [12]. Plates were incubated at 37ºC for 24 – 48 hours for the Nutrient Agar and Mannitol Salt Agar. Sabouraud Dextrose Agar was incubated at 37ºC for 5 days. After incubation, plates were observed for microbial growth. Emerging colonies on Nutrient Agar, Mannitol Salt Agar and Sabouraud Dextrose Agar plate were counted and discrete colonies were sub-cultured onto sterile nutrient agar and Sabouraud Dextrose Agar plates to obtain pure isolates (Cheesebrough, 2000) [2].

Identification of Bacterial and Fungal Isolates

The pure bacteria isolates were characterized by Gram's staining and Biochemical tests such as catalase test, indole test, methyl red test, citrate test, coagulase test, Voges Proskauer test and sugar fermentation tests. Identity of the isolates was matched with the Bergy's Manual of Determinative Bacteriology and ABIS online for confirmation. Fungal Isolates were identified using their morphological features such as colony color, shape, texture and size of colony followed by microscopic examination (Conidial shape, arrangement of hyphae and type of spore) of their wet mounts prepared with lactophenol cotton blue. The results were later compared by referencing fungal characteristics in the book of fungi identification manual (Sarah *et al*., 2016) [14].

Antibiotics sensitivity test

The disk diffusion method of antibiotics testing according to the Clinical laboratory standard institute. Twenty-four (24) hours old culture were standardized using the 0.5 McFarland standard (CLSI, 2020). This was done by matching the turbidity of the isolates in sterile 4mL normal saline to the 0.5McFarland standard. Sterile swab sticks were then dipped into the standardized isolates and swabbed uniformly on the surface of the dried Mueller-Hinton agar plates. The bacterial isolates were tested against already prepared commercial antibiotics: Ciproflox (10µg), Augmentin (30µg), Tarivid (10µg), Streptomycin (30µg), Reflacine (10µg) Nalididixic Acid (30µg), Ceporex (10µg), Septrin (30µg), Norfloxacin (10µg), Levofloxacin (20µg), Ampiclox (20µg) Chloramphenicol (30µg), Amoxil (20µg), Rifampicin (20µg), Erythromycin (30µg) and Ampicilin (30µg). The plates were held at room temperature for 3-5mins to allow drying. The antibiotics discs were placed on the plates, and the plates were incubated for 18-24 hours at 37 ºC. The diameters of zone of inhibition were recorded to millimeter and classified as resistant (R), intermediate (I) and susceptible (S) according to published interpretive chart (CLSI, 2020) [4].

Results

Results of the microbial counts from the various canned beverages is as presented in Table 1. Results of the total heterotrophic bacterial counts ranged from 1.03±0.02 to 3.8±0.14 $x10^4$ CFU/cm^2 and the total fungal counts ranged from 1.2±0.14 to 7.5±0.7 $x10^3$ CFU/cm^2 for the different cans respectively. Results also showed that the microbial counts in the different beverages varied. The total heterotrophic bacterial and fungal counts from the various can surfaces, showed significant differences ($p \geq 0.05$). Identified bacterial isolates associated with the different beverage cans include; *Bacillus* sp., *Streptococcus* sp., *Enterobacter* sp., *Staphylococcus* sp., *Micrococcus* sp., *Pseudomonas* sp. and *Enterococcus* sp. The fungal isolates were *Mucor* sp., *Aspergillus* sp., *Mucor* sp. *Penicillium* sp., *Trichoderma* sp. *Rhizopus* sp. and *Candida albicans.* Results showing the percentage distribution of bacterial isolates from the beverage cans is presented in Figure and are as follows; *Bacillus* sp., (20%), *Streptococcus* sp. (20%), *Enterobacter* sp. (10%), *Staphylococcus* sp., (20%), *Micrococcus* sp. (10%), *Pseudomonas* sp. (10%) and *Enterococcus* sp. (10%). Results show that *Bacillus* sp., *Streptococcus* sp. and *Staphylococcus* sp. were the most distributed bacterial isolates while *Enterobacter* sp.,

Micrococcus sp. and *Pseudomonas* sp. were the least distributed isolates amongst the study cans. The percentage distribution of fungal isolates is as presented in Figure 1. *Candida albicans* (28.6%) and *Mucor* sp. (28.6%) were the most predominant fungal isolates followed by *Trichophyton* sp. (14.3%), *Penicillium* sp. (14.3%), and *Aspergillus* spp. (14.2%).

Results of the susceptibility pattern of Gram-positive and Gram-negative bacterial Isolates is as presented in Table 1, 2 and 3 respectively. Results showed that *Bacillus* sp. was more susceptible to Levofloxacin, Gentamycin, Ciprofloxacin, Septrin and showed more resistance to Ampiclox, Streptomycin and Amoxil. *Staphylococcus* sp. was more susceptible to Levofloxacin, Chloramphenicol, Septrin, Streptomycin and showed more resistance to Gentamycin, Amoxil and Ampiclox. *Enterococcus* was susceptible to Ampiclox, Erythromycin, Gentamycin and resistant to Levofloxacin, Ciprofloxacin and Septrin. *Pseudomonas* sp. was more susceptible to Augmentin, Ciproflox, Streptomycin, Nalidixic acid and was resistant to Ampicilin, Tarivid and Gentamycin.

Table 1: Mean Microbial population (Cfu/cm^2) of Beverage Cans

Samples	THB (x10^{-4})	TFC (x10^{-3})
N1: Heneiken	1.9±0.07^{d}	2.8±0.14 ^{d}
N2: Grand	3.1±0.07^{b}	1.2±0.14^{e}
N3: Hero	3.8±0.14^{a}	1.4±0.07^{e}
N4: Amstel	3.2±0.07 ^{b}	5.8±0.07 ^{b}
N5: Maltina	2.7±0.07^{c}	2.7±0.07 ^{d}
N6: star	2.8±0.07^{c}	2.1±0.07 ^{de}
N7: Beta	1.03±0.02^{e}	7.5±0.7^{a}
N8: Dubic	2.9±0.07^{bc}	4.2±0.07 ^{c}
N9: Smirnoff	3.1±0.07 ^{b}	1.8±0.07^{e}
P-Value	0.00	0.00

THB-Total Heterotrophic Bacteria
TFC-Total Fungal Count
*Means with similar superscript down the group showed no significant difference (p>0.05)

Table 2: Susceptibility Pattern of *Bacillus* sp. and *Staphylococcus* sp. Isolated from Canned Beverages

	***Bacillus* (2)**			***Staphylococcus* (2)**		
Antibiotics (conc.µg)	**R n (%)**	**I n (%)**	**S n (%)**	**R n (%)**	**I n (%)**	**S n (%)**
Chloramphenicol(30µg)	0(0.00)	2(100)	0(0.00)	0(0.00)	0(0.00)	2(100)
Ampiclox (10 µg)	2(100)	0(0.00)	0(0.00)	1(50)	1(50)	0(0.00)
Levofloxacin (20 µg)	0(0.00)	0(0.00)	2(100)	0(0.00)	0(0.00)	2(100)
Erythromycin (30µg)	1(50)	1(50)	0(0.00)	0(0.00)	1(50)	1(50)
Gentamycin (10 µg)	0(0.00)	0(0.00)	2(100)	1(50)	1(50)	0(0.00)
Ciprofloxacin (10µg)	0(0.00)	0(0.00)	2(100)	0(0.00)	1(50)	1(50)
Streptomycin (20µg)	2(100)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	2(100)
Amoxil (20 µg)	2(100)	0(0.00)	0(0.00)	2(100)	0(0.00)	0(0.00)
Pefloxacin (30 µg)	0(0.00)	1(50)	1(50)	0(0.00)	1(50)	1(50)
Septrin (25 µg)	0(0.00)	0(0.00)	2(100)	0(0.00)	0(0.00)	2(100)

Key-R: Resistant, I: Intermediate, S: Susceptible

Table 3: Susceptibility Pattern of *Micrococcus* sp. and *Enterococcus* sp. Isolated from Canned Beverages

	***Enterococcus* (1)**			***Micrococcus* (1)**		
Antibiotics (conc.µg)	**R n(%)**	**I n(%)**	**S n(%)**	**R n(%)**	**I n(%)**	**S n(%)**
Ampiclox (10µg)	0(0.00)	0(0.00)	1(100)	1(100)	0(0.00)	0(0.00)
Levofloxacin (20 µg)	1(100)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(100)
Erythromycin (30µg)	0(0.00)	0(0.00)	1(100)	1(100)	0(0.00)	0(0.00)
Gentamicin (10µg)	0(0.00)	0(0.00)	1(100)	1(100)	0(0.00)	0(0.00)
Ciprofloxacin (10µg)	1(100)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(100)
Streptomycin (20µg)	0(0.00)	1(100)	0(0.00)	1(100)	0(0.00)	0(0.00)
Amoxil (20 µg)	0(0.00)	0(0.00)	1(100)	0(0.00)	0(0.00)	1(100)
Pefloxacin (30 µg)	0(0.00)	0(0.00)	1(100)	0(0.00)	1(100)	0(0.00)
Septrin (25 µg)	1(100)	0(0.00)	0(0.00)	1(100)	0(0.00)	0(0.00)

Key-R: Resistant, I: Intermediate, S: Susceptible

Table 4: Susceptibility Pattern of *Pseudomona*s sp. and *Enterobacter* sp. Isolated from Canned Beverages

	***Pseudomonas* (1)**			***Enterobacter* (1)**		
Antibiotics (Conc.µg)	**Rn (%)**	**In (%)**	**Sn (%)**	**Rn (%)**	**In (%)**	**Sn (%)**
Augmentin (30µg)	0 (0.00)	0(0.00)	1(100)	0(0.00)	0(0.00)	1(100)
Ciproflox (10 µg)	0 (0.00)	0(0.00)	1(100)	0(0.00)	0(0.00)	1(100)
Septrin (30µg)	1 (100)	0(0.00)	0(0.00)	1(100)	0(0.00)	0(0.00)
Ampicillin (30µg)	1 (100)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(100)
Ceporex (10 µg)	1 (100)	0(0.00)	0(0.00)	1(100)	0(0.00)	0(0.00)
Tarivid (10 µg)	1 (100)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(100)
Streptomycin (30µg)	0 (0.00)	0(0.00)	1(100)	0(0.00)	0(0.00)	1(100)
Nalidixic acid (20µg)	0 (0.00)	0(0.00)	1(0.00)	1(100)	0(0.00)	0(0.00)
Gentamicin (30µg)	1 (100)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(100)
Pefloxacin (20 µg)	1 (100)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(100)

Key-R: Resistant, I: Intermediate, S: Susceptible

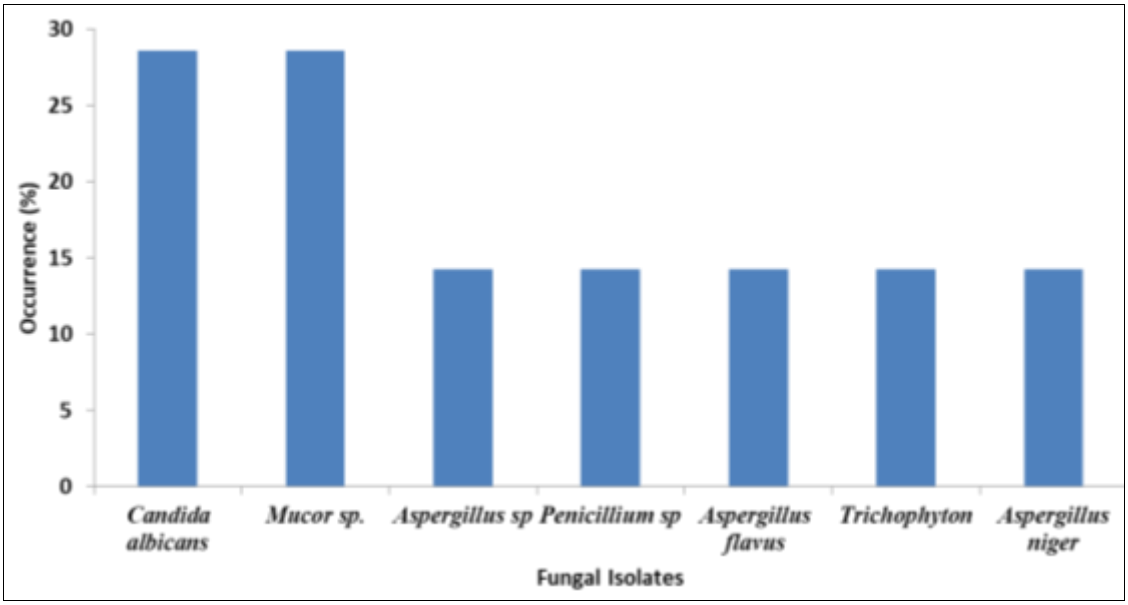

Fig 2: Percentage Occurrence of Fungi Isolates from the samples

Discussion

The findings from this study has sufficiently demonstrated that surfaces of canned drinks are exposed to contamination by microorganisms. The study also showed that both non-alcoholic and alcoholic beverage cans are exposed equally, to viable bacteria on their surfaces. This agrees with the findings of Ezelote *et al*., 2022, who in their study, isolated different species of bacteria and fungi. Also, the results from the present study is further confirmed by the findings of Kigighai and Jonathan (2012) [10], who carried out microbiological survey of non-alcoholic carbonated beverages. In their research, they reported the isolation of different species of bacteria including *Staphylococcus*, *Bacillus* and *Pseudomonas* species all of which poses potential public health challenges.

As regards to the findings from this study, the total heterotrophic bacterial counts of beverage cans showed that "Hero" had the highest counts followed by Smirnoff from Staff Club. Beta had the least count of total heterotrophic bacteria and this may be due to low request of malt/non-alcoholic beverages in the sample location thereby reducing human contact with certain drinks. It could also be attributed to improved condition of storage, while the high total heterotrophic count in Hero and Smirnoff may be due to constant contact with humans following higher demand. Amstel and Dubic however, had the highest count for total heterotrophic fungi.

Although the microbial counts recorded in this study were high, the counts recorded in similar study were higher than those recorded in this study (Ezemba *et al.*, 2021) [7]. The mean range of the total heterotrophic bacterial load from all samples in this study ranged from 1.03±0.02 to 3.8±0.14 CFU/cm^2. The range of the total heterotrophic bacterial load recorded by Ezemba *et al.* (2021) [7] were 2.0x10^1 to 4.2x10^1 CFU/ml using cans from refrigerator and packs sold in Federal University of Technology Owerri. This may be due to the difference in lifestyle of the vendors and the storage environment where the cans were kept. Furthermore, the total heterotrophic fungal count in this study was low in contrast to the count reported by Ezelote *et al*. (2022).

Staphylococcus sp. isolated from beverage cans in this study has been reported to cause various infections in humans such as boils, diarrhea and food poisoning (Ezemba *et al*., 2021) [7]. The bacteria and fungi on the cans could be due to dirty environments and improper handling of the cans

Ten bacteria isolates belonging to four genera were identified in this study; *Enterobacter* sp. 1(10%), *Pseudomonas* sp. 1 (10%), *Staphylococcus* sp. 2(20%), *Bacillus* sp. 2(20%), *Enterococcus* sp. 1(10%), *Micrococcus* sp. 1(10%), *Streptococcus* sp. 2(20%). Also, seven fungal isolates belonging to five genera were isolated; *Candida* sp. (18.2%) *Trichoderma* sp. (9.1%), *Penicillium* sp. (27.3%), *Mucor* sp. (18.2%), *Aspergillus* sp. (27.3%).

The percentage occurrence of bacterial species isolated from the surface of canned beverages was analyzed in this study. *Bacillus* sp., *Staphylococcus* sp. and *Streptococcus* sp. had the highest occurrence (20% respectively). *Bacillus* sp. have been reported to cause certain foodborne diseases with *Staphylococcus* sp. also being implicated in abscess, impetigo and scalded skin syndrome (Enemour *et al*., 2013; Christopher, 2015) [6, 3]. For fungi, *Aspergillus* sp. and *Pennicilium* sp. had the highest percentage occurrence.

Susceptibility testing was performed to determine the pattern of susceptibility of the bacterial isolates from surfaces of beverage cans to standard antibiotics and also determine the health implications of the bacterial isolates. Results showed that *Staphylococcus* sp. was more susceptible to Levofloxacin, Chloramphenicol, Septrin, Streptomycin and showed more resistance to Gentamycin, Amoxil and Ampiclox. *Bacillus* sp. was more susceptible to Levofloxacin, Ciprofloxacin, Gentamicin and showed resistance to Ampiclox, Streptomycin and Amoxil. *Pseudomonas* sp. was susceptible Augmentin, Ciproflox, Streptomycin, Nalidixic acid and was resistant to Ampicilin, Tarivid and Gentamycin as seen in a similar study (Ezemba *et al.*, 2021) [7]. Some bacteria isolated from the surfaces of the canned beverages in this study were found to be resistant to two or more antibiotics. However, some were susceptible to the drugs. Antibiotic resistance is no longer an emerging trend as many microorganisms have acquired multi-resistant ability to several antibiotics, the consequences of antibiotic resistance on public health are significant as resistance impacts negatively on treatment options. Abuse and misuse of antibiotics has been identified as major factors

contributing significantly to this trend. Therefore, it is necessary to ensure that the surfaces of canned drinks are properly washed to avoid fomite-mediated transmission of infectious agents to humans during the consumption of canned beverages.

Conclusion and Recommendation

The findings from this study has demonstrated the presence of bacterial and fungal isolates on surfaces of canned beverages that impacts public health significantly. Percentage occurrence from bacterial isolates showed that *Streptococcus* sp., *Staphylococcus* sp. and *Bacillus* sp. were the most dominant bacterial species isolated while *Aspergillus* sp. and *Penicillium* sp. were the predominant fungi isolated in all samples. Some of the bacteria isolated from this study, showed resistance to more than one antibiotics although, some also showed susceptibility to the drugs. Washing of canned drinks surfaces is likely to minimize the occurrence these microbes from the can surfaces and improved hygiene and handling by vendors is recommended. Usage of straws instead of drinking directly from the cans is also recommended and could minimize potential public health outbreaks.

Conflict of Interest

Not available

Financial Support

Not available

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