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Antifungal Resistance Features of Fungal Isolates from Canned Expired Beverages Identified in Supermarkets in Port Harcourt

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A B S T R A C T

Following the sale of expired can beverage in the market and subsequent confiscation, few retailers still engage in sales and consumers ignorantly purchase and consume the product. Consequently, the consumed beverages result to food poisoning and consumers resort to antibiotic therapy which sometimes has been marked unsuccessful due to resistant microbes. One hundred (100) canned expired beverage samples were soughted- out from the counter of some supermarket in Oroekpo community of Rivers State, Nigeria and analyzed for fungal load, identity, resistance and biofilm properties using standard microbiological technique. Result showed a mean heterotrophic fungal load of 118 x 10^2 cfu/lm, a macroscopic and microscopic identity of four fungal genera namely; *Mucor* sp., *Penicillium* sp., *Aspergillus flavus* and *Aspergillus niger* from the samples. The result, further showed that the concentrations of clotrimazole antifungal drug were sensitive against *Mucor* sp., *Aspergillus flavus, Penicillium* spp. and *Aspergillus niger* showing varying sensitivity percentage between 100 and 67 %. Similarly, *Mucor* sp., *Aspergillus niger* and *Aspergillus* sp. were most sensitive to concentrations of fluconazole. Thus, the isolation and identification of micconazole resistance fungal in expired can beverages is a pointer to a potential public health risk, following fungal biofilm feature which resist most common antifungal drugs. Hence, more appropriate and effective antifungal drug is hereby proposed.

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INTRODUCTION

Due to the presence of expired products in Nigerian Market, expired beverages have been sported and confiscated however, they still persist and remains a major challenge in Nigeria (Vanguard News, 2017). The consumption of these expired products can lead to food poisoning and many more ill-health (Oyewale, 2018). According to Oyewale (2018), the period of the expiration is at a phase in which the product losses its quality. The expiration of some products have been reported to induce microbial cell mutation following the presence of chemical constituents in the product (Cheung & Mehta, 2020).

According to Cheung and Mehta (2020) the beverages contains ingredients such as malted barley, water brewer's yeast (*Saccharomyces* spp.), sucrose, sorghum, hop extract, all of which loss their strength and quality after expiration and hence becomes un-consumable. At the period of expiry, the active ingredients in the product have become disabled, and are likely to be consumed in an inactive state if not identified (Oyewale, 2018). Similarly, Ihechu et al. (2022) also noted that the ignorant consumption of expired product is believed to cause symptoms such as diarrhea, vomiting and other gastrointestinal issues. Gastrointestinal effects cannot be over emphasized, following the ignorance observed by consumers in their inability to check for expiration date (Ihechu et al., 2022). following this circumstance, consumers of expired product resort to antibiotic therapy (Lior &Bjerrum, 2014). Thus, consumers are at risk with poor therapeutic measures that yield no result or response, following increased antibiotic resistant cells.

LITERATURE REVIEW

The overuse or mis-use of antibiotics thus, promotes life threatening infections were the causative agent resist the effect of the administered antibiotics (Lior &Bjerrum, 2014). The likely resistance of fungi to antifungal therapy is off special concern, due to the baker's yeast genetic mutation which yields mold (Raghavan et al., 2019). Although studies have shown the differences in antifungal resistance among several species of fungi, are due to host body machinery or drug factors (Pal et al., 2018).

The hop extract, an ingredient with antifungal property may be sensitive to the baker's yeast, a beverage constituent which disable the yeast with a proliferation of mold that maybe pathogenic to consumers. Hence, the proliferation of mold and several super infections that may emerge (Nionelli et al. (2018). The presence of molds in expired can beverage calls for antifungal screening due to constant development of fungal resistance to antifungal efficiency.

The resistance of antibiotics may transcend borders which calls for public health emergency and the need for caution and effective antifungal therapy. An antibiogram is therefore need to combat the abnormalities in canned expired beverage/ consumables with a bid to address the menace. The study seeks to evaluate the antifungal resistance features of fungi isolates recovered from can expired beverage with a view of providing early warning sign to consumers who ignorantly, purchase and consume expired beverages.

METHODOLOGY

Collection of Canned Expired Beverages

Canned expired beverage samples were soughted- out from the counter of some supermarket in Oroekpo community of Obio/Akpor Local Government, Rivers State, Nigeria. As the expired beverages were confirmed, they were then picked / collected and not purchases as it were. Hundred (100) of the samples were then taken to the Department of Biology Laboratory, Faculty of Natural and Applied Sciences, Ignatius Ajuru University, Rumuolumeni Port Harcourt, Nigeria and stored in refrigerator at 40 degrees centigrade.

Determination of the Fungal Load in the Canned Expired Beverages

1. **Preparation of the Beverage Samples**

The samples were serially diluted through a dilution factor of 10^{-1} and then to 10^{-2} dilution for the study as carried out by Werner (2023) aimed at reducing the number of viable cells of fungal. The expired beverage dilution factor of 10^{-2} was adopted for the study.

2. **Preparation of the Sabauraud dextrose Media**

Sabauraud dextrose agar (SDA), an agar for the growth and selective isolation of fungal organisms, was adopted for the study as carried out by Charkraboty and Pal (2008). The preparation of the Sabauraud agar involved dissolving them into a distilled water as instructed by the manufacturer and then autoclave. Following, the autoclave of the substance, it was allowed to cool ($50^{o}C$) before dispensed into sterile petri dishes were it solidify.

3. **Enumeration of the Fungal Load**

Enumeration of the fungi load in the expired beverage samples involved the adoption of the spread plate method (Beuchat, 1992). The method involved aseptic inoculation of 0.1ml of the 10^{-2} diluted sample on the freshly prepared sabauraud agar media (SAM) with the use of 1ml pipette. Following the inoculation, a sterile jockey stick was used to spread the inoculum evenly on the media. The SAM plates were returned, in a room temperature for 4 days for fungi colonial development and growths. Visual observed colonial growths were counted as colony forming unit per mill. The colonies, thereafter were purified, characterized and identified for antifungal and biofilm assay.

4. **Morphological / Colonial Characterization of the Fungal Isolates**

The morphological features of the recovered fungal isolates involved macroscopic and microscopic examination of the colonies. Macroscopic examination entailed the description of the colony appearance, size, shape etc of the isolate on sabauraud agar media (SAM) plates (Beuchat, 1992) while microscopic examination involved the use of a light microscope to view a wet preparations of the isolated fungi in a clean glass slide as adopted by Ezeonuegbu, (2022). All observations noted were compared with pictorial atlas.

Antifungal Sensitivity Testing

1. **Preparation of Antifungal Drug**

Three commonly administered or dispensed, antifungal drugs namely; miconazole, clotrimazole and fluconazol were adopted for the study and purchased from pharmacist in the study area. The antifungal drugs were noted 500mg per tablet. Each class of drug, 500 mg were prepared by serial dilution into

9ml normal saline to obtain an effective dose. Thus, the concentrations as carried out by Ostrosky-Zeichner et al. (2020) were obtained; miconazole 5.6, miconazole 0.56, miconazole 0.056, clotrimazole5.6, clotrimazole 0.56, clotrimazole 0.056, fluconazo 5.6, fluconazo 0.56, and fluconazole 0.056mg. all summed up to nine (9) concentrations.

2. Preparation of Muller Hilton Media

In preparing the media, a measured quantity of Muller Hilton agar was dispensed into conical flask with the addition of a measured quantity of water, stired, cocked properly and autoclaved as instructed by the manufacturer before use (Charkraboty & Pal 2008). Following, the autoclave of the media, the media was allowed to cool to 50^{o}C before been dispensed on sterile petri dishes were it solidify for sample inoculation.

3. Determination of Fungal Resistivity

Antifungal sensitivity and resistivity test were determined using the minimum inhibitory concentration test procedure as adopted by Ostrosky-Zeichner et al. (2020) and the pour plate technique as adopted by Beuchat (1992). Thus, the test involved the mixture of the various concentrations of the antifungal drug with a Muller Hinton agar media, and by the use of pour plating technique, the mixtures were dispensed into sterile petri dishes. The mixture components were allowed to cool/solidify. Following the solidification of the media, a sterile wire loop was used to inoculated the fungal into the freshly, prepared media (component) and incubated at 35^{0}C for five days. Thereafter, antifungal features were determined by measuring the zone of inhibition in diameter and with the use of clinical and laboratory standard method with the responses classed into sensitivity, intermediate and resistance.

4. Biofilm Formation Screening

The screening for biofilm formation was carried out to determine the level of fungi resistance to the antifungal drug. Thus, resistance is indicated by the development of a biofilm cover. The screening adopted the use of Congo red agar media as reported by Freeman et al. (1989) and Amadi-Ikpa et al. (2020). The anti-fungal isolates were inoculated into freshly prepared Congo red agar media and the media composition incubated for 22-24 hours at a temperature of 27 ^{o}C. Black colored appearance around the fungal after incubation indicated biofilm production whereas an absence indicated no biofilm formed (Shrestha et al., 2022).

5. Statistical Analysis

The descriptive statistical tool was adopted to summarize data recovered in the study. The mean of the fungal load obtained were considered (Amadi-Ikpa et al., 2020).

RESULT AND DISCUSSION

Enumeration of Fungal

The result in Table.1 showed the fungal load in the expired beverage samples. Heterotrophic fungal on Sabauraud dextrose media showed a mean counts of 118 x 10^{2} CFU/ml. Statistically, the mean counts were not significantly different from WHO (2020) permissible counts of fungal in drinking water.

Table 1. Mean Counts of Fungal from the Beverages (Expired)

Media Plate	Expired Beverages (CFU/ml)	WHO (2020)
SDA (THF)	7 X 10^2	10^2

Key; CFU/ml = colony forming unit per mill, THF= Total heterotrophic fungi, SDA Sabauraud dextrose agar, WHO= World Health Organization.

Colonial/ Macroscopic Characterization of the Isolated Fungi

Table 2 showed the macroscopic and microscopic characterization of the fungi isolates recovered. Four (4) fungi were recovered from the expired can beverage samples. The fungi *Mucor* sp. were noted whitish in colour with a large size and cottony texture microscopically showing swells. *Penicillin* sp. were observed with a tint of greenish-blue appearance and a powdery textured surface. The fungi appeared long and branched under the microscope The fungi *Aspergillus flavus* and *Aspergillus niger* appeared with shades of yellow-green and dark brown in color respectively, with a small/circular sized colony. On viewing under the microscope the *A. flavus* had hyphae that was branched while that of *A. niger* is separated.

Table 2. Macroscopic and Microscopic Characterization of the Fungi Isolate

Isolates	Macroscopic				Microscopic	Identification
	Colour	Size/ shape	Growth rate	Texture		
1	Whitish	Large/ Circular	Slow	Cottony/ woolly	The tip of the sporangiospore swells	*Mucor* sp.
2	Blue-green	Small/ Circular	Fast	Powdery	Branched septate that is long and smooth	*Penicillin* sp.
3	Yellow	Small	Rapid	Powdery	The hyphae appear to be branched	*Aspergillus flavus*
4	Dark brown	Circular	fast	powdery	The hyphae appear to be separated	*Aspergillus niger*

Frequency of Fungal Prevalence

Table 3 showed The percentage frequency prevalence of the fungal isolates recovered from the expired beverage. *Mucor* sp. had a percentage frequency prevalence of 19 and *Penicillium* sp. 43 % prevalence. *Aspergillus flavus* and *Aspergillus niger* had 33 and 5% respectively.

Table 3. Fungal Prevalence Across the Drinks

Media	**Frequency of Isolates in Expired Beverages**	**% Frequency Prevalence**
Mucor sp.	4	19
Penicilium sp.	9	43
Aspergillus flavus.	7	33
Aspergillus niger	1	5

Antifungal Resistivity Assay

The result in Table 4 showed that all concentrations of clotrimazole were active or sensitive against *Mucor* sp., *Aspergillus niger, Penicillium* sp. and *Aspergillus flavus* showing varying sensitivity percentage between 100 and 67 %. Similarly, *Mucor* sp., *Aspergillus niger*. and *Aspergillus flavus*. were most sensitive to concentrations of fluconazole. However, *Penicilium* sp. had high level resistance to concentrations of fluconazole at a resistivity level of 100%. Some level of sensitivity was observed with *Aspergillus niger, Penicillium* sp. and *Aspergillus flavus* with respect to concentrations of Miconazole, Consequently, *Mucor* sp. showed some level of resistance to concentrations of Miconazole.

Table 4. Antifungal Resistivity Assay

Antifungal Drugs	***Mucor* sp.**			***Aspergillus niger***			***Penicilium* sp.**			***Aspergillus flavus***		
	S	I	R	S	I	R	S	I	R	S	I	R
Clotrimazole	3(89)	1(11)	0(0)	1(100)	0(0)	0(0)	6(67)	3(33)	0(0)	5(70)	2(30)	0(0)
	2(78)	2(22)	0(0)	1(100)	0(0)	0(0)	6(67)	3(33)	0(0)	6(85)	1(15)	0(0)
	2(50)	1(25)	1(25)	1(100)	0(0)	0(0)	7(78)	2(22)	0(0)	6(85)	1(15)	0(0)
Fluconazole	2(50)	1(25)	1(25)	1(100)	0(0)	0(0)	0(0)	0(0)	9(100)	5(70)	2(30)	0(0)
	3(89)	1(11)	0(0)	1(100)	0(0)	0(0)	0(0)	0(0)	9(100)	5(70)	2(30)	0(0)
	3(89)	1(11)	0(0)	1(100)	0(0)	0(0)	0(0)	0(0)	9(100)	7(100)	0(0)	0(0)
Miconazole	0(0)	0(0)	4(100)	1(100)	0(0)	0(0)	7(78)	2(22)	0(0)	5(70)	2(30)	0(0)
	0(0)	0(0)	4(100)	1(100)	0(0)	0(0)	7(78)	2(22)	0(00)	6(85)	1(15)	0(0)
	0(0)	0(0)	4(100)	1(100)	0(0)	0(0)	5(56)	4(44)	0(0)	6(85)	1(15)	0(0)

Keys ; R= Resistance, S= Sensitive, I = Intermediate

Biofilm Formation Capacity

Table 5 showed the reaction of the fungi to Congo red agar media. All fungi were able to express biofilm feature. Basically, biofilm feature was noted with *Mucor* sp., *Aspergillus niger, Penicillium* sp. and *Aspergillus flavus.*. *Mucor* sp. expressed heavy load of biofilm property unlike the others that showed weak biofilm strength.

Table 5. Biofilm Formation Features

Fungi	**Biofilm Strength**
Aspergillus niger	+
Penicillium sp.	+
Aspergillus flavus	+
Mucor sp.	++++

Key;
weak biofformers+
light bioformers ++
heavy bioformers+++

RESULT AND DISCUSSION

Viable counts of fungi as reported in this study were above the stipulated number of heterotrophic fungi in drinking water as documented by WHO (2020). The WHO (2020) report is in line with the set standard by Vijayalakshini et al. (2020) were they noted that the permissible limit of heterotrophic fungi in beverage should not exceed 100 colony forming unit per ml. Moreso, the fungi counts in this study clearly ruled out the standard by Vijayalakshini et al. (2020). Hence, the expired beverage maybe in a fermentative state were the fungal proliferated. The isolation of heavy load of heterotrophic fungal in the expired beverage is a pointer to a potential public health risk. The identification and recovery of *Mucor* sp. *Aspergillus niger Penicillium* spp. and *Aspergillus* flavus which are mostly human pathogens were also encountered by Juvonem et al. (2019) in various consumable drinks.

However, this study contradicts the study carried out by Lawlor et al. (2021) were the fungi *Zygosaccharomyces* sp., *Sachromyces brehanomyces, Hanseniaspora* sp, *Hansenula* sp., and *Pichia* sp. were noted the most encountered species isolated in beverage involved in spoilage. As reported in this study, the high prevalence of *Penicillium* sp and least prevalence of *Aspergillus niger* are also reported by Yamaguchi et al. (2007). Yamaguchi et al. (2007) noted high prevalence of *Penicillium* sp. in treated and bottle mineral water where fungi such as *Cladosporium cladosporiodes, Aiternaria alternate, Cladosporium,* and *Rhizopus* were also isolated. Thus elevated levels of *Penicillium* sp. maybe attributed to the ability of the fungi is able to withstand competition from other indigenous microorganisms with higher growth rate (Lawlor, 2021).

The resistance of the *Mucor* sp. and *Penicillium* sp. to several concentrations of miconazole and fluconazole respectively may suggest the ability of the fungi to form biofilm. The formation of biofilm is worrisome due to the difficulty in control of infections associated with biofilm. Biofilm microbes are embedded in self-produced matrix composed of extracellular DNA, lipids, proteins and carbohydrates. This embedded structure is responsible for the resistance offered by the fungi (Freeman et al., 1989). Generally, the capacity for *Mucor* sp. and other fungi isolated in this study to form biofilm quite agreed with reports by Caruso (2020) that noted microbes in water environment which the

beverage offers have the capacity to form biofilm which protects the microbes against harsh condition invariably provided by expiry of the beverage.

CONCLUSIONS AND RECOMMENDATIONS

The study identified *Mucor* sp. and *Penicillium* sp. isolates are capable of causing infections in a low immune compromised consumer following the resistance to Fluconazol and Miconazole. The expired can beverage could serve as a transmission point for severe infections. The study solicits for anti-biofilm chemotherapeutic drugs that has the capacity to ward off invading resistant fungi.

FURTHER STUDY

This study still has limitations so that further research is still needed on the topic of "Antifungal Resistance Features of Fungal Isolates from Canned Expired Beverages Identified in Supermarkets in Port Harcourt".

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