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Bacterial Contamination of Fermented Saps of *Raphia hookari* obtained from selected Markets in Epe, Lagos Nigeria

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Abstract

Raphia hookari fermented sap also known as raffia wine is an alcoholic drink enjoyed in Africa, Asia and South America. It may be described as ready-to-eat food as it is often sold in plastic bottles or cups without further processing. The use of unhygienic packaging material or non-potable water may lead to contamination by pathogenic organisms. This study examines the bacterial profile and the antibiotic resistance of isolates obtained from raffia wine sold in Epe, Lagos, Nigeria. Four samples were obtained from different locations and analysed. The samples were spread plated on Plate count agar, Salmonella-Shigella agar, Mannitol salt agar and MacConkey agar. The Kirby Bauer method was used to evaluate the antimicrobial susceptibility of the isolates. The results showed varying levels of bacterial contamination. A mean viable count of 1.0 x 10^7 CFU/mL was obtained on plate count agar, 8.1 x 10^5 CFU/mL on mannitol salt agar, 1.9 x 10^4 CFU/mL on MacConkey agar, and no isolates were obtained on Salmonella-Shigella agar. The frequency of occurrence of Escherichia coli and Staphylococcus aureus isolated were 54.8 % and 45.2%, respectively. The antibiogram showed that Staphylococcus aureus was highly resistant to chloramphenicol (85.7%), ampiclox (64.3%), and erythromycin (42.8%). E. coli was highly resistant to erythromycin (100%), chloramphenicol (82.4%), and amoxicillin (57.1%). The presence of pathogenic bacteria in raffia wine indicates potential adulteration and a decrease in the alcoholic content. This highlights the need for public awareness about food safety practices.

Keywords: Antibiotic resistance, Fermented beverages, Food pathogen, Raffia wine, Ready-To-Eat.

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INTRODUCTION

Foods and drinks consumed without undergoing additional processing to lower the microbial load are referred to as ready-to-eat foods (Mengistu *et al.,* 2022). Ready-to-eat foods are convenient meals for present-day lifestyles but with associated risks. Most microbial fermented drinks are considered ready-to-eat foods, they are also sources of income as families sell them on the streets to augment their financial needs. Ready-to-eat (RTE) may be fruit and its products, animal products such as meat and eggs as well as drinks like cocktails and fermented beverages, e.g., raffia wine.

Raffia wine also known as palm wine is a traditional alcoholic beverage popularly known to the Sub-Saharan people of Africa. Djeni *et al*. (2022) noted that the wine is made from the palm sap of palm trees which could either be raphia palm (*Raphia hookeri*) or the oil palm (*Elaeis guineensis)* or ron palm *(Borassus aethiopum).* Raffia wine is usually consumed at celebratory events or casual gatherings. In Africa, raffia wine is used for culinary purposes, eye treatment, improve milk production by lactating mothers, and pouring libations (Amadi, 2022).

Consumption of contaminated fermented *Raphia hookeri* sap can be a public health threat, as food poisoning and death could occur. The World Health Organisation stated that consuming contaminated food can result in or transmit more than 200 diseases (WHO, 2021). According to Elbehiry *et al.* (2023) foodborne diseases are on the increase, so are antimicrobial resistant pathogens. Samtiya *et al*. (2022) and Almansour *et al.* (2023) highlighted the crucial roles that food plays in the effective transmission of antimicrobial resistance (AMR) factors to consumers' digestive tracts. The most common bacteria in ready-to-eat foods are *Salmonella*, *Listeria monocytogenes*, *Escherichia coli*, *Campylobacter jejuni*, *Staphylococcus aureus*, *Bacillus cereus*, and *Clostridium perfringens*. These organisms are associated with high health risk (Mengistu *et al*., 2022) attributed to cross contamination between food handlers and contaminated machinery and packaging materials.

Although now bottled in 25L gallons, 50/75 cl polyethylene terephthalate (PET) bottles, or gourd cups (Nwaiwu and Itumoh, 2017), raffia wine can easily be contaminated. These plastic bottles are usually exposed to contamination because they are mostly obtained from waste disposal units, restaurants or picked up along the roads. Hence are potential sources of foodborne pathogens. Besides, the original quality of the wine may be adulterated with untreated water in an attempt to increase quantity (Nwaiwu and Itumoh, 2017). Some other factors such as tapping palm sap with repeatedly used instruments without proper sterilisation and unhygienic storage, can facilitate the microbial contamination of raffia wine. Raffia wine is a widely consumed staple beverage, however, the unsanitary methods employed in its packaging for retail, coupled with a lack of health awareness and poor adherence to food production regulations, pose significant health risks. Production, packing, and distribution of raffia wine are highly unregulated in Nigeria, as thorough controls for ready-to-eat foods are hardly implemented, thereby exposing consumers to food pathogens. Also, based on the belief that raffia wine is highly alcoholic, there is a dearth in knowledge on the microbial safety of raffia wine.

MATERIALS AND METHODS

Study Area: The study was conducted in Epe town (6.34'59.99^{o}N; 3.58'59.99^{o}E) Lagos, Nigeria. Four (4) locations, Ilara, Iragushi, Mojoda, and Ibowon were investigated. The samples collected were conveyed to the Microbiology Laboratory, Biological Sciences Department, Augustine University, Ilara-Epe, Lagos, Nigeria.

Isolation and Identification of Isolates: The raffia wine samples were serially diluted in sterile deionized water, and appropriate dilutions were plated on plate count agar (PCA); Salmonella-Shigella agar (SSA), MacConkey agar (MAC) and Mannitol Salt agar (MSA) using spread plate method. The inoculated plates were incubated for 24 hours at 37^0C. Spherical elevated pink-coloured colonies on MacConkey agar were presumed to be *E. coli,* while yellow colonies on MSA were assumed to be *S. aureus*. Pure cultures were obtained by sub-culturing on nutrient agar plates.

The bacterial isolates were characterised phenotypically. Gram's reaction was assayed by making smears from the cultured plates aseptically on clean glass slides. The smears were heat fixed before they were stained with crystal violet for a minute and washed off with water. Lugol iodine was applied to the smears for a minute and then washed off with water. The smears were decolourised with a few drops of alcohol for 30 seconds and washed immediately with water. They were counterstained with safranin for 1 minute and rinsed with water for 1 minute. The slides were air-dried. A drop of immersion oil was dropped on the smear and was examined under the oil immersion lens of the binocular microscope. Gram- positive bacterial cells were coloured purple under the microscope, while Gram-negative bacteria appeared reddish pink (Abdu *et al.,* 2017).

The indole test aim to detect tryptophan-degrading organisms that convert tryptophan to indole. A distinct colony of the isolate was transferred into a test tube holding 5mL of peptone water using a wire loop. Five drops of Kovac's indole reagent were added, and the mixture was gently shaken. The organisms were incubated at 37°C for 24 hours. A positive reaction is indicated by the formation of red coloration at the top layer (Cheesbrough, 2006).

The generation of acids during the breakdown of sugar was detected using the Methyl Red test as described by Geletu *et al.* (2022). The isolates were inoculated into 5 mL MRVP broth and incubated at 37°C for 48 hours. Following the incubation, 2 drops of methyl red were added to 1mL of the broth in a test tube. A positive test is indicated by red colouration, whereas yellow colouration indicates a negative test. To ascertain the production of acetoin, Voges-Proskauer test was carried out. Five drops of 40% KOH and fifteen drops of 15% alpha-naphtha in alcohol were added to the incubated broth and gently mixed. A positive test result is indicated by the emergence of a red colour within an hour (Cheesbrough, 2006).

Citrate test assay the ability of the isolates to utilize citrate. A single colony of the isolates was streaked on Simmon's citrate agar and incubated for 48 hours at 37°C. The formation of a deep blue colour indicates a positive reaction (Cheesbrough,2006).

Antibiotic Sensitivity Testing: The antibiotic sensitivity pattern of the isolates was determined using a disc diffusion assay (Geletu *et al.,* 2022). Distinct colonies from the overnight culture of the test isolates were suspended in normal saline till the turbidity of the inoculum was equivalent to 0.5 McFarland standard. Each standardized inoculum was then inoculated by swabbing on Mueller-Hinton agar plates. The antibiotic-impregnated discs were aseptically placed on the surface of the bacterial lawn and allowed to pre-diffuse into the agar before incubation at 37°C for 24 hours. A panel of 10 antibiotics was tested: erythromycin (ERY, 10 µg), ampiclox (APX, 30 µg), Chloramphenicol (CHL, 30 µg), gentamicin (GEN, 10µg), pefloxacin (PEF, 10 µg), amoxicillin (AMX, 30 µg), Ceftriaxone (CTR, 25 µg), and ciprofloxacin (CIP, 10 µg). The antimicrobial susceptibility was measured as the inhibition zone diameter in millimeters around the antibiotic disc and interpreted according to the Clinical and Laboratory Standards Institute(2020).

The Multidrug antibiotic resistance (MAR) index, was used to estimate the frequency at which an isolate shows resistance to three or more classes of antibiotics tested. It was estimated according to Krumperman's proposed formula: *a*/*b*, where '*a*' represents the number of antibiotics to which an isolate was resistant, and *'b*' represents the total number of antibiotics tested (Ayandele *et al.,* 2020). A MAR index greater than 0.2 indicates that the isolate was obtained from high-risk sources where antibiotics are often used.

RESULTS AND DISCUSSION

The mean counts of the bacterial isolates from raffia wine sold in Epe is shown in Table 1. The result indicated that wine from Mojoda had the highest total viable bacterial count at 2.6 x 10^7 CFU/mL, whereas the lowest count was from the raffia wine obtained from Ilara at 3.4 x 10^4 CFU/mL. The highest *Staphylococcus* count was noted in the raffia wine from Ilara at 3.2 x 10^6 CFU/mL and the lowest count was observed in the Mojoda raffia wine sample. The highest coliform count was obtained from Iragusin raffia wine sample at 5.5 x 10^4 CFU/mL and the least growth was observed in the Ilara raffia wine sample (8.0 x 10^3). The presence of these organisms indicates that consumers are at high risk of foodborne infections. Additionally, the survival of enteric bacteria in the raffia wine samples may be linked to the reduction of alcoholic content as a result of their dilution with water.

Presumptive *S. aureus* was identified as distinct colonies with a yellowish halo on MSA and are gram-positive bacilli, indole-negative but methyl, Voges-Proskaueer and catalase positive. The *E. coli* was identified as gram-negative bacilli, indole-positive, citrate-negative, methyl red-positive, and Voges-Proskaue negative. The frequency of occurrence shown in Figure 1 indicates that the raffia wine from Mojoda had the highest number of *S. aureus* while Iragushi had the highest number of *E. coli.* Coincidentally, Out-Bassey *et al.* (2017) observed that *Raphia hookeri was* highly contaminated with enteric bacterial pathogens, (20%) than *Elaeis guineensis* (15%). The presence of *E. coli* and *S. aureus* indicates a lack of hygiene in the handling of these beverages. Contamination could likely have occurred due to the use of non-potable water, unsterilized distribution bottles, or improper handling during the production process. The reuse of PET bottles normally obtained from restaurants, waste disposal out-fits or picked on the road could be the source of contamination. Besides, *S. aureus* and *E.coli* are normal flora of the skin and gut, respectively, hence, contamination could have been due to improper handling by processing personnel or the use of non-potable water. According to Amare *et al*. (2019), the hands of food handlers could serve as a primary vehicle for transferring pathogens from faeces, the nose, and skin to food, leading to cross-contamination. The prevalence of both *E. coli* and *S. aureus* in food and beverages has been documented by Nwaiwu *et al.* (2020) and Odo *et al.* (2021).

The results of the antibiotic susceptibility tests (Table 2) showed that 85.7% of *S. aureus* strains exhibited resistance to at least two classes of antibiotics. Specifically, 14.3% and 28.5% of the *S. aureus* strains were resistant to fluoroquinolone. Additionally, resistance was observed in 42.8% of strains to macrolides, 28.5% to aminoglycosides, and 14.3% to cephalosporin. The isolates also demonstrated resistance to penicillin and its combination with cloxacillin, showing rates of 57.1% and 64.3%, respectively. Notably, the *S. aureus* strains exhibited a high resistance to chloramphenicol at 85.7%. In contrast, all *E. coli* strains were susceptible to ciprofloxacin, with only 5.8% showing resistance to pefloxacin, despite both drugs being fluoroquinolones. Furthermore, all strains were resistant to macrolides, with 82.4% resistant to chloramphenicol and 29.4% resistant to aminoglycosides. While all strains were susceptible to cephalosporins, resistance rates for penicillin were 23.5% and 11.8%. Amare *et al.* (2019) similarly noted that *E. coli* and *S. aureus* demonstrate significant resistance to penicillin. The

isolates demonstrated resistance to multiple classes of antibiotics, highlighting the increase in the prevalence of multidrug-resistant pathogens. In this study, 52% of the isolates exhibited a multidrug resistance (MAR) index greater than 0.3 (Table 3).

Table 1: Total Viable Counts of Raffia Wine from Epe, Lagos (CFU/mL)

Samples	PCA	MSA	SSA	MAC
Irangushi	1.5×10^7	4.0×10^4	Nil	5.5×10^4
Ibonwon	1.0×10^4	3.1×10^4	Nil	1.2×10^4
Mojoda	2.6×10^7	2.2×10^4	Nil	5.2×10^4
Ilara	3.4×10^4	3.2×10^6	Nil	8.0×10^3
Mean count	1.0×10^7	8.1×10^6	-	1.9×10^4

Key: PCA- Plate Count Agar; MSA- Manitol Salt Agar; SSA Salmonella Shigella agar and MAC- MacConkey agar. Values are means of triplicate determinations.

Table 2. Antibiotic Susceptibility Test of *S. aureus* and *E. coli* (%)

Drugs	*Staphylococcus* (n=14)	*E. coli* (n=17)
Erythromycin (10 µg)	42.8	100
Ampiclox (30 µg)	64.3	11.8
Chloramphenicol (30 µg)	85.7	82.4
Gentamicin (10 µg)	28.5	29.4
Pefloxacin (10 µg)	28.5	5.8
Amoxicillin (10 µg)	57.1	23.5
Ceftriaxone (25 µg)	14.3	0.0
Ciprofloxacin (10µg)	14.3	0.0

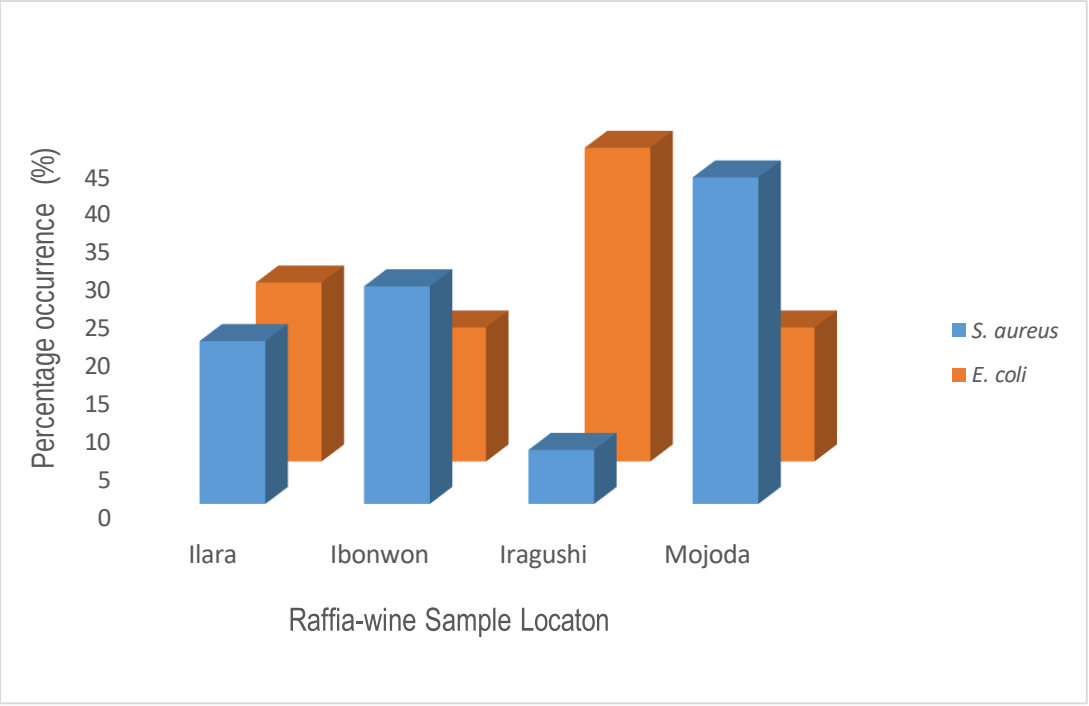

Figure 1. Frequency of Occurrence of *S. aureus and E. coli* in Raffia-wine.

Table 3. The Antibiotic Resistance Patterns and MAR Index

No. of Antibiotics	Resistance Pattern	No. of Strain	MAR Index
	S. aureus		
3	APX*CHL*AMX	1	0.37
3	APX*CHL*PEF	1	0.37
3	ERY*APX*CHL	1	0.37
4	ERY*APX*CHL*AMX	3	0.5
5	APX*CHL*GEN*PEF*AMX	1	0.63
5	ERY*CHL*PEF*AMX*CTR	1	0.63
6	ERY*APX*CHL*GEN*AMX*CPX	1	0.75
6	APX*CHL*GEN*AMX*CTR*CPX	1	0.75
	E. coli		
3	ERY*CHL*GEN	2	0.37
3	ERY*CHL*AMX	1	0.37
4	ERY*APX*CHL*AMX	1	0.5
5	ERY*APX*CHL*GEN*AMX	1	0.63
5	ERY*APX*CHL*PEF*AMX	1	0.63

The isolates showed a total of 13 MDR patterns. *Staphylococcus aureus* exhibited 8 while *E. coli* showed 5 MDR patterns. All isolates were resistant to at least two antibiotics, with a maximum resistance to six antibiotics. Among the tested *S. aureus*, 71.4% showed multidrug resistance, while *E. coli* resistance was 35.3% across various antibiotic classes. The phenomenon of multidrug resistance in bacteria is more often than not attributed to the accumulation of resistance (R) plasmids or transposons carrying genes that code for resistance to specific agents, along with the activity of multidrug efflux systems (Nikaido, 2009). Consequently, the indiscriminate use of antibiotics can exacerbate the issue of multidrug resistance (Boireau *et a*l., 2018; Popoola *et al.,* 2024). In Nigeria, the ease of acquiring over-the-counter antibiotics is a common practice that should be discouraged to mitigate the prevalence of multidrug resistance.

CONCLUSION

Staphylococcus aureus and E. coli were isolated from all the sampled raffia wine, indicating poor hygiene**.** This study highlights potential public health hazards due to the isolation o f MDR pathogens. Therefore, caution must be exercised when consuming raffia wine.

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