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Plasmid-Mediated Multidrug Resistance in Bacteria from *Hibiscus sabdariffa* (*Zobo*) Beverages: Public Health Implications for University Communities in Nigeria

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Abstract

Foodborne pathogens and antimicrobial resistance (AMR) pose escalating global health challenges, particularly in low- and middle-income countries, where the informal food and beverage sectors remain largely unregulated. *Hibiscus sabdariffa*–based beverages, popularly known as *zobo* in Nigeria, are widely consumed for their nutritional and medicinal properties. However, traditional preparation may predispose them to microbial contamination. This study examined the presence of plasmid-mediated multidrug resistance bacteria in zobo drinks sold within Edo State University Iyamho, Nigeria. Bacteria isolation from five zobo drink samples was carried out using standard microbiological methods. Isolated bacteria were assessed for multidrug resistance (MDR) using the Kirby-Bauer disc diffusion method. The presence of extrachromosomal DNA, *hlb* and *pbp* genes were detected using polymerase chain rection (PCR). The zobo drinks revealed the presence of pathogenic as well as opportunistic bacteria, including *Staphylococcus aureus*, *Bacillus subtilis*, *Staphylococcus epidermidis*, *Micrococcus luteus*, and *Enterococcus faecalis*. Antibiotic susceptibility testing revealed MDR with resistance indices ranging from 0.4 to 0.6. Plasmid curing experiments indicated that resistance was largely plasmid-mediated with resistance indices ranging from 0.0 to 0.2. Plasmid profiling confirmed the presence of extrachromosomal DNA in isolated bacteria. Molecular assays further amplified the *hlb* (beta-hemolysin) and *pbp* (penicillin-binding protein) genes in bacteria isolates, underscoring the virulence and resistance potential of the isolates. These findings highlight

zobo drinks as potential reservoirs of AMR bacteria, with implications for food safety and public health in densely populated university settings and calls for the urgent need for improved hygiene practices and AMR surveillance in informal food sectors.

Keywords: Zobo Drinks, Multidrug Resistance Bacteria, Plasmids, Antibiotic Resistance Genes, Public Health.

Introduction

Foodborne diseases remain a major contributor to global morbidity and mortality, with the World Health Organization estimating that unsafe food causes over 600 million illnesses annually (Keddy *et al.,* 2025). The burden is disproportionately higher in developing countries, where informal food and beverage markets play a central role in nutrition and livelihoods (Holliday *et al.,* 2025). Of particular concern is the global rise in antimicrobial resistance (AMR) and the role of mobile genetic elements, especially plasmids, in the dissemination of resistance traits among bacterial populations across diverse ecological niches (Elbehiry *et al.,* 2025).

The spread of AMR through the food chain is a "One Health" issue, involving complex interactions between humans, animals, and the environment. The cycle begins with the use of antibiotics in agriculture and aquaculture, which exerts selective pressure, leading to the emergence of antimicrobial-resistant bacteria (Farrukh *et al.,* 2025). These AMR, along with free-floating antimicrobial resistance genes (ARGs), can then be transmitted to humans through the consumption of contaminated food.

A comprehensive review by Farrukh et al. (2025) reported alarmingly high resistance levels in *E. coli* and *Staphylococcus aureus* isolated from food. *E. coli* showed complete (100%) resistance to penicillin and high resistance to tetracycline (72-100%) and ampicillin (89.5-100%) while *Staphylococcus aureus* showed 98.5% resistance to penicillin. Recent research reveals that specific plasmids are uniquely associated with different non-typhoidal *Salmonella* serotypes such as IncHI2 in *S. Typhimurium*, the pESI megaplasmid in *S. Infantis*, and multiple plasmids in *S. Indiana,* each driving distinct antimicrobial resistance risks (*Ishihara et al*., 2025; Zhou *et al.,* 2026). These findings underscore that plasmid-mediated resistance is serotype-specific, requiring tailored surveillance and intervention strategies for each high-risk *Salmonella* type in the food chain.

A study by Barker et al. (2025) found *Listeria monocytogenes* carrying a novel 120, 420 bp plasmids with resistance genes for trimethoprim, lincosamides, and macrolides likely acquired from other Gram-positive species highlighting its potential to gain resistance via plasmids in the food chain. Another study by Balbuena-Alonso et al. (2022) on genomic analysis confirmed that foodborne *E. coli* strains act as a major

reservoir for plasmid-mediated transfer of resistance and virulence genes, with plasmids readily exchanged between food and clinical strains even when direct pathogen transmission is uncommon.

Hibiscus sabdariffa, commonly referred to as roselle, is a plant of significant nutritional and medicinal importance (Gomaa *et al.,* 2025). In Nigeria, beverages prepared from its calyces are popularly known as *zobo* (Adeosun and Umoren, 2025). *Zobo* is consumed across socio-economic strata due to its affordability, refreshing taste and reported health benefits. Despite these benefits, *zobo*'s traditional preparation often involving manual handling, non-sterile equipment, and storage in plastic containers or sachets renders it highly susceptible to microbial contamination. Previous studies have reported the presence of diverse bacterial genera in *zobo* drinks, including *Staphylococcus, Escherichia, Bacillus* (Ike *et al.,* 2025; Samuel *et al.,* 2024) and *Pseudomonas* (Samuel *et al.,* 2024). Many of these organisms are opportunistic pathogens that can produce toxins or cause gastrointestinal infections, particularly in individuals with compromised immune systems (Fagbemi *et al.,* 2023). More concerning is the detection of antibiotic-resistant strains, which may act as reservoirs for resistance determinants transferable to other pathogens (Ado *et al.,* 2022). The informal nature of *zobo* production, combined with limited regulatory oversight, exacerbates these risks.

Despite the widespread consumption of zobo in Nigerian universities, there is a lack of baseline data on its bacteriological quality and the molecular mechanisms underlying resistance among contaminating bacteria. This gap hampers the effectiveness of public health interventions and leaves university communities vulnerable to foodborne outbreaks.

The present study addresses this gap by conducting a bacteriological assessment of *zobo* drinks sold within Edo State University Iyamho. Specifically, the study seeks to isolate and identify bacterial contaminants, determine their antibiotic susceptibility patterns, and assess the presence and role of plasmids in mediating resistance.

MATERIALS AND METHODS

Sample Collection, Bacteria Isolation and Enumeration

Five (5) independent *Hibiscus sabdariffa* (zobo) drinks samples were purchased from different vendors operating on campus (Edo State University, Iyamho, Nigeria) to capture variability in preparation and handling practices. Sample were transported in ice box to the laboratory, and processed within two hours of collection to minimize changes in microbial load (Chauhan and Jindal, 2020). Nutrient agar (NA), MacConkey agar (MA) and Salmonella-Shigella agar (SSA) were prepared according to the manufacturer's instructions (Oxoid, UK) and sterilized by autoclaving at 121°C for 15 minutes. Total viable counts were determined using the pour plate method. Serial dilutions (10^{-1} to 10^{-6}) of each sample were prepared in sterile physiological saline, and aliquots were plated in triplicate. Plates were incubated at 37 °C for 24–48 h, and colony-forming units (CFU/mL) were calculated. Distinct colonies were sub-cultured to obtain pure isolates. Pure isolates were inoculated on slants and kept for further studies (Ado et al., 2022).

Identification of Bacterial Isolates

Isolates were characterized by cultural, Gram staining and standard biochemical assays (catalase, coagulase, oxidase, indole, citrate utilization, urease, and sugar fermentation tests). Identification was confirmed using reference keys from Bergey's Manual of Determinative Bacteriology. Representative isolates were preserved in NA slants and stored at 4 °C for downstream analyses (Cheesbrough, 2006).

Antibiotic Susceptibility Testing

Antimicrobial susceptibility was assessed using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar, in accordance with CLSI, 2021. A standardized inoculum of each bacterial isolate was prepared and turbidity of the suspension was adjusted to match the 0.5 McFarland standard**,** which corresponds to approximately 1.5×10^8 CFU/mL. Sterile Mueller-Hinton agar plates were used for the susceptibility testing. A sterile cotton swab was used to evenly spread the inoculum across the entire surface of the agar plate and were allowed to dry. A panel of commonly prescribed antibiotics including streptomycin, gentamicin, erythromycin, septrin, pefloxacin, ciprofloxacin, rifampicin, ampicillin, zinacef, and amoxicillin were aseptically placed on the agar. Zones of inhibition were measured after 24 h incubation at 37 °C. The multidrug resistance (MDR) index was calculated as the ratio of the number of antibiotics to which an isolate was resistant, to the total number tested (CLSI, 2021). The **0.2** threshold established by Krumperman (1983) based on the rationale that an isolate from a healthy host would only be exposed to a limited number of antibiotics, making resistance to more than 20% of tested agents unlikely was adopted.

Whereby > 0.2 = High-risk / Clinical significance indicating the isolate originated from a high-risk environment with frequent antibiotic exposure (e.g., a patient, livestock, or food product where antibiotics are heavily used).

Plasmid Isolation and Curing

Plasmid DNA was extracted using the alkaline lysis method described by Gautam (2022). Extracted plasmid DNA was stored at -20°C until further analysis. Presence of plasmid was confirmed by electrophoresis of 5 µL of the extracted DNA on a 1% (w/v) agarose gel containing 0.5 µg/mL ethidium bromide. This was run at 80 V for 60 minutes in 1× TAE buffer, and visualized under UV light. Plasmid curing was performed by exposing the isolates to acridine orange (50 µg/mL) in nutrient broth, followed by incubation at 37°C for 48 hours. Post-curing susceptibility testing was repeated to determine plasmid-mediated resistance.

Molecular Detection of Resistance and Virulence Genes

DNA extraction from isolated bacteria was carried out following the method of Purushothaman et al. (2025) using a DNA extraction kit (QIAamp mini-DNA kit). Polymerase chain reaction (PCR) assays were conducted to detect selected resistance and virulence

determinants. Primers targeting the *pbp* (penicillin-binding protein) forward (ATCATACTAGCCATAAAGCG) and reverse (CCAATAATTTTACATAGCTAAAG) (Jayakar *et al.,* 2019) and *hlb* (beta-hemolysin) forward (GTGCACTTACTGACAATAGTGC) and reverse (GTTGATGAGTAGCTACCTTCAGT) (Kmieciak *et al.,* 2016; Shahzad *et al.,* 2024) were used. PCR conditions included initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation (95 °C, 30 s), annealing (55–60 °C, 30 s), and extension (72 °C, 1 min), with a final extension at 72 °C for 10 min. Amplicons were resolved on 1.5% agarose gels and visualized under UV illumination.

RESULTS

Bacterial Load in Zobo Samples

The total viable bacterial counts of five *zobo* drink samples is presented in Table 1. For estimation of total aerobic bacterial load on NA, counts ranged from 6 × 10^3 to 48 × 10^3 CFU/mL with a mean of 22.80 ± 17.34 × 10^3 CFU/mL.

Table 1: Total viable bacterial counts (10^3 CFU/mL) in zobo drink samples across vendors

Samples	NA	MAC	SSA
Admin	15.0	2.0	1.0
Hall 1	33.0	59.0	1.0
Hall 2	48.0	4.0	1.0
Hall 6	6.0	3.0	2.0
Hall 7	12.0	1.0	2.0
Mean	22.80±17.34	13.80±25.62	01.20±0.45

Bacterial Species Present in Zobo Drink Samples

Morphological, Gram staining, and biochemical characterization revealed the presence of multiple bacterial genera. The predominant isolates included *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, *Micrococcus luteus*, and *Enterococcus faecalis*. These organisms are recognized opportunistic pathogens and have been implicated in foodborne illnesses and spoilage. Cultural and morphological features of these isolates are summarized in Table 2.

Antibiotic Susceptibility Profiles

Disk diffusion assays demonstrated widespread resistance among isolates. Before plasmid curing, resistance was most pronounced against amoxicillin, ampicillin, and zinacef. The multidrug resistance index (MDRI) ranged from 0.40 to 0.60 (where an index > 0.30 indicates significant resistance burden) (Table 3). Following plasmid curing, susceptibility patterns shifted significantly, with several isolates regaining sensitivity which highlights the role of plasmid-borne determinants in resistance (Table 4).

Table 2: Morphological and biochemical characterization of isolates

PARAMETERS	N1	N2	N3	N4	N5
CULTURAL					
Shape	Circular	Irregular	Circular	Circular	Circular
Colour	White	Cream	Yellow	Yellow	Cream
Size	Large	Large	Medium	Medium	Medium
Elevation	Flat	Flat	Convex	Raised	Flat
Transparency	Opaque	Opaque	Opaque	Translucent	Transparent
MORPHOLOGICAL					
Gram stain	Positive	Positive	Positive	Positive	Positive
Cell type	Cocci	Rod	Cocci	Cocci	Cocci
Cell arrangement	Chains	Single	Clusters	Clusters	Scattered
Spore staining	-	+	-	-	-
BIOCHEMICAL					
Urease	-	-	-	-	-
Indole	-	-	-	-	-
Citrate	-	+	+	+	-
Catalase	+	-	+	+	-
Coagulase	+	-	-	-	
Oxidase	-	+	+	-	-
H_2S	-	-	-	-	-
Motility	-	-	-	+	-
OXIDATIVE FERMENTATION					
Glucose	+	+	A	-	A
Sucrose	-	+	-	-	A
Lactose	+	-	A	-	A
Isolate's identity	*Staphylococcus aureus*	*Bacillus subtilis*	*Staphylococcus epidermidis*	*Micrococcus luteus*	*Enterococcus faecalis*

Table 3: Antibiotic resistance profiles before plasmid curing.

Gram positive (+ve)	CPX	S	SXT	E	PEF	CN	APX	Z	AM	RD	MDRI
Staphylococcus aureus	S	S	S	S	S	R	R	R	R	S	0.40
Bacillus subtilis	S	S	R	R	R	S	S	R	R	S	0.50
Staphylococcus epidermidis	S	S	S	S	S	R	R	R	R	S	0.40
Micrococcus luteus	S	S	S	R	S	R	R	R	R	R	0.60
Enterococcus faecalis	S	S	S	R	R	S	R	R	R	R	0.60

Note: CPX = Ciprofloxacin, S = Streptomycin, SXT= Septrin, E= Erythromycin, PEF = Pefloxacin CN= Gentamicin, APX = Ampicillin, Z = Zinacef, AM = Amoxicillin, RD = Rifampicin, Resistance (R) = zone diameter ≤10 mm; Sensitivity (S) = zone diameter ≥ 18mm MDRI = Multidrug resistance index

Plasmid Profiling

Agarose gel electrophoresis confirmed the presence of extrachromosomal DNA of approximately 800 bp in isolated bacteria (Figure 1) which correlates with observed MDR phenotypes reported in Table 3. This supports the hypothesis that resistance determinants may be plasmid-borne.

Table 4: Antibiotic resistance profiles after plasmid curing.

Gram positive (+ve)	CPX	S	SXT	E	PEF	CN	APX	Z	AM	R	MDR
Staphylococcus aureus	S	S	S	S	S	S	S	S	S	S	0.00
Bacillus subtilis	S	S	S	S	S	S	S	S	S	S	0.00
Staphylococcus epidermidis	S	S	S	S	S	S	R	S	S	S	0.10
Micrococcus luteus	S	S	S	S	S	S	R	R	S	S	0.20
Enterococcus faecalis	S	S	S	S	S	S	R	R	S	S	0.20

Key: CN= Gentamicin, APX = Ampicillin, Z = Zinacef, AM = Amoxicillin, R = Rifampicin, CPX = Ciprofloxacin, PEF = Pefloxacin, S = Streptomycin, S= Septrin, E= Erythromycin, Resistance (R) = ≤10 mm, Sensitivity (S) ≥ 18mm

Molecular Detection of Resistance and Virulence Genes

PCR amplification revealed the presence of the *hlb* (beta-hemolysin) gene in *Staphylococcus aureus* isolate (Figure 2), confirming their pathogenic potential. Additionally, the *pbp* (penicillin-binding protein) gene was detected in selected isolates including *Bacillus subtilis*, *Staphylococcus epidermidis* and *Micrococcus luteus* (Figure 3), supporting the observed resistance to beta-lactam antibiotics.

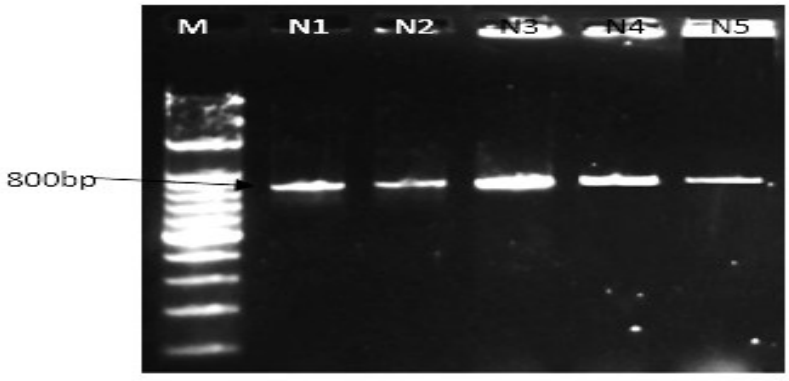


Figure 1: **Agarose gel electrophoresis of plasmid profile of isolated bacteria from zobo drinks**

Lanes: M = molecular size marker (base pairs, bp); N1 = *Staphylococcus aureus*; N2 = *Bacillus subtilis*; N3 = *Staphylococcus epidermidis*; N4 = *Micrococcus luteus*; N5 = *Enterococcus faecalis*

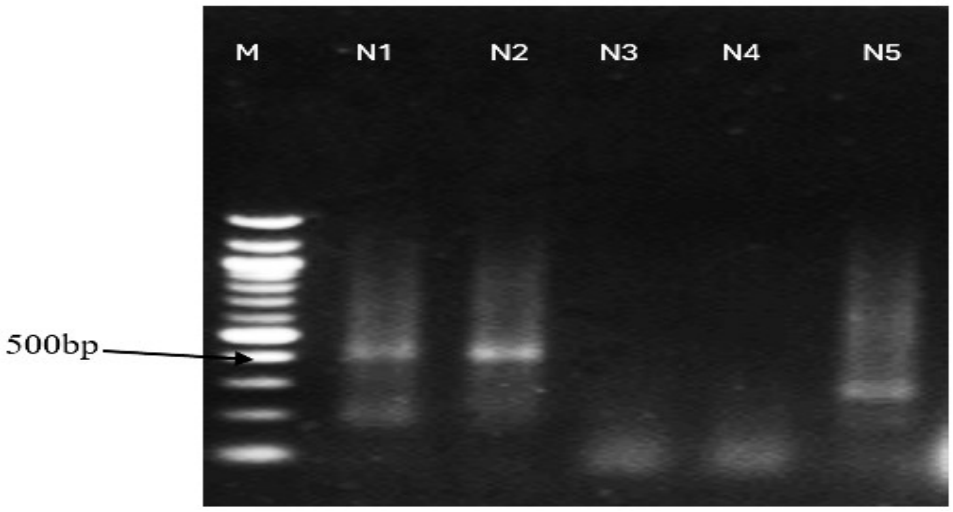


Figure 2: **Agarose gel electrophoresis of PCR amplification of the *hlb* gene of isolated bacteria from zobo drinks**

Lanes: M = molecular size marker (base pairs, bp); N1 = *Staphylococcus aureus*; N2 = *Bacillus subtilis*; N3 = *Staphylococcus epidermidis*; N4 = *Micrococcus luteus*; N5 = *Enterococcus faecalis*

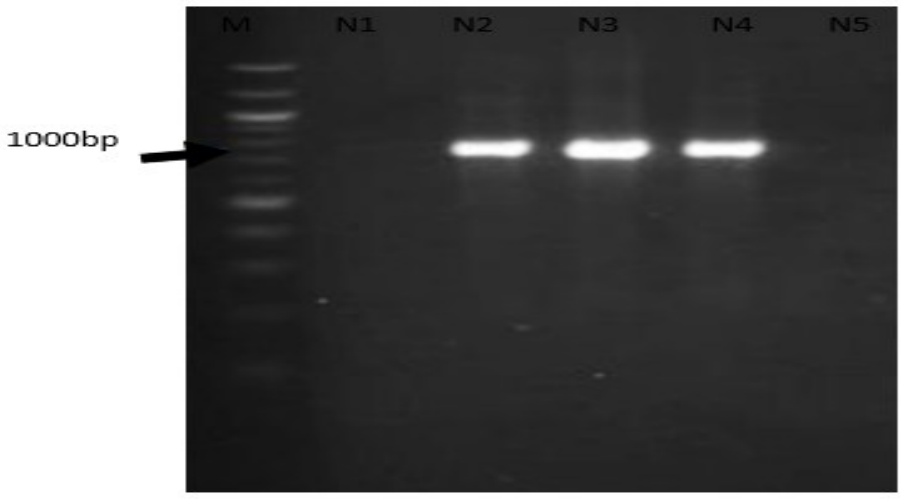


Figure 3: **Agarose gel electrophoresis of PCR amplification of the *pbp* gene of isolated bacteria from zobo drinks**

Lanes: M = molecular size marker (base pairs, bp); N1 = *Staphylococcus aureus*; N2 = *Bacillus subtilis*; N3 = *Staphylococcus epidermidis*; N4 = *Micrococcus luteus*; N5 = *Enterococcus faecalis*

DISCUSSION

The present study provides evidence that *zobo* drinks sold within Edo State University, Iyamho, are contaminated with diverse bacterial species. Total viable counts exceeded acceptable thresholds for ready-to-eat drinks. Notably, all samples exceeded the WHO recommended limit of $< 1 \times 10^2$ CFU/mL for heterotrophic plate count in drinking water (WHO, 2017), and Nigerian Industrial Standard (NIS) for packaged water ($< 1 \times 10^2$ total viable count) (NIS, 2007) indicating poor microbiological quality across all vendor locations. These organisms are well-recognized opportunistic pathogens, capable of producing toxins and causing gastrointestinal or systemic infections, particularly in immune-compromised individuals. These findings align with previous reports from Nigeria and other low- and middle-income countries, where informal beverages frequently harbor diverse microbial populations (Bonfoh *et al.,* 2024; Egbueri *et al.,* 2025; Oduori *et al.,* 2022; Roy *et al.,* 2023). Ugwuanyi et al. (2015) reported *Enterococcus* spp. in locally prepared zobo beverages, attributing contamination to poor hygiene during production and vending. *Staphylococcus aureus* and *Bacillus subtilis* have also been frequently isolated from *zobo* samples in Auchi and Lagos, where contamination was attributed to poor hygiene and inadequate storage conditions (Oleghe *et al.,* 2022; Uwandu *et al.,* 2022; Ike *et al.,* 2025). Similarly, *Enterococcus faecalis* and *Micrococcus luteus* have been documented in traditional drinks in West Africa, underscoring the ubiquity of these organisms in informal food systems (Adekoya *et al.,* 2019; Nwaiwu *et al.,* 2020; Ghosh *et al.,* 2024). *Staphylococcus epidermidis* and *Micrococcus luteus*, typically are harmless skin commensals. The occurrence of *Micrococcus luteus* and *Bacillus subtilis* aligns with findings by Awe & Abdulmumini (2019), who identified such commensals in traditional drinks, suggesting environmental or human-source contamination pathways. While metabolic activities of *Micrococcus luteus* can contribute to potential food spoilage, *Staphylococcus epidermidis* can become opportunistic pathogen especially in immunocompromised individuals or those with implanted medical devices due to its ability to form biofilms that protect it from the immune system and antibiotics. Their presence in zobo drinks indicate contamination likely from handling with unclean hands or equipment and highlights the critical need for strict hygiene practices to prevent potential infections caused by these bacteria.

The observed resistance before plasmid curing illustrates a MDR phenotype in bacteria isolated from zobo drink samples. These bacteria's resistance mechanisms may include production of enzymes like β-lactamases that degrade penicillins (Ampicillin, Amoxicillin), target site modifications, efflux pumps reducing intracellular drug concentration, or altered cell permeability, which are common in Gram-positive bacteria. The MDR profiles observed in this study mirror global trends in AMR surveillance, where resistance to commonly prescribed antibiotics such as ampicillin, amoxicillin, and erythromycin is widespread (Marino *et al.,* 2025; Mazhar *et al.,* 2025; Sajish *et al.,* 2025). The MDR index values (0.4–0.6) are comparable to those reported in studies of street foods in Ghana and India (Mensah *et al.,* 2025; Onohuean *et al.,* 2025; Sinha *et al.,* 2025), suggesting that informal beverages may

serve as reservoirs of resistance across diverse geographic contexts. University campuses represent densely populated environments where contaminated beverages can facilitate rapid transmission of pathogens (Meisterling *et al.,* 2022).

The presence of MDR bacteria in food products like zobo drinks indicates potential health risks to consumers, including infections that could be difficult to treat with routine antibiotics. This could lead to treatment failures and increased healthcare burdens (Ifedinezi *et al.,* 2024). Food and beverages contaminated with resistant bacteria may act as reservoirs for resistance genes, facilitating their transmission through the food chain to humans, exacerbating the global antimicrobial resistance crisis. Overuse and misuse of these antibiotics in clinical and non-clinical settings create selective pressure that encourages the survival and spread of resistant strains. This is a major factor driving resistance development worldwide. In the context of global AMR surveillance, these findings underscore the previously overlooked role of informal food systems as reservoirs of resistance, emphasizing the need for enhanced hygiene practices, regulatory oversight, and targeted interventions in high-risk settings.

Plasmid profiling confirmed the presence of extrachromosomal DNA among the isolates, which may encode virulence or resistance traits. These plasmids may carry antibiotic resistance genes observed in antibiotic susceptibility tests. The presence of plasmid-mediated antibiotic resistance in *zobo* isolates could imply a high risk for horizontal gene transfer among bacteria, facilitating the rapid spread of resistance in populations. This demonstration adds a novel dimension to existing literature. While earlier studies have documented bacterial contamination in street foods and drinks, few have integrated molecular assays to confirm the genetic basis of resistance (Fusaro *et al.,* 2024; Onohuean *et al.,* 2025; Salamandane *et al.,* 2022; Samtiya *et al.,* 2022; Sivakumar *et al.,* 2021). Plasmid curing experiments demonstrated that resistance was possibly plasmid-borne, highlighting the potential for horizontal gene transfer to amplify resistance within microbial communities (Vesel *et al.,* 2025; Wachino, 2025). These findings align with the findings of Bush and Bradford (2019), who noted plasmid-linked MDR in foodborne *Staphylococci* and *Streptococci*.

The detection of *pbp* and *hlb* genes in bacterial isolates further highlights the pathogenic potential of these isolates linking resistance to virulence in a manner that complicates treatment outcomes (Jhalora & Bist, 2025). It provides direct evidence of virulence and resistance determinants, situating *zobo* drinks within the broader global discourse on mobile genetic elements and AMR dissemination. The presence of *hlb* gene in *Staphylococcus aureus* is consistent with well-documented hemolytic potential of *S. aureus* and suggests that among the zobo contaminants, *S. aureus* poses a heightened virulence risk. The presence of *pbp* gene correlates strongly with the phenotypic beta-lactam resistance observed in antibiotic susceptibility testing, where 80% of isolates were resistant to ampicillin, amoxicillin, and cefuroxime. This concordance between genotypic and phenotypic data supports the conclusion that *pbp*-mediated mechanisms are likely contributing to the high beta-lactam resistance burden in Gram-positive bacteria contaminating zobo drinks.

Intervention studies assessing the impact of hygiene training for vendors, improved storage practices, and regulatory oversight could provide actionable strategies for reducing contamination. In addition, collaboration with public health authorities to incorporate informal beverages into AMR surveillance frameworks would ensure that these overlooked reservoirs are addressed in national and global strategies.

CONCLUSION

In conclusion, this study demonstrates that *zobo* drinks sold within Edo State University, Iyamho harbor potential pathogenic bacteria with plasmid-mediated multidrug resistance and virulence genes. These findings highlight the dual risks of foodborne illness and AMR dissemination in university settings, where consumption is widespread, and population density facilitates transmission. By linking local beverage safety to global AMR concerns, this work highlights the urgent need for hygiene interventions, regulatory frameworks, and surveillance strategies tailored to informal food systems. Addressing these challenges is critical to safeguarding public health and curbing the spread of AMR.

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