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SCREENING AND CHARACTERIZATION OF MULTIDRUG RESISTANT *ENTEROCOCCI* FROM DOMESTIC EFFLUENTS WITHIN NASARAWA STATE, NIGERIA.

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Abstract

Enterococci are predominantly pathogens inhabiting the intestinal mucosa of animals and humans. The exchange of Plasmid or transposon mobility facilitates horizontal gene transfer among enterococci which favours the development of drug-resistant strains. This study was aimed to determine the frequency and characterize multidrug resistant Enterococci isolated from various domestic effluents in Nasarawa State, Nigeria. A total of 150 samples of domestic effluents from households, dairy farms, restaurants, water factories and bakeries were obtained. Standard cultural and molecular techniques were employed for isolation and identification. Antimicrobial susceptibility profile of *Enterococcus* species was determined using the Kirby-Bauer disc diffusion method and Multidrug resistance (MDR) was determined. All isolates were cured of their plasmids using acridine orange technique. The percentage occurrence of *Enterococcus* species across all the five sources were: *E. faecalis* (78.0%), *E. faecium* (17.3%), *E. durans* (1.3%), *E. hirae* (1.3%) and *E. gallinarum* (0.6%). MDR for *E. faecalis*, the highest percentage was found in Household (81.8%) with MDR index of 0.6 while *E. faecium* recorded the highest percentage in Household (83.3%) with MDR index of 0.7, other species isolated recorded a higher frequency in water factory (100%) with MDR index of 0.9. The presence of plasmid genes (pTEF, vanA, pAD1, pRUM and pAD1) were detected and the isolates' percentage reduction in antibiotic resistance before and after plasmid curing range from 28.5-100.0 %. The presence of multiple resistant indices in *Enterococcus* spp. indicates the possibility

of outbreaks of pathogenic and multidrug resistant enterococcal infections from domestic effluents.

Keywords: Antibiotics, Effluents, *Enterococcus* spp., Multidrug resistance, Plasmids.

Introduction

Enterococci are Gram-positive, ovoid shaped bacteria found in pairs or chains of various length; they are mostly facultative anaerobes, non-capsulated and are lactic acid bacteria with an ideal growth temperature range of 35 - 37 °C. However, some species may thrive in temperatures as low as 10°C or as high as 45°C. These bacteria can also adapt to high sodium chloride (NaCl) concentrations of 6.5% and can hydrolyze bile esculin. They are chemoorganotrophs, catalase negative and some species show haemolytic activity (Generalov, 2017; Růžičková, Vitezova and Kushkevych, 2020; Vanamala et al., 2022). Humans' and animals' gastrointestinal tracts are home to the genus Enterococci, which is also an important pathogen that can result in endocarditis, bacteraemia, infections of surgical wounds, and newborn sepsis. At least twelve (12) species of Enterococci can cause infections, however most of clinical infections can be due to either *Enterococcus feacalis* or *Enterococcus feacium*, but occasionally can be caused by *Enterococcus raffinosus, Enterococcus gallinarum*, *Enterococcus durans*, *Enterococcus casseliflavus*, *Enterococcus avium*, *Enterococcus pseudoavium*, *Enterococcus malodoratus*, *Enterococcus mundtii*, and *Enterococcus hirae* (Abamecha, Wondafresh and Abdissa, 2015; Krawczyk *et al*., 2021; Farsi *et al*., 2023).

Water usage introduces several organic, inorganic, and synthetic components such as metals, chemicals, fertilizers, dirt, oil, plant and animal wastes into wastewater. This makes effluents from various sources, including domestic waste, restaurants, hospitals, dairy farms, and sachet water factories, a potential medium for pathogenic bacteria like the Enterococci. These organic compounds are typically biodegradable and contain nutrients that can be metabolized by microorganisms (Chahal *et al*., 2016). Furthermore, antimicrobial drugs have been widely used in human and veterinary medicine to kill pathogenic bacteria (Caneschi *et al*., 2023; Prescott, 2024). According to Kümmerer in 2003, antibiotic market usage is thought to range from 100,000 to 200,000 tons globally. Thus, exerting pressure for the resistance in harmful bacteria to develop and spread. Enterococci have the capacity of acquiring high level of resistance to some antibiotics, making the treatment options limited for clinicians. Notably, because of how these bacteria are spread to people through water-related activities as well as the spread of genes that code for antimicrobial resistance (AR) to other bacteria, resistance to antimicrobials that are introduced into surface water by human and agricultural waste is of public health concern (Cho *et al.*, 2020; Almeida-Santos 2021). Furthermore, Enterococci can express acquired resistance through mutations or through the uptake of foreign genetic material (Krause *et al*., 2022). Noteworthy, Plasmid exchange or transposon mobility facilitates horizontal gene transfer among enterococci. To create composite mobile elements, these components may interact with the bacterial chromosome and one another. Plasmids with a wide host range can transmit genetic information, which necessitates tight cell interaction (Emamalipour *et al*., 2020). Partridge *et al*., in 2018 stated that most of the gene mobility in enterococci is caused by three different types of transposons: transposons from the Tn3 family, as well as composite and conjugative transposons.

For decades, the development of drug-resistant strains has been of great public health concern to Clinical professionals because both Gram-positive and Gram-negative bacteria can acquire transferable

genetic material from Enterococci, which has an unusually broad host range (Klemm *et al*., 2018). Thus, there is need for sustained surveillance of *Enterococcus* species to enhance comprehensive prevention and control measures. Furthermore, Enterococci are part of the leading cause of endocarditis, urinary tract infections, abscess, soft tissue infection and bacteraemia. *E. feacalis* and *E. feacium* are the common causes of these life-threatening infections. Hence, the study of the frequency of occurrence of Enterococci from domestic effluents remains one of the important sources of information that will help in reducing the occurrence of this organism and underatnding the antibiotic susceptibility pattern of the isolate (Fiore *et al*., 2019; Khan *et al*., 2024). Molecular characterizations of this organism will also enhance our understanding of the strains in Nigeria and the world at large. Therefore, this study aimed at determining the occurrence and characterization of multi drug resistant Enterococci isolated from various domestic effluents in Nasarawa State, Nigeria.

Materials and Methods

Study Area

The study was conducted in the three senatorial zones of Nasarawa State, Nigeria. Effluent samples from households, restaurants, bakeries, water factories and dairy farms were collected from one major community in each of the senatorial zones namely; Akwanga (8°54'38.77''N 8°24'23.58''E), Keffi (8°50'47.44''N 7°52'24.74''E) and Lafia (8°29'38.04''N 8°30'55.15''E).

Sample Collection

A total of One hundred and fifty (150) samples from all the senatorial zones (Akwanga, Keffi and Lafia), comprising thirty (30) samples each (from households, restaurants, bakeries, water factories and dairy liquid effluent) were collected at two weeks intervals over two sampling periods specifically from 16th - 23rd June and 24th to 30th June, 2022. During each sampling period, fifteen (15) samples were collected from each site using sterile 250mls bottles. The bottles were directly submerged into the surface of the effluents to collect the sample. bi-weekly within two (2) sampling periods from 16^{th} – 30^{th} of June 2022 respectively. Fifteen (15) samples from each of the sampling sites were collected using sterile 250ml bottles by directly dipping the bottle into the surface of the effluent. The samples were packed into an ice box and immediately transported to the laboratory for analysis.

Isolation and Identification of *Enterococci* spp.

The isolation of Enterococcus spp. Was carried out by the method described by Aryal *et al.*, (2019). Briefly, using a sterile pipette, 0.5 ml of each sample was aseptically added to a test tube containing 9.5 ml of Tryptic Soy Broth (TSB) and incubated for 18 hours at 37°C. After incubation, tubes that showed bacterial growth were streaked onto Bile Aesculin Azide Agar plates and incubated at 37°C for 24 hours. Presumptively isolated Enterococci colonies were Gram stained, and confirmed for catalase production, salt tolerance (ability to grow in 6.5% NaCl broth) and hydrolysis of esculin in the presence of bile. Preliminary identification of *Enterococci* species was carried out using sugar fermentation test based on arginine, mannitol, sorbitol, and melibiose biochemical reaction.

Antimicrobial Susceptibility Testing for the *Enterococci* spp.

The Kirby-Bauer disc diffusion method for susceptibility testing was used to examine the susceptibility of all the isolates using standard protocol as earlier described by the Clinical and Laboratory Standards Institute (2014). Briefly, each isolate (0.5 McFarland) was aseptically streaked on Mueller-Hinton agar plate, and the respective antibiotics discs were placed on Mueller-Hinton agar plates using sterile forceps and incubated at

37°C for 24 hours. Clinical and laboratory standards institute (CLSI, 2014) established limits for inhibition zone diameter were compared. The Multi Drug Resistance Index (MDRI) was determined by calculating the ratio between the number of antibiotics the isolate is resistant to and the total number of antibiotics the isolate is exposed to; thus, MDRI = $^{a}/_{b}$ Where; 'a' represent the number of antibiotics to which the test isolate is resistant to and 'b' represents the total number of antibiotics to which the test isolate has been exposed.

Molecular Identification of *Enterococci* spp.

The bacterial DNA extraction and quantification was carried out with minor modification in the method earlier described by Abimiku *et al.,* (2016). Briefly, ten (10) milliliters of an overnight broth culture of the bacterial isolate in 1mL Luria Bertani (LB) were spun at 14000rpm for 3 min. The supernatant was discarded, and the harvested cell pellet was resuspended in 1 mL sterile distilled water and transferred into 1.5 mL centrifuge tube and centrifuged at 14000rpm for 10 min. The supernatant was discarded carefully. The pellet was re-suspended in 100 µL of distilled water. The tube was centrifuged again at 14000 g for 10 min, and the supernatant was discarded carefully. The cells were re-suspended in 500µl of normal saline and heated at 95^{0}C for 20 min. The heated bacterial suspension was cooled on ice for 10mins and spun for 3 min at 14000rpm. The supernatant containing the DNA was transferred to a 1.5ml microcentrifuge tube and stored at -20°C for other downstream reactions. Further, the extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. A 2µl of the extracted DNA was loaded onto the lower pedestal, the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the "measure" button.

DNA Amplification by multiplex Polymerase Chain Reaction (PCR)

The multiplex polymerase chain reaction (mPCR) was carried out by an established procedure as earlier adapted by Abimiku *et al.,* (2019). Briefly, this was performed in 25 µl total volume using the ABI 9700 Applied Biosystems thermal cycler, containing 2.5 µl $MgCl_2$ (50 mM), 2.5 µl 10 × buffer, 0.9 µl of each primer (2.7 µM), 8 mM of dNTPs, 0.5 µl Taq DNA polymerase and 5 µl of DNA template. Amplification was carried out in the following program: an initial denaturation step at 95°C for 5 minutes, followed by 35 denaturation cycles at 94°C for 1 min, annealing at 55°C for 30s and extension at 68°C for 30s, and a final extension step at 68 °C for 7 minutes.

Plasmid DNA Extraction

A technique previously described by Carattoli *et al*., (2005) was used to extract the bacterial DNA. Each bacterial isolate was inoculated into 10 mL of Luria-Bertani (LB) broth supplemented with the specific antibiotics corresponding to the plasmid-borne resistance markers identified prior to curing. The cultures were incubated at 37°C for 16 hours with shaking to select for plasmid-harbouring cells. The antibiotic selection was based on the following resistance profiles: Hygromycin B for pTEF1, Streptomycin for pAD1, Vancomycin for vanA and Erythromycin for pRUM. Subsequently, the cultures were centrifuged at 8000 rpm. The pelleted cells were then transferred to a micro centrifuge tube and re-suspended in 250 ml of re-suspension solution. Exactly, 250 µl of lysis solution was added and mixed thoroughly by inverting the tube 4-6 times until solution was viscous and slightly clear. The supernatant was transferred to GeneJET spin column by decanting, centrifuged for 1 minute and the flow-through was discarded. The GeneJET spin column was transferred into fresh 1.5 ml microcentrifuge tube and 50µl of elution buffer was added to the center of the column to elute plasmid DNA. This was incubated for 2 minutes at room temperature, centrifuged for 2 minutes, and

the purified plasmid DNA was stored at -20°C.

Detection of number and sizes of plasmid DNA by Agarose Gel Electrophoresis (DNA Quantification).

Briefly, this was carried out with modification on the established protocol as earlier described by Armstrong and Schulz (2015) and Green and Sambrook (2019). The DNA fragment was resolved using 2% agarose gel. This was made by adding 2g of agarose to 10ml of tris-borate Ethylene-Diamine-Tetraacetate buffer and 90ml of sterile distilled water in a 250ml beaker flask, then heated for two (2) minutes. The dissolved agarose solution was added to, and thoroughly mixed with, 7µl of ethidium bromide. Casting combs were added after the gel was poured onto a small horizontal gel electrophoresis tank. The gel was given 30 minutes to set. Tris-Borate-Ethylene-Diamine-Tetraacetic Acid (TBE) buffer at one time concentration (1X) was added to the reservoir until the agarose gel was completely coated. With gentle mixing, exactly 8µl of the gel tracking dye (bromophenol blue) was applied to 10µl of each sample. Each well of the agarose gel was loaded with 10 µl of the DNA samples, after which electrophoresis was carried out at 100–200 mA for one hour. The gels were loaded with samples of the gel. Afterwards, the gel was then removed from the buffer and was viewed under UV-transilluminator, photographed with a Polaroid camera and the electrophoresis documenting system was used to document it.

Plasmid curing

The isolates were cured of their plasmids using the acridine orange technique as described by Igwe *et al*., (2013). Using a sterile pipette, 200µL of bacterial culture (0.5 McFarland standard) was introduced into each of the different dilutions and incubated at 37°C. After 24 h the susceptibility test and Subminimal Inhibitory Concentration (SIC) of acridine orange was determined. Subsequently, individual bacterial colonies were streaked separately onto Mueller Hinton agar with and without antibiotics (ampicillin (10 µg), ampiclox (20µg), rifampicin (20µg), erythromycin (30µg), gentamicin (10µg), chloramphenicol (30µg), norfloxacin (10µg), Levofloxacin (20µg) and ciprofloxacin (10µg)) and the plates were incubated at 37°C for 24 h. The presence of colonies on a media devoid of antibiotics and the lack of the same colonies on a medium with antibiotics were regarded as cured strain.

Statistical Analysis

All data from the results of this study were presented in frequencies and percentages. Crosstabs (χ^2 analysis) was computed to assess frequency differences of *Enterococcus* species with respect to their statistical significances to sources of domestic effluents, resistance to antibiotics and multidrug resistance index. All analyses were conducted with Statistical Package for Social Sciences version 26 (IBM SPSS Inc. IL, Chicago, USA). Two-tailed *P*-values of <0.05 were significant.

Results

Occurrence of *Enterococcus* species Isolated from domestic effluents

Out of 148 isolates obtained from residential wastewater samples, five Enterococci species: *E. faecalis, E. faecium, E. durans, E. hirae, and E. gallinarum* were identified. Regardless of the sampling location, *Enterococcus faecalis* predominated in effluent samples, with the following percentages: 86.7% (n = 26), 83.3% (n = 25), 66.0% (n = 20), 73.3% (n = 22), and 80% (n = 24) in the dairy farms, homes, water factories, and bakeries, respectively. *E. faecium*, which constituted 3.3% (n = 1), 16.7% (n = 5), 30% (n = 9), 16.7% (n = 5), and 20% (n = 6) in dairy farm, households, water factory, restaurant, and bakery effluents, respectively, was the second most prevalent species. The abundance of the remaining species in wastewater was substantially lower, with *E. gallinarum* having 3.3% (n = 1), *E. durans* having

6.67% (n = 2) and *E. hirae* having 6.67% (n = 2) in dairy farm effluent as seen in Table 1. There was significant difference in the occurrence of individual *Enterococcus* species isolated from domestic effluents.

Table 1: Prevalence and distribution of *Enterococcus* species isolated from various domestic effluents.

Effluent Type	N	*E. faecalis* n (%)	*E. faecium* n (%)	*E. hirae* n (%)	*E. durans* n (%)	*E. gallinarum* n (%)	χ^2	*P*
Dairy Farm	30	26(86.7)	1(3.3)	2(6.67)	0	0	334.53	<0.001[x]
Household	30	25(83.3)	5(16.7)	0	0	0		
Water Factory	30	20(66.0)	9(30)	0	0	1(3.3)		
Restaurants	30	22(73.3)	5(16.7)	0	2(6.67)	0		
Bakery	30	24(80.0)	6(20.0)	0	0	0		
Total	150	117(78.0) $\chi^2 = 0.99$, $P = 0.911$[y]	26(17.3) $\chi^2 = 7.54$, $P = 0.057$[y]	2(1.3%)	2(1.3)	1(0.6)		

N = Total no. of samples; n = No. of positive samples; [x]= Values with significant difference; [y] = Values without significant difference.

As shown in Table 2, the isolated bacteria were tested against 10 antimicrobial agents belonging to 5 antibiotic groups. Among the 117 tested *E. faecalis* isolates, 37(31.6%) were resistant to Gentamycin, 30(25.6%) to Chloramphenicol, 62(53%) to Amoxicillin, 1(0.6%) to Levofloxacin, 59(50.4%) to Norfloxacin, 61(52.1%) to Rifampicin, 8(6.8%) to Ciprofloxacin, 50(42.7%) to Erythromycin and 46(39.3%) to Streptomycin. For the 26 tested *E. faecium,* 6(23.1%) were resistant to Gentamycin and Streptomycin, 7(26.9%) to Amoxicillin and Rifampicin, 4(15.4%) to Chloramphenicol, 10(38.5%) to Norfloxacin, 1(3.8%) to Ciprofloxacin and 8(30.8%) to Erythromycin. The low abundant species isolates have 3(60%) resistant to Gentamycin and Chloramphenicol, 2(40%) to Levofloxacin, Norfloxacin, Rifampicin and 4(80%) to Amoxicillin. There was a significant difference (P<0.05) in some reactions of the isolates to certain antimicrobial agents.

Table 2: Antimicrobial resistance in isolates of *Enterococcus* species

Antibiotics	*E. faecalis (N=117)*		*E. feacium (N=26)*		*Other species (N=5)*			
	No. of Antibiotics Resistant Isolates	% Resistance	No. of Antibiotics Resistant Isolates	% Resistance	No. of Antibiotics Resistant Isolates	% Resistance	χ^2	*P*
Gentamycin	37	31.6	6	23.1	3	60.0	557.93	<0.001[x]
Chloramphenicol	30	25.6	4	15.4	3	60.0		
Amoxicillin	62	53.0	7	26.9	4	80.0		
Levofloxacin	1	0.6	0	0	2	40.0		
Norfloxacin	59	50.4	10	38.5	2	40.0		
Rifampicin	61	52.1	7	26.9	2	40.0		
Ciprofloxacin	8	6.8	1	3.8	0	0		

Erythromycin	50	42.7	8	30.8	2	40.0
Streptomycin	46	39.3	6	23.1	3	60.0
Ampiclox	58	49.6	9	34.6	4	80.0
	$\chi^2 = 106.45$, $P < 0.001^x$		$\chi^2 = 14.66$, $P = 0.023^y$		$\chi^2 = 0.08$, $P = 0.961^y$	

N= No. of isolates screened; x= Values with significant difference; y = Values without significant difference

Multidrug resistant pattern of Enterococcus faecalis isolates The E. faecalis isolates gotten from this study were tested for multidrug resistance in Table 3 according to the different sources where they are isolated. The highest percentage (81.8%) was found in E. feacalis from Household, with 18 isolates which were resistant to 6 antibiotics (CHc, AMXp, NBf, RDm, Sa, APXp) belonging to five groups of antimicrobials which included cephalosporin, penicillin, fluoroquinolone, macrolide and aminoglycoside and a multidrug resistant index (MDR) of 0.6. The lowest percentage (5.6%) was found in E. faecalis from Dairy farm with 1 isolate which was resistant to four antibiotics (CNa, CHc, AMXp, APXp) belonging to 3 groups of antimicrobials which include aminoglycoside, cephalosporin and penicillin and a multidrug resistant index (MDR) of 0.4.

Table 3: Multi-drug resistance index of *Enterococcus faecalis* isolates from various effluents

Sources	N	Antibiotics	No. of Isolates (%)	MDRI	χ^2	*P*
Dfl	18	CN^a, CH^c, AMX^p, NB^f, RD^m,E^mS^a,APX^p	10(55.6)	0.8	7.00	0.030^x
		CN^a, CH^c, AMX^p, RD^m, S^a APX^p	7(38.9)	0.6		
		CN^a, CH^c, AMX^p, APX^p	1(5.6)	0.4		
Hfl	22	CN^a, CH^c, AMX^p, NB^f, RD^m,APX^p	4(18.2)	0.6	8.91	0.003^x
		CH^c,AMX^p,NB^f,RD^m,S^a,APX^p	18(81.8)	0.6		
Wfl	9	CN^a, CH^c, AMX^p, RD^m,E^m,S^a,APX^p	1(11.1)	0.7	2.00	0.368^y
		CH^c,AMX^p,NB^f,CPX^f,E^m,S^a,APX^p	2(22.2)	0.7		
		CH^c,AMX^p,NB^f,CPX^f,E^m	4(44.4)	0.5		
		CN^a,CH^c,AMX^p,APX^p	2(22.2)	0.4		
Rfl(5)	5	CN^a,AMX^p,LEV^f,NB^f,RD^m,E^m,S^a,APX^p	2(40.0)	0.8	1.80	0.180^y
		AMX^p,NB^f,RD^m,E^m,S^a	1(20.0)	0.5		
		AMX^p,NB^f,RD^m,E^m	2(40.0)	0.4		
Bfl	8	CN^a, CH^c, AMX^p,NB^f, RD^m,S^a,APX^p	1(12.5)	0.7	3.25	0.197^y
		CN^a,AMX^p,LEV^f,NB^f,RD^m,E^m,APX^p	2(25.0)	0.7		

N= No. of multidrug resistant isolates; MDRI=Multidrug resistant index; CN=Gentamycin; CH=Chloramphenicol; AMX=Amoxicillin; LEV= Levofloxacin; NB=Norfloxacin; RD=Rifampicin; CPX=Ciprofloxacin; E=Erythromycin; S=Streptomycin; APX=Ampiclox; a= Aminoglycoside; c = Cephalosporin; p = Penicillin; m = Macrolides; f = flouroquinolones; Dfl= *E. faecalis* from Diary Farm*;* Hfl= *E. faecalis* from Household; Rfl= *E.faecalis* fromRestaurant; Bfl= *E. faecalis* from Bakery,^{x}= Values with significant difference; ^{y} = Values without significant difference.

Multidrug resistant pattern of *Enterococcus faecium* isolates

As shown in Table 4, *E. faecium* isolates gotten from this study were tested for multidrug resistance according to the different sources they were isolated. The highest percentage (83.3%) was found in *E. faecium* from Household, with five isolates which were resistant to seven antibiotics (CN^a, CH^c, AMX^p, NB^f, RD^m, S^a, APX^p) belonging to five groups of antimicrobials which include aminoglycoside, cephalosporin, penicillin, fluoroquinolones and macrolide and the lowest percentage (16.7%) was recorded in *E. faecium* from Household with 1 isolate which was resistant to five antibiotics (CN^a, CH^c, AMX^P, RD^m, APX^P) belonging to four groups of antibiotics which include aminoglycoside, cephalosporin, penicillin and macrolide and a multidrug resistant index (MDR) of 0.5.

Table 4: Multi-drug resistance index of *Enterococcus faecium* isolated from various effluents

Sources	N	Antibiotics	No. of Isolates (%)	MDRI	χ^2	*P*
Hfc	5	CH^c,CH^c,AMX^p,NB^f,RD^m,S^a,APX^p	4(83.3)	0.7	2.67	0.102^y
		CN^a,CH^c,AMX^p,RD^m,APX^p	1(16.7)	0.5		
Wfc	6	CH^C,AMX^p,NB^f,CPX^f,E^m,APX^p	3(50.0)	0.6	1.00	0.607^y
		CN^a.CH^c,AMX^p,APX^p	2(33.3)	0.4		
		CH^c,NB^f,E^m	1(16.7)	0.3		
Rfc	2	CN^c,AMX^p,RD^m,E^m,S^a,APX^p	1(50.0)	0.6	0.00	1.000^y
		CN^a,CH^c,NB^f,RD^m,E^m	1(50.0)	0.5		
Bfc	2	CH^c,AMX^p,LEV^{fc},NB^f,E^m,S^a,APX^p	1(50.0)	0.7	0.00	1.000^y

N= No. of multidrug resistant isolates; MDRI=Multidrug resistant index; CN=Gentamycin; CH=Chloramphenicol; AMX=Amoxicillin; LEV= Levofloxacin; NB=Norfloxacin; RD=Rifampicin; CPX=Ciprofloxacin; E=Erythromycin; S=Streptomycin; APX=Ampiclox; a = Aminoglycoside; c = Cephalosporin; p = Penicillin; m = Macrolides; f = flouroquinolones; Hfc= *E. faecium* from Household; Rfc= *E.faecium* from Restaurant; Bfc=*E. faecium* from Bakery; ^{y} = Values without significant difference

Multidrug resistant pattern of other *Enterococci* species

As shown in Table 5, other species are isolated which included *Enterococcus hirae, Enterococcus durans* and *Enterococcus gallinarum* and were also tested for multidrug resistance. The isolate with the highest percentage was found in water factory effluent sample (100.0%) with one isolate resistant to nine antibiotics (CN^a, CH^c, AMX^p, LEV^f, NB^f, RD^m, E^m, S^a, APX^p) belonging to five (5) groups of antibiotics which included aminoglycoside, cephalosporin, penicillin, fluoroquinolones and macrolide with a multidrug resistant index (MDR) of 0.9 and a low percentage of 33.3% was recorded across all the 3 isolates from dairy farm.

Table 5: Multi-drug resistance index of other *Enterococcus* species isolated from various effluents

Sources	N	Antibiotics	No. of Isolates (%)		χ^2	*P*
DO	2	CN^a,CH^c,AMX^p,RD^m,E^m,S^a,APX^p	1(50.0)	0.7	0.00	1.000^y
		CH^c,AMX^p,NB^f,RD^m,S^a,APX^p	1(50.0)	0.6		
WO	1	CN^a,CH^c,AMX^p,LEV^f,NB^f,RD^m,E^m,S^a,APX^p	1(100.0)	0.9		

N= No. of multidrug resistant isolates; MDRI=Multidrug resistant index; CN=Gentamycin; CH=Chloramphenicol; AMX=Amoxicillin; LEV= Levofloxacin; NB=Norfloxacin; RD=Rifampicin; CPX=Ciprofloxacin; E=Erythromycin; S=Streptomycin; APX=Ampiclox; a = Aminoglycoside; c = Cephalosporin; p = Penicillin; m = Macrolides; f = fluoroquinolones; DO= other species from Dairy Farm; WO= other species from water factory; RO= other species from Restaurant; ^{y} = Values without significant difference.

Plasmid profile of *Enterococcus* isolates

In Table 6, the plasmid profile of all the isolates with the highest Multidrug resistant index (Plate 1) was determined. One plasmid was discovered across all the five isolates that were subjected to plasmid profiling. The isolates according to their various sources and their plasmids were Hfc11 (pTEF1), WO13(*vanA*), Dfl(pAD1), Bfc1(pRUM) and Rfl2(pAD1).

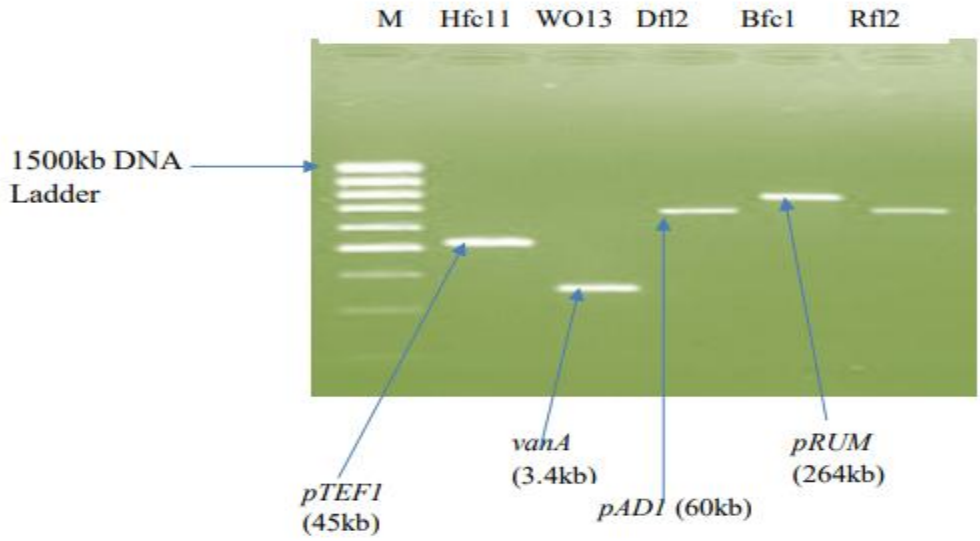


Plate 1: Agarose gel electrophoresis of Plasmids DNA from antibiotic resistant *Enterococcus* species with their respective bands and sizes.

Table 6: Plasmids profile of antibiotic-resistant genes of *Enterococcus* species

Isolates	Number of plasmids	Plasmids (kb)
Hfc11	1	pTEF1 (45kb)
WO13	1	*vanA* (3.4kb)
Dfl2	1	pAD1 (60kb)
Bfc1	1	pRUM (264kb)
Rfl2	1	pAD1 (60kb)

Hfc11= *E. feacium* from household; WO13= other species from water factory; Dfl2= *E. feacalis* from dairy farm; Bfc2= *E. faecium* from bakery*;* Rfl2= *E. faecalis* from Restaurant.

Antibiotic resistant pattern of some multidrug resistant *Enterococcus* species before and after plasmid curing.

In Table 7, the pattern of the drug resistance of isolates prior to and following plasmid curing was determined. *E. faecium* from household (Hfc11) effluent which was resistant to CN, CH, AMX, NB, RD, APX before plasmid curing became susceptible to all the antibiotics, *E faecalis* from dairy farm (Dfl2) effluent which was resistant to CN, CH, AMX, NB, RD, S, APX before plasmid curing became susceptible to all the antibiotics and *E. faecalis* from restaurant (Rfl2) effluent which was resistant to CN, AMX, NB, RD, E, S, APX before plasmid curing became susceptible to all the antibiotics. On the contrary, *Enterococcus gallinarum* from water factory (WO13) that was resistant to CH, AMX, NB, CPX, E, S, APX was resistant to CPX and NB after plasmid curing and *E. faecium* from bakery (Bfc1) effluent, which was resistant to CN, CH, AMX, RD, S, APX was still resistant to CN, CH, S and RD.

Table 7: Drug resistant pattern of some multidrug resistant *Enterococcus* spp. before and after plasmid curing.

Isolates	Resistant Profile before curing	Resistant profile after curing	% Reduction in resistance
WO13	CH, AMX, NB, CPX, E, S, APX	CPX, NB	28.57
Dfl2	CN, CH, AMX, NB, RD, S, APX	-	100.00
Bfc1	CN, CH, AMX, RD, S, APX	CN, CH, S, RD	66.67
Rfl2	CN, AMX, NB, RD, E, S, APX	-	100.00

Hfc11= *E. feacium* from household; WO13= other species from water factory; Dfl2= *E. feacalis* from dairy farm; Bfc2= *E. faecium* from bakery; Rfl2= *E. faecalis* from Restaurant.

Discussion

One of Nigeria's main environmental issues is water contamination. Domestic effluents are one of the many sources of this contamination. Poor-quality effluents that are capable of containing microorganisms that could infect the population have been produced as a result of ineffective wastewater treatment or failure to eliminate contaminants (Ndubuisi *et al.,* 2017). This study identified five *Enterococcus* species from 148 isolates obtained from domestic effluents, with *Enterococcus faecalis* being the most prevalent species across all effluent types. *E. faecalis* constituted between 66.0% and 86.7% of isolates from effluents sourced from dairy farms, homes, water factories, restaurants, and bakeries. These findings are consistent with previous studies that have reported *E. faecalis* as the dominant enterococcal species in various environmental samples, including wastewater (Iweriebor *et al*., 2015; Gotkowska-Płachta, 2021; Emurotu *et al*., 2023). Additionally, this is also similar to the report of Boehm and Sassoubre (2014) whose study reported *E. faecalis* and *E. faecium* as the most abundant in species isolated in waste water and water contaminated with fecal matter. The high prevalence of *E. faecalis* could be due to its strong ability to survive in diverse environments, including those with high nutrient loads and varying oxygen levels, such as wastewater. This specie is also well-known for its persistence under harsh environmental conditions, including exposure to bile salts, detergents, and antibiotics (Fisher and Phillips, 2009; Boeder *et al*., 2024). The significant abundance of *E. faecalis* in wastewater presents a public health concern because this species is a major cause of healthcare-associated infections (Toru *et al*., 2018; Gotkowska-Płachta, 2021; Said *et al*., 2024). In contrast, *E. faecium* was the second most prevalent species but was significantly less abundant than *E. faecalis*, with its highest presence being 30% in water factory effluent. Previous studies have shown that *E. faecium* is typically less abundant in environmental samples but can still be a critical opportunistic pathogen, especially in immunocompromised individuals (Zaheer *et al*., 2020; Lebreton *et al*., 2014). The comparatively lower occurrence of *E. faecium* might be attributed to its less robust environmental adaptability compared to *E. faecalis*. The lower prevalence of *E. gallinarum*, *E. durans*, and *E. hirae* in dairy farm effluents suggests that these species may not be as well-adapted to survive in wastewater environments. Their reduced abundance could also be due to differences in host specificity and environmental conditions that do not favour their survival (Boss *et al*., 2016; Ben Said *et al*., 2017). The variation in species distribution across different effluent sources highlights the potential influence of environmental factors and human activities on microbial diversity (Boss *et al*., 2016).

The antimicrobial susceptibility testing of the *Enterococcus* isolates revealed high resistance rates to several antibiotics, with 53% of *E. faecalis* isolates resistant to Amoxicillin, 52.1% resistant to Rifampicin, and 50.4% resistant to Norfloxacin. These findings are consistent with prior reports of increasing antimicrobial resistance among enterococci, particularly in clinical and environmental settings (Iweriebor *et al*., 2015; Ben Said *et al*., 2017; Zaheer *et al*., 2020; Emurotu *et al*., 2023). The resistance of *E. faecalis* to multiple antibiotics, including first-line treatments such as Amoxicillin, poses a significant challenge for infection control and highlights the potential for wastewater to serve as a reservoir for resistant strains (Toru *et al*., 2018; Gotkowska-Płachta, 2021; Zaheer *et al*., 2020; Emurotu *et al*., 2023). The observed resistance patterns may be linked to the widespread use of antibiotics in both healthcare and agricultural settings, leading to the isolation of resistant strains (Toru *et al*., 2018; Gotkowska-Płachta, 2021). The high levels of resistance to Norfloxacin and Rifampicin could reflect the selective pressure exerted by the frequent use of these antibiotics in both human and veterinary medicine (Ben Said *et al*., 2017; Gotkowska-Płachta, 2021). The presence of multidrug-resistant (MDR) *Enterococcus* species in wastewater underscores the need for improved regulation of antibiotic use and wastewater treatment processes to mitigate the spread of resistant organisms (Ikehata, 2012; Kalli *et al*., 2023). The lower levels of resistance observed in *E. faecium* isolates (e.g., 38.5% resistant to Norfloxacin, and 30.8% resistant to Erythromycin) compared to *E. faecalis* suggest species-specific differences in resistance mechanisms. It is possible that *E. faecium* encounters fewer selective pressures in the environment compared to *E. faecalis*, or that it harbors fewer mobile genetic elements conferring resistance (Sanderson *et al*., 2020). These differences may have significant implications for the treatment of *E. faecium* infections, as resistance patterns can vary widely between species.

The multidrug resistance (MDR) patterns of the *Enterococcus* species in this study were particularly concerning. The study showed that about 82% of *E. faecalis* isolates from household effluents were resistant to six antibiotics across five different groups, resulting in an MDR index of 0.6. This is consistent with findings from other studies, which have documented high MDR indices among *Enterococcus* isolates from wastewater environments (Adeniji *et al*., 2021; Gotkowska-Płachta, 2021). The high MDR index suggests that these strains have been exposed to multiple antibiotics, likely through household and medical wastewater, leading to the selection of highly resistant strains. The detection of plasmids in all multidrug-resistant isolates further supports the role of plasmid-mediated resistance in these environments. The plasmid curing experiments revealed that resistance to multiple antibiotics could be lost following plasmid removal in several isolates, particularly in *E. faecium* and *E. faecalis* from household effluents. As reported by Boeder *et al*. (2024), Li and Zhang (2023), and Hashimoto *et al*. (2023), plasmids carry key resistance genes, which could be responsible for the multidrug-resistant phenotypes observed in these isolates. However, in some cases, resistance persisted even after plasmid curing, such as in *E. gallinarum* from water factory effluent. This indicates the presence of chromosomal resistance mechanisms or other mobile genetic elements, such as transposons or integrons, that contribute to the persistence of resistance despite plasmid loss (Waddington *et al*., 2022). The ability of *Enterococcus* species to harbour multiple mechanisms of resistance increases the complexity of managing infections caused by these organisms and highlights the need for continued surveillance and research into the mechanisms underlying antimicrobial resistance.

Conclusion

The present study proved that domestic effluents are important sources of multidrug-resistant *Enterococcus* species in the environment, and a strong correlation between the various sources of effluents and antibiotics was found. This has significant public health implications, particularly in areas where untreated wastewater may contaminate drinking water sources and infect humans. The high levels of resistance observed in this study underscore the potential for wastewater to act as a reservoir for resistant organisms that can spread to humans, animals, and the wider environment. Furthermore, the detection of plasmid-mediated resistance highlights the potential for horizontal gene transfer between bacteria in wastewater environments, facilitating the spread of resistance genes across different species and settings. Hence, a clear recognition that environmental reservoirs, such as wastewater, play a critical role in the global dissemination of antimicrobial resistance.

Conflict of Interest

All authors have no conflict of interest to declare.

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