



## Investigation of Antibiotic-Resistant *Enterococci* in Farm Animals and Vegetables: A case study of Selected Towns in Ekiti State, Nigeria

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### Abstract

*Enterococci* can be found in unprocessed and ready-to-eat farm produce. The extensive use of antimicrobials in agricultural practices has influenced natural selection in *Enterococci*. Human consumers can be infected with these antibiotic-resistant strains with resulting clinical complications. In this study, the resistance and virulence potential of *Enterococcus* spp. from farm animals and food-vegetables in Ekiti, Nigeria was investigated. Ninety-six samples: poultry rectal swabs ( $n = 35$ ), swine faecal droppings ( $n = 18$ ) and vegetables ( $n = 43$ ) were processed for the isolation of *Enterococcus* spp. Isolated *Enterococcus* spp. were further identified by different biochemical tests. Susceptibility to antibiotics was investigated using the Kirby-Bauer disk diffusion test on Mueller Hinton agar. A total number of 118 isolates were obtained from which 96 (81%) *Enterococci* were recovered, with 63 (66%) *E. faecium*, while 33 (34%) were identified as other species. Twenty-eight (28%) of the isolates tested were susceptible to Augmentin; resistance to cloxacillin was observed among all isolates while resistance to gentamicin, ofloxacin, vancomycin and erythromycin was observed among 97%, 82%, 75%, and 72%, respectively. Thirty-nine (41%) exhibited the ability to produce biofilms. Ninety-three (97%) isolates expressed multiple drug resistance to three or more antimicrobials with 15 different antimicrobial resistance patterns. AUG/CXC/ERY/GEN/OFL/VAN was the most predominant ( $n = 36$ ) resistant pattern. This study therefore, emphasises farm animals and food-vegetables to be important carriers of *Enterococcus* spp., with a high prevalence of multidrug resistance, which could be transferred through the food chain implicatively serving as threat to the public health.

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## 1.0 Introduction

*Enterococci* are ubiquitous microorganisms with representative organisms (residing in the gastrointestinal tracts of humans and animals, in plants, sewage, water, soil, and foods) implicated in disease causation and antibiotic resistance (Grassotti *et al.*, 2018; Costa *et al.*, 2019; Bhardwaj, 2019; Xu *et al.*, 2023; EPA, 2024). Within this genus, two species - *Enterococcus faecium* and *Enterococcus faecalis*, are recognized aetiological agents of nosocomial infections causing endocarditis, bloodstream infections, wound infections, and urinary tract infections (Osman *et al.*, 2016; Brinkwirth *et al.*, 2021). These strains have been reported to share close genetic identity with *Enterococci* of animal origin (Yilmaz *et al.*, 2016). Faecal contamination of water and vegetables by *Enterococci* from soil treated with fertilizers of animal origins and urban sewage can consequently lead to the secondary invasion of the intestinal tract in both domestic and wild animals (Ramos *et al.*, 2020). As a result, *Enterococci* and other bacteria have been found in vegetables, fruits, and ready-to-eat food due to cross-contamination throughout the production process, causing gastroenteritis in humans when such contaminated foods are ingested (Osafo *et al.*, 2022).

The use of antibiotics in humans has been shown to select for antibiotic-resistant bacterial strains. Antibiotics are also used extensively in livestock. In the United States, over 13 million kilograms, or approximately 80% of total quantity of antibiotics consumed annually are in livestock (Van Boeckel *et al.*, 2015). Consequently, antimicrobial-resistant *Enterococci* have been

reported in livestock, raising concerns that these antibiotic-resistant could transfer resistance genes to other bacteria, including normal flora and pathogens that cause human illness. These bacteria include tetracycline-resistant *Enterococci*, Vancomycin - resistant *Enterococci*, and others (Wist *et al.*, 2020; Ramos *et al.*, 2020). *Enterococci* are major colonizers of the animal intestinal tract, and certain *Enterococci* strains may be source-specific, making them suitable as bacterial source-tracking indicators (Urakawa *et al.*, 2021).

While there is substantial evidence pointing to the presence and antibiotic resistance of *Enterococci* in various environments across the globe, the situation in Nigeria, particularly in agricultural regions like Ekiti State, is not well understood. Ekiti State is a significant agricultural hub, engaging in animal husbandry and the prominent use of animal dung in crop and vegetable production, which are then sold and distributed across the country. The distribution of these agricultural products across the country is an impetus for understanding the local prevalence, resistance patterns, and virulence of *Enterococci* strains in this area. In Nigeria, previous studies have reported the prevalence and characteristics of antibiotic-resistant *Enterococci* across the clinical, food, and animal spectrums (Oguntoyinbo and Okueso, 2013; Amaechi and Nwankwo, 2015; Anyanwu and Obetta, 2015; Ayeni *et al.*, 2016). However, there is paucity of data specifically addressing the resistance and virulence profiles of *Enterococcus* spp. from farm animals and vegetables in Ekiti State. Therefore, this study aims to investigate the resistance and virulence potential of

*Enterococcus spp.* from farm animals and food-vegetables in Ekiti, Nigeria. This research will contribute to the broader comprehension of antimicrobial resistance patterns in agricultural settings and provide data that could inform public health strategies for the control of antibiotic-resistant *Enterococci* spread in Nigeria and beyond.

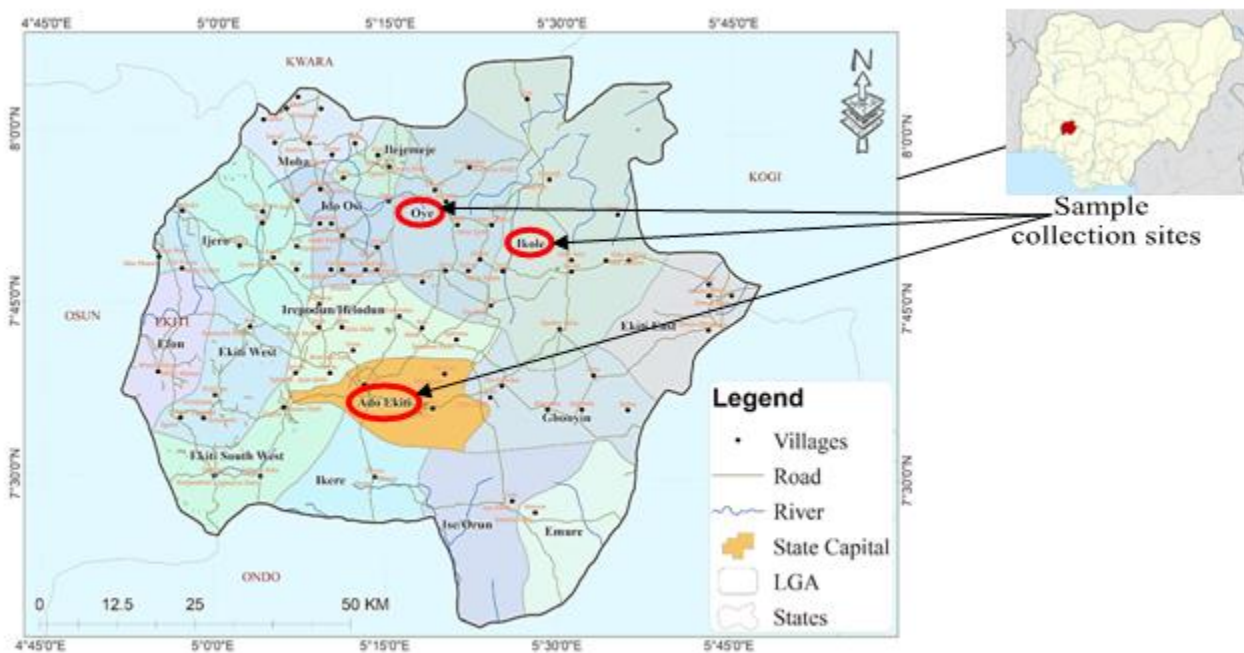
## 2.0 Materials and Methods

### 2.1 Study locations

Four towns in Ekiti State were selected for sample collection in this study: Oye Ekiti, Ilupeju Ekiti (within Oye Local Government Area), Ikole Ekiti and Ado Ekiti. The samples collected at these locations included poultry rectal swabs, pig faecal shedding, and vegetables.

### 2.2 Sample collection

Poultry rectal swabs, pig faecal shedding and vegetables were collected from farms and markets in Oye Ekiti, Ilupeju Ekiti, Ikole Ekiti and Ado Ekiti. A total of 99 samples were originally retrieved from 35 chickens, 18 pigs, and 43 food vegetables for processing. Analyses were performed at the Microbiology Laboratory, Federal University Oye Ekiti, Ekiti State, Nigeria. Samples were collected using standard procedures. All samples were collected using sterile techniques to prevent contamination, as early as possible, and were promptly transported to the laboratory for processing. To preserve sample integrity, samples were stored at 4°C until further analysis.



**Figure 1:** Map showing the sample collection sites in Ekiti, State Nigeria

### 2.3 Isolation and identification of Enterococci

Samples were brought to the laboratory within 2 hours of collection. Rectal swabs were inoculated into Tryptone Soy Broth (TSB) for enrichment. The broth for each sample was incubated at 37°C for 2-3 hours, after which each broth was

checked for changes in turbidity. Using an inoculating loop, each incubated broth was streaked onto freshly prepared Slanetz and Bartley (S&B) medium plates for *Enterococci*. The plates were then incubated at 44°C for 24-48 hours. After 48-hour incubation, distinct red

colonies were picked individually and transferred onto sterile nutrient agar slants, which were then incubated at 37°C for 18 hours. Slants containing organisms were stored in the refrigerator at 4°C for further use. Identification was carried out using Gram staining and conventional biochemical tests. Appropriate positive and negative control strains were used to validate the biochemical assays. The bacteria isolates identified as *E. faecium* and *Enterococcus* spp. were further analysed for susceptibility to selected antibiotics and for the phenotypic expression of virulence traits.

#### 2.4 Antibiotic susceptibility testing

The Kirby Bauer disk diffusion method was used in this study, involving the measurement of zones of inhibition on Mueller-Hinton agar (MHA) plates to determine susceptibility to standard antibiotics. The susceptibility of all 92 *Enterococci* isolates to gentamicin (GEN, 10 µg), erythromycin (ERY, 5 µg), cloxacillin (CXC, 5 µg), ofloxacin (OFL, 5 µg), vancomycin (VAN, 30 µg) and Augmentin (AUG, 30 µg) was tested, and results were interpreted based on CLSI (Clinical and Laboratory Standard Institute) guidelines (CLSI, 2020). Plates were incubated at 37°C for 24 hours, and zones of inhibition were recorded. Multidrug resistance (MDR) was attributed to isolates that exhibited resistance to at least one agent in three or more classes of antibiotics tested. The *Escherichiacoli* ATCC 25922 quality control strain was used to validate media and disc potency.

#### 2.5 Assay for virulence traits

The Congo red agar (CRA) method described by Freeman *et al.* (1989) was used in this study. Congo red powder (0.8g/L) was prepared as a concentrated aqueous solution and sterilized in the autoclave at 121°C for 15 minutes, separately from other medium constituents [MHA (38 g/L) and Sucrose (50 g/L)] and was thereafter added

when the agar had cooled to 55°C. Plates were inoculated with isolates and incubated aerobically for 24 hours at 37°C. Colour changes were observed after incubation. The control strains used were *Staphylococcus aureus* ATCC 25923 (positive control) and *Escherichiacoli* ATCC 25922 (negative control).

### 3.0 Results

#### 3.1 Isolation rate of *Enterococcus* species

A total of 118 isolates were recovered, with 68 isolates from livestock and 50 from vegetables. Among the animal isolates, 46 (67.6%) were *Enterococci*, comprising 30 (65.2%) from poultry birds and 16 (34.8%) from pigs (Table 1). In vegetables, all ( $n = 50$ ) yielded *Enterococci*: 20 (40.0%) from leafy greens (amaranth green, water leaf, jute leaf, pumpkin leaf, bitter leaf, and scent leaf), 20 (40.0%) from fruit vegetables (scotch bonnet pepper, tomatoes, cayenne pepper, bell pepper, garden egg, and cucumber), 7 (14.0%) from podded vegetables (okra and green peas), 3 (6.0%) from cruciferous vegetables (cabbage), and none from bulb (onions) and root vegetables (carrot). The biochemical scheme differentiated the isolates into 31 (67%) *E. faecium* and 15 (33%) *Enterococcus* spp. in livestock, while in vegetables, 32 (64%) *E. faecium* and 18 (36%) *Enterococcus* spp. with an overall predominance of 63 (65.6%) *E. faecium* recorded (See Table 2).

#### 3.2 Antibiotic susceptibility profile

Table 3 shows the antimicrobial susceptibility profile of *Enterococci* isolates in this study. All isolates were resistant to at least one antimicrobial agent tested. Twenty-eight (29%) of the isolates tested were susceptible to Augmentin. Resistance to Cloxacillin was observed among all isolates, while resistance to Gentamicin, Ofloxacin, Vancomycin and Erythromycin was observed among 96.9%, 82.3%, 75.0%, and 71.9%, respectively. None of

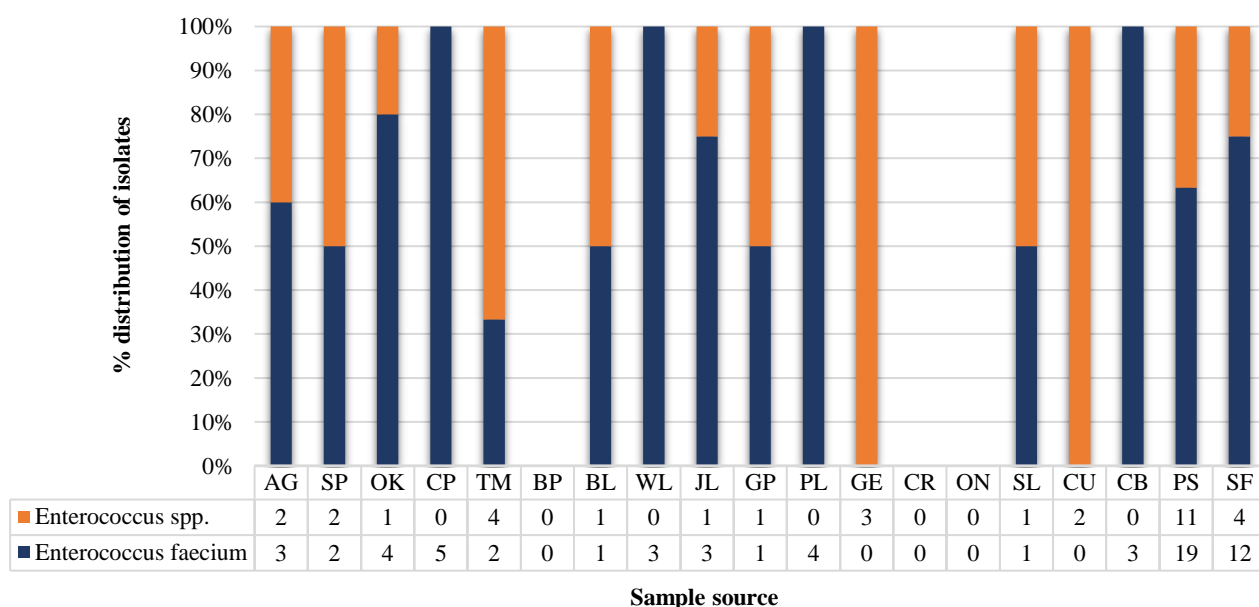
the vegetable isolates were sensitive to Augmentin, while 28 (60.9%) of animal isolates showed sensitivity to Augmentin. Over seventy percent (33/46) resistance against Ofloxacin was observed among farm animals, with a higher observation (46: 92.0%) in vegetables. Forty (87.0%) animal isolates showed resistance to Vancomycin, while 32 (64.0%) was revealed among vegetable isolates. Among all resistant strains, 93 (96.9%) exhibited multiple drug resistance (resistance to at least 3 antibiotics), presenting as 15 different patterns of multidrug resistance. AUG/CXC/ERY/GEN/OFL/VAN was the most predominant (n = 36; 38.7%) resistant pattern (Tables 2 and 3).

### 3.3 Detection of virulence traits

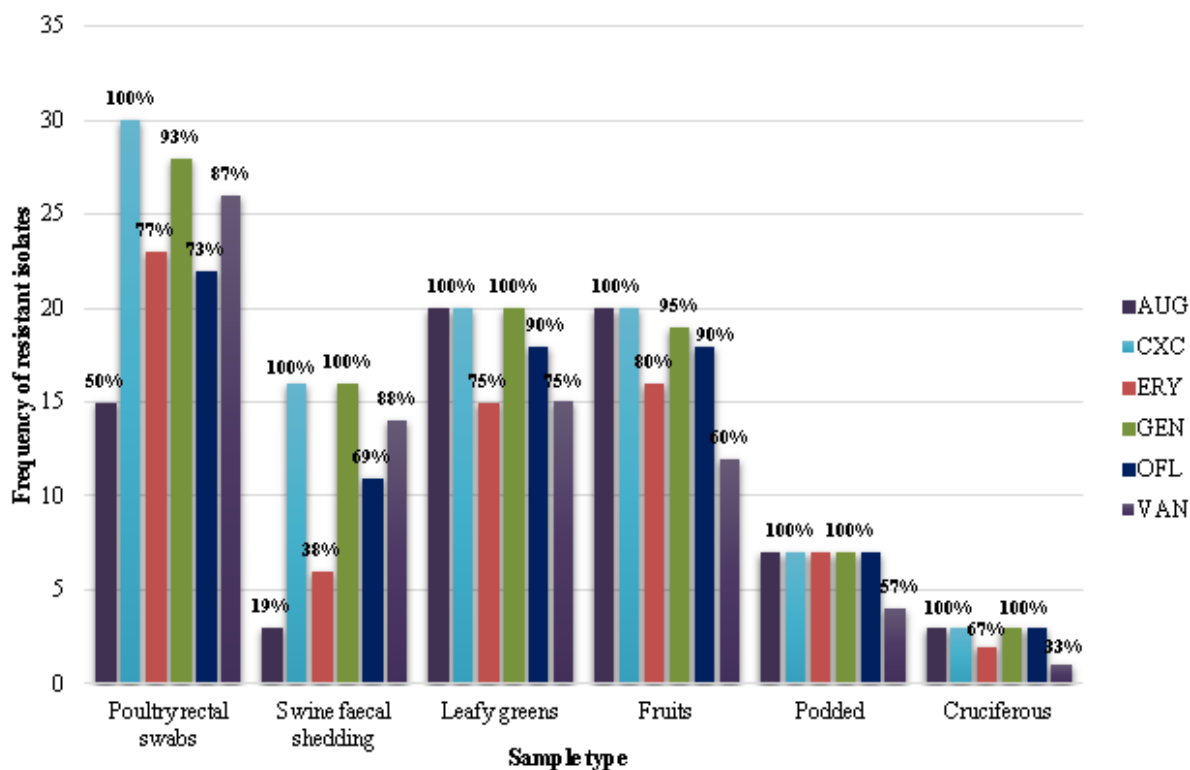
Biofilm production ability was detected in 39 (40.6%) *Enterococcus* isolates (Table 4). The majority (68.8%) of *Enterococci* isolates from faecal shedding produced biofilms, while only 9 (30.0%) of rectal swab expressed the trait. In vegetables, biofilm expression was highest (42.9%) in podded vegetable isolates.

**Table 1:** Distribution of samples for isolation of Enterococci

Sample source	Frequency (%)
Poultry rectal swabs	35 (36.5)
Swine faecal droppings	18 (18.8)
Vegetables	43 (44.8)
TOTAL	96 (100.0)



**Figure 2:** Occurrence of Enterococci isolated from farm animals and vegetables. Abbreviations: AG – Amaranth green, SP – Scotch bonnet pepper, OK – Okra, CP – Cayenne pepper, TM – Tomatoes, BP – Bitter leaf, WL – Water leaf, JL – Jute leaf, GP – Green pea, PL – Pumpkin leaf, GE – Garden egg, CR – Carrot, ON – Onions, SL – Scent leaf, CU – Cucumber, CB – Cabbage, PS – Poultry rectal swab, SF – Swine faecal shedding.



**Figure 3:** Antibiotic resistance profile of Enterococci isolated from farm animals and vegetables. Abbreviations: AUG-Augmentin; CXC- Cloxacillin; ERY- Erythromycin; GEN- Gentamicin; OFL - Ofloxacin; VAN- Vancomycin.

**Table 2:** Multidrug resistance patterns of *Enterococci* isolated from farm animals and vegetables

MDR patterns	No. of isolates (%)
CXC/OFL/VAN	1 (1.1)
CXC/ERY/VAN	1 (1.1)
CXC/GEN/OFL	1 (1.1)
CXC/GEN/VAN	4 (4.3)
CXC/ERY/GEN/VAN	3 (3.2)
CXC/ERY/GEN/OFL	2 (2.2)
CXC/GEN/OFL/VAN	4 (4.3)
AUG/CXC/GEN/OFL	5 (5.3)
AUG/CXC/GEN/VAN	3 (3.2)
AUG/CXC/ERY/GEN	1 (1.1)
AUG/CXC/ERY/GEN/OFL	12 (12.9)
AUG/CXC/ERY/GEN/VAN	2 (2.2)
AUG/CXC/GEN/OFL/VAN	6 (6.5)
CXC/ERY/GEN/OFL/VAN	12 (12.9)
AUG/CXC/ERY/GEN/OFL/VAN	36 (38.7)
<b>TOTAL</b>	<b>93 (100.0)</b>

**Table 3:** Distribution of multiple antibiotic resistance patterns by samples

MDR patterns	Rectal swab	Faecal dropping	Vegetables
CXC/OFL/VAN	1	0	0
CXC/ERY/VAN	1	0	0
CXC/GEN/OFL	0	1	0
CXC/GEN/VAN	3	1	0
CXC/ERY/GEN/VAN	3	0	0
CXC/ERY/GEN/OFL	2	0	0
CXC/GEN/OFL/VAN	1	3	0
AUG/CXC/GEN/OFL	1	0	4
AUG/CXC/GEN/VAN	3	0	0
AUG/CXC/ERY/GEN	0	0	1
AUG/CXC/ERY/GEN/OFL	1	0	11
AUG/CXC/ERY/GEN/VAN	1	0	1
AUG/CXC/GEN/OFL/VAN	0	2	4
CXC/ERY/GEN/OFL/VAN	8	4	1
AUG/CXC/ERY/GEN/OFL/VAN	9	0	27
<b>TOTAL</b>	34	11	49

**Table 4:** Biofilm producing traits expressed by *Enterococcus* isolated from farm animals and food-vegetables in Ekiti, Nigeria

Source of samples	Total no. tested	No. (%) of isolates		
		Biofilm producers	Indeterminate	Non-biofilm producers
Poultry rectal swabs	30	9 (30.0)	5 (16.7)	16 (53.3)
Swine faecal shedding	16	11 (68.8)	1 (6.3)	4 (25.0)
Leafy greens	20	8 (40.0)	0 (0.0)	12 (60.0)
Fruits	20	5 (25.0)	0 (0.0)	15 (75.0)
Podded	7	3 (42.9)	0 (0.0)	4 (57.1)
Cruciferous	3	3 (100.0)	0 (0.0)	0 (0.0)

#### 4.0 Discussion

This study aimed to determine the carriage of antibiotic resistant *Enterococci* in pigs and poultry, and to assess the presence of these bacteria in vegetables, and fruits. Bacteria, with reduced susceptibility to antibiotics have been frequently detected in animals, farm produce, and other related environments (Jezak and Kozajda, 2022). Consistent with this study, previous reports have shown that animals and farm produce play crucial roles as carriers and agents in the spread and persistence of antimicrobial resistance within communities (Mukuna *et al.*, 2023). In addition to the detection of antibiotic-

resistant *Enterococci* in vegetables, the use of organic manure as fertilizer in vegetables farms could significantly contribute to the spread and persistence of antimicrobial resistance. *Enterococci* are typically implicated as causative agents in nosocomial infections (Simon *et al.*, 2021). By implication, the transfer of antimicrobial-resistant and virulent strains of *Enterococci* of animal origin to humans through the ingestion of contaminated food poses a major public health threat, contributing to the spread and persistence of antibiotic-resistance bacteria, generally in the community (Woolhouse *et al.*, 2015; Almansour *et al.*, 2023). Investigating

antibiotic-resistant *Enterococci* in farm animals and vegetables is embedded with the likelihood to reveal important insights into the role of farm produce as reservoirs and disseminators of resistant and virulent strains.

In this study, 96 antibiotic-resistant bacteria belonging to the genus *Enterococci* were isolated. The prevalence of *Enterococci* (36.5%) among poultry birds aligns with findings from Ilshah, Ogun State (Ayeni *et al.*, 2016) and studies in Poland and Zambia (Dolka *et al.*, 2017; Mwikuma *et al.*, 2023). However, Eldaly *et al.* (2019) and Mudenda *et al.* (2022) reported higher prevalence, while Calonico *et al.* (2018) observed a lower prevalence. The *Enterococci* isolation rate among pigs was 18.8%, slightly lower in prevalence compared with Calonico *et al.*, (2018). A significant proportion (44.8%) of *Enterococci* was also isolated from vegetables, presenting a potential food safety concern if these vegetables are not properly cooked. Previous studies have also reported *Enterococci* in vegetables, though in varying proportions. For instance, Costa *et al.* (2022) reported a lower prevalence (39.5%), while Kim *et al.* (2020) observed a higher prevalence (56.0%) in leafy vegetables. These differences may be attributed to factors such as antibiotic use, disparities in sampling techniques, sample variety, location, and methodology.

In this study, *E. faecium* was the predominant species (65.6%) isolated from farm animals and vegetables, exhibiting a high rate of antimicrobial resistance. This finding is consistent with reports from other studies conducted in various parts of the world (Jackson *et al.*, 2011; Kwon *et al.*, 2012; Klibi *et al.*, 2013; Ali *et al.*, 2014; Iweriebor *et al.*, 2015, 2016; Ben *et al.*, 2016; Steoien-Pysniak *et al.*, 2016; Kim *et al.*, 2020). This phenomenon can be attributed to *E. faecium*'s commensal nature and the ability to thrive under different

selective pressures, including antimicrobial use in animal production environments. However, some studies have reported the dominance of other species in Nigeria (Bashiru *et al.*, 2017), Malaysia (Tan *et al.*, 2018) and in South Africa (Badul *et al.*, 2021). This may be as a result of the species-specific adaptations to different environments. The preponderance of *E. faecium* in healthy human samples has also been established by Barreto *et al.* (2009).

In recent years, the incidence of high resistance among *Enterococci* has increased at an alarming rate (Hollenbeck and Rice, 2012; Iweriebor *et al.*, 2015; Novais *et al.*, 2013; Molechan *et al.*, 2019). In this study, *Enterococci* isolates from farm animals and vegetables generally exhibited outstanding resistance against several antibiotics, particularly cloxacillin (100.0%) and Gentamicin (96.9%). Resistance to ofloxacin (82.3%), vancomycin (75.0%), Augmentin (70.8%), and erythromycin (71.9%) was also concerning. The consistent and irregular use of antimicrobials in livestock production guarantees selective pressure, which is reported to boost the emergence of resistant bacterial strains. In Nigeria, the excessive use of macrolides, penicillin, and aminoglycosides in animal production has been reported (Oluwasile *et al.*, 2014; Adesokan *et al.*, 2015; Olonitola *et al.*, 2015; Ayeni *et al.*, 2016; Alhaji and Isola, 2018; Adebowale *et al.*, 2020; Emeje *et al.*, 2022), which may explain the significant resistance observed to these classes of antibiotics. The presence of VRE (Vancomycin-resistant *Enterococci*) in this study is consistent with reports of its presence in animals and humans (Alemayehu and Hailemariam, 2020). Considering the history of antibiotics use in the agricultural sector, VRE has become a constant threat to public health and overall population (O'Driscoll and Crank, 2015). Orababa and Colleagues (2021) have also shown in their study



that the highest prevalence of VRE is in the western part of Nigeria. Studies have also shown that the use of macrolides, such as tylosin in pigs could confer co-selection of Vancomycin resistance among *Enterococci* since the genes encoding the two resistances were located on the same plasmid (Aarestrup, 2000).

Multidrug-resistant strains of *Enterococci* were detected at a very high rate (96.9%) in this study, similar to findings from Malaysia (Tan *et al.*, 2018). MDR isolates showed resistance to as few as three and as many as six different antimicrobials. Over half of these isolates exhibited the MDR phenotype in vegetables, with a significant frequency (36.2%) in pigs, and 11.7% in poultry birds. This observation differs from a previous study by Ngbede *et al.* (2016), where MDR was more predominant among poultry isolates, possibly due to varying patterns of antibiotic administration in livestock. The MDR pattern exhibited by isolates in this study aligns with previous studies on *Enterococci* from animals, humans, and food in different parts of the world (Ristori *et al.*, 2012; Chung *et al.*, 2014; Iweriebor *et al.*, 2015, 2016; Pruksakom *et al.*, 2016). The emergence of MDR in *Enterococci* may be facilitated by their ubiquitous nature, genome plasticity, and uncontrolled widespread use of antibiotics (Prieto *et al.*, 2016; Emeje *et al.*, 2022).

Despite the commensal nature of most *E. faecium* strains in humans and animals, the high level of resistance to antibiotics and the presence of multiple drug resistance calls for serious concern (Feiyang Ma *et al.*, 2021; WHO, 2023; Cebeci *et al.*, 2024). From our study, it was also discovered that *Enterococci* isolates from farm animals and vegetables exhibited the ability to produce biofilms (40.6%), which are major contributors to pathogenesis and antibiotic resistance. This finding is consistent with a study on the biofilm

formation ability of dairy isolates in Serbia (Popović *et al.*, 2018). A high proportion (68.8%) of pig isolates, 30% of poultry isolates, and about 40% of vegetable isolates produced biofilms in the current study. However, Castaño-Arriba *et al.* (2020) reported a higher occurrence (100.0%) of biofilm formation in red meat and poultry preparations. The rate of biofilm formation in this study was lower compared to the rate of antibiotic resistance, suggesting that resistance in the study cannot be entirely attributed to the isolates' ability to form biofilms. It is likely that these isolates adopt alternative mechanisms for antimicrobial resistance, such as the acquisition of resistant genes from adjacent bacterial plasmids or through selective pressures induced by various factors (Grudlewdka-Buda *et al.*, 2023).

## 5.0 Conclusion and recommendation

This study demonstrates a high level of antibiotic resistance exhibited by *Enterococci* of animal and food-vegetable origin in the study environment, particularly to four major groups of antibiotics, viz;  $\beta$ -Lactams (Cloxacillin), aminoglycosides (Gentamicin), glycopeptides (Vancomycin) and macrolides (Erythromycin). Moreover, the study highlights vegetables as potential reservoirs for antibiotic-resistant *Enterococcus*, which leads to food borne infections (Rahman *et al.*, 2021). The detection of MDR isolates detected in this study could endanger public health as the same class of antibiotic is currently in use as treatment options for most bacterial diseases in humans. Due to the persistence of *Enterococci* in harsh environments, their eradication in both agricultural and clinical settings remains challenging.

Routine cleaning of animal production facilities can reduce the resilience of *Enterococci* and other pathogens in poultry and piggery houses.

Implementing strict hygiene protocols in slaughterhouses is also necessary in controlling faecal contamination of the meat with *Enterococci* and other zoonotic bacteria, thereby preventing consequent transmission. Furthermore, it is important to promote antimicrobial stewardship to limit the use of antibiotics in vegetable farming; this will reduce the risk of transmission of antibiotic resistance bacteria to consumers and healthy adults. Likewise, adherence to stringent hand hygiene practices and thorough cleaning is imperative to prevent nosocomial infections by *Enterococci* and other resistant organisms in clinical settings.

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