FUDMA Journal of Sciences (FJS)
ISSN online: 2616-1370
ISSN print: 2645 - 2944
Vol. 10 No. 18, September, 2026, pp 156 – 161
DOI: https://doi.org/10.33003/fjs-2026-1018-5638

Occurrence, Safety Assessment and Enterocinogenic Potential of Enterococcus Species Recovered from Retail Kilishi, a Traditional Ready-to-Eat Meat Product in Nigeria

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Received: 29 Jul. 2026
Accepted: 07 Aug. 2026
Published: 24 Sep. 2026

ABSTRACT
This study investigated the occurrence, safety characteristics and enterocinogenic potential of *Enterococcus faecium* isolated from retail Kilishi, a traditional Nigerian ready-to-eat dried meat product. A total of 201 retail Kilishi samples were homogenized in buffered peptone water, cultured on De Man, Rogosa, and Sharpe (MRS) agar, and phenotypically characterized isolates via Gram staining, catalase testing, and bile-esculin hydrolysis. Species specific PCR assays targeting *E. faecium* confirmed 54 distinct enterococci isolates. These confirmed isolates were systematically screened for phenotypic virulence traits (haemolysin, gelatinase, and aggregation substance) alongside critical virulence genes (*gelE*, *esp* and *asa1*) and antimicrobial resistance genes (*ermB*, *vanA* and *vanB*) via multiplex PCR. Antibiotic susceptibility profiles were found using the Kirby-Bauer disc diffusion method against CLSI guidelines. Isolates exhibiting ideal safety characteristics were subsequently screened for production of enterocin-like substances against *Listeria monocytogenes*. *E. faecium* predominated, accounting for 72.2% of the confirmed isolates, while *E. faecalis* constituted 27.8%. Most isolates exhibited low virulence potential, and four *E. faecium* isolates lacked the tested virulence genes and major antimicrobial resistance genes. Although resistance to some clinically important antibiotics was observed among a proportion of isolates, *E. faecium* KEC79 remained susceptible to all antibiotics evaluated and demonstrated the highest enterocin-like substance production (5120 AU mL^{-1}) together with the strongest inhibitory activity against *Listeria monocytogenes*. Retail kilishi therefore, harbours safe enterocin-producing *E. faecium* strains. Following comprehensive safety assessment, KEC79 in particular is found to be a promising candidate for biopreservation applications. These findings highlight traditional meat products as valuable reservoirs of technologically important enterococci while emphasizing the necessity of comprehensive safety screening before their use in food systems.

Keywords: *Enterococcus faecium*; Kilishi; Enterocin; Antimicrobial Resistance; Virulence Determinants; *Listeria monocytogenes*

INTRODUCTION

The growing consumer preference for minimally processed foods with fewer synthetic preservatives has accelerated the search for natural antimicrobial compounds capable of improving food safety without compromising product quality (Aspri et al., 2022). Bacteriocins produced by lactic acid bacteria are promising candidates due to their targeted activity against foodborne pathogens, particularly Listeria monocytogenes (Cotter et al., 2013). Although nisin remains the only bacteriocin widely approved for commercial food application; nevertheless, increasing interest has focused on the discovery of novel bacteriocins possessing broader activity spectra and improved stability under diverse food processing conditions (Cleveland et al., 2001; Cotter et al., 2013).

Members of the genus Enterococcus occupy a unique position in food microbiology. They are common inhabitants of the gastrointestinal tract of humans and animals and frequently occur in foods of animal origin, particularly fermented and dried meat products (Franz et al., 1999; Klein, 2003). Many enterococcus strains synthesize enterocins, a diverse group of ribosomally synthesized antimicrobial peptides that exhibit potent inhibitory activity against important foodborne pathogens, particularly L. monocytogenes (Giraffa, 2002; Franz et al., 2007).

Despite these beneficial technological attributes, the application of enterococci in foods remains controversial because certain strains, especially clinical isolates, possess virulence determinants and transferable antimicrobial resistance genes that may compromise food safety (Hanchi et al., 2018). Consequently, international recommendations emphasize that candidate enterococcal strains intended for food application should undergo rigorous safety assessment (Foulquié Moreno et al., 2006), including evaluation of virulence characteristics, antimicrobial susceptibility and the presence of clinically relevant resistance determinants, before being considered for technological use (Franz et al., 2001; Eaton and Gasson, 2001).

Among enterococcal species, E. faecium has attracted particular interest because food-associated strains generally exhibit lower virulence potential than E. faecalis while frequently producing enterocins with strong anti-listerial activity (Holzapfel et al., 2018). Numerous enterocins produced by E. faecium, including enterocin A, enterocin B and enterocin P, have demonstrated promising inhibitory activity against L. monocytogenes in meat and dairy products, highlighting their potential as natural biopreservatives (Franz et al., 2007; Furlaneto-Maia et al., 2020).

Kilishi is a traditional Nigerian ready-to-eat dried meat product prepared through slicing, seasoning, drying and

roasting of beef. Although these processing steps reduce microbial loads and enhance shelf stability, post-processing handling, open-market retail conditions and prolonged ambient storage may permit contamination with spoilage organisms and foodborne pathogens (Dahiru & Maigari, 2019). While previous investigations have reported the occurrence of enterococci in various fermented and processed meat products, information regarding the occurrence, safety characteristics and enterocinogenic potential of E. faecium recovered from retail kilishi remains limited. The present study therefore investigated the occurrence of Enterococcus species in retail kilishi, evaluated the safety characteristics of E. faecium isolates through phenotypic and molecular assessment of virulence and antimicrobial resistance, and identified strong enterocin-producing strains.

MATERIALS AND METHODS

Study Area

This study was conducted in Kano metropolis, Kano state, covering important kilishi production and retail areas including Agadasawa, Yola, Dala, Jakara, Sabon Titi and environs *(12.0000°N, 8.5167°E)*. The study employed a laboratory based experimental study with purposive sample collection from various outlets within the mentioned sampling areas.

Sample Collection

A total of 201 retail Kilishi samples were purchased from the study area using aseptic procedures. Samples were individually packaged in sterile polyethylene bags, transported to the laboratory in insulated containers and analysed immediately or stored at 4°C for no longer than 24 h prior to microbiological analysis (Daminabo *et al*., 2013).

Isolation and Phenotypic Identification of *Enterococcus* Species

Twenty-five grams of each sample was aseptically homogenized in sterile buffered peptone water and serially diluted. Appropriate dilutions were cultured on De Man, Rogosa, and Sharpe (MRS) agar and incubated at 34 °C for 24 hours for the isolation of presumptive enterococci following standard microbiological procedures. Representative colonies displaying typical enterococcal morphology were purified and subjected to Gram staining, catalase test, bile-esculin hydrolysis, growth in 6.5% NaCl broth, growth at 10°C and 45°C, and carbohydrate fermentation tests for species differentiation according to established protocols (Yildirim *et al*., 2014).

Molecular Confirmation of *Enterococcus* Species

Genomic DNA was extracted from purified isolates using the traditional phenol-chloroform method (Mahuku, 2004). Species confirmation was performed by polymerase chain reaction (PCR) using species-specific primers targeting *E. faecium*. PCR amplification was carried out under optimized cycling conditions, and amplification products were resolved by electrophoresis on 1.5% agarose gel stained with ethidium bromide and visualized under ultraviolet illumination.

Determination of Phenotypic Virulence Factors

The isolates were evaluated for selected phenotypic virulence characteristics including haemolysin production, gelatinase and aggregation substance activity according to the methods used by Barbosa *et al*. (2010). Haemolytic activity was assessed on 5% sheep blood agar, gelatinase production on nutrient gelatin medium, while aggregation substance was evaluated by the auto-aggregation assay. Appropriate positive and negative controls were included in the analyses.

Detection of Virulence Genes

PCR assays were performed to detect the virulence genes *gelE*, *esp* and *asa1* according to the method used by Mariam (2021). Amplifications were carried out using previously described primers and reaction conditions. PCR products were analysed by agarose gel electrophoresis, and representative amplicons were compared with a molecular size marker to confirm the expected product sizes.

Antibiotic Susceptibility Testing

Antimicrobial susceptibility was determined by the Kirby–Bauer disc diffusion method on Mueller–Hinton agar in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines. The antibiotic panel included representatives of clinically important antimicrobial classes commonly used for enterococcal susceptibility testing. Zone diameters were interpreted as susceptible, intermediate or resistant according to CLSI breakpoints (CLSI, 2026).

Detection of Antimicrobial Resistance Genes

Selected antimicrobial resistance genes, including *ermB*, *vanA* and *vanB*, were detected by PCR using gene-specific primers according to the method used by Mariam (2021). Amplification products were analysed by agarose gel electrophoresis and interpreted according to the expected fragment sizes.

Screening for Enterocin Production

Isolates were screened for enterocin production by incubation in MRS broth for 24 hours at 37°C to obtain the cell free supernatants (CFS), using method described by Rashid *et al.* (2023). The cell-free supernatants were obtained from the overnight cultures by centrifugation, followed by pH neutralization and catalase treatment to eliminate the inhibitory effects of organic acids and hydrogen peroxide. Antimicrobial activity against *Listeria monocytogenes* ATCC 19115 was expressed as arbitrary units per millilitre (AU mL^{-1}), and the CFS exhibiting the highest activity was considered for potential application.

Statistical Analysis

Experiments were performed in triplicate where applicable, and data were expressed as mean ± standard deviation. Descriptive statistics, including frequencies and percentages, were used to summarize the occurrence and distribution of *Enterococcus* species. Associations between categorical variables were evaluated using the Chi-square test. Statistical analyses were performed using GraphPad Prism version 10, and differences were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

Occurrence and Identification of *Enterococcus* Species

A total of 201 retail kilishi samples were analysed, yielding 98 presumptive enterococcal isolates. Following biochemical characterization and molecular confirmation, 54 isolates were identified as members of the genus *Enterococcus*, corresponding to a confirmation rate of 55.1% among presumptive isolates. Species identification revealed that *E. faecium* predominated, accounting for 39 (72.2%) of the confirmed isolates, whereas *E. faecalis* constituted 15 (27.8%) (Table 1). After preliminary identification by conventional biochemical methods, representative isolates

with desirable safety and activity profiles were confirmed by PCR amplification and sequencing (Figure 1).

Safety Assessment of the Isolates

Phenotypic screening demonstrated considerable variation in virulence-associated characteristics among the isolates. Although haemolytic activity, gelatinase production and aggregation substance were detected in a proportion of isolates, several *E. faecium* strains including KEC14, KEC22, KEC79 and KEC97 lacked all detectable virulence traits. PCR screening further showed that the prevalence of the tested virulence genes (*gelE*, *esp* and *asa1*) was generally low among the selected food isolates (Table 2).

Antimicrobial susceptibility testing revealed variable resistance patterns across the isolates. Resistance was most frequently observed against selected antibiotics, whereas susceptibility to clinically important agents remained high for several isolates and the selected isolates showed susceptibility to all tested antibiotics. PCR analysis showed that the major resistance genes investigated (*ermB*, *vanA* and *vanB*) were absent from the selected candidate isolates (Table 2).

Screening for Enterocinogenic *E. Faecium*

Following safety evaluation, four *E. faecium* isolates (KEC14, KEC22, KEC79 and KEC97) that lacked the tested virulence determinants and resistance genes were selected for assessment of enterocin production. All four isolates exhibited CFS inhibitory activity against *Listeria monocytogenes* ATCC 19115. However, marked differences in antimicrobial potency were observed (Table 3). Isolate KEC79 produced the highest enterocin-like substance activity (5120 AU mL^{-1}) and generated the largest inhibition zone against the indicator organism (Table 3). The remaining isolates produced lower antimicrobial activities (2048 to 2560 AU mL^{-1}), indicating strain-dependent variation in enterocin production.

Selection of *E. faecium* KEC79

The selection of KEC79 for subsequent purification and characterization was based on the combined assessment of safety and technological attributes rather than antimicrobial activity alone. In addition to producing the highest enterocin activity via zone of inhibition produced against indicator bacteria, the isolate lacked the tested virulence genes (*gelE*, *esp* and *asa1*), was negative for the investigated resistance genes (*ermB*, *vanA* and *vanB*), and exhibited susceptibility to all antibiotics evaluated. Collectively, these characteristics identified KEC79 as the most suitable candidate for subsequent studies on purification, biochemical characterization and food application (Table 4).

Table 1: Occurrence and Identification of Enterococcus Species Recovered From Retail Kilishi

Parameter	Number	Percentage (%)
Samples analysed	201	100
Presumptive isolates	98	48.8
Confirmed Enterococcus	54	26.9*
E. faecium	39	72.2†
E. faecalis	15	27.8†

*Percentage of presumptive isolates confirmed as *Enterococcus*.
†Percentage of confirmed isolates.

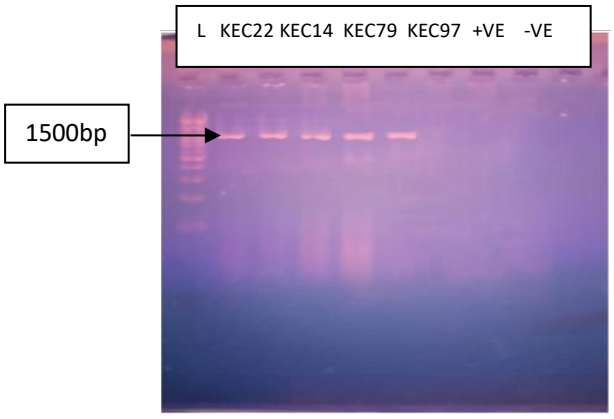

Figure 1: Agarose gel electrophoresis showing species-specific PCR amplification of representative *Enterococcus* isolates. Lane L: DNA ladder; lanes KEC14-KEC97: isolates; +: positive control; -: negative control.

Table 2: Safety Profile of Selected *E. Faecium* Isolates

Isolate	Virulence Genes (gelE/esp/asa1)	Antibiotic Susceptibility	Resistance Genes (ermB/vanA/vanB)
KEC14	All negative	Susceptible to all	All negative
KEC22	All negative	Susceptible to all	All negative
KEC79	All negative	Susceptible to all	All negative
KEC97	All negative	Susceptible to all	All negative

Table 3: Enterocin-like substance activity of selected isolates

Isolate	Activity (AU/mL)	Inhibition Zone vs L. monocytogenes (mm)
KEC79	5120	18.5 ± 1.0^{a}
KEC14	2560	17.5 ± 1.2^{a}
KEC22	2150	16.0 ± 0.8^{b}
KEC97	2048	13.5 ± 1.5^{c}

Means sharing different superscripts differ significantly ($P < 0.05$).

Table 4: Comparative Safety Characteristics and Enterocinogenic Potential of Selected *E. Faecium* Isolates

Characteristic	KEC14	KEC22	KEC79	KEC97
Species	E. faecium	E. faecium	E. faecium	E. faecium
Haemolysis	Negative	Negative	Negative	Negative
Gelatinase	Negative	Negative	Negative	Negative
Biofilm formation	Negative	Negative	Negative	Negative
Virulence genes (gelE, esp, asa1)	All negative	All negative	All negative	All negative
Antibiotic susceptibility	Susceptible to all	Susceptible to all	Susceptible to all	Susceptible to all
Resistance genes (ermB, vanA, vanB)	All negative	All negative	All negative	All negative
Enterocin-like substance activity (AU/mL)	2560	2150	5120	2048
Inhibition zone vs L. monocytogenes (mm)	17.5 ± 1.2	16.0 ± 0.8	18.5 ± 1.0	13.5 ± 1.5
Final Ranking	2	3	1	4

Only isolates meeting the predefined safety criteria (absence of tested virulence genes and resistance genes + full antibiotic susceptibility) were included. KEC79 was selected for subsequent purification and characterization based on its superior safety profile and highest enterocin production.

Figure 2: Representative agar well diffusion assay showing anti-*Listeria* activity

Discussion

The present study investigated the occurrence, safety characteristics and enterocinogenic potential of *Enterococcus* species recovered from retail kilishi, a traditional ready-to-eat dried meat product widely consumed in Nigeria. The findings demonstrate that although enterococci were readily recovered from retail kilishi, the majority of confirmed isolates were *E. faecium*, several exhibited favourable safety characteristics, and a small number possessed strong anti-listerial activity. The predominance of *E. faecium* (72.2%) over *E. faecalis* agrees with numerous investigations of fermented and dried meat products, where *E. faecium* is frequently the dominant enterococcal species because of its superior adaptation to environments characterised by low water activity, elevated salt concentrations and nutrient limitation (Giraffa, 2002). The processing of kilishi, involving salting, spice incorporation, drying and roasting, likely creates selective pressures that favour stress-tolerant *E. faecium* populations.

The predominance of *E. faecium* is technologically important because food-associated strains generally possess greater potential for bacteriocin production and lower virulence than *E. faecalis* (Franz *et al*., 2007). Consequently, the species composition observed in this study provides an encouraging starting point for identifying isolates with possible application as protective cultures or sources of natural antimicrobial peptides.

A principal objective of this study was to determine whether food-derived enterococci from kilishi possess characteristics compatible with future food applications. Although phenotypic virulence traits were detected in a proportion of isolates, several *E. faecium* strains lacked haemolytic activity, gelatinase production, aggregation substance and the virulence genes examined, indicating considerable heterogeneity within the isolate population (Fisher & Phillips, 2009). The comparatively lower virulence observed among *E. faecium* isolates is consistent with previous reports showing that food-derived strains generally harbour fewer virulence determinants than isolates originating from clinical settings (Franz *et al*., 2001; Eaton & Gasson, 2001). In contrast, *E. faecalis* is widely regarded as the more virulent species because it more frequently carries genes encoding aggregation substance, cytolysin, gelatinase, enterococcal surface protein and other factors that facilitate adhesion, colonisation and tissue invasion (Semedo *et al*., 2003).

The absence of *gelE*, *esp* and *asa1* in the selected candidate isolates is particularly noteworthy because these genes are among the most frequently investigated virulence determinants during safety assessment of enterococci intended for food use. Although the absence of these markers cannot categorically establish safety, it substantially reduces concerns regarding pathogenic potential and satisfies an important prerequisite for considering food-derived enterococci as technological cultures (Vankerckhoven *et al*., 2008).

The antimicrobial susceptibility profiles observed in this study further *emphasise* the importance of strain-level safety

assessment. While resistance to some antibiotics occurred among portions of the isolate collection, the candidate isolates selected for enterocin production were susceptible to clinically relevant antibiotics and lacked the investigated resistance genes (*ermB*, *vanA* and *vanB*) (Werner *et al*., 2013). These findings are encouraging because transferable antimicrobial resistance remains one of the principal obstacles limiting the use of enterococci in food biotechnology (Hollenbeck & Rice, 2012). The resistance detected among some isolates likely reflects selective pressures arising from antimicrobial use in food-animal production, environmental dissemination of resistant bacteria and horizontal gene transfer within the gastrointestinal tract (Arias & Murray, 2012). The complete susceptibility profile of KEC79 is therefore of particular significance. In addition to lacking the investigated virulence determinants and resistance genes, its susceptibility to all tested antibiotics enhances confidence in its suitability as a candidate food-grade culture and reduces concerns regarding dissemination of clinically important resistance determinants (Ogier and Serror, 2008).

One of the most significant findings of this study was the identification of several *E. faecium* isolates capable of producing enterocin-like substances active against *Listeria monocytogenes*. Although all four selected isolates demonstrated antimicrobial activity, KEC79 consistently exhibited the highest enterocin production and strongest inhibition of the indicator organism, indicating substantial strain-dependent variability within food-associated *E. faecium* populations. Cintas *et al.* (1997) obtained a novel enterocin from *E. faecium* P13 isolated from a Spanish dry-fermented sausage with wide spectrum of activity against a diverse array of foodborne pathogens.

Variation in enterocin production among isolates has been widely reported and reflects differences in bacteriocin structural genes, regulatory systems, plasmid content and environmental adaptation. Such diversity *emphasises* the importance of systematic screening rather than assuming equivalent antimicrobial potential among isolates of the same species. The identification of a highly productive food-derived *E. faecium* isolate supports previous reports describing traditional fermented and dried foods as valuable reservoirs of bacteriocin-producing lactic acid bacteria (Belgacem *et al*., 2010; Yildirim *et al*., 2014; da Costa *et al*., 2021). Consequently, retail kilishi may represent an underexplored source of technologically valuable microorganisms with potential applications in natural food preservation (Gálvez *et al*., 2007).

The identification of KEC79 as a strain combining favourable safety characteristics with high anti-listerial activity provides a strong scientific basis for subsequent purification, biochemical characterisation and food application studies (Ennahar *et al*., 2000). This stepwise selection strategy substantially reduces safety concerns while increasing the likelihood of identifying strains possessing genuine industrial relevance. Finally, it must be acknowledged that only three virulence genes and three antimicrobial resistance genes were investigated, and whole-genome sequencing was not performed. Additional genomic analyses would provide a more comprehensive assessment of safety and facilitate identification of the enterocin structural genes responsible for antimicrobial activity. The identification of KEC79 highlights the feasibility of sourcing safe, bacteriocin-producing *E.faecium from* traditional meat products, which may contribute to clean-label preservation strategies.

CONCLUSION

This study demonstrates that retail kilishi constitutes a reservoir of predominantly *E. faecium* strains exhibiting diverse safety and technological characteristics. Comprehensive phenotypic and molecular safety assessment showed that while some isolates possessed virulence-associated traits or antimicrobial resistance, several *E. faecium* isolates exhibited favourable safety profiles characterised by the absence of the investigated virulence determinants (*gelE*, *esp* and *asa1*) and major antimicrobial resistance genes (*ermB*, *vanA* and *vanB*). Among these, isolate KEC79 demonstrated the highest enterocin-like substance production together with complete susceptibility to the antibiotics tested, identifying it as the most suitable candidate for further technological evaluation.

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