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A survey of antibiotic resistance and virulence factors in *Enterococcus* species isolated from poultry farms in Benin City, Nigeria

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Abstract:

Background: Enterococci are commensal bacteria resident in the gastrointestinal tract of humans and animals. However, their increasing resistance to clinically important antimicrobial agents remain a global threat. The objective of this study is to determine the prevalence, antimicrobial resistance profile and virulence factors of *Enterococcus* isolated from selected poultry farms in Benin City, Nigeria.
Methodology: Sixty samples (20 feed, 20 water and 20 faecal samples) were randomly collected from five selected poultry farms in different commercial farming areas between August and September 2020. The samples were first enriched in Tryptone Soy Broth (TSB) and then cultured on Bile Aesculin Azide (BAA) agar aerobically at 37°C for 18-24 hours. Black colonies on BAA agar were presumptively identified as *Enterococcus* and confirmed by conventional biochemical tests and Analytical Profile Index (API) rapid ID 32 STREP. The antibiotic susceptibility of the isolates was determined by the Kirby-Bauer disc diffusion method. The virulence factors and biofilm formation were evaluated using standard bacteriological and microtitre plate methods.
Results: In total, *Enterococcus*-positive samples were 32/60 (53.3%) with a total of 45 *Enterococcus* isolates. The speciation of the *Enterococcus* isolates based on API rapid ID 32 STREP were *Enterococcus faecium* 15/45 (33.3%), *Enterococcus faecalis* 12/45 (26.7%), *Enterococcus durans* 8/45 (17.8%), *Enterococcus casseliflavus* 5/45 (11.1%) and *Enterococcus hirae* 5/45 (11.1%). The isolates showed the highest antibiotic resistance to ampicillin (100.0%), fosfomycin (95.6%) and penicillin G (88.9%) and the least resistance to ciprofloxacin (22.2%) and chloramphenicol (28.9%). The virulence factors of *Enterococcus* species observed were gelatinase, β-hemolytic and hyaluronidase activity, biofilm, and S-layer formation. The degree of biofilm formation by the *Enterococcus* species was strong biofilm formation (19/45, 42.2%), moderate biofilm formation (10/45, 22.2%), weak biofilm formation (11/45, 24.4%) and no biofilm formation (5/45, 11.1%).
Conclusion: Findings from this study emphasized on the potential health implications associated with antimicrobial resistance and phenotypic virulence factors of *Enterococcus* in poultry products.

Keywords: Antibiotic resistance; *Enterococcus*; Poultry; Virulence factors; Benin City

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Une enquête sur la résistance aux antibiotiques et les facteurs de virulence chez les espèces d'*Enterococcus* isolées dans des élevages de volailles à Benin City, Nigeria

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Résumé:

Contexte: Les entérocoques sont des bactéries commensales résidant dans le tractus gastro-intestinal des humains et des animaux. Cependant, leur résistance croissante aux agents antimicrobiens cliniquement importants reste une menace mondiale. L'objectif de cette étude est de déterminer la prévalence, le profil de résistance aux antimicrobiens et les facteurs de virulence d'*Enterococcus* isolés dans des élevages de volailles sélectionnés à Benin City, au Nigeria.
Méthodologie: Soixante échantillons (20 échantillons d'aliments, 20 d'eau et 20 échantillons de matières fécales) ont été collectés de manière aléatoire dans cinq élevages de volailles sélectionnés dans différentes zones d'élevage commercial entre août et septembre 2020. Les échantillons ont d'abord été enrichis dans du bouillon tryptone soja (TSB), puis cultivés sur Gélose bile-esculine-azide (BAA) en aérobiose à 37 °C pendant 18 à 24 heures. Les colonies noires sur gélose BAA ont été présumées identifiées comme étant Enterococcus et confirmées par des tests biochimiques conventionnels et par l'indice de profil analytique (API) ID rapide 32 STREP. La sensibilité aux antibiotiques des isolats a été déterminée par la méthode de diffusion sur disque de Kirby-Bauer. Les facteurs de virulence et la formation de biofilm ont été évalués à l'aide de méthodes bactériologiques et de plaques de microtitrage standard.
Résultats: Au total, les échantillons positifs pour *Enterococcus* étaient de 32/60 (53,3 %) avec un total de 45 isolats d'*Enterococcus*. La spéciation des isolats d'*Enterococcus* basée sur l'API rapid ID 32 STREP était *Enterococcus faecium* 15/45 (33,3%), *Enterococcus faecalis* 12/45 (26,7%), *Enterococcus durans* 8/45 (17,8%), *Enterococcus casseliflavus* 5/45 (11,1%) et *Enterococcus hirae* 5/45 (11,1%). Les isolats présentaient la résistance aux antibiotiques la plus élevée à l'ampicilline (100,0%), à la fosfomycine (95,6%) et à la pénicilline G (88,9%) et la moindre résistance à la ciprofloxacine (22,2%) et au chloramphénicol (28,9%). Les facteurs de virulence des espèces d'*Enterococcus* observés étaient la gélatinase, l'activité β-hémolytique et hyaluronidase, le biofilm et la formation de couche S. Le degré de formation de biofilm chez l'espèce *Enterococcus* était une forte formation de biofilm (19/45, 42,2%), une formation modérée de biofilm (10/45, 22,2%), une faible formation de biofilm (11/45, 24,4%) et aucune formation de biofilm (5/45, 11,1%).
Conclusion: Les résultats de cette étude mettent l'accent sur les implications potentielles sur la santé associées à la résistance aux antimicrobiens et aux facteurs de virulence phénotypique d'Enterococcus dans les produits de volaille.

Mots-clés: Résistance aux antibiotiques; Entérocoque; La volaille; Facteurs de virulence; Bénin Ville

Introduction:

Enterococcus is an autochthonous microbiota of the gastrointestinal and skin flora tract of birds, humans and diverse animal species (1). Enterococci are Gram-positive, non-spore-forming, catalase-negative and facultative anaerobic bacteria. In domestic animals, especially in the poultry industry, enterococcal probiotics are beneficial in infection control, improving the immune system and growth promotion (2). Globally, the poultry industry is one of the fastest and largest growing agro-based protein production industries. The intense desire to meet up with the high demand for poultry products usually involve the usage of enterococcal probiotic supplements (3). However, despite their intrinsic potentials in the food industry, they are not generally recognized as safe (GRAS), and their presence could also be attributed to faecal contamination (4).

The activities of *Enterococcus,* like other opportunist pathogens, can also trigger an infection in animals and humans when it invades other mucosal and skin surfaces, especially in cases of reduced host immunity (5). The foremost species responsible for enterococcal-related infections in humans are *E. faecalis* and *E. faecium,* and they are usually associated with urinary tract infections, liver infections, endocarditis and septicemia (1). The ability of these microorganisms to cause can be attributed to several virulence factors (6). However, their resistance to various antibiotics notably enhances the pathogenic strength expressed by these virulence factors (7). This makes the absence of transferable antibiotic resistance an essential criterion for selecting enterococci as probiotic food supplements (8,9).

Enterococci of food origin have not been explicitly determined as immediate causes of clinical infections (10). Still, the presence of antibiotic-resistant enterococci has been reported in retail poultry meats (11). This tends to be a potential risk of transmitting antimicrobial resistance genes to humans when consumed (10). Antimicrobial resistance in enterococci also enhances their ability to withstand a variety of host defenses including innate immune system (6). Although the strains of enterococci linked with clinical infections may vary from animal-related strains, antibiotic-resistant strains that are genetically related have been linked to both animals and human colonization (12, 13).

The exchange of vancomycin resistance between animals and humans has also been noticed *in vitro* and *in vivo* (14). The surveillance of antimicrobial resistance (AMR) in poultry production and the use of specific therapeutic agents are, therefore, imperative

concerning public food safety and environmental health concerns (15). The objective of this study is to determine the antibiotic resistance profile and phenotypic virulence properties associated with *Enterococcus* species isolated from poultry farms in Benin City, Edo State, Nigeria.

Materials and method:

Description of study area:

The samples were collected from five poultry farms in Benin City, Edo State, Nigeria. The five poultry farms were selected by simple random sampling from the different commercial farming areas within Benin City; Ekenwan road (Farm A), Sapele road (Farm B), Aruogba (Farm C), New Benin (Farm D) and Ugbowo (Farm E).

Sample collection:

Sixty samples were randomly collected from each poultry farm (12 random samples from each farm) between July and September 2020. The samples include 20 feed samples, 20 water samples and 20 faecal samples. Sterile containers were used to collect the water, feeds and fecal samples from the various farms and transported immediately to the Applied Microbial Processes and Environmental Health Research Group (AMPEHREG) laboratory, University of Benin, for analysis within 4 hours of sample collection.

Ethical consideration:

The samples were collected as recommended in "Institutional Animal Care and Use Committee" guidelines on ethics concerning the usage of animals and animal products for research purposes according to Suckow and Lamberti (16).

Enrichment and isolation:

Enrichment and isolation were carried out according to the method previously described by Sanlibaba et al., (17). Ten grams of the samples (feed and faecal) and 10 ml of water samples were introduced into 90 ml sterile distilled water. An aliquot of 1 ml from each stock solution was aseptically pipetted into 9 ml tryptone soy broth (TSB, Merck, Darmstadt, Germany). The TSB was incubated at 37^{o}C for 18–24 hours. Subsequently, a loopful of bacterial culture in the TSB was streaked on bile aesculin azide (BAA) agar (TM Media, Rajasthan, India). The culture plates were incubated for 18-24 hours at 37^{o}C.

Black colonies on BAA agar were considered to be presumptive *Enterococcus* isolates. The colonies were sub-cultured on fresh BAA agar and incubated for another 18-24 h at 37^{o}C. Presumptive *Enterococcus* colonies that were recovered were purified on nutrient agar for 18-24 hours at 37^{o}C. Purified isolates were stored on nutrient agar (Lab M, Lancashire, United Kingdom) slants until needed for further analysis.

Characterization and identification of *Enterococcus*:

Morphological characteristics and biochemical tests were determined using purified isolates as previously described (17,18). The purified isolates on Nutrient agar were characterized using Gram reaction with potassium hydroxide (3% KOH), oxidase test, catalase test, temperature tolerance range assay (10^{o}C, 45^{o}C), sodium chloride (NaCl) tolerance assay and Pyrrolidonyl-beta-naphthylamide (PYR) test. Following the manufacturer's instruction, the isolates were subsequently confirmed using Analytical Profile Index (API) rapid 32 STREP strips (BioMerieux, France).

Antimicrobial susceptibility screening:

Enterococcus isolates were screened for antibiotic resistance using the Kirby-Bauer disc diffusion method. Suspension of the test isolates with of 0.5 McFarland's approximated turbidity was pipetted and aseptically spread on Mueller-Hinton agar plates (Lab M, Lancashire, United Kingdom). The antibiotics discs (Mast Diagnostics, Merseyside, United Kingdom) were aseptically placed on the Mueller-Hinton agar culture plates. The antibiotics tested include penicillin G (10 units), ampicillin (10µg), rifampin (5µg), erythromycin (15µg), vancomycin (30µg), ciprofloxacin (5µg), chloramphenicol (30µg), fosfomycin (200µg) and nitrofurantoin (300 µg).

The culture plates were incubated at 37^{o}C for 18-24 hours. The diameter of inhibition zones was measured and interpreted using the Clinical and Laboratory Standards Institute (CLSI) guidelines (19,20).

Multiple antibiotic resistance index:

Multiple antibiotic resistance index (MARI) was determined according to the formula of Chitanand et al., (21) as simplified by Ogofure and Igbinosa (22); MARI = y/nx, where 'y' is the number of resistant isolates, 'n' is the number of isolates, and 'x' is the number of antibiotics tested. MAR index higher than 0.2 indicates that the organisms originate from high-risk sources of contamination and are, therefore of public health significance.

Determination of phenotypic virulence:

The colonies were cultured on TSA (Merck, Darmstadt, Germany) and re-suspended in 20 ml TSB. The turbidity of the suspension was adjusted to 10^{6} cells/ml using McFarland guidelines for virulence determination. Gelatinase production was determined on gelatin medium. The β-haemolytic activity

was determined on sheep blood agar plate. Hyaluronidase activity was evaluated by spot inoculation using brain heart infusion broth supplemented with 1.0 g of agar-agar. The presence of surface-layer (S-layer) was assessed by streaking cultures on TSA plates, augmented with 0.1 mg/ml Coomassie brilliant blue R 250 (Merck, Darmstadt, Germany). All experiments were performed in triplicates and assessed in accordance with the method previously described (23).

Biofilm characterization:

The biofilm formation potential of the *Enterococcus* isolates was assessed quantitatively using the microtitre plate method. Suspension of overnight cultured *Enterococcus* (20µl) were re-standardized to 0.5 McFarland turbidity, inoculated into 96-wells microtitre plates containing 200 µl of nutrient broth and incubated at 37^{o}C for 18-24 hours. Constituents of respective wells were removed, plates were rinsed with sterile phosphate buffered saline (PBS) and air-dried. The plates were then stained with 1% crystal violet (200 µl) for 30 mins. Respective wells were rinsed with de-ionized water to remove the crystal violet and then dried at 28±2^{o}C. Crystal violet dye that bound to adherent cells was solubilized using 150 µl of absolute ethanol.

The optical density (OD) of the plates was determined at a wavelength 570 nm with a micro-plate reader (Synergy MxBiotekR, USA). The OD of each triplicate result, negative and positive controls was calculated. Isolates were classified as strong (ODi>0.12), moderate (ODi=0.1<0.12), weak (ODc<ODi <0.1) and non-biofilm producer (ODi<ODc), accordingly as previously described (24,25).

Statistical analysis:

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 21.0 and Microsoft Excel 2013. Mean values were expressed using descriptive statistics.

Results:

A total of 60 samples which comprised of 20 feeds, 20 water and 20 faecal samples obtained from 5 different poultry farms in Benin City, Nigeria, was assessed in this study. In overall, the total positive samples for *Enterococcus* isolates were 32/60 (53.3%). The frequency of *Enterococcus* isolation from the different samples is shown in Table 1 with 60.0% (12/20) from feeds, 75.0% (15/20) from water and 25.0% (5/20) from faeces

Table 1: Frequency of *Enterococcus* isolation from the different samples

Sample types	No of samples	No of *Enterococcus* positive samples (%)
Feed	20	12 (60.0)
Water	20	15 (75.0)
Faeces	20	5 (25.0)
Total	60	32 (53.3)

Fig 1 shows the frequency of *Enterococcus* isolation from the samples in each poultry farm, with 66.7% (8/12) in Farm A, 50.0% (6/12) in Farm B, 41.7% (5/12) in Farm C, 66.7% (8/12) in Farm D and 41.7% (5/12) in Farm E. Table 2 shows the phenotypic characterization and speciation of the 45 *Enterococcus* isolates based on API rapid ID 32 STREP. The frequency of *Enterococcus faecium* is 33.3% (15/45), *Enterococcus faecalis* 26.7% (12/45), *Enterococcus durans* 17.8% (8/45), *Enterococcus casseliflavus* 11.1% (5/45) and *Enterococcus hirae* 11.1% (5/45).

The antibiotic resistant profile of *Enterococcus* species is shown in Table 3, with resistance to penicillin G (88.9%, 40/45), rifampin (75.6%, 34/45), erythromycin (77.8%, 35/45), vancomycin (68.9%, 31/45), ciprofloxacin (22.2%, 10/45)**,** chloramphenicol (28.9%, 13/45), ampicillin (100%, 45/45), fosfomycin (95.6%, 43/45) and nitrofurantoin (86.7%, 39/45).

The multiple antibiotic resistance index (MARI) of *Enterococcus* species is shown in Table 4. It was observed that a total of 38/45 (84.4%) isolates demonstrated resistance to at least five antibiotics. In comparison, all the isolates (45/45, 100.0%) demonstrated resis-

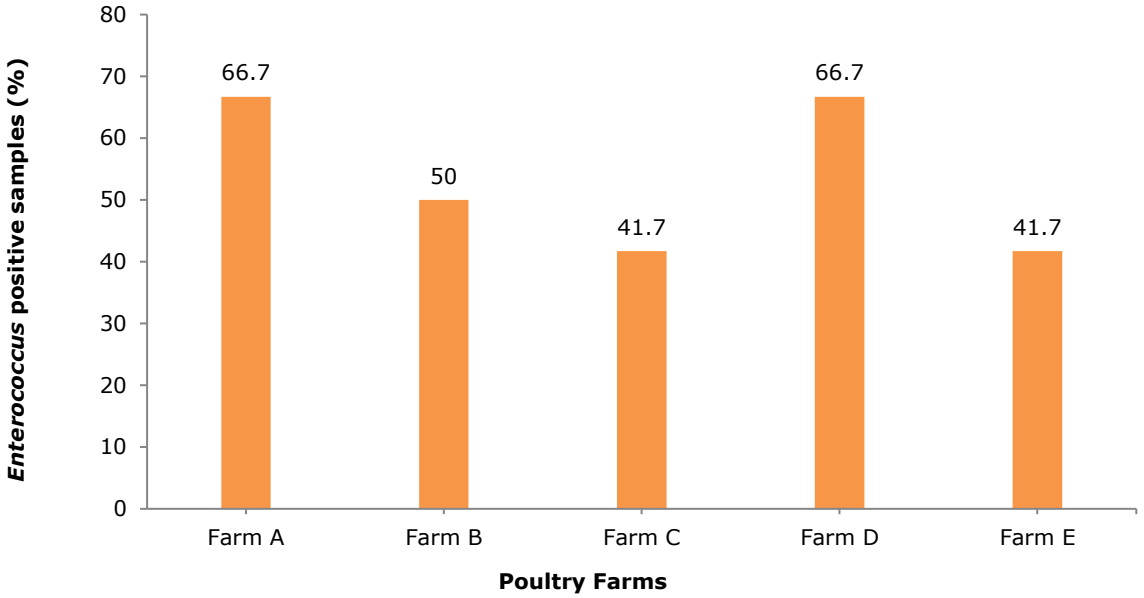

Fig 1: Frequency of occurrence of *Enterococcus* positive samples in each poultry farms

Table 2: Phenotypic characterization of the isolated *Enterococcus* species by conventional biochemical tests and API ID STREP

Group of isolates	Conventional biochemical tests						API ID 32 STREP	Number of isolates (%)
	Gram reaction (3% KOH)	Temperature of growth		Growth in 6.5% NaCl	PYR Test	Genus Identification		
		10 °C	45 °C					
Group A	+	+	+	+	+	*Enterococcus* spp.	*E. faecalis*	12 (26.7)
Group B	+	+	+	+	+	*Enterococcus* spp.	*E. faecium*	15 (33.3)
Group C	+	+	+	+	+	*Enterococcus* spp.	*E. durans*	8 (17.8)
Group D	+	+	+	+	+	*Enterococcus* spp.	*E. casseliflavus*	5 (11.1)
Group E	+	+	+	+	+	*Enterococcus* spp.	*E. hirae*	5 (11.1)
Total								**45 (100.0)**

KOH: Potassium hydroxide; PYR: Pyrrolidonyl-β- naphthylamide; NaCl: Sodium chloride
Group A: AF, BD, 2AF, CF, DD, 2AW, 2EW2, 4DW2, 2BF, 4BF2, DF, 3BW1
Group B: DW, 2EW1, 4AW2, 4DW1, AW, AD, CW, 4EW1, BF, CD, 2DF, 3DF1, ED, 2DW, 3DF2
Group C: 3BW2, 3DW1, 3CW1, 4AF2, 4CW1, 4AW1, 4CW2, 4EW2
Group D: 3DW2, 4BF1, EF, 3AF1, 3CW2
Group E: 4AF1, EW, 3AF2, 4EW1, BW

tance to at least three antibiotics used in this study. The MARI of the *Enterococcus* species ranged from 0.3–0.9, with 38/45 (84.4%) isolates having a MARI of ≥ 0.5. A total of 2 of 45 (4.4%) of the isolates were resistant to four antibiotics with a MARI of 0.4 while 5/45 (11.1%) were resistant to three antibiotics with MARI of 0.3. All the isolates in the study (45/45, 100%) had MARI of ≥ 0.3.

The virulence factors of *Enterococcus* species are shown in Fig 2. The virulence factors observed in *Enterococcus faecalis* include gelatinase activity in 10/12 (83.3%), β-haemolytic activity in 12/12 (100.0%), hyaluronidase activity in 11/12 (91.7%) and S-layer formation in 12/12 (100,0%).

The virulence factors observed in *Enterococcus faecium* include gelatinase activity in 11/15 (73.3%), β-haemolytic activity in 10 of 15 (66.7%), hyaluronidase activity in 13 of 15 (86.7%) and S-layer formation in 15 of 15 (100.0%) isolates.

The virulence factors observed in *Enterococcus durans* include gelatinase activity in 3/8 (37.5%), β-haemolytic activity in 4/8 (50%), hyaluronidase activity in 4/8 (50%) and S-layer formation in 7/8 (87.5%) isolates.

The virulence factors observed in *Enterococcus hirae* include gelatinase activity in 1/5 (20.0%), β-haemolytic activity in 2/5 (40.0%), hyaluronidase activity in 2/5 (40%) and S-layer formation in 4/5 (80.0%).

Table 3: Antibiotic susceptibility profile of *Enterococcus* species isolated from poultry in selected farms in Benin City, Nigeria

Antibiotics	Antibiotic susceptibility profile (%)															Resistant strains (%)
	***E. faecalis* (n=12)**			***E. faecium* (n=15)**			***E. durans* (n=8)**			***E. casseliflavus* (n=5)**			***E. hirae* (n=5)**			
	R	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	
Penicillins																
PEN (10 units)	12 (100)	0	0	11 (73.3)	0	4 (26.7)	8 (100)	0	0	4 (80)	0	1 (20)	5 (100)	0	0	40 (88.9)
AMP (10µg)	12 (100)	0	0	15 (100)	0	0	8 (100)	0	0	5 (100)	0	0	5 (100)	0	0	45 (100)
Ansamycins																
RIF (5µg)	9 (75)	0	3 (25)	12 (80)	0	3 (20)	5 (62.5)	0	3 (37.5)	3 (60)	0	2 (40)	5 (100)	0	0	34 (75.6)
Macrolides																
ERY (15µg)	10 (83.3)	1 (8.3)	1 (8.3)	14 (93.3)	0	1 (6.7)	5 (62.5)	3 (37.5)	0	2 (40)	2 (40)	1 (20)	4 (80)	1 (20)	0	35 (77.8)
Glycopeptides																
VAN (30µg)	9 (75)	2 (16.7)	1 (8.3)	10 (66.7)	2 (13.3)	3 (20)	6 (75)	2 (25)	0	3 (60)	1 (20)	1 (20)	3 (60)	2 (40)	0	31 (68.9)
Fluoroquinolones																
CIP (5µg)	1 (8.3)	4 (33.3)	7 (58.3)	5 (33.3)	2 (13.3)	8 (53.3)	2 (25)	2 (25)	4 (50)	1 (20)	1 (20)	3 (60)	1 (20)	1 (20)	3 (60)	10 (22.2)
Phenicols																
CHL (30µg)	5 (41.7)	3 (25.0)	4 (33.3)	4 (26.7)	8 (53.3)	3 (20)	1 (12.5)	6 (75)	1 (12.5)	1 (20)	3 (60)	1 (20)	2 (40)	2 (40)	1 (20)	13 (28.9)
Fosfomycins																
FOS (200µg)	12 (100)	0	0	15 (100)	0	0	7 (87.5)	1 (12.5)	0	4 (80)	0	1 (20)	5 (100)	0	0	43 (95.6)
Nitrofurans																
NIT (300µg)	9 (75)	2 (16.7)	1 (8.3)	13 (86.7)	2 (13.3)	0	8 (100)	0	0	5 (100)	0	0	4 (80)	1(20)	0	39 (86.7)

PEN: Penicillin G (10 units); RIF: Rifampin (5µg); ERY: Erythromycin (15µg); VAN: Vancomycin (30µg); CIP: Ciprofloxacin (5 µg); CHL: Chloramphenicol (30µg); AMP: Ampicillin (10µg); FOS: Fosfomycin (200µg) and NIT: Nitrofurantoin (300 µg). Values in parenthesis represent percentage (%).

Table 4: Resistance phenotypes and multiple antibiotic resistance index of *Enterococcus* species

Isolates Code	Number of antibiotics	Resistance phenotype	MARI
AW, CF, 4DW2	8	PEN^R - RIF^R - ERY^R - VAN^R - CHL^R - AMP^R - FOS^R - NIT^R	0.9
BW	8	PEN^R - RIF^R - VAN^R - CIP^R - CHL^R - AMP^R - FOS^R - NIT^R	0.9
CW, ED, 3AF2, 4BF2	8	PEN^R - RIF^R - ERY^R - VAN^R - CHL^R - AMP^R - FOS^R - NIT^R	0.9
DF, EF, 3DF1, 4EW2, 4EW3	8	PEN^R - RIF^R - ERY^R - VAN^R - CIP^R - AMP^R - FOS^R - NIT^R	0.9
AF	7	PEN^R - RIF^R - ERY^R - VAN^R - CHL^R - AMP^R - FOS^R	0.8
AD, BF, BD, DD, EW, 3BW2, 3DF2, 3DW1	7	PEN^R - RIF^R - ERY^R - VAN^R - AMP^R - FOS^R - NIT^R	0.8
DW	7	PEN^R - RIF^R - VAN^R - CIP^R - AMP^R - FOS^R - NIT^R	0.8
3BW1	7	PEN^R - ERY^R - VAN^R - CHL^R - AMP^R - FOS^R - NIT^R	0.8
CD	7	PEN^R - ERY^R - VAN^R - CIP^R - AMP^R - FOS^R - NIT^R	0.8
4AW2	7	RIF^R - ERY^R - CIP^R - CHL^R - AMP^R - FOS^R - NIT^R	0.8
4CW2	7	PEN^R - ERY^R - VAN^R - CHL^R - AMP^R - FOS^R - NIT^R	0.8
2BF, 4AF2, 4EW1	6	PEN^R - RIF^R - ERY^R - AMP^R - FOS^R - NIT^R	0.7
2EW2, 4AW1, 4BF1	6	PEN^R - RIF^R - VAN^R - AMP^R - FOS^R - NIT^R	0.7
3AF1	6	RIF^R - ERY^R - VAN^R - AMP^R - FOS^R - NIT^R	0.7
3DW2	5	PEN^R - CHL^R - AMP^R - FOS^R - NIT^R	0.6
4AF1	5	PEN^R - RIF^R - ERY^R - AMP^R - FOS^R	0.6
4CW1	5	PEN^R - VAN^R - AMP^R - FOS^R - NIT^R	0.6
4DW1	5	RIF^R - ERY^R - AMP^R - FOS^R - NIT^R	0.6
2DF	4	ERY^R - AMP^R - FOS^R - NIT^R	0.4
3CW1	4	PEN^R - CIP^R - AMP^R - NIT^R	0.4
2AF, 2DW	3	PEN^R - AMP^R - FOS^R	0.3
2AW	3	ERY^R - AMP^R - FOS^R	0.3
2EW1	3	RIF^R - AMP^R - FOS^R	0.3
3CW2	3	PEN^R - AMP^R - NIT^R	0.3

PEN: Penicillin G (10 units); RIF: Rifampin (5µg); ERY: Erythromycin (15µg); VAN: Vancomycin (30µg); CIP: Ciprofloxacin (5 µg); CHL: Chloramphenicol (30µg); AMP: Ampicillin (10µg); FOS: Fosfomycin (200µg) and NIT: Nitrofurantoin (300 µg); MARI: Multiple antibiotic resistance index

The virulence factors observed in *Enterococcus casseliflavus* include gelatinase activity in 3/5 (60.0%), β-haemolytic activity in 2/5 (40.0%), hyaluronidase activity in 3/5 (60%) and S-layer formation in 5/5 (100%). In total, the virulence factors formation of *Enterococcus* species observed were gelatinase activity in 28/45 (62.2%), β-hemolytic activity in 30/45 (66.7%), hyaluronidase activity in 33/45 (73.3%) and S-layer formation in 43/45 (95.6%) isolates.

The frequency distribution of biofilm forming *Enterococcus* species is shown in Fig 3. Biofilm formation ability observed in *E. faecalis* includes strong biofilm formation in 5/12 (41.7%), moderate biofilm formation in 3/12 (25.0%), weak biofilm formation in 4 of 12 (33.3%) and no biofilm formation in nil isolate.

Biofilm formation capacity observed in *E. faecium* includes strong biofilm formation in 6/15 (40.0%), moderate biofilm formation in 4/15 (26.7%), weak biofilm formation in 3/15 (20.0%) and no biofilm formation in 2/15 (20.0%) isolates.

Biofilm formation capacity in *E. durans* includes strong biofilm formation in 3/8 (37.5%), moderate biofilm formation in 1/8 (12.5%), weak biofilm formation in 2/8 (25.0%) and no biofilm formation in 2/8 (25.0%) isolates.

Biofilm formation capacity observed in *E. hirae* includes strong biofilm formation in 2/5 (40.0%), moderate biofilm formation in 1/5 (20.0%), weak biofilm formation in 1/5 (20.0%) and no biofilm formation in 1/5 (20.0%).

Biofilm formation capacity observed in *E. casseliflavus* includes strong biofilm formation in 3/5 (60.0%), moderate biofilm formation in 1/5 (20.0%), weak biofilm formation in 1/5 (20.0%) and no biofilm formation in nil isolate.

In total, the frequency of biofilm formation observed in *Enterococcus* species was 42.2% (19/45) for strong biofilm formation, 22.2% (10/45) for moderate biofilm formation, 24.4% (11/45) for weak biofilm formation 11.1% (5/45) for no biofilm formation.

Discussion:

Enterococci are widely known for their probiotic potential in birds including poultry. However, the presence of antibiotic-resistant strains of enterococci remains a global health concern as it tends to influence animal pathology. In this study, *Enterococcus* species were detected in the feeds, water and faecal samples of poultry birds. Previous studies have equally reported the detection of enterococci in feeds, water and faecal samples poultry (26,27).

The detection of enterococci in faecal samples could be attributed to the fact that enterococci are gastrointestinal flora of animals including poultry (4). Furthermore, Lata et al., (28) reported that enterococci in water could indicate fecal contamination. The water samples investigated in this study could have been exposed to enterococcal contamination through unclean water trough or enterococci dissemination through air. This affirmed pre-

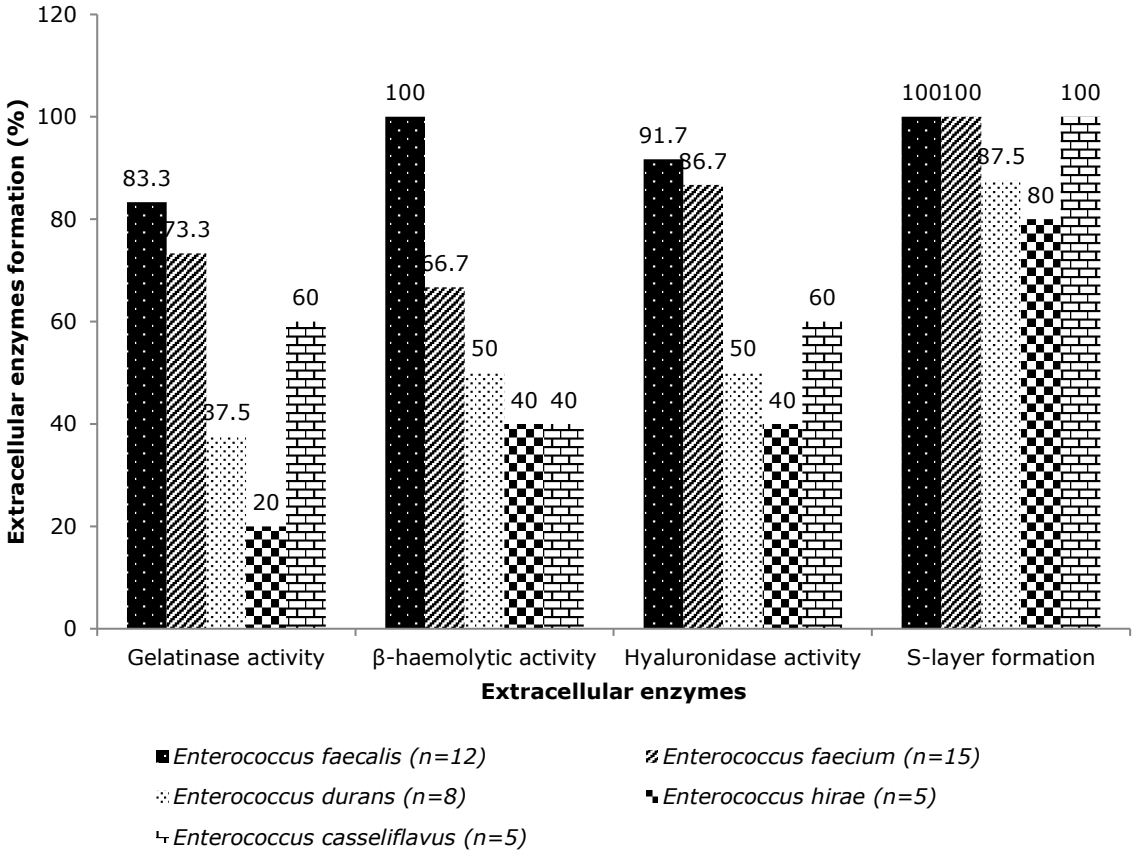

Fig 2: Distribution of phenotypic virulence factors of the *Enterococcus* isolates

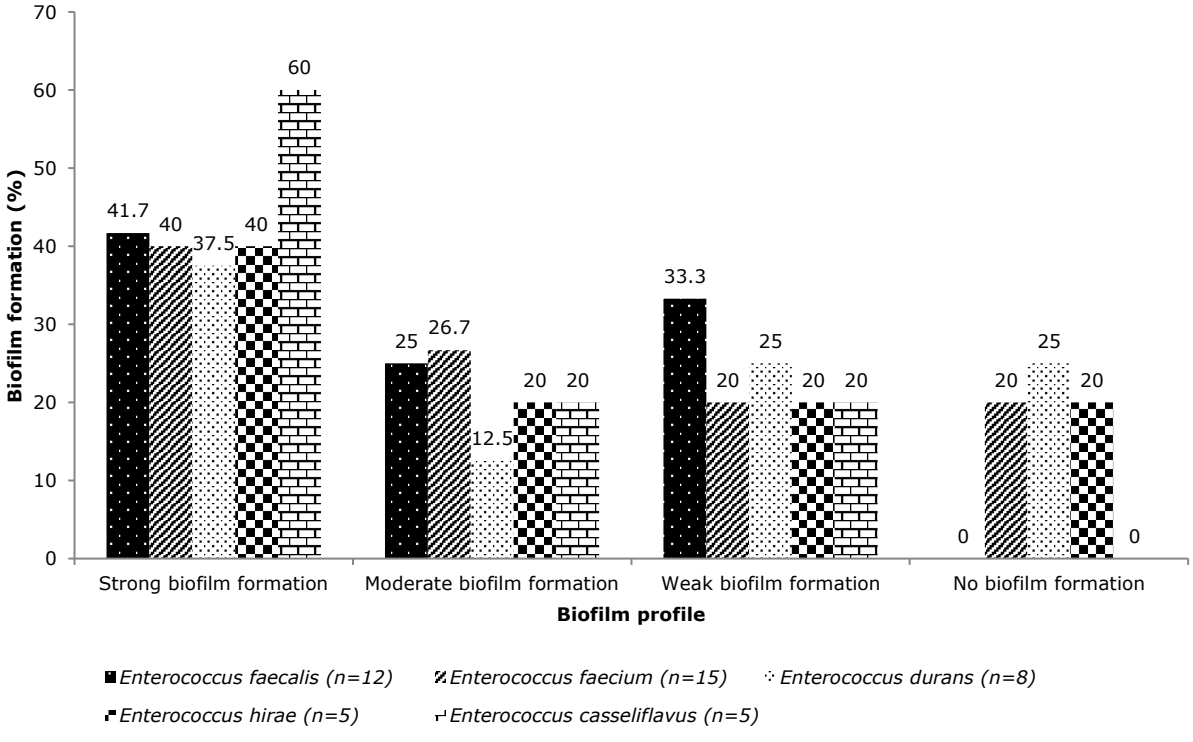

Fig 3: Biofilm formation distribution of *Enterococcus* species

vious studies which reported that microorganisms associated with faecal discharge, including enterococci, can be disseminated through air (29). The presence of enterococci in the feeds could be attributed to their usage as probiotic supplements and contaminations arising from faecal matters. Several studies have reported using enterococci strains as probiotic supplements in animal feeds for growth promotion and disease control (30,31).

The most prevalent *Enterococcus* spp isolated in this study were *E. faecium* (33.3%) and *E. faecalis* (26.7%), followed by *E. durans* (17.8%), *E. casseliflavus* (11.1%) and *E. hirae* (11.1%). This agrees with previous studies that reported the detection of *E. faecium*, *E. faecalis, E. durans*, *E. casseliflavus* and *E. hirae* in poultry and its environment in which *E. faecium* and *E. faecalis* are the most prevalent species (26,29). Although enterococci may be involved in the pathology of birds, enterococci from food animals have not been exclusively implicated as pathogenic in human because investigations have attributed resulting infections in human to nosocomial and community-associated strains (10). Nevertheless, enterococci isolated from poultry and several other food chains can still adversely affect human and animal health as they could influence the acquisition and dissemination of antibiotic resistance (32).

In this study, it was observed that enterococci demonstrated high resistance to ampicillin (100%), fosfomycin (95.6%), penicillin G (88.9%) and nitrofurantoin (86.7%) while the least resistance was demonstrated to chloramphenicol (22.2%) and ciprofloxacin (28.9%). In agreement with this study, it has been previously envisaged that enterococci show significantly high resistance to β-lactam antibiotics and lower towards quinolones (6). Contrary to this study, enterococci isolates investigated in the study by Bertelloni et al., (27) reported a lower resistance to chloramphenicol (19.1%) compared to the 22.2% observed in our study. However, the resistance rate of enterococci in the study to nitrofurantoin (48.7%), ampicillin (29.6%), rifampicin (22.6%) and vancomycin (10.0%) were lower than the rate reported in our study. In agreement with our study, previous studies reported significant resistance of enterococci to erythromycin, penicillin and ampicillin (33,34).

The different antimicrobial resistance rates of *Enterococcus* in these studies could be due to variations in geographical locations and intensity of antibiotics usage in different settings (35). Unrestrained use of antimicrobial agents is acknowledged as the most essential factor contributing to the development of resistant microorganisms which could spread to humans via the food chain.

The multiple antibiotic resistance index (MARI) of *Enterococcus* species in this study, showed that 84.4% of the isolates were resistant to at least five antibiotics, while all the isolates (100.0%) were resistant to at least three antibiotics. The MARI of the *Enterococcus* species ranged from 0.3 - 0.9 in which all the isolates demonstrated MARI of ≥ 0.3. The MARI is a good risk assessment tool, and MARI > 0.2 indicates that isolates are from high-health risk sources where frequency of antibiotic use is high (36). The MARI in all the enterococci isolates in our study was greater than the 0.2 threshold value, further intensifying the possibility of antibiotic resistance dissemination.

The virulence factors investigated in this study showed that 62.2% of the enterococci isolates demonstrated gelatinase activity, 66.7% β-haemolytic activity, 73.3% hyaluronidase activity and 95.6% showed S-layer formation. The virulence factors detected in this study have been implicated in previous investigation on enterococci isolates from animal products meant for human consumption and its environment (37). In addition, the degree of biofilm formation in the enterococci isolates showed that 42.2% were strong biofilm forming, 22.2% moderate biofilm forming, 24.4% weak biofilm forming while only 11.1% were non-biofilm forming enterococci isolates.

The linkage of enterococci from food origin with virulence production, which is an effector molecule that enhances pathogenicity further increases their clinical significance as opportunistic pathogens. This agrees with previous studies which emphasized that the demonstration of biofilm and other virulence factors in enterococci of non-clinical origin increases their chances of causing infections (37,38). This makes it essential for enterococci originating from food sources to be monitored regarding potential antibiotic resistance (39). This is to strategize on how to minimize their potential threat to animal and human health. In view of this, proper monitoring and surveillance of virulence traits and antimicrobial resistance exchange among animal, human or indirectly through environmental interface could help reduce the possible health risks associated with using enterococci as probiotics in poultry.

Conclusion:

Our study shows that the poultry environment is a potential reservoir of virulent enterococci with antibiotic-resistant capabilities.

The linkage of the isolated enterococci with extracellular virulence properties, biofilm potential and resistance to multiple antibiotics signal that enterococci of non-clinical origin remain possible route of disseminating antimicrobial resistance and virulence traits to human microbiota. Therefore, it remains fundamental to emphasize proper hygiene practices and antibiotic use in poultry farms. Furthermore, the use of probiotic supplements in poultry feeds should also be strictly monitored.

Contribution of authors:

IEO conceptualized the study and designed the laboratory methods; IBO, AO and NCN were involved in material preparation, data collection and analysis; IBO, AO and NCN prepared the initial manuscript draft; IBO, AO and IEO revised the manuscript. All authors read and approved the final manuscript.

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