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Research Paper

Influence of Sex on the Prevalence and Virulence Genes of *Enterococcus* Species in Selected Livestock and Companion Animals Raised In South-South Nigeria

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ABSTRACT
The influence of sex on the prevalence of Enterococcus species in selected livestock and companion animals in South-South Nigeria was assessed using standard methods, involving buffered peptone water, trypticase soy broth, bile esculin azide agar and tuf and virulence gene primers. A total of 169 animals comprising 48 companion and 121 livestock animals (10 cats, 38 dogs, 20 cattle and 101 chickens) were examined. Sixty-nine (69) (35 males and 34 females) Enterococcus species were confirmed representing 26 E. faecalis, 17 E. faecium and 26 undifferentiated Enterococcus. Among the males, chicken had the highest counts (42.90%), followed by dog (34.30%), cattle (17.10%) and cats (5.70%). Furthermore, among the female animals, chicken also had the highest number of positive Enterococcus (41.20%), followed by dog (26.50%), cattle (23.50% and cats (8.80%). Confirmed isolates produced the expected base pair of 419 and 543 with virulence genes gelE and ccf respectively. The detection of gelE in isolates from males reveals highest number with chickens (43.8%), followed by cattle (25.0%), dog (18.8%) and cat (12.5%), while for the isolates from female animals, the highest was also chicken (47.1%), followed by dog (29.4%), cattle (17.6%) and cats (5.9%). The detection of ccf in isolates from males reveals highest number with chickens (46.15%), followed by dog (30.77%), cattle (15.38%) and cat (7.69%). For female animals, the highest value was also observed in chicken (50.0%) while cattle and dog had 25.00% each. The presence of virulence bearing Enterococcus in livestock and companion animals portend danger for humans.

Keywords: *Companion animals, Enterococcus species, livestock, virulence,*

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I. INTRODUCTION

Although there are positive impacts of some bacteria, the negative impact of bacterial diseases on man and livestock animals cannot be over emphasized. Bacterial infections have a large impact on public health that calls for more attention (Doron and Gorbach, 2008). One bacterium of upmost public health concerns is *Enterococcus.* According to Wikipedia (2022), *Enterococcus* is a large genus of lactic acid bacteria of the phylum Bacillota. Enterococci are gram-positive cocci that often occur in pairs (diplococci) or short chains, and are difficult to distinguish from streptococci on physical characteristics alone. In addition, enterococci are indigenous flora of the gastro-intestinal tracts of animals and humans (Billington *et al.* 2014). Billington *et al.* (2014) also emphasized that enterococci are a clinically significant cause of bloodstream infections (BSI), particularly in the nosocomial setting. The one with higher prevalence is *Enterococcus faecalis* which is associated with urinary focus, genitourinary malignancy, and abnormal genitourinary anatomy. However, *E. faecium* infections are associated with a gastrointestinal focus.

Orababa *et al*. (2021) stated that enterococci are opportunistic pathogens and are one of the most important bacteria in hospital-acquired infections. Their high prevalence in livestock and companion animal species has also become a concern. It has also been discovered that they are resistant to antibiotics such as vancomycin. This has led to life-threatening and difficult-to-treat nosocomial infections. The members of the genus *Enterococcus* belong to the family *Enterococcaceae* and are single/paired, catalase-negative, Gram-positive, non–spore-forming, facultatively anaerobic bacteria (Ciftci *et al.*, 2009; Teixeira and

Merquior, 2013). They are mainly found as normal flora in the intestine of both animal and man (Bibalan *et al*., 2015). They are also found in the female genital tract (Kunurya *et al*., 2020), plants, food, and soil (Cassenego *et al*., 2011). *Enterococcus faecium* and *E faecalis* are the most common species in this group of bacteria with *E faecalis* accounting for approximately 90% of infections caused by members of this genus (Gordon *et al*., 1991).

Different virulent genes have been associated with *Enterococcus* species. Stępień-Pyśniak *et al.* (2019) explained that the aggregation substance *ccf* is a sex pheromone plasmid-encoded surface protein. Some of *E. faecalis* strains with all sex pheromone genes (cpd, cob, and *ccf*) exhibited the presence of the agg and asa1 genes as well (Stępień-Pyśniak *et al*., 2019). In addition, Vankerckhoven *et al*., (2004) reported that *gelE* was present as a silent gene in *E. faecalis* (77.8%), and *E. faecium* (58.6). Ferguson *et al.* (2016) reported that among the virulence genes analysed, *gelE* was the most frequently detected and widely distributed among *E. faecalis* strains.

Some researchers have implicated the influence of sex on the prevalence of *Enterococcus* species and virulence genes among livestock and companion animals. Not much of this has been done with animal and companion animals in the South-South region of Nigeria using molecular procedures for confirmation. This necessitates this research to get the necessary information needed for proper public health principles since this disease is zoonotic.

II. MATERIALS AND METHODS

Sample collection

A total of 169 animals that comprised of 48 companion and 121 livestock animals were sampled over a period of 3 months and used for this study. The distribution of the examined livestock and companion animals is presented in Table 1.

Isolation of *Enterococcus* species

Samples from rectal and cloacal areas were collected using a sterile swab and enriched with 5 ml buffered peptone water (BPW) followed by incubation at 37^{o}C for 24 h. They were further enriched in trypticase soy broth (TSB) by adding 1 ml of the BPW to 5 ml TSB and incubated at 37^{o}C for 24 h. A loopful of each sample was thereafter, streaked onto bile esculin azide (BEA) agar plate and incubated at 37^{o}C for 24 h. The presumptive identification of *Enterococcus* species was based on the hydrolysis of the esculin in the media into glucose and aesculetin resulting in the medium turning dark brown or black (Pillay *et al*., 2018).

Table 1: Total number of isolates collected from companion and livestock animals

Category	Animal species	Male	Female	Total
Companion	Cat	6	4	10
	Dog	23	15	38
Livestock	Cattle	6	14	20
	Chicken	19	82	101
	Total	54	115	169

DNA Extraction Procedure

The DNA was extracted from the isolate using the boiling method as shown below:

1. Isolates were resuspend a loopful of bacteria in 150ml of dH_20
2. And then boiled for 10 min
3. After which they were placed on ice for 5 min
4. Centrifuged for 5 min at 10,000 g and then
5. The supernatant was transferred to fresh Eppendorf tubes.

Confirmation and identification of *Enterococcus* species and detection of *gelE* and *ccf* virulence genes

To confirm the presence of *Enterococcus* species in the isolates, DNA extracted from all the isolates were subjected to polymerase chain reaction (PCR) using primers for the *tuf* gene (Table 2). The DNA was subjected to the following cocktail mix and condition for the PCR. This consists 25ul of 10× PCR buffer (2.5ul), 25mM Mgcl2 (1.0ul), 5pMol forward primer (1.0ul), 5pMol reverse primer (1.0ul), DMSO (1.0ul), 2.5Mm DNTPs (2.0ul), Taq 5u/ul (0.1ul), 10ng/µl DNA (3.0ul), dH2O(13.4ul) (Pillay *et al.*, 2018).

To identify the number of *E. faecalis* present in the positive samples after the PCR using the *tuf* gene, the primers, SodA - *E. faecalis* (Table 2) were used using the PCR cocktail described above and PCR condition earlier reported by Pillay *et al.* (2018). Primers for SodA - *E. faecium* (Table 2) were also used to detect the presence of *E. faecium* using the PCR cocktail described above and the PCR condition reported by Pillay *et al.*

(2018). To detect the presence of virulence genes *gelE* and *ccf*, their respective primers (Table 2) were also used with the PCR cocktail described above and the PCR condition explained by Pillay *et al.* (2018).

Gel electrophoresis

The presence or absence of bands were assessed using gel electrophoresis employing a Portable Gel hood built in Blue LED (470nm) by Royal Biotech/Biolympics (www.royalbiotech.com) 1.5% agarose gel at a constant voltage and 1X TBE for approximately 1 h. They were visualized by Ethidium bromide staining and photographed under ultraviolet light. The ladder used is 100 base pair ladder from thermo scientific.

Table 2: Primer Sequences for identifying enterococcus species and virulence genes in companion and livestock animal species

Gene name	Types	Sequence	Product size (bp)
tuf	Forward	5'-TACTGACAAACCATTCATGATG-3'	112 bp
	Reverse	5'-AACTTCGTCACCAACGCGAAC-3'	
SodA - *E. faecalis*	Forward	5'-ACTTATGTGACTAACTTAACC-3'	360 bp
	Reverse	5'-TAATGGTGAATCTTGGTTTGG-3'	
SodA - *E. faecium*	Forward	5'-GAAAAAACAATAGAAGAATTAT-3'	215 bp
	Reverse	5'-TGCTTTTTTGAATTCTTCTTTA-3'	
gelE	Forward	5'-ACC CCG TAT CAT TGG TTT-3'	419 bp
	Reverse	5'-ACG CAT TGC TTT TCC ATC-3'	
ccf	Forward	5'-GGG AAT TGA GTA GTG AAG AAG-3'	543 bp
	Reverse	5'-AGC CGC TAA AAT CGG TAA AAT-3'	

Source: Pillay *et al.* (2018)

Statistical analysis

Data generated in this study were subjected to statistical analysis using IBM SPSS statistics (version 16). Descriptive statistics was used to generate the frequencies of the prevalence of the *Enterococcus* species and virulence genes. Furthermore, Chi-square tests were used to test the significance of the prevalence of genes detected from the different sex and species of animals used in this study. A binary logistic regression analysis was also used to test the relationship between genes that were detected in this study. The model used for the study is the presence (1) and absence (0) of each gene used to detect species and virulence factor. Test results were considered significant at $p < 0.05$.

Ethical considerations

This study was approved by the appropriate Ethics Committee of the University of Port Harcourt, Port Harcourt, Nigeria and the research was carried out in accordance with the ethical standards established in the 1964 Declaration of Helsinki and its subsequent amendments.

III. RESULTS

Confirmation of *Enterococcus* species

A total of one hundred and sixty-nine (169) samples were collected from the field and characteristic colonies of *Enterococcus* on BEA agar were prepared and confirmed with polymerase chain reaction using specific primers. A total of 69 positive isolates (40.8%) were confirmed using the *tuf* gene. Out of this value, 26 isolates (37.68%) were confirmed as *E. faecalis*, 17 isolates (24.64%) were confirmed as *E. faecium* while 26 (37.68%) were undifferentiated *Enterococcus* species.

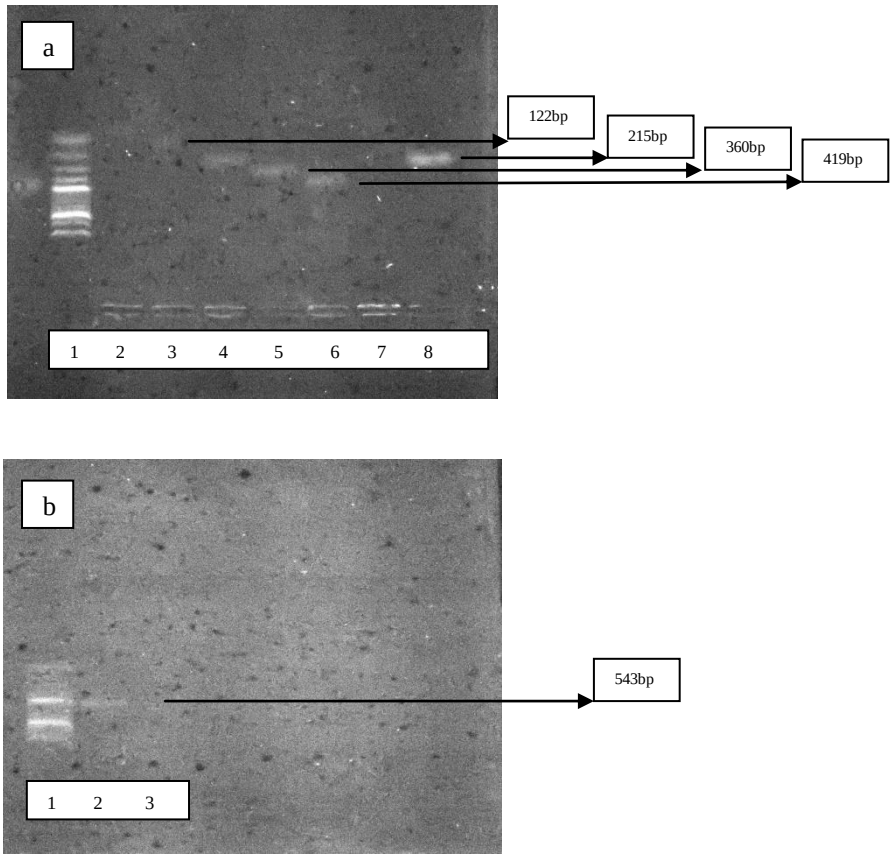

Figure 1: (a) Gel electrophoresis bands showing lane 1 for 100 bp DNA ladder, lane 2 negative control, lane 3 for tuf gene (112 bp), lane 5 for SodA gene for E. faecalis (360 bp) and lane 4 and 8 E. faecium (215 bp), lane 6 for gelE (419 bp). (b) Gel electrophoresis bands showing lane 1 for 100 bp DNA ladder and lane 2 for ccf gene (543 bp).

Figure 2 shows the number of isolates from male and female companion and livestock animals that were positive for *Tuf* gene which was used to detect the presence of *Enterococcus*. Positive samples were higher in dogs and chickens while the reverse was observed for cats and cattle. There were 35 positive males having the highest number from chicken (42.9%). This was followed by dog (34.3%), cattle (17.1%) and the least recorded with cats (5.7%). A similar trend was observed among females. The highest positive isolates were observed among chickens (41.2%). The percentage for dogs, cattle and cats were: 26.5%, 23.5% and 8.8% respectively.

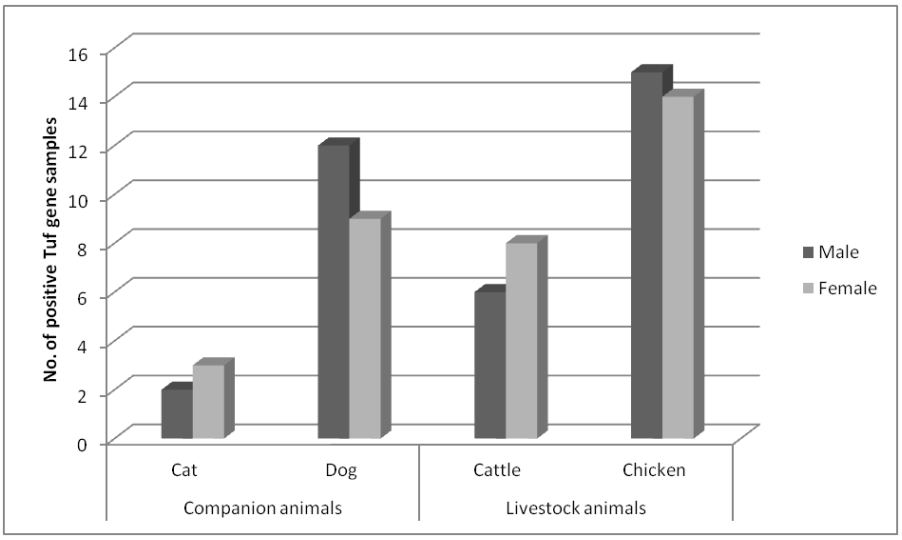

Figure 2: Positive *Tuf* gene among all isolates from male and female of companion and livestock animals

Identification of *E. faecalis, E. faecium* and other *Enterococcus* species

Figure 3 shows isolates from male and female of companion and livestock animals that were positive for *E. faecalis.* Positive male isolates for *E faecalis* were higher in cats, dogs and chickens but were lower in cattle. The highest male positive isolates for *E. faecalis* were observed in chicken (35%), followed by dog (28.6%). Values for cattle and cat are 21.4% and 14.3% respectively. For the female animals, the highest value of occurrence of *E. faecalis* was observed in cattle and chicken both having 33.3% each. This was followed by dogs (25.0%) and finally 8.3% that was observed for cats. Figure 4 shows isolates from male and female companion and livestock animals positive for *E. faecium.* The highest prevalence among males was observed in chicken and dog with 33.3% frequency. Cattle had 22.2% and the least occurrence was observed among cats (11.1%). However, in female animals, chicken had the highest prevalence value of 37.5%. Cattle and dog had the same prevalence value of 25.0% and the least was observed among cats (12.5%). Figure 5 shows isolates from male and female of companion and livestock animals positive for other *Enterococcus spp.* Other *Enterococcus* spp. were only found among cattle, chicken and dog with the highest value of 53.8% observed among chicken. This was followed by dog (38.5%) and 7.7% among cattle. For female animals, the highest value was also observed in chicken (53.8%), followed by dog (30.8%) and cattle (15.4%).

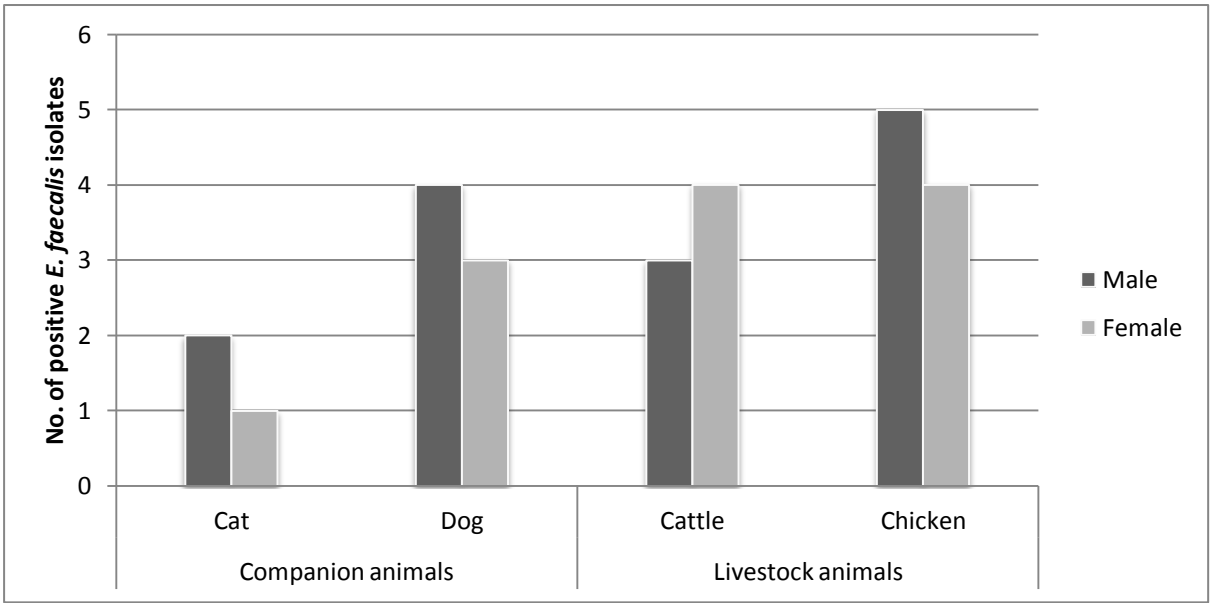

Figure 3: Positive *E. faecalis* isolates from male and female of companion and livestock animals

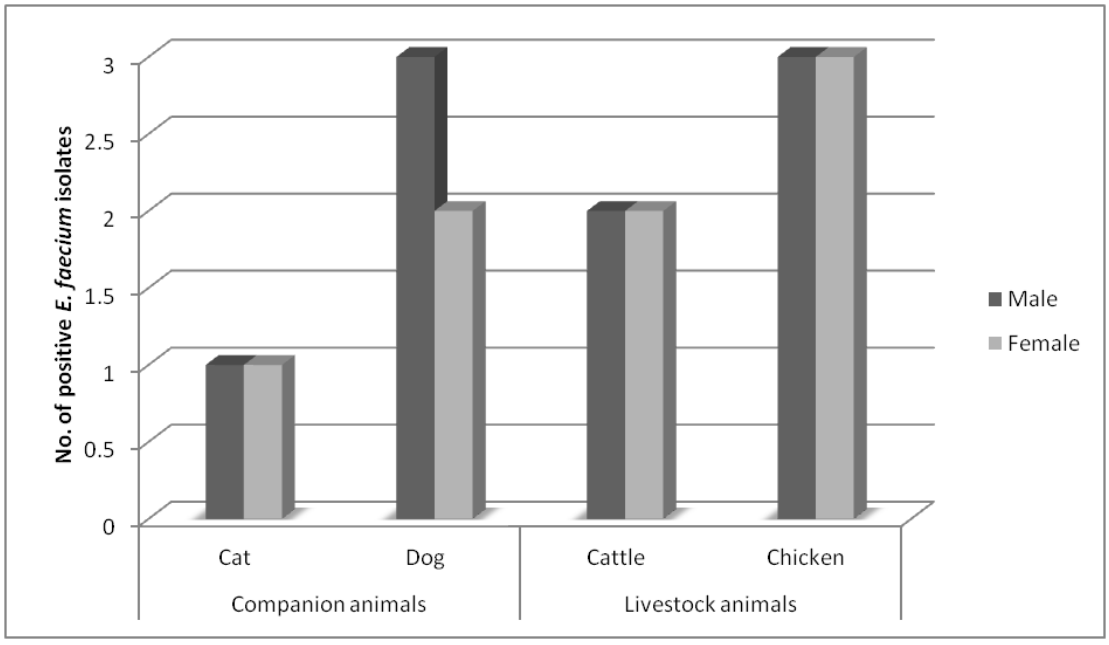

Figure 4: Positive *E. faecium* isolates from male and female of companion and livestock animals

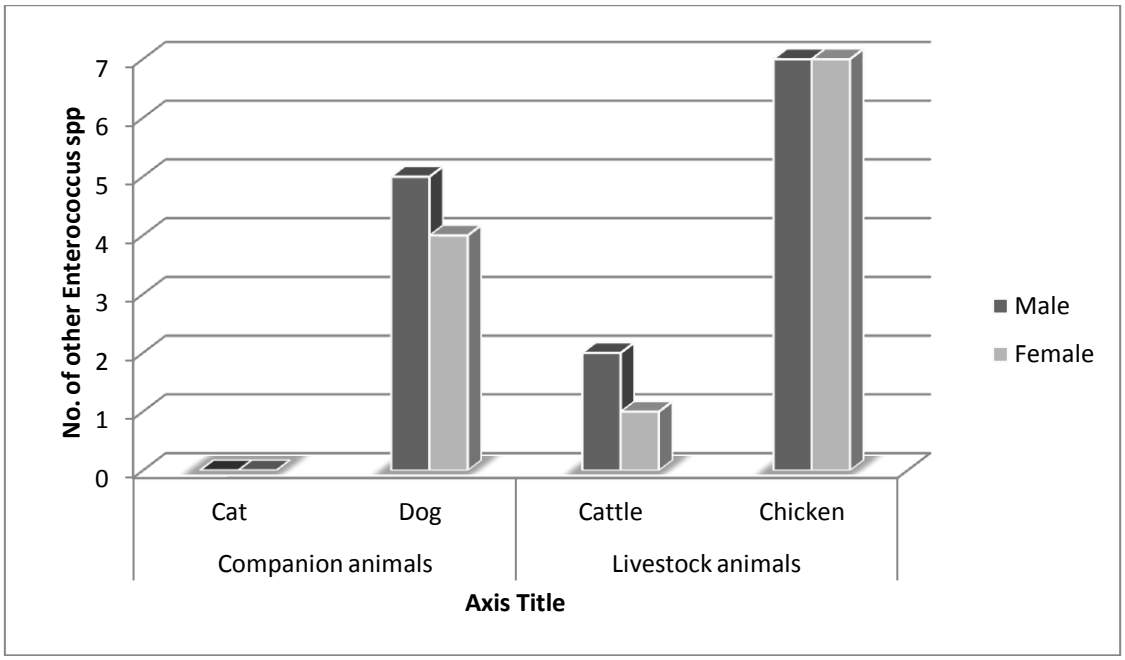

Figure 5: Positive undifferentiated *Enterococcus* spp. isolates from male and female of companion and livestock animals

Detection of virulence genes

Figure 6 shows the prevalence of *gelE* virulence gene in livestock and companion animals used in this study. For males, the highest prevalence was observed among chickens (43.8%). This was followed by cattle (25.0%), dog (18.8%) and cat (12.5%). For female animals, the highest value was also observed in chicken (47.1%), followed by dog (29.4%), cattle (17.6%) while the least was among cats (5.9%).

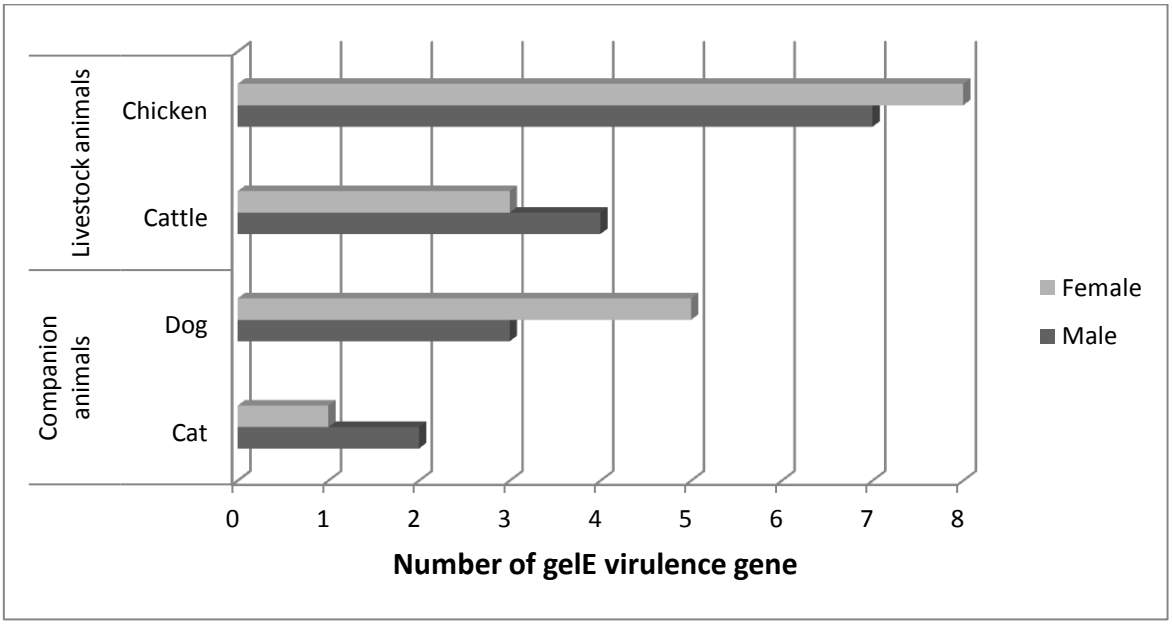

Figure 6: Prevalence of *gelE* virulence gene in livestock and companion animals

Figure 7 shows the prevalence of *ccf* virulence gene in livestock and companion animals used in this study. For males, the highest prevalence was observed among chickens (46.15%). This was followed by dog (30.77%), cattle (15.38%) and cat (7.69%). For female animals, the highest value was also observed in chicken (50.0%). Cattle and dog had the same prevalence value of 25.00%. The *ccf* genes were not detected in isolates from cat.

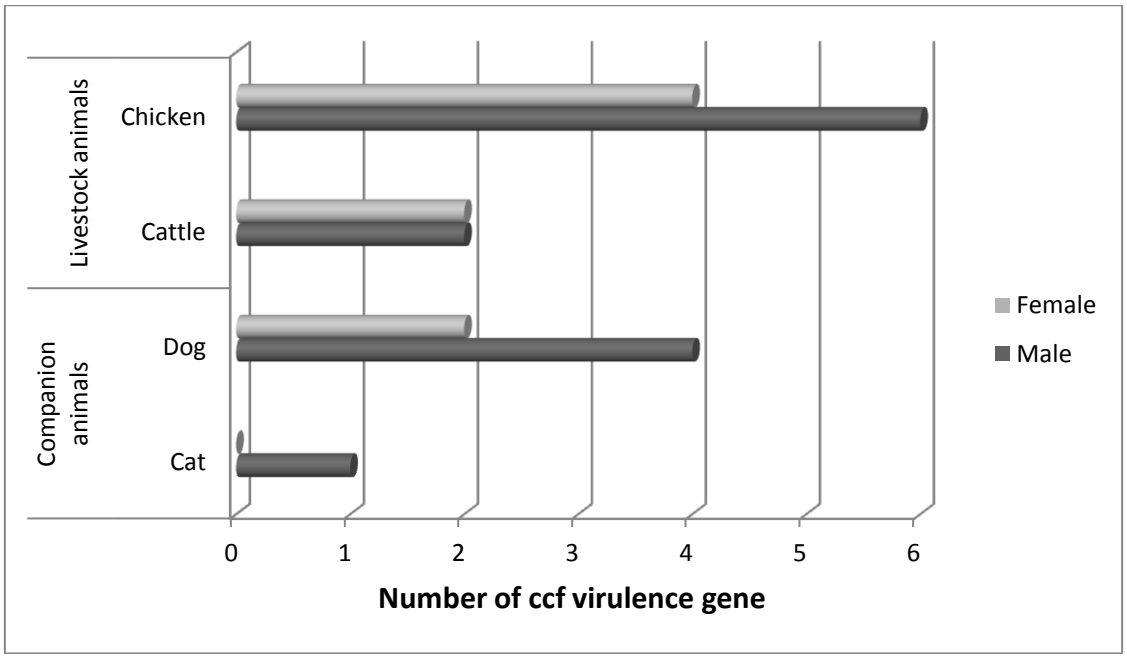

Figure 7: Prevalence of *ccf* virulence gene in livestock and companion animals

Table 3 shows the results of the Chi square test, likelihood ratio and Fischer's exact test for significant effect of *Enterococcus* species and virulence genes across gender in animal species. The chi square test was not significant ($p>0.05$) for *E. faecalis* 0.687, *E. faecium* 0.833, other species 0.096, *gelE* 0.730 and *ccf* 0.893. Similar trend was observed in the results of the likelihood ratio and Fishers exact test. Values for likelihood ratio are 0.887 for *E. faecalis,* 1.000 for *E. faecium* and 0.059 for other species. Also, for the virulence genes, that for *gelE* is 0.727 and *ccf* is 0.886. The Fishers exact test value was 0.805 for *E. faecalis,* 0.833 for *E. faecium,* 0.101 for the undifferentiated species, 0.725 for *gelE* and 0.941 for *ccf.*

Table 3: Chi square test, likelihood ratio and Fischer's exact test for significant effect of enterococcus species and virulence genes across gender in animal species

Statistical test	*E. faecalis*	*E. facium*	Other species	*gelE*	*ccf*
Chi square test	0.687	0.833	0.096	0.730	0.893
Likelihood ratio	0.887	1.000	0.059	0.727	0.886
Fishers exact test	0.805	0.833	0.101	0.725	0.941

Table 4 shows a null model output from binary logistic regression showing association of virulence genes and *Enterococcus* species with gender of animal species. The result was not significant ($p>0.05$) for all the *Enterococcus* species and *gelE*. However, *ccf* was significant ($p<0.05$). The value of B (regression coefficient or the slope of the regression line) which explains the rate of change of one variable as a function of changes in the other was -0.201 with a standard error of 0.498 for *E. faecalis,* -0.118 with a standard error of 0.559 for *E. faecium and* 0.604 with a standard error of 0.312 for the undifferentiated species. For the virulent genes, the value of regression coefficient for *gelE* is -0.087 with a standard error of 0.241 and *ccf* with a regression coefficient value of -0.759 with a standard error of 0.258. The negative values of regression coefficient observed for *E. faecalis, E. faecium, gelE* and *ccf* show the direction of the change. In addition, the exponential of regression coefficient produced which is the odd ratio explains if there is association between the *Enterococcus* species and gender which was tested at 95% confidence interval. Exp (B) value for *E. faecalis was* 0.818 and that for *E. faecium was* 0.889 and the undifferentiated species had the value of 1.829. For the virulence genes, that of *gelE* was 0.917 and for *ccf,* 0.468.

Table 4: Null model output from binary logistic regression showing association of virulence genes and *Enterococcus* species with gender of animal species

Species or gene	B	SE	P	Exp (B)
E. faecalis	-0.201	0.498	0.687	0.818
E. faecium	-0.118	0.559	0.833	0.889
Undifferentiated species	0.604	0.312	0.053	1.829
gelE	-0.087	0.241	0.718	0.917
ccf	-0.759	0.258	0.003	0.468

IV. DISCUSSION

This research confirmed 40.80% (69 of 169) of the examined companion animals and livestock were positive for *Enterococcus* species, representing 53.62% males and 46.38% females. Positive samples were higher in chicken when compared to other animal species. The variation in the prevalence of *Enterococcus* species across these animals could be attributed to the population size. Pillay *et al.* (2018) in their work on the prevalence of virulence genes in *Enterococcus* species isolated from companion animals and livestock in South Africa reported that 86% of their samples were positive for the *tuf* gene used in confirming the presence of *Enterococcus* species. Wada *et al.* (2020) reported a prevalence of *Enterococcus* species of 88.9% in the South-South region of Nigeria. Adesida *et al.* (2017) while working on carriage of multidrug resistant *E. faecium* and *E. faecalis* among apparently healthy humans in Nigeria also reported 73% of the prevalence of *Enterococcus* species. Other authors have however, reported lower prevalence in shrimps (2.78 to 12.50%) and humans (5.90%) in South-South Nigeria (Olawale *et al.* (2011; Igbinosa and Beshiru (2019). The variation observed between males and females in the prevalence of *Enterococcus* species in this work was not consistent with a higher prevalence of 57.40% in females compared to the 46.60% in males reported by Ezeah *et al.* (2020).

A total of 37.68% of positive *Enterococcus* species were *E. faecalis*, with 53.85% of this value found among male animals while 46.15% were observed in female animals. The highest male positive samples for *E. faecalis* were observed in chicken (35%). For the female animals, the highest value of occurrence of *E. faecalis* was observed in cattle and chicken samples. Pillay *et al.* (2018) reported 80% *Enterococcus faecalis* in their study, mostly in chicken cloacal samples. They however, did not detect *Enterococcus faecalis* in cattle samples. Wada *et al.* (2020) also reported that *E. faecalis* was the most reported with a prevalence of 62.98% while Adesida *et al.* (2017) put their figure as 44.6%. Orababa *et al.* (2021) reported 40.7% *Enterococcus faecalis* while Olawale *et al.* (2011) reported 85.7%. However, Oladipo *et al.* (2013) reported lower *E. faecalis* prevalence with a figure of 7.6%. Other values reported are 70% (Billington *et al.* 2014) and 88.7% (Komiyama *et al*., 2016).

Enterococcus faecium varied across sex and species of the animals used in this research with 24.64% of the positive *Enterococcus* species being *E. faecium*. Out of this value, 52.94% were from males while 47.06% were from females. Pillay *et al.* (2018) did not detect *E. faecium* in cattle examined. Wada *et al.* (2020) identified *E. faecium* with a prevalence of 21.70% which is consistent with the findings of this study. Similarly, Billington *et al.* (2014) reported a prevalence value of 25.00%. In addition higher *E. faecium* prevalence of 59.5% was reported by Orababa *et al*., (2021). A similar range of 55.4% prevalence of *E. faecium* was reported by Adesida *et al.* (2017). Other species of *Enterococcus* detected in all examined animals with the exception of cats were 37.68%. Pillay *et al.* (2018) reported higher prevalence of undifferentiated *Enterococcus* spp. However, Billington *et al.* (2014) reported a very low prevalence (5%) of undifferentiated *Enterococcus* species in their work.

The highest prevalence of *gelE* virulence gene was observed among chickens. This is in agreement with the report of Pillay *et al.* (2018) who reported that *Enterococcus* isolated from chicken samples had the highest incidence of virulence genes. They also reported that isolates from dogs had the second highest prevalence of *gelE* gene which is consistent with the results of this study. According to Waters *et al.* (2003), Gelatinase (*GelE*) is a secreted Zn-metalloprotease of *E. faecalis* subsp. *liquefaciens* that has been implicated as one such factor. Kiruthiga *et al.* (2020) reported that *gelE* was significantly predominant in *E. faecalis* (85.39%) than *E. faecium* (60.78%) ($p < 0.0001$). They further reported that 39.3% of enterococci [*E. faecalis* (37.1%), *E. faecium* (43.1%)] were gelatinase producers.

In addition, Vankerckhoven *et al*., (2004) reported that *gelE* was present as a silent gene in *E. faecalis* (77.8%), and *E. faecium* (58.6). Ferguson *et al.* 2016 reported that among the virulence genes they analysed, *gelE* was the most frequently detected and widely distributed among *E. faecalis* strain which is consistent with previous studies. It has been reported that virulence factors (VFs) among strains of enterococci play a vital role in pathogenesis. Cogue *et al.* (1995) stressed that the *gelE* gene has been shown to be present more frequently in clinical isolates than in non-clinical strains. Furthermore, Singh *et al.* (1995**)** reported that a *gelE* mutant *E. faecalis* strain showed reduced virulence in a mouse peritonitis model and in a *Caenorhabditis elegans* model of bacterial virulence **(**Sifri *et al.*, 2002**).**

The variation in the prevalence of *ccf* gene across sex and animal species reported in this study is consistent with the report of other authors (Pillay *et al.,* 2018; Orababa *et al.,* 2021; Stępień-Pyśniak *et al.,* 2019). Pillay *et al.* (2018) reported that chickens had the highest prevalence of *ccf* gene. Stępień-Pyśniak *et al.* (2019) stated that sex pheromone determinants (cpd, cob, *ccf*) were widespread in the analysed *E. faecalis* isolates (100% strains). They further stated that *ccf* gene was also detected in all but one *E. faecium*.

The chi square test was not significant ($p>0.05$) across species and gender for the prevalence of *Enterococcus* species and virulence genes in this study. In the contrary, Pillay *et al.* (2018) reported that the prevalence of *E. faecalis* from source was statistically significant ($p < 0.05$) compared to *E. faecium* which was statistically insignificant ($p > 0.05$). This report was also supported by Orababa *et al.* (2021). The null model

output from binary logistic regression showing association of virulence genes and *Enterococcus* species with gender of animal species was not significant ($p>0.05$) for all the *Enterococcus* species and *gelE*. However, *ccf* was significant ($p<0.05$). Pillay *et al*. (2018) reported that their results from binary logistic regression were statistically insignificant ($p > 0.05$) for all the genes except for *E. faecium* and the gelatinase gene (*gelE*) in animal species. However, Orababa *et al.* (2021) reported significant values in their results.

V. CONCLUSION

The presence of *Enterococcus* spp. was confirmed among the livestock and companion animals' species sampled in this study. Variations in the degree of prevalence across species and sex were observed but not significant ($p<0.05$) except for the prevalence of *ccf* which was significant ($p<0.05$). The influence of sex of animal species on prevalence of *Enterococcus* was also observed. Chicken had the highest infection of *Enterococcus* species and the virulence genes considered in this study while male animals recorded slightly higher infection of *Enterococcus* species and the virulence genes. The results of this study call for the need to intensify the control infectious diseases especially, *Enterococcus* in Nigerian in order to reduce the prevalence, mortality, morbidity, and cost of care associated with infectious diseases.

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