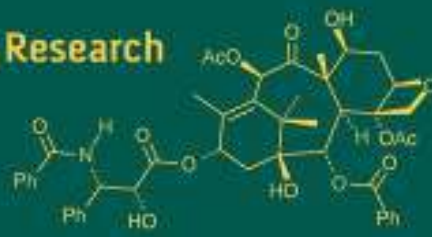
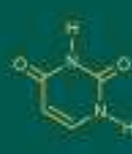


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Biofilm-associated virulence in *Enterococcus* isolates from poultry: Comparative gene distribution and statistical gene-phenotype associations

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Abstract

Enterococcus species have emerged as notable opportunistic microorganisms within the food chain due to their remarkable environmental persistence, physiological adaptability, ability to generate biofilms, and carriage of specific virulence-associated genes. This study focused on the detection of five major virulence genes *gelE*, *asa1*, *esp*, *cylA*, and *hyl* and evaluation of biofilm-forming ability among *Enterococcus* isolates derived solely from retail poultry meat samples. A total of 150 poultry meat samples were collected from retail outlets, of which 84 showed presumptive positivity, and 80 were molecularly confirmed as *Enterococcus*. Biofilm formation was assessed using Congo Red Agar (CRA), while virulence gene identification was confirmed through PCR. The prevalence of virulence genes among isolates was highest for *gelE* (88.75%), followed by *asa1* (40.47%), *esp* (15%), and *cylA* (11.25%), with no *hyl* gene observed. Biofilm formation was detected in 90% of isolates, yet statistical evaluation using Chi-square and Fisher's exact tests revealed a significant association only with *cylA* ($p = 0.042$), contrary to general expectations about *gelE* and *asa1*, which although frequent among strong biofilm producers, did not show statistical significance ($p > 0.05$). These findings highlight the persistence potential of *Enterococcus* in poultry processing environments and reinforce the importance of improved sanitation strategies to prevent biofilm-mediated contamination. The study concludes by emphasizing the need for advanced molecular tracking, quantitative biofilm assessment, and expanded sampling in future investigations.

Keywords: *Enterococcus* spp., biofilm formation, virulence genes, statistical association, poultry meat contamination

1. Introduction

Enterococci are Gram-positive, facultatively anaerobic microorganisms that commonly inhabit the intestinal tract of poultry and other animals. During poultry slaughter and processing, contamination of meat products may occur, frequently facilitated by exposure to intestinal microflora. Owing to their ability to survive adverse environmental conditions, such as extreme temperatures, high salinity, and desiccation, these organisms are capable of thriving during processing, storage, and even under conditions of inadequate sanitation. Their biofilm-forming capability provides additional survival advantages, particularly on food-contact surfaces such as stainless steel and plastic used in processing lines. These biofilms can protect bacterial cells against commonly applied sanitizers and stressors, facilitating persistence and potential cross-contamination (Chavez, 2007; Son *et al.*, 2024) [2, 17].

Several virulence genes have been implicated in the pathogenicity and survival of *Enterococcus* within food systems. Among these *gelE*, encoding gelatinase, has been widely reported; *asa1* contributes to aggregation during colonization; *esp* encodes enterococcal surface protein, which enhances adherence and biofilm development; while *cylA* is associated with cytolysin production, which may support colonization under stress. However, the expression and regulatory impact of these genes on biofilm development remain poorly understood. Previous studies often described descriptive associations between virulence markers and biofilm phenotype but lacked statistical support, reducing biological inference reliability.

Therefore, this study aimed to investigate not only the presence of virulence-associated genes but also their correlation with biofilm formation through statistical assessment.

2. Materials and Methods

2.1 Sampling

A total of 150 poultry meat samples were collected aseptically from retail outlets over six months. Samples were transported under refrigeration conditions and processed within four hours to minimize contamination bias.

2.2 Isolation and Biochemical Identification

Isolation of enterococci was performed using selective media including Slanetz-Bartley agar and Citrate Azide Tween Carbonate agar. Colonies exhibiting characteristic

morphology were subjected to biochemical tests such as PYR and catalase reaction for further confirmation.

2.3 Molecular Confirmation and PCR Analysis

Genomic DNA from *Enterococcus* isolates was extracted using the snap-chill method. PCR amplification of the *tuf* gene (genus-level) followed published protocols by Dutka-Malen *et al.* (1995) [4]. Primer sequences and specifications are listed in Table 1.

PCR reactions (25 µl) contained 12.5 µl 2X master mix, 1 µl of each primer (10 pmol), 5 µl DNA template, and nuclease-free water. Cycling conditions for *tuf* included: 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 50 °C for 90 s, 72 °C for 60 s; final extension at 72 °C for 10 min. PCR products were resolved on 2% agarose gels and visualized using a gel documentation system.

Table 1: PCR primer sequences used for molecular confirmation

Sr. No.	Target gene	Primer sequence (5' — 3')	Product Size (base pairs)	Reference
1.	<i>Tuf</i>	Ent1-TACTGACAAACCATTTCATGATG Ent2-AACTTCGTCAACCAACGCGAAC	112	Ke <i>et al.</i> (1999) [7]

2.4 Optimization of PCR for Virulence Associated Genes of *Enterococcus* species isolates

The isolates of *Enterococcus* spp. were screened for the detection of major virulence-associated genes by targeting *asa1*, *gelE*, *cylA*, *esp* and *hyl* genes (Table 2). The DNA from isolates was extracted by boiling method. A loopful of pure culture was suspended in 100 µl of DNase and RNase free Milli-Q water in a sterilized eppendorf tube. The suspension was vortexed and then heated at 95°C for 10 min in the thermal cycler. This was then centrifuged at 10000 rpm for 6 min in order to settle down the cell debris. The

upper aqueous phase was transferred to another eppendorf tube and this was used as a DNA template for PCR. The reaction mixture was prepared in 200 µl PCR tubes which contained 12.5µl PCR master mix (2X), 1 µl each of forward and reverse primer (10pmol), 5.5 µl nuclease free water and 5 µl templates. The details of thermal profiling of PCR are mentioned in Table 3described by Pangtey (2017) [12] with slightly modifications. The final amplified product was analysed by agarose gel electrophoresis on 2% agarose gel and visualized under gel documentation system.

Table 2: Description of primer used for detection of virulence associated genes of *Enterococcus* spp. (Vankerckhoven *et al.*, 2004) [19]

Sr. No.	Target- Gene	Primer sequence (5' — 3')	Product Size (base pairs)	Reference
1.	<i>asa1</i>	F : GCACGCTATTACGAACATATGA R: TAAGAAAGAATCATCACACGA	375 bp	Vankerckhoven <i>et al.</i> (2004) [19]
2	<i>gelE</i>	F :TATGACAATGCTTTTGGGAT R:AGATGCACCCGAAATAATATA	213 bp	
3	<i>cylA</i>	F: ACTCGGGGATTGATAGGC R:GCTGCTAAAGCTGCGCTT	688 bp	
4	<i>Esp</i>	F: AGATTTCATCTTTGATTCTTGG R: AATTGATTCTTTAGCATCTGG	510 bp	
5	<i>Hyl</i>	F: ACAGAAGAGCTGCAGGAAATG R:GACTGACGTCCAAGTTTCCAA	276bp	

Table 3: PCR condition for detection of virulence associated genes

Cycling Conditions		Temperature	Time
Initial Denaturation		94°C	2 min
35 cycles	Denaturation	94°C	1 min
	Annealing	55.5 °C	2 min
	Extension	72°C	1 min
	Final Extension	72°C	10 min

2.5 Biofilm Detection

Biofilm formation was assessed using the Congo Red Agar method. Colonies were visually examined and categorized as strong biofilm producers if black and dry in appearance, moderate producers when exhibiting slightly black mucoid colonies, and weak or non-producers upon presentation of red or smooth colony morphology.

2.6 Statistical Analysis

Statistical analysis was performed using Chi-square test, and Fisher's exact test when cell frequencies were fewer than

five, to determine whether virulence gene presence significantly influenced biofilm formation. A threshold of $p < 0.05$ was used to measure statistical significance.

3. Results

3.1 Prevalence of *Enterococcus*

Using cultural methods for primary isolation, the overall prevalence of *Enterococcus* spp. in poultry meat samples was found to be 56% (84/150) (Fig. 1 and 2). Comparable findings were reported by Banik *et al.* (2018) [1], who documented a prevalence of 62.5%. In contrast, a

considerably higher prevalence of 91.60% was observed by Naas *et al.* (2021) ^[10], likely attributable to inadequate hygienic practices during meat processing (Laban *et al.*, 2021) ^[8]. Conversely, a lower prevalence of 39.21% (40/102) reported by Talebi *et al.* (2015) ^[18] may reflect the implementation of improved hygienic standards during slaughter and processing.

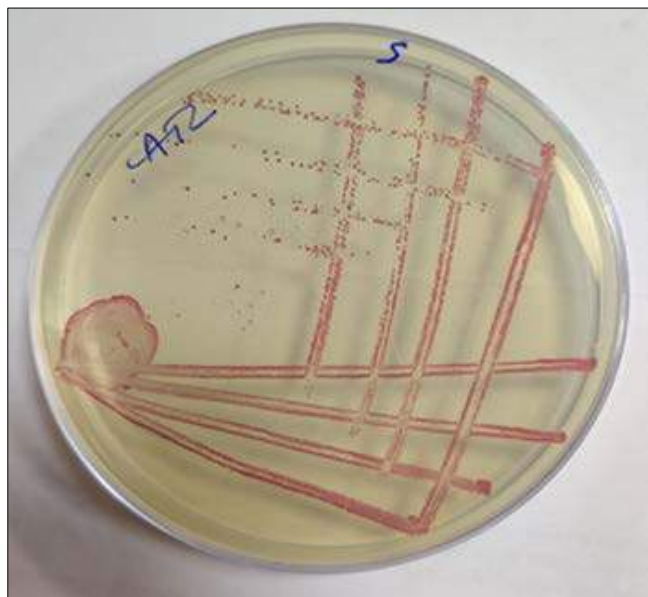


Fig 2: Red colonies of *Enterococcus* on Citrate Azide Tween Carbonate medium (SB) Medium

Of the 84 culturally confirmed *Enterococcus* isolates, 80 (95.23%) were further verified as *Enterococcus* spp. by PCR (Fig. 3). The prevalence observed in the present study is comparable to the findings of Manson *et al.* (2019) ^[9], who reported a 98.23% detection rate of *Enterococcus* spp. in chicken meat. In contrast, moderate prevalence levels such as 49% have been reported by Rozanska *et al.* (2015) ^[13], while Onaran *et al.* (2019) ^[14] recorded a lower prevalence of 30%, likely attributable to improved hygienic practices during meat processing.

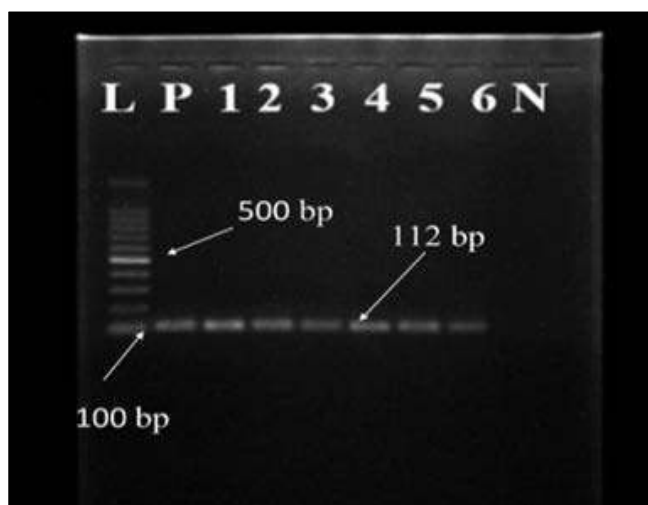


Fig 3: Agarose gel showing amplification product of *tuf* gene (112 bp)

3.2 Virulence Gene Distribution

Among the confirmed isolates, detection of the *gelE* gene was highest (71/80; 88.75%), followed by *asa1* 34/80

(40.47%), *esp12/80* (15%), and *cylA* 9/80 (11.25%), while the *hyl* gene was not detected in any isolate.

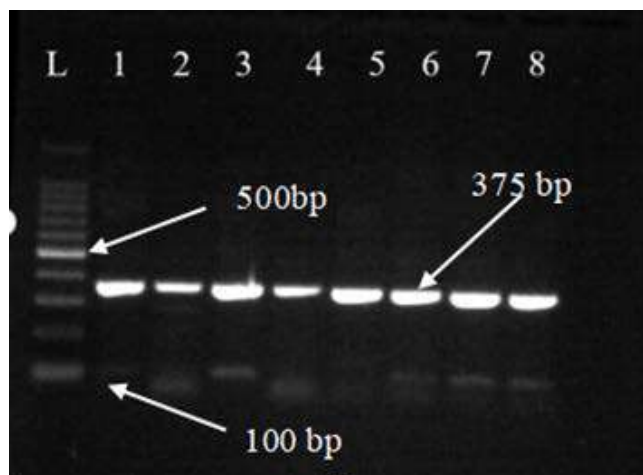


Fig 4: Agarose gel showing amplification product of *asa* gene (375 bp)

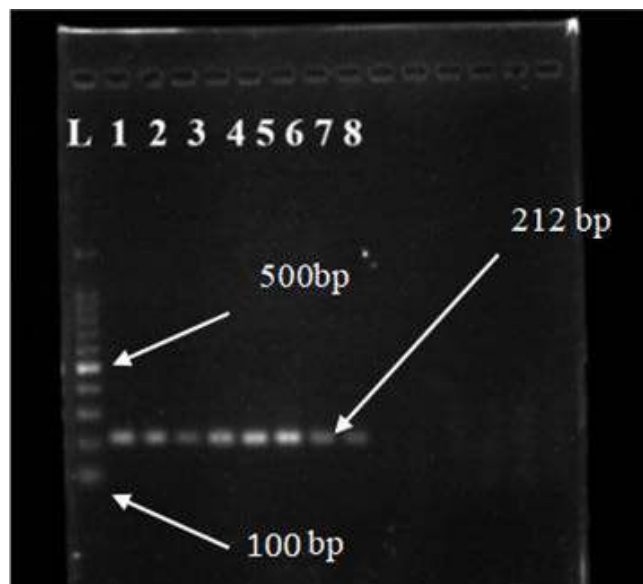


Fig 5: Agarose gel showing amplification product of *gelE* gene (212 bp)

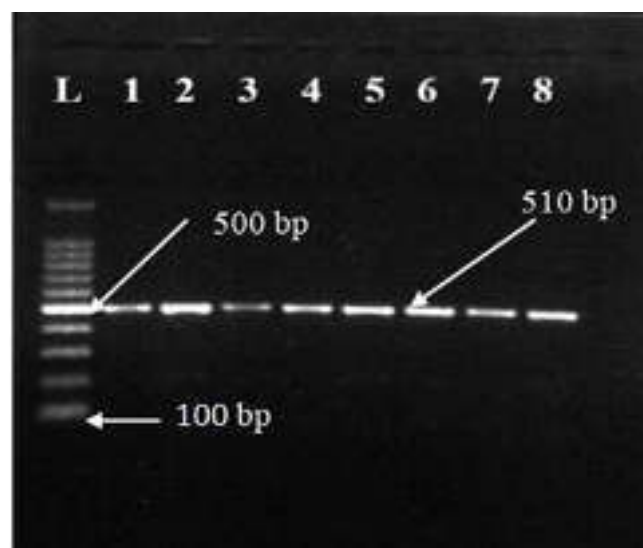


Fig 6: Agarose gel showing amplification product of *esp* gene (510 bp)



Fig 7: Agarose gel showing amplification product of *cyl* gene (688 bp)

Table 4: Frequency distribution of virulence genes detected among *Enterococcus* isolates (n = 80)

Virulence gene	Positive isolates (n)	Percentage (%)	Negative isolates (n)	Percentage (%)
<i>gelE</i>	71	88.75%	9	11.25%
<i>asa1</i>	34	40.47%	50	59.53%
<i>Esp</i>	12	15.00%	68	85.00%
<i>cylA</i>	9	11.25%	71	88.75%
<i>Hyl</i>	0	0.00%	80	100.00%

3.3 Biofilm Formation

Biofilm testing revealed that out of 80 isolates, 72 (90%)

demonstrated biofilm formation, with a majority exhibiting strong production.

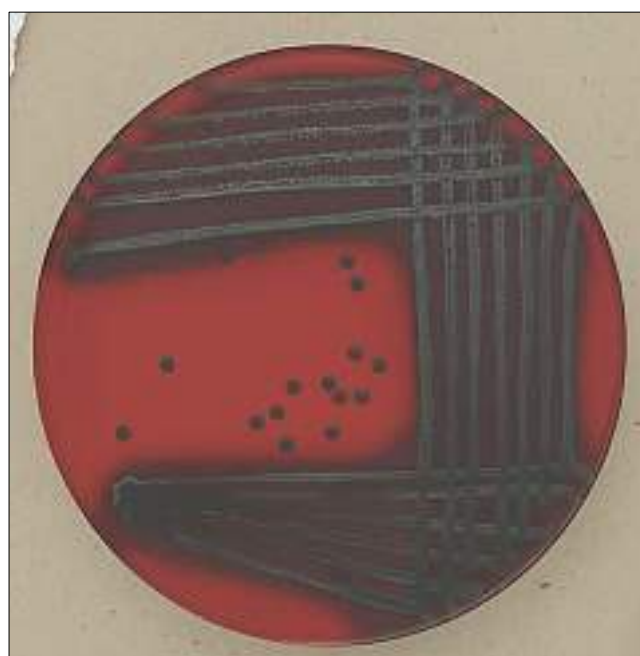


Fig 8: Biofilm formation on CRA

Correlation between virulence gene prevalence and biofilm phenotype was analyzed through contingency analysis. Results showed that *gelE*, *asa1*, and *esp*, despite their presence among biofilm-positive isolates, did not exhibit statistically significant correlation, while *cylA* was

significantly associated with biofilm development ($p = 0.042$). The contingency values used to calculate these results were presented in Table 4. The actual statistical metrics, including Chi-square and Fisher's exact test values, were reported in table 5,

Table 5: Contingency table of virulence gene presence versus biofilm phenotype among *Enterococcus* isolates (n = 80)

Gene	Gene+ Biofilm+	Gene+ Biofilm-	Gene- Biofilm+	Gene- Biofilm-	Total isolates
gelE	64	7	8	1	80
asa1	30	4	42	4	80
esp	9	3	63	5	80
cylA	6	3	66	5	80
hyl	0	-	-	-	0

Table 6: Statistical correlation results of virulence genes and biofilm formation

Virulence gene	χ^2 value	DF	p-value (Chi-square)	Fisher's exact test p-value	Interpretation
gelE	2.714	1	0.099	0.124	Not significant
asa1	0.380	1	0.538	0.626	Not significant
Esp	3.686	1	0.055	0.068	Borderline
cylA	4.140	1	0.042*	0.048*	Significant
Hyl	-	-	-	-	Not applicable

Interpretation Key: $p < 0.05$ considered statistically significant indicates statistically significant association, *esp* shows borderline correlation but does not reach significance threshold

Note: Analysis performed using Chi-square test; Fisher's exact test was used where expected cell counts were < 5 . Only *cylA* demonstrated significant association with biofilm formation ($p < 0.05$).

4. Discussion

The present study identified a high prevalence of biofilm formation among *Enterococcus* isolates derived from poultry meat, indicating a strong environmental persistence capability in food system structures. While earlier literature commonly suggested an association of *gelE* and *asa1* with biofilm production, the lack of statistical support in this research emphasizes the need to reassess previous assumptions. Although *gelE* was highly prevalent, it did not correlate significantly with biofilm presence. The only gene demonstrating a statistically supported association with biofilm phenotype was *cylA*, indicating that cytolysin-associated mechanisms may play a more direct role in biofilm facilitation within food-borne *Enterococcus* populations.

Comparison with literature demonstrates alignment with findings of Son *et al.* (2024) [17], who reported similarly high biofilm prevalence in poultry-derived isolates. However, the absence of significant association between *gelE* and biofilm contrasts with Yang *et al.* (2023) [20], suggesting geographic, strain-level, or method-related discrepancies. It is possible that gene expression and regulatory controls differ between isolates, and PCR-based detection does not capture transcription or activity level, limiting interpretation.

These findings hold important implications for food processing safety, as biofilm-forming enterococci may withstand cleaning procedures and consequently contribute to cross-contamination. Given that poultry meat remains one of the most widely consumed protein sources, the persistence of biofilm-producing strains represents a potentially underestimated risk. The reliance on qualitative biofilm detection methods such as CRA also presents limitations, and future research should incorporate quantitative assessment techniques such as micro-titer plate assays coupled with absorbance analysis.

5. Conclusion

This study demonstrated that enterococci isolated from poultry meat predominantly possess virulence traits and

exhibit strong biofilm-forming ability; however, only the presence of the *cylA* gene was statistically associated with biofilm formation. The high prevalence of *gelE* and *asa1* did not translate into significant biofilm predictive reliability. These findings highlight the need for more advanced molecular diagnostics and surface decontamination strategies in poultry processing units. Quantitative biofilm evaluation and genome-level analysis are recommended for future studies to improve mechanistic understanding and accurately characterize risk potential.

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