Research Article

LGU Journal of Life Sciences

ISSN (P): 2519-9404
ISSN (E): 2521-0130

Isolating virulent and antibiotic-resistant strains of *Enterococcus* sp. from food and veterinary clinic samples from different regions of Punjab, Pakistan

*Umaima Rubbani, *Sadia Tahir, Iqra Arooj, Aamina Sajid*

Department of Microbiology and Molecular Genetics, Faculty of Life Sciences, The Women University Multan, Multan, Pakistan

***Correspondence:**

sadiatahir_pu@hotmail.com

DOI: 10.54692/lgujls.2026.10102

Received: 1st March, 2025

Revised: 14th June, 2026

Accepted: 15th June, 2026

Citation: Rubbani, U, Tahir, S, Arooj, I, Sajid A. (2026). Isolating virulent and antibiotic-resistant strains of Enterococcus sp. from food and veterinary clinic samples from different regions of Punjab, Pakistan. LGU Journal of Life Sciences. 10 (01): 5-17.

Keywords: *Enterococci,* veterinary clinics, animal-related food, virulence traits, antimicrobial resistance

Abstract

Enterococci play a vital role in the spread of nosocomial infections and can also transfer virulence, along with genes encoding antimicrobial resistance, to other bacteria. To juxtapose isolates' virulence traits, samples from veterinary clinics and animal-related food, as well as from cages of pet animals and birds, were randomly collected from Punjab, Pakistan, brought to the workplace, and kept at 4°C until further processing. Bile esculin agar was employed to select *Enterococci*, which were then verified by their biochemical and morphological traits. The motility test, biofilm formation, cytolysin activity, antibacterial analysis, and gelatinase formation test were among the virulence characteristics of the isolated *Enterococcus* strains that were further examined. 73% of the isolated strains were found to be motile, 24.4% exhibited β-hemolysis, and 75.6% exhibited γ-hemolysis. Furthermore, 25.6% of the isolated strains produced moderate amounts of biofilm, whereas 37.8% of the strains produced strong amounts. However, when tested against various antibiotics included in our study, 28% (23 of 82) of the isolates were MDRs, and 56% of the strains tested positive for gelatin production. Given that *Enterococcus* is thought to be the third most important cause of nosocomial infections, this conclusion can be drawn. These bacteria have the potential to cause infection and potentially serious health problems, as evidenced by the fact that some of the isolated strains in this study displayed virulence traits and were resistant to different antibiotics. As a result, it is recommended that livestock environments and veterinary clinics must be continuously observed.

Introduction

Enterococci are widely spread gram-positive as well as facultative anaerobic bacteria that live in the intestines of humans as well as other mammals (Boeder *et al*., 2024). The genus Enterococcus is linked to the extent to prosper and sustain in a variety of settings, and it is found to be the resident bacterium of the GIT of both humans and animals (Aung *et al*., 2023). These species are predominantly characterized by their capability to withstand high concentrations of salt, such as 6.5% of NaCl concentration, along with an extensive range of temperatures and pH values (Geraldes *et al*., 2022). Two subtypes and 55 species have been distinguished up till now by using 16S rRNA. These species aid in the development of probiotic or initiator bacterial suspensions during food processing (Elnar and Kim, 2026). Moreover, some Enterococci—mainly *Enterococcus faecium* and *Enterococcus faecalis*—form obstructive elements that could stop foodborne pathogens from spreading and microorganisms from putrefying. Considering their lack of general acceptance as safe, their presence is typically indicative of fecal matter rather than their significance in food manufacturing. Nonetheless, there is no evidence linking hospital-acquired infections to enterococci found in food (Dong *et al*., 2024). There are currently about fifty species in the genus

 LGUJLS is Licensed by CC BY 4.0

Enterococcus, of which *E. faecium* as well as *E. faecalis* are two of the most frequently species being grown and that represents more than 80% of all enterococci isolates. Despite reports that two of the top four causes of nosocomial infections worldwide are Enterococcus faecium as well as *Enterococcus faecalis* (Al Bshabshe *et al.,* 2024). Furthermore, in contrast to *E. faecalis* along-with *E. faecium*, other Enterococcal species, comprising of *E. hirae, E. avium, E. durans, etc* are considered to be more opportunistic (Fahim *et al*., 2025).

Enterococcal species carry various virulent genes, comprising of *gelE*, *asa1, esp, cylA, efaA, hly*, and *ace* (Wathij and Kadhim, 2025). They are renowned for their capacity to transfer antibiotic resistance to other bacteria and disseminate genetic information via mobile genetic elements (Nasiri and Hanifian, 2022). One of the most prevalent nosocomial pathogens is enterococci, which can be transmitted from one person to the other via food or the environment causing endocarditis, bacteremia, infections of the soft tissues or wounds, and, most commonly, urinary tract infections (Massella *et al*., 2025). One virulence factor known to contribute to antibiotic resistance in Enterococcus species is their tendency to form biofilms. Antimicrobial concentrations 10–1000 times more than those required to kill planktonic bacteria can persist in a mature biofilm (Ghazvinian *et al*., 2024). The genes *gelE, esp* and *asa1* are involved in the formation of biofilms (Șchiopu *et al.,* 2023). The bioactive substances including hemoglobin, collagen, gelatin, casein, as well as others can be broken down by the enzyme gelatinase. Moreover, hemolysis in human red blood cells can be induced by cytolysin, which is encoded by *cylA* (Kim et al., 2022). Antimicrobial resistance (AMR) is among the major dangers to global healthcare systems. According to a United Nations International Committee report, MDR-affected infections are predicted to kill 10 million people by 2050—more than all cancer-related deaths (Alara and Alara, 2024). Enterococci infections have become more concerning in recent years because of their propensity to become resistant to an advance range of antibiotics (Bocella *et al*., 2021). Our research sought to ascertain the prevalence of virulent genes and enterococcal strains resistant to antibiotics in isolates obtained from veterinary practices and animal-related foods. Our study highlights the presence of specific virulent characteristics in isolates obtained from food and veterinary clinics.

Material and methods

Isolation and Identification

Eighty-six food samples including meat, milk as well as yogurt and samples from five different veterinary clinics including cage door, stethoscope, mouth gauge, thermometer *etc* were randomly collected and enriched in PBS which were then placed in shaking incubator at approximately 37ºC for about a day.

By spreading on a bile esculin agar plate, isolates were obtained. Samples were diluted, and was spread across the culture plate and were kept at 37ºC for 24 hours (Paschoalini *et al*., 2023). For veterinary clinic sample, approximately 10µl of the PBS were spread on media plates (KuKanich *et al.,* 2012). *Enterococcus* strains that hydrolyze aesculin to aesculetin were selected for additional purification while to detect uncontaminated colonies, other methods including catalase production test, 6.5% NaCl resistance test, and Gram staining were performed (Al-Azawi, 2023)**. S**elected strains were further checked on MSA (Cappucino and Sherman, 2014), MacConkey agar as well as CLED agar (Cheesbrough, 2000).

Virulence profiling

Motility test

Motility of the enterococcal isolates were scrutinized using semi solid medium for motility test. To achieve that, a straight needle was used to very slightly penetrate the center of the medium with isolate, avoiding damage to the surrounding medium or media tubes. The tubes were then incubated for 24 hours at 37ºC. Following that, bacterium's growth was noticed whether in its unaltered state or close to the medium (Ngbede *et al.,* 2017).

Biofilm Formation Assay

Qualitative biofilm formation test was performed by using cultured tubes filled with tryptone soy broth (TSB) which were inoculated with bacterial strains and were kept at 37°C for approximately 2 days. After that, broth was decanted from the tubes followed by gently washing of the tubes to avoid damage of biofilm formation. Test tubes were later on dried with hot air until fixation of biofilm followed by staining with crystal violet stain and water and ethanol to remove excess amount of stain which were dried naturally later on (Khalil *et al.,* 2022).

Hemolysin production

This test was performed by autoclaving BHI agar and later supplementing the agar with 5% blood when cooled to approximately 7°C followed by distributing equally in media dishes. Later, the isolates were streaked on the media dishes containing agar with blood and left for 24 hours at 37°C in the incubator. Hemolytic action of the streaked organisms was later classified as α, β and γ hemolysis (Gulhan *et al.,* 2015).

Gelatinase Production Assay

In the assay for producing gelatinase, 1 microliter of an overnight grown culture of Enterococcus was appended to BHI agar along with 3 grams per 100 milliliters of gelatin. After being maintained at 4°C for four hours, the cultures underwent incubation at 37°C for two to three days. An opaque halo that developed

around the colonies appeared to be a good result (Abdelrahman *et al*., 2024).

Antibiotic Susceptibility test

Kirby-Baur method was done to check the antimicrobial susceptibility analysis which includes autoclaving of MH agar and broth followed by addition of test strain in the broth and incubating them for 1 day and later the OD of the test strain was set to 0.5 standard at 625nm. Antibiotics that were chosen to check the antibiotic susceptibility testing were erythromycin, vancomycin, cefotaxime and ampicillin. Sterilized cotton swabs were used to prepare the lawn of the test organisms on the surface of agar followed by addition of antibiotic discs on the plates which were further incubated for 24 hours at 37°C (Arumugam *et al.,* 2017).

Results

Isolation and Identification

Eighty-two samples were isolated from the total samples being collected including food samples and veterinary clinic samples which were selected dependent on the ability to grow on bile aesculin agar.

Strains were further characterized on the basis of the cell morphology, gram's reaction, capacity to develop in the presence of high salt concentration (6.5%), catalase test and ability to grow on CLED agar, MSA and MacConkey agar (Table 1 and Table 2).

Table 1: Isolation and characterization of *Enterococci* strains from veterinary clinics and cages

Isolates from veterinary clinics and cages	Gram's reaction	Biochemical tests					Salt tolerance test
		Cell Morphology	Catalase test	Mannitol utilization test	CLED agar	Lactose Utilization test	
$H_1M_{(1)}$	+ve	Cocci	-	-	+	+	+
$H_1M_{(2)}$	+ve	Cocci	-	-	+	+	+
$H_1T_{(1)}$	+ve	Cocci	-	-	+	+	+
$H_1T_{(2)}$	+ve	Cocci	-	+	+	+	+
H_1C_1	+ve	Cocci	-	+	+	+	+
H_1C_2	+ve	Cocci	-	+	+	+	+
H_4S	+ve	Cocci	-	-	+	+	+
H_4O	+ve	Cocci	-	-	+	+	+
H_4T	+ve	Cocci	-	-	+	+	+
H_4C	+ve	Cocci	-	-	+	+	+
H_4E	+ve	Cocci	-	+	+	+	+
$H_4M_{(1)}$	+ve	Cocci	-	+	+	+	+
$H_4M_{(2)}$	+ve	Cocci	-	-	+	+	+
H_3A	+ve	Cocci	-	-	+	+	+
H_3M	+ve	Cocci	-	-	+	+	+
H_3TC	+ve	Cocci	-	-	+	+	+
H_3T	+ve	Cocci	-	-	+	+	+
H_3O	+ve	Cocci	-	-	+	+	+
C_2	+ve	Cocci	-	-	+	+	+
C_4	+ve	Cocci	-	-	-	-	+
C_7	+ve	Cocci	-	+	-	+	+
C_8	+ve	Cocci	-	-	-	+	+
C_9	+ve	Cocci	-	-	-	+	+
C_{10}	+ve	Cocci	-	-	+	-	+
C_{11}	+ve	Cocci	-	-	+	+	+
H_2E	+ve	Cocci	-	-	+	+	+
H_5M	+ve	Cocci	-	-	+	+	+
H_5S	+ve	Cocci	-	+	+	+	+
$H_5T_{(1)}$	+ve	Cocci	-	-	+	+	+
$H_5T_{(2)}$	+ve	Cocci	-	-	+	+	-

Table 2: Isolation and characterization of *Enterococci* strains from animal related food

Isolates from food samples	Gram's reaction	Biochemical tests					Salt tolerance test
		Cell Morphology	Catalase test	Mannitol utilization test	CLED agar	Lactose Utilization test	
S_1M_{27}	+ve	Cocci	-	-	+	+	+
S_1M_{28}	+ve	Cocci	-	-	+	+	+
S_1M_{30}	+ve	Cocci	-	+	-	-	-
S_2Y_{13}	+ve	Cocci	-	-	+	+	+
S_4C_1	+ve	Cocci	-	-	+	+	+
S_4C_2	+ve	Cocci	-	-	+	+	+
$S_4C_{3(1)}$	+ve	Cocci	-	-	+	+	+
$S_4C_{3(2)}$	+ve	Cocci	-	-	+	+	+
$S_4C_{4(1)}$	+ve	Cocci	-	-	-	+	-
$S_4C_{4(2)}$	+ve	Cocci	-	-	-	+	+
$S_4C_{5(1)}$	+ve	Cocci	-	-	-	+	+
$S_4C_{5(2)}$	+ve	Cocci	-	-	-	+	+
S_4C_6	+ve	Cocci	-	-	+	+	+
$S_4C_{7(1)}$	+ve	Cocci	-	-	-	+	+
$S_4C_{7(2)}$	+ve	Cocci	-	-	-	+	+
$S_4C_{8(1)}$	+ve	Cocci	-	-	-	+	+
$S_4C_{8(2)}$	+ve	Cocci	-	-	-	+	+
S_4C_9	+ve	Cocci	-	-	+	+	+
S_4C_{10}	+ve	Cocci	-	-	+	+	+
S_3BM_3	+ve	Cocci	-	-	+	+	+
S_4C_{11}	+ve	Cocci	-	-	+	+	+
S_4C_{12}	+ve	Cocci	-	-	+	+	+
S_4C_{13}	+ve	Cocci	-	+	-	+	+
$S_4C_{14(1)}$	+ve	Cocci	-	-	-	+	+
$S_4C_{14(2)}$	+ve	Cocci	-	-	-	+	+
$S_4C_{15(1)}$	+ve	Cocci	-	-	+	+	+
$S_4C_{15(2)}$	+ve	Cocci	-	-	+	+	+
S_4C_{16}	+ve	Cocci	-	-	+	+	+
$S_4GM_{17(1)}$	+ve	Cocci	-	+	-	-	+
$S_4GM_{17(2)}$	+ve	Cocci	-	-	+	+	-
S_4C_{18}	+ve	Cocci	-	-	+	+	+
S_1M_{25}	+ve	Cocci	-	-	-	+	+
BM1	+ve	Cocci	-	+	+	+	+
BM2	+ve	Cocci	-	+	+	-	+
BM3	+ve	Cocci	-	-	+	+	+
BM4	+ve	Cocci	-	+	+	-	+
BM5	+ve	Cocci	-	+	+	+	+
BM6	+ve	Cocci	-	+	+	+	+
M1	+ve	Cocci	-	-	+	+	+
M2	+ve	Cocci	-	+	+	-	+
M2.1	+ve	Cocci	-	+	+	-	+
M3	+ve	Cocci	-	+	+	+	+
Y1	+ve	Cocci	-	+	+	+	+
Y3	+ve	Cocci	-	-	+	-	+
Y4	+ve	Cocci	-	+	+	-	+
CH2	+ve	Cocci	-	+	+	+	+
CH3	+ve	Cocci	-	-	+	+	+
B1	+ve	Cocci	-	+	+	-	+
B2	+ve	Cocci	-	+	+	+	+

B3	+ve	Cocci	-	-	+	+	+
CH1	+ve	Cocci	-	+	+	-	+
Y4.1	+ve	Cocci	-	-	+	+	+

Assays for virulence traits

The results of the hemolysis assay, biofilm formation assay, and motility test were accustomed to determine the virulence of the isolated strains from veterinary clinics and cages as well as from animal related food are displayed in Table 3 and Table 4. Of the strains obtained, about 73.6 percent were motile, and 24.4% showed β hemolysis and 75.6% showed γ hemolysis. Moreover, 25.6% of the isolated strains produced biofilm in a moderate amount, compared to 37.8% of strong producers (table 2).

Table 3: Assays for identifying virulence traits of isolated *Enterococcus* sp. from veterinary clinics and cages

Isolates from veterinary clinics and cages	Motility test result	Hemolysis test result	Biofilm activity test result
$H_1M_{(1)}$	+	Γ	Strong
$H_1M_{(2)}$	-	Γ	Strong
$H_1T_{(1)}$	+	Γ	Strong
$H_1T_{(2)}$	+	Γ	Strong
H_1C_1	+	Γ	Strong
H_1C_2	-	Γ	Strong
H_4S	+	Γ	Moderate
H_4O	+	γ	Strong
H_4T	+	γ	Moderate
H_4C	+	γ	Strong
H_4E	+	γ	-
$H_4M_{(1)}$	+	β	Moderate
$H_4M_{(2)}$	+	γ	Moderate
H_3A	+	γ	Weak
H_3M	+	γ	Strong
H_3TC	+	γ	Strong
H_3T	+	γ	Strong
H_3O	+	γ	Weak
C_2	+	γ	Strong
C_4	+	β	Strong
C_7	-	β	Strong
C_8	-	γ	Strong
C_9	+	γ	Strong
C_{10}	+	γ	Strong
C_{11}	+	γ	Strong
H_2E	+	γ	Weak
H_5M	+	β	Weak
H_5S	+	γ	Strong
$H_5T_{(1)}$	+	β	Weak
$H_5T_{(2)}$	+	γ	Weak

Table 4: Assays for identifying virulence traits of isolated *Enterococcus sp* from animal related food

Isolates from food samples	Motility test result	Hemolysis test result	Biofilm activity test result
S_1M_{27}	+	Γ	Moderate
S_1M_{28}	+	Γ	Weak
S_1M_{30}	+	Β	Moderate
S_2Y_{13}	+	Γ	-
S_4C_1	+	Γ	Weak
S_4C_2	+	Γ	Moderate
$S_4C_{3(1)}$	+	Γ	Weak

$S_4C_{3(2)}$	+	B	Moderate
$S_4C_{4(1)}$	+	Γ	Weak
$S_4C_{4(2)}$	+	B	Weak
$S_4C_{5(1)}$	+	Γ	Weak
$S_4C_{5(2)}$	+	B	Weak
S_4C_6	+	Γ	Strong
$S_4C_{7(1)}$	+	Γ	Weak
$S_4C_{7(2)}$	+	B	Strong
$S_4C_{8(1)}$	+	Γ	Weak
$S_4C_{8(2)}$	+	B	Strong
S_4C_9	+	B	Strong
S_4C_{10}	+	Γ	Strong
S_3BM_3	+	Γ	Moderate
S_4C_{11}	+	β	Moderate
S_4C_{12}	+	β	Moderate
S_4C_{13}	+	β	Moderate
$S_4C_{14(1)}$	+	β	Moderate
$S_4C_{14(2)}$	+	β	Moderate
$S_4C_{15(1)}$	+	β	-
$S_4C_{15(2)}$	+	γ	-
S_4C_{16}	+	γ	Moderate
$S_4GM_{17(1)}$	+	β	Moderate
$S_4GM_{17(2)}$	+	γ	Weak
S_4C_{18}	+	γ	Moderate
S_1M_{25}	+	β	Weak
BM1	-	γ	Strong
BM2	-	γ	-
BM3	-	γ	Strong
BM4	-	γ	Moderate
BM5	-	γ	Weak
BM6	+	γ	Strong
M1	-	γ	Moderate
M2	-	γ	-
M2.1	+	γ	Moderate
M3	-	γ	Strong
Y1	-	γ	Weak
Y3	-	γ	Strong
Y4	-	γ	-
CH2	-	γ	Weak
CH3	-	γ	Moderate
B1	-	γ	Weak
B2	-	γ	-
B3	-	γ	Strong
CH1	-	γ	Strong
Y4.1	-	γ	Weak

Gelatinase production assay

Gelatinase production of isolated strains were checked for gelatin production and results were recorded in table 5 and 6. According to which, of the total 50 strains that were tested, 56% strains were tested positive for gelatin production.

Table 5: Detection of gelatinase production by isolated *Enterococcus sp* from animal related food

Sample Isolate from food samples	Gelatinase production
S_1M_{27}	+
S_4C_{10}	+
S_4C_{12}	+
S_1M_{28}	-
$S_4GM_{17(1)}$	N/A
S_4C_9	+
S_4C_{16}	+
S_1M_{25}	+
S_4C_{13}	+
$S_4C_{3(1)}$	+
S_4C_{12}	-
S_4C_{13}	-
S_1M_{25}	-
S_3BM_3	N/A
$S_4C_{4(2)}$	+
$S_4C7_{(1)}$	+
S_4C_6	-
BM1	-
BM2	+
BM3	N/A
BM4	+
BM5	+
BM6	N/A
M1	-
M2	+
M2.1	+
M3	-
Y1	-
Y3	-
Y4	+
Y4.1	+
CH2	-
CH3	N/A
B1	+
B2	+
B3	-
CH1	+

Table 6: Detection of gelatinase production by isolated *Enterococcus sp* from animal related food

Sample Isolate from veterinary clinics and cages	Gelatinase production
C_9	+
C_7	N/A
H_3TC	+
C_5	N/A
H_5S	-
H_3A	+
H_4C	-
H_3T	-
H_3M	+
H_2E	+

H_4O	+
C_2	+
C_8	+

Antibiotic Susceptibility test

Four antibiotics including ampicillin, vancomycin, erythromycin, and cefotaxime were used to test the isolated strains' susceptibility to antibiotics. Of the total 82 strains, 74.3% were resistant to cefotaxime, 58.5% of the strains exhibited erythromycin resistance, 15.8% were resistant to vancomycin and 36.9% displayed resistance to ampicillin. The results mentioned above indicate that about 29.2% of the strains were multidrug resistant bacteria. Table 7 and 8 contains a record of every outcome. Furthermore, Figure 1 summarizes the antibiotic resistance profiles of various isolates.

Table 7: Antibiotic susceptibility testing of *Enterococcus sp.* from animal related food against different antibiotics

Isolates from food samples	Zone of Inhibition			
	Cefotaxime	Erythromycin	Vancomycin	Ampicillin
S_1M_{27}	Resistant	Resistant	Susceptible	Susceptible
S_1M_{28}	Resistant	Intermediate	Susceptible	Resistant
S_1M_{30}	Resistant	Intermediate	Intermediate	Susceptible
S_2Y_{13}	Intermediate	Susceptible	Susceptible	Susceptible
S_4C_1	Resistant	Resistant	Susceptible	Susceptible
S_4C_2	Resistant	Resistant	Susceptible	Resistant
$S_4C_{3(1)}$	Intermediate	Intermediate	Susceptible	Susceptible
$S_4C_{3(2)}$	Resistant	Resistant	Resistant	Resistant
$S_4C_{4(1)}$	Resistant	Resistant	Susceptible	Susceptible
$S_4C_{4(2)}$	Resistant	Susceptible	Susceptible	Susceptible
$S_4C_{5(1)}$	Resistant	Resistant	Resistant	Resistant
$S_4C_{5(2)}$	Resistant	Resistant	Susceptible	Susceptible
S_4C_6	Resistant	Resistant	Intermediate	Resistant
$S_4C_{7(1)}$	Resistant	Resistant	Resistant	Resistant
$S_4C_{7(2)}$	Resistant	Resistant	Intermediate	Resistant
$S_4C_{8(1)}$	Resistant	Resistant	Intermediate	Resistant
$S_4C_{8(2)}$	Resistant	Resistant	Resistant	Susceptible
S_4C_9	Resistant	Resistant	Susceptible	Susceptible
S_4C_{10}	Resistant	Intermediate	Susceptible	Susceptible
S_3BM_3	Intermediate	Intermediate	Susceptible	Susceptible
S_4C_{11}	Resistant	Resistant	Intermediate	Resistant
S_4C_{12}	Resistant	Resistant	Intermediate	Susceptible
S_4C_{13}	Intermediate	Intermediate	Susceptible	Susceptible
$S_4C_{14(1)}$	Intermediate	Intermediate	Intermediate	Susceptible
$S_4C_{14(2)}$	Intermediate	Intermediate	Intermediate	Susceptible
$S_4C_{15(1)}$	Resistant	Intermediate	Intermediate	Susceptible
$S_4C_{15(2)}$	Resistant	Resistant	Intermediate	Resistant
S_4C_{16}	Resistant	Resistant	Intermediate	Susceptible
$S_4GM_{17(1)}$	Resistant	Susceptible	Susceptible	Susceptible
$S_4GM_{17(2)}$	Intermediate	Susceptible	Susceptible	Resistant
S_4C_{18}	Resistant	Resistant	Susceptible	Susceptible
S_1M_{25}	Intermediate	Intermediate	Susceptible	Susceptible
BM1	Resistant	Susceptible	Susceptible	Intermediate
BM2	Resistant	Susceptible	Susceptible	Resistant
BM3	Resistant	Resistant	Resistant	Resistant
BM4	Resistant	Intermediate	Intermediate	Resistant
BM5	Resistant	Resistant	Susceptible	Susceptible

BM6	Resistant	Resistant	Susceptible	Susceptible
M1	Intermediate	Susceptible	Resistant	Resistant
M2	Resistant	Resistant	Resistant	Resistant
M2.1	Resistant	Susceptible	Susceptible	Resistant
M3	Resistant	Resistant	Intermediate	Intermediate
Y1	Intermediate	Resistant	Intermediate	Resistant
Y3	Resistant	Resistant	Intermediate	Intermediate
Y4	Resistant	Resistant	Resistant	Resistant
Y4.1	Resistant	Intermediate	Susceptible	Susceptible
CH2	Resistant	Intermediate	Intermediate	Resistant
CH3	Resistant	Resistant	Resistant	Susceptible
B1	Resistant	Resistant	Intermediate	Resistant
B2	Intermediate	Resistant	Susceptible	Intermediate
B3	Resistant	Intermediate	Susceptible	Intermediate
CH1	Resistant	Intermediate	Susceptible	Resistant

Table 8: Antibiotic susceptibility testing of *Enterococcus sp.* from veterinary clinics and cages against different antibiotics

Strains from veterinary clinics and cages	**Zone of Inhibition**			
	Cefotaxime	**Erythromycin**	**Vancomycin**	**Ampicillin**
$H_1M_{(1)}$	Intermediate	Susceptible	Intermediate	Susceptible
$H_1M_{(2)}$	Intermediate	Resistant	Susceptible	Susceptible
$H_1T_{(1)}$	Resistant	Intermediate	Resistant	Susceptible
$H_1T_{(2)}$	Resistant	Resistant	Intermediate	Resistant
H_1C_1	Resistant	Resistant	Intermediate	Resistant
H_1C_2	Intermediate	Resistant	Susceptible	Susceptible
H_4S	Resistant	Intermediate	Resistant	Susceptible
H_4O	Resistant	Resistant	Susceptible	Resistant
H_4T	Resistant	Resistant	Susceptible	Susceptible
H_4C	Resistant	Resistant	Intermediate	Susceptible
H_4E	Resistant	Resistant	Intermediate	Resistant
$H_4M_{(1)}$	Resistant	Susceptible	Susceptible	Susceptible
$H_4M_{(2)}$	Resistant	Resistant	Susceptible	Susceptible
H_3A	Resistant	Resistant	Susceptible	Susceptible
H_3M	Resistant	Resistant	Intermediate	Resistant
H_3TC	Resistant	Resistant	Intermediate	Susceptible
H_3T	Resistant	Resistant	Susceptible	Susceptible
H_3O	Resistant	Resistant	Intermediate	Resistant
C_2	Resistant	Resistant	Susceptible	Susceptible
C_4	Intermediate	Resistant	Susceptible	Susceptible
C_7	Intermediate	Intermediate	Intermediate	Susceptible
C_8	Intermediate	Intermediate	Susceptible	Susceptible
C_9	Susceptible	Susceptible	Susceptible	Susceptible
C_{10}	Intermediate	Intermediate	Susceptible	Susceptible
C_{11}	Resistant	Resistant	Resistant	Susceptible
H_2E	Resistant	Resistant	Intermediate	Resistant
H_5M	Intermediate	Intermediate	Resistant	Resistant
H_5S	Intermediate	Susceptible	Susceptible	Susceptible
$H_5T_{(1)}$	Resistant	Resistant	Susceptible	Susceptible
$H_5T_{(2)}$	Resistant	Susceptible	Susceptible	Susceptible

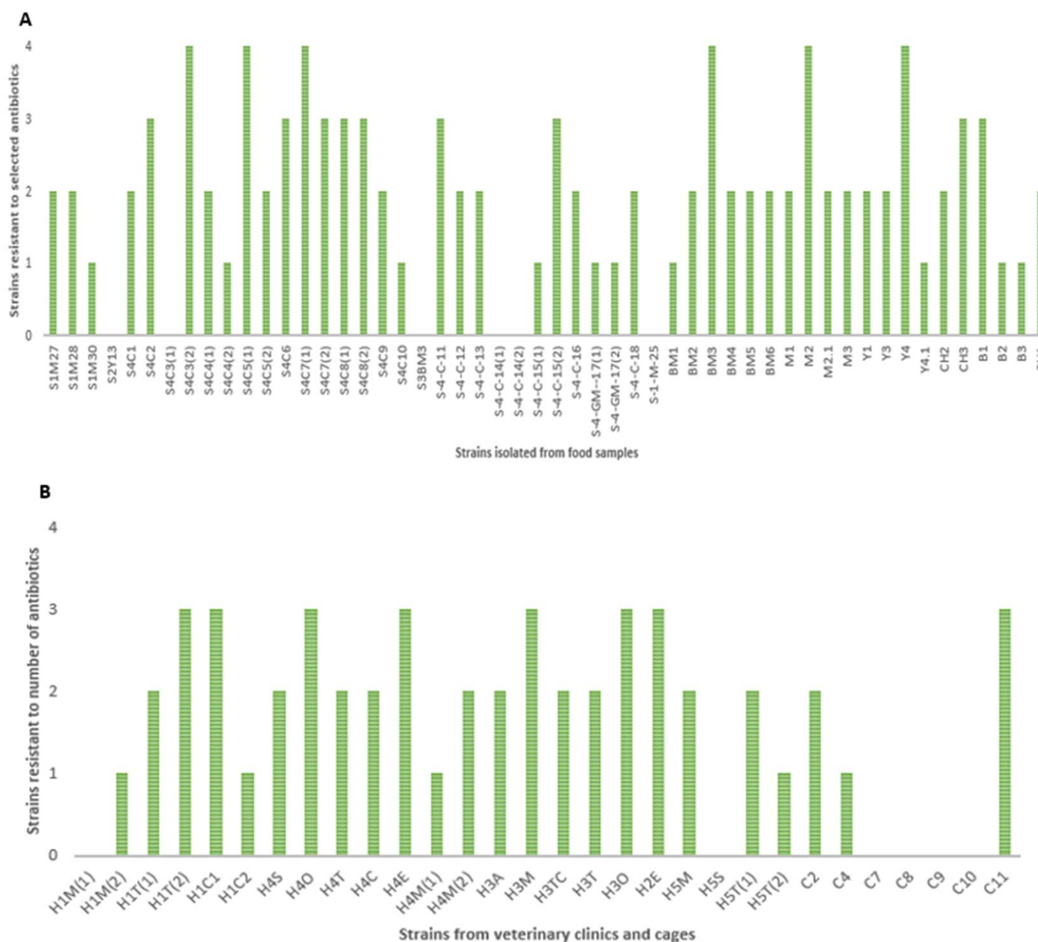

Figure 1: Resistance profiles of strains isolated from (A) Food samples and (B) Veterinary clinics and cages.

Discussion

The predominant type of bacteria found in food is Enterococcus spp. This is majorly because of their resistance to harsh production conditions as well as their high degree of resilience to food storage conditions (Chajecka-Wierzchowska *et al*., 2016). Acute nosocomial infections can be caused by enterococci, specifically *E. faecalis* (Heidari *et al*., 2017).

Food samples as well as samples from veterinary clinics were collected from various locations throughout Punjab, Pakistan. *Enterococcus* specific medium such as Bile Aesculin Agar was used for identification of isolates which was further confirmed by their morphology as well as biochemical traits. 29.3%, 78% and 85.4% of the isolated strains from food and veterinary clinics demonstrated positive growth on MSA, CLED agar, and lactose utilization test respectively. All of the isolated strains were catalase-negative. Nearly all of the outcomes adhered to Bergey's manual scheme. 95% of the isolated strains showed positive growth when tested with an elevated 6.5% NaCl concentration. Molan and Hussien conducted the same test earlier in 2024, and the isolates from those experiments likewise showed growth in the existance of 6.5% NaCl concentration. The 5% of strains that did not respond well to a 6.5% NaCl solution could have been the result of incorrect experimental conditions or environmental factors (Molan and Hussien, 2024).

The purpose of this motility test experiment was to ascertain whether or not the bacteria could move through a liquid or semisolid medium by conducting a motility test to look into their ability to accelerate through liquids. 73% of the Enterococci strains in our investigation showed motility in semi-solid media. The majority of motile Enterococcus spp. isolates (51.6%) came from food samples, with veterinary

clinic samples coming in second. According to some previous studies, majority of motile *Enterococci* are *E. gallinarum* and *E. casseliflavus*, which were commonly obtained from clinical specimens and were implicated in a prohibitive range of human trespassing infections, particularly in those who were immunodeficient or chronically sick (Abamecha *et al*., 2015). Additionally, *E. innesii* were found to be motile in the Gooch *et al*. study from 2021 (Gooch *et al* in 2021). One of the most prominent features is the production of biofilms, which is intricated in the pathogenic mechanisms of enterococcal infections (Chajęcka-Wierzchowska *et al.,* 2016). Antecedent studies have claimed that isolates of *Enterococcus faecalis* that are not source-dependent are more likely than isolates of other species to form biofilms. In our investigation, 25.6% of the isolated strains generated moderate amounts of biofilm, whereas 37.8% of the strains produced high levels. According to the study of Popovic et al in 2018, 40 percent of the isolated strains developed biofilms.

Enterococci producing hemolytic enzymes cause infections and cause virulence to infections in animals and humans (Gulhan *et al*., 2015). The motive of this assessment was to identify any characteristics of virulent traits present in strains that were isolated. According to our investigation, β hemolysis was observed in 75.6% of strains, β hemolysis in 24.4%, and α hemolysis in none. In 2020, Margalho et al examined the hemolysin activity of Enterococcus strains and discovered that 56.8% of their Enterococcus strains exhibited hemolysis, with 12.0% designated as β-hemolytic and 44.9% as α-hemolytic, accordingly.

The appearance of MDR has increased interest in enterococci in recent years (Saha and Sarkar, 2021). Disc diffusion method was used to check the multi-drug resistance of the testing species against antibiotics including cefotaxime, erythromycin, vancomycin and ampicillin. Results depicted that out of total 82 strains being isolated, 74.3%, 58.5%, 15.8% and 36.9% of the strains were resistant to cefotaxime, erythromycin, vancomycin and ampicillin respectively. A definition of multi-drug resistance is an isolate's capacity to withstand three or more antibiotics. So, in our study approximately 28% that is 23 of 82 strains were resistant to multiple drugs being tested and of the total strains being categorized as MDRs, approximately 42% of them were of poultry origin. Hence, we agree with the previous study carried out in 2023, that poultry was a major source of multi drug resistant *Enterococcus sp.* (Mwikuma *et al*., 2023).

The enzyme's capability to breakdown gelatin, collagen, and many other peptides with bioactive properties gives a clue that it's involved in the initiation and spread of *E. faecalis*-related inflammatory processes. In our study, gelatin hydrolyzing activity was observed in 56% of isolates. The large-scale gelatinase activity between enterococci in this investigation is most likely elaborated with certainty that majority of enterococcal strains are recovered from proteinaceous substances, such as meat and milk. However, 35.7% *E. faecalis* strains manifest gelatinase activity in the study of Aldarose *et al* (Aldarose *et al*. 2019).

Conclusion

In conclusion, it is suggested that the surroundings of the livestock could serve as the primary conduit for the release of resistant along with virulent strains of Enterococci, which may infest food and lead to problems with the environment and public health. Moreover, we found that a high prevalence of Enterococci which can result in dangerous pathological conditions. Certain isolates in this study displayed virulence traits and had the capacity to endure various antibiotics, indicating their capacity for triggering infection along with serious health problems. This highlights the significance of regular monitoring of the livestock environment as well as veterinary clinics.

References

1. Abamecha A, Wondafrash B, Abdissa A (2015). Antimicrobial resistance profile of Enterococcus species isolated from intestinal tracts of hospitalized patients in Jimma, Ethiopia. BMC research notes, **8**(1), 213-219.
2. Abdelrahman KA, Kashef MT, Aziz RK, Hashem YA (2024). Genotype–phenotype correlation of fecal Streptococcus regulator (fsr) locus with gelatinase activity and biofilm formation intensity in clinical *E. faecalis* isolates. *Future Journal of Pharmaceutical Sciences*, *10*(1), 37-44.
3. Al Bshabshe A, Algarni A, Shabi Y, Alwahhabi A, Asiri M, Alasmari A, Noreldin N (2024). Characterization and Antimicrobial Susceptibility Patterns of Enterococcus Species Isolated from Nosocomial Infections in a Saudi Tertiary Care Hospital over a Ten-Year Period (2012–2021). Diagnostics, *14*(11), 1190.
4. Aladarose BE, Said HS, Abdelmegeed ES (2019). Incidence of virulence determinants among enterococcal clinical isolates in Egypt and its association with biofilm formation. Microbial Drug Resistance, **25**(6), 880-889.
5. Alara JA, Alara OR (2024). An Overview of the Global Alarming Increase of Multiple Drug Resistant: A Major Challenge in Clinical Diagnosis. Infectious Disorders-Drug Targets (Formerly Current Drug Targets-Infectious Disorders), **24**(3), 26-42.

6. Al-Azawi IH (2023). The Molecular Identification of agg and hyl genes of Enterococcus faecalis isolated from different clinical sampl: Molecular Detection of agg and hyl genes in Enterococcus faecalis isolates. AL-QADISIYAH MEDICAL JOURNAL, **19**(2), 65-68.
7. Arumugam U, Stalin N, Rebecca GP (2017). Isolation, molecular identification and antibiotic resistance of Enterococcus faecalis from diseased Tilapia. Internationa Journal of Current Microbiolology and Applied Sciences, **6**(6), 136-146.
8. Aung MS, Urushibara N, Kawaguchiya M, Ohashi N, Hirose M, Kudo K, Kobayashi N. (2023). Antimicrobial resistance, virulence factors, and genotypes of Enterococcus faecalis and Enterococcus faecium clinical isolates in Northern Japan: identification of optrA in ST480 E. *faecalis. Antibiotics*, **12**(1), 108.
9. Boccella M, Santella B, Pagliano P, De Filippis A, Casolaro V, Galdiero M, Franci G (2021). Prevalence and antimicrobial resistance of Enterococcus species: a retrospective cohort study in Italy. Antibiotics, **10**(12), 1552.
10. Boeder AM, Spiller F, Carlstrom M, Izídio GS (2024). Enterococcus faecalis: implications for host health. World Journal of Microbiology and Biotechnology, **40**(6), 1-16.
11. Cappuccino JG, Sherman N (2014). Microbiology: A Laboratory Manual, (edn 10) revised.
12. Chajęcka-Wierzchowska W, Zadernowska A, Łaniewska-Trokenheim Ł (2016). Virulence factors, antimicrobial resistance and biofilm formation in Enterococcus spp. isolated from retail shrimps. LWT-Food Science and Technology, **69**(2016), 117-122.
13. Cheesbrough M. *District Laboratory Practice In Tropical Countries, Part 2*, Cambridge University Press: United kingdom, 2000.
14. Dong S, Li L, Hao F, Fang Z, Zhong R, Wu J, Fang X (2024). Improving quality of poultry and its meat products with probiotics, prebiotics, and phytoextracts. Poultry Science, *103*(2), 103287.
15. Elnar AG, Kim GB (2026). Understanding the ambiguity behind Enterococcus as a probiotic ally or a pathogenic adversary. Critical reviews in microbiology, *52*(2), 350-376.
16. Fahim NAI, Masud RI, Salam S, Hasan MAE, Rahman AMT, Punom SA, Rahman MT (2025). Role of Enterococcus in spreading antimicrobial resistance genes and its public health significance. Ger. J. Vet. Res, *5*(1), 95-12.
17. Geraldes C, Tavares L, Gil S, Oliveira M (2022). Enterococcus virulence and resistant traits associated with its permanence in the hospital environment. Antibiotics, **11**(7), 857.
18. Ghazvinian M, Asgharzadeh Marghmalek S, Gholami M, Amir Gholami S, Amiri E, Goli HR (2024). Antimicrobial resistance patterns, virulence genes, and biofilm formation in enterococci strains collected from different sources. BMC Infectious Diseases, *24*(1), 274.
19. Gooch HC, Kiu R, Rudder S, Baker DJ, Hall LJ, Maxwell A (2021). Enterococcus innesii sp. nov., isolated from the wax moth Galleria mellonella. International journal of systematic and evolutionary microbiology, *71*(12), 005168.
20. Gulhan T, Boynukara B, Ciftci A, Sogut MU Findik A (2015). Characterization of Enterococcus faecalis isolates originating from different sources for their virulence factors and genes, antibiotic resistance patterns, genotypes and biofilm production. Iranian journal of veterinary research, **16**(3), 261-266
21. Heidari H, Hasanpour S, Ebrahim-Saraie HS, Motamedifar M (2017). High incidence of virulence factors among clinical Enterococcus faecalis isolates in Southwestern Iran. Infection & chemotherapy, **49(**1), 51-56.
22. Khalil MA, Alorabi JA, Al-Otaibi LM, Ali SS, Elsilk SE (2022). Antibiotic resistance and biofilm formation in Enterococcus spp. isolated from urinary tract infections. Pathogens, **12**(1), 34.
23. Kim HJ, Youn HY, Kang HJ, Moon JS, Jang YS, Song KY, Seo KH (2022). Prevalence and virulence characteristics of Enterococcus faecalis and Enterococcus faecium in bovine mastitis milk compared to bovine normal raw milk in South Korea. Animals, **12**(11), 1407.
24. KuKanich KS, Ghosh A, Skarbek JV, Lothamer; Ludek Zurek KM (2012). Surveillance of bacterial contamination in small animal veterinary hospitals with special focus on antimicrobial resistance and virulence traits of enterococci. Journal of the American Veterinary Medical Association, **240**(4), 437-445.
25. Margalho LP, van Schalkwijk S, Bachmann H, Sant'Ana AS (2020). Enterococcus spp. in Brazilian artisanal cheeses: Occurrence and assessment of phenotypic and safety properties of a large set of strains through the use of high throughput tools combined with multivariate statistics. Food control, *118*, 107425.
26. Massella E, Russo S, Filippi A, Garbarino CA, Ricchi M, Bassi P, Luppi A (2025). Does bovine raw milk represent a potential risk for vancomycin-resistant Enterococci (VRE) transmission to humans?. Antibiotics, *14*(8), 814.
27. Molan NK, Hussien AR (2024). Isolation and Identification of Enterococcus spp. Resistant to Macrolides Antibiotics. Iraqi Journal of Science, 65(8), 4271-4280

28. Mwikuma G, Kainga H, Kallu SA, Nakajima C, Suzuki Y, Hang'ombe BM (2023). Determination of the prevalence and antimicrobial resistance of *Enterococcus faecalis* and *Enterococcus faecium* associated with poultry in four districts in Zambia. Antibiotics, *12*(4), 657.
29. Nasiri M, Hanifian S (2022). Enterococcus faecalis and Enterococcus faecium in pasteurized milk: Prevalence, genotyping, and characterization of virulence traits. Lwt, 153, 112452.
30. Ngbede EO, Raji MA, Kwanashie CN, Kwaga JKP (2017). Antimicrobial resistance and virulence profile of enterococci isolated from poultry and cattle sources in Nigeria. Tropical animal health and production, **49**(3), 451-458.
31. Paschoalini BR, Nuñez KVM, Maffei JT, Langoni H, Guimarães FF, Gebara C, Silva NCC (2023). The emergence of antimicrobial resistance and virulence characteristics in Enterococcus species isolated from bovine milk. Antibiotics, **12**(8), 1243.
32. Popović N, Dinić M, Tolinački M, Mihajlović S, Terzić-Vidojević A, Bojić S, Veljović K (2018). New insight into biofilm formation ability, the presence of virulence genes and probiotic potential of Enterococcus sp. dairy isolates. Frontiers in microbiology, **9**(2018), 78-88.
33. Saha M, Sarkar A (2021). Review on multiple facets of drug resistance: a rising challenge in the 21st century. Journal of xenobiotics, **11**(4), 197-214.
34. Șchiopu P, Toc DA, Colosi IA, Costache C, Ruospo G, Berar G, Todea DA (2023). An overview of the factors involved in biofilm production by the enterococcus genus. International Journal of Molecular Sciences, **24**(14), 11577.
35. Wathij H, Kadhim, (2025). Molecular Detection and Virulence Gene Profiling of Enterococcus spp. in Pediatric Diarrheal Cases. Babcock University Medical Journal, *8*(2), 12-25.