UNIVERSITI PUTRA MALAYSIA

OCCURRENCES AND ANTIMICROBIAL RESISTANCE PROFILE OF *Enterococcus* spp. ISOLATED FROM BROILERS IN SELANGOR

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OCCURRENCES AND ANTIMICROBIAL RESISTANCE PROFILE OF *Enterococcus* spp. ISOLATED FROM BROILERS IN SELANGOR

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A project paper submitted to the
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CERTIFICATION

It is hereby certified that i/we have read this project paper entitled " Occurrences and Antimicrobial Resistance Profile Of *Enterococcus* spp. Isolated From Broilers in Selangor", by Nur Amirah Binti Ismail and in my/our opinion, it is satisfactory in terms of scopes, quality, and presentation as partial fulfillment of the requirement for the course VPD 4901 -Project

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ABSTRAK

Abstrak daripada kertas projek yang dikemukan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus.

VPD 4901-Projek Ilmiah Akhir Tahun

KEMUNCULAN DAN KERINTANGAN ANTIMIKROB DALAM *ENTEROCOCCUS* SPP. YANG DIISOLASI DARIPADA AYAM PEDAGING DI SELANGOR

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Enterococcus spp. adalah bakteria gram-positif, anaerobik fakultatif yang merupakan salah satu mikroflora utama dalam saluran pencernaan burung dan mamalia. *Enterococcus* spp. adalah bakteria oportunis dan kebelakangan ini telah mula muncul dalam ayam. Isu lain yang membangkitkan kebimbangan adalah risiko kesihatan awam dengan penularan viiitandard serta kerintangan antimikrob (AMR) melalui pemakanan daging ayam. Tujuan kajian ini adalah untuk menentukan kehadiran *Enterococcus* spp. pada ayam pedaging dan mengkaji profil kerintangan antimikrob *Enterococcus* spp. yang diasingkan di Selangor. Sejumlah 30 sampel calitan kloakal telah dikumpulkan dari ayam pedaging di sebuah ladang ayam di Selangor. Semua sampel disimpan di dalam media terpilih untuk mengasingkan bakteria ini. Identifikasi lanjut dilakukan menggunakan pewarnaan gram, ujian katalase, dan siri ujian biokimia lain seperti ujian '*bile esculin*', '*soluble hemolysin*', 6.5% NaCl,

arabinosa, mannitol, laktosa, dan ujian gula tambahan untuk identifikasi lanjut. Seterusnya, kaedah Kirby-Bauer digunakan untuk menentukan profil kerintangan antimikrob daripada kultur yang telah diasingkan. Secara keseluruhan, kehadiran *Enterococcus* spp. daripada ayam pedaging ialah 36.7% daripada jumlah ayam. Daripada 25 kultur asli yand didapati, 60% sampel adalah positif untuk *Enterococcus* spp. Berdasarkan ujian biokimia, 33.3% dikenalpasti sebagai *Enterococcus faecalis (E. faecalis*), diikuti oleh 20% *E. gallinarum*, 13.3% *E. faecium*. 13.3% adalah *E. cecorum*. *Enterococcus cecorum* baru-baru ini dikenal pasti sebagai strain baharu yang memberi kesan kepada sistem 'musculoskeletal' ayam. Isolat lain yang diperoleh diklasifikasikan di bawah *Enterococcus* spp. (lain-lain). Dalam ujian kerintangan antimikrob (AST), 100% didapati rintang terhadap erythromycin (5µg) dan tetracycline (30µg). Enam puluh isolat (60%) kebal terhadap enrofloxacin (5µg), dan 27% tahan terhadap vancomysin (5µg). Sementara itu, majoriti isolat sensitif terhadap amoxicillin (10µg) pada 87%. Kesimpulannya, *Enterococcus* spp. yang diisolasikan dari ayam pedaging . membentuk 36.7% daripada jumlah isolat keseluruhan. Hasil menunjukkan bahawa ayam adalah sumber potensi *Enterococcus* spp. yang rintang terhadap pelbagai antimikrob (AMR) dan membawa risiko ketahanan antimikrob dalam kesihatan awam. Penyelidikan lanjut perlu dilakukan untuk menyiasat faktor yang menyebabkan penyakit yang berkaitan dengan enterococcus (EAD) dalam ayam dan strategi untuk mengurangkan AMR dengan memastikan kecekapan penggunaan antimikrob.

Kata Kunci: *Enterococcus*, ayam broiler, ketahanan pelbagai dadah, ketahanan antimikrob, penyakit berkaitan dengan *Enterococcus*.

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4901-Final Year Project

OCCURRENCE AND ANTIMICROBIAL RESISTANCE IN *Enterococcus* spp. ISOLATED FROM BROILERS IN SELANGOR

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Enterococcus spp. is a gram-positive, facultative anaerobic bacteria that is one of the main microflora of the gastrointestinal tract in birds and mammals. Enterococci is an opportunistic pathogen and has recently become an emerging health issue in the poultry industry. Another rising concern is the public health risk for zoonotic transmission as well as antimicrobial resistance (AMR) enterococci via poultry meat consumption. The aim of this study is to determine the occurrence of *Enterococcus* spp. from broilers and to examine the antimicrobial resistance profile of the isolated *Enterococcus* spp. in Selangor. A total of 30 cloacal swabs were collected from broilers from a poultry farm in Selangor. All samples were processed by plating onto selective media to isolate presumptive enterococci. Further identification was carried out using Gram staining, catalase test, and a series of other biochemical tests such as bile esculin test, soluble hemolysin, 6.5% NaCl, arabinose, mannitol, lactose, and additional sugar tests for species identification. Next, the Kirby-Bauer disc

diffusion method was used to determine the antimicrobial susceptibility profile of the isolated strains. Overall, the occurrence of *Enterococcus* spp. was 36.7% of total birds . From 25 pure cultures obtained, 33.3% of isolates were identified as *Enterococcus faecalis* (*E. faecalis*), followed by 20% of *E. gallinarum*, 13.3% of *E. faecium*. 13.3% were *E. cecorum*. *Enterococcus cecorum* has recently been identified as the newly pathogenic strain that affects the musculoskeletal system of birds. Other isolates obtained were classified under *Enterococcus* spp (others). In the antimicrobial susceptibility testing (AST), 100% of the isolates were found to be resistant to erythromycin (5µg) and tetracycline (30µg). Sixty percent (60%) resistance was found against enrofloxacin (5µg), and 27% were resistant to vancomycin (5µg) . Meanwhile, the majority of the isolates were susceptible to amoxicillin (10µg) at 87%. In conclusion, there are high occurrences of *Enterococcus* spp. isolated from broilers which constitutes 60% of the total isolates. The results show that poultry is a potential source of multiple-drug resistant enterococci and presents a risk of AMR enterococci in public health. Further investigation should be done to investigate predisposing factors leading to enterococci-associated diseases (EAD) in the poultry industry and strategies to minimize AMR by ensuring the efficiency of antibiotic usage.

Keyword: *Enterococcus,* broiler, multiple-drug resistance, antimicrobial-resistance, enterococci-associated-diseases

2.0 INTRODUCTION

Enterococci is a gram-positive cocci or diplococci and is classified under the lactic acid bacteria. It is one of the building microflora of the intestinal tracts of birds as well as mammals. They are non-spore forming and facultative anaerobes. Among the frequently encountered Enterococci species in birds include, *Enterococcus faecalis* (*E.faecalis*), *Enterococcus faecium* (*E.faecium*), *Enterococcus avium* (*E.avium*), *Enterococcus gallinarum* (*E.gallinarum*), and *Enterococcus cecorum* (*E.cecorum*). Enterococci are categorised under opportunistic pathogens which will turn pathogenic once the immune system of the host is compromised. Recently, enterococci-associated diseases (EAD) have surfaced in the past few decades, especially in the poultry industry. Transmission of enterococcal infection between birds occurs via fecal-oral route, aerosol routes as well as direct contact. Some of the symptoms portrayed by the birds include general appearances of sick birds like depression, lethargy, ruffled feathers, diarrhea and decreased egg production. *E. cecorum* shows prominent musculoskeletal disorders. Most of the lesions observed are also species related. Among generalised lesions observed include multiple organ enlargements, pericarditis, perihepatitis, spondylitis, myocarditis and osteomyelitis. If left untreated, most affected birds can die.

Enterococci also poses risk for zoonotic transmission via foodborne. Although rare, clinical signs were observed in humans from mild

uncomplicated wound to severe endocarditis. With the re-emergence of antimicrobial-drug-resistance (AMR) bacteria, poultry meat can be a reservoir for AMR- Enterococci. AMR-enterococci can also be transmitted to humans and other animals via direct contact with contaminated sources and food chains.

The risk of enterococcal infection will definitely impact farm in terms of arising health issues among birds and economic wise, while the potential spread of AMR-enterococci is a note-worthy concern in the public health sector.

Thus, the objectives of this study are :

To determine the occurrence of *Enterococcus* spp. and,

To investigate the antimicrobial resistance profile of isolated *Enterococcus* spp. from fecal samples of broilers in Selangor.

2.0 LITERATURE REVIEW

2.1 *Enterococcus* spp.

Enterococci are classified under Gram-positive cocci that exist in pairs (diplococci) or short chains. They are nonspore-forming, catalase and oxidase-negative, and facultative anaerobic bacteria which means they can undergo respiration in both oxygen-rich and oxygen-poor environments. They are ubiquitous in the environment. All enterococci are non-motile, except for *E. gallinarum* and *E. casseliflavus* (Taban et al., 2014). These mesophilic bacteria are able to grow within temperatures of 10 ℃ to 45℃ and a range of pH from 4.4 to 9.6. It grows in 6.5% NaCl and can grow in broth supplemented with 40% bile salts and hydrolyze esculin. These are traits to differentiate enterococci from streptococci as they are both categorized under lactic acid bacteria (Ben Braïek & Smaoui, 2019). Over 60 species of *Enterococcus* are known based on their variation of biochemical and morphological characteristics. *Enterococcus* genus make up the intestinal microflora of mammals and avian. In birds, there are a few identified species; mainly: *E. faecalis, E. faecium, E. hirae, E. cecorum, E. durans, E. avium, E. casseliflavus, E. gallinarum, E. raffinosus and E. columbae* (from pigeons)*, E. alce-dini*s (from common kingfishers) (Dolka et al., 2017). In a research done on the frequency of occurrence of *Enterococcus* in poultry, the most predominant species were identified as *E. faecalis* (74.7%), *E. faecium* (10.1%),*E. gallinarum* (5.5%), *E. hirae* (4.6%), and *E. cecorum* (4.1%). The remaining strains were *E. casseliflavus* (0.8%), *E. avium* (0.1%) in the heart of

a 23-week-old laying hen, and *E. columbae* (0.1%) in a 2-week-old goose. The percentage of *Enterococcus* spp. occurrence in broilers was the highest at 88.1%, followed by laying hens (5.3%), turkeys (3.9%), breeding hens (2.2%) and geese (0.4%) (Stępień-Pyśniak et al., 2016). Another retrospective report in France stated that broiler production was ranked first with 71.5% compared to other sectors, with 53.1% of species identified as *E.cecorum* and 24.3% as *E.faecalis* (Souillard et al., 2022) . As a natural intestinal microflora, they do not typically cause disease but instead benefit the host with their probiotic effect. However, they can turn pathological when the host's immune system is compromised (Fisher & Phillips, 2009)

2.2 Prevalence of *Enterococcus* spp.

For the past decades, enterococci-associated diseases (EAD) have recently emerged in the poultry industry, particularly broilers. A retrospective study was done in Poland that concluded from 2014 to 2015, 82.6% of *Enterococcus spp.* isolated from animals was from poultry. 61.2% of strains were found from commercial broiler chickens (CB), 7.2% from commercial layers), 3.6% from broiler breeder chickens, and 1.7% from other chickens. (Dolka et al., 2017). The increasing prevalence of EAD is multifactorial. This includes husbandry management, nutrition, genetics, farm herd health plan, and exposure to other infectious agents (Borst et al., 2016). In Zambia, the prevalence of Enterococci was 31.1%, with *E. faecalis* having a prevalence of 37.9%, which was slightly higher than *E. faecium* with 10.5%. Other species

were also identified but vary slightly depending on the type of management, source of chicks, sampling procedures, and isolation and identification process (Mwikuma et al., 2023). *Enterococcus* spp. was present in cloacal swabs of broilers and layers collected from farms in Malaysia. In contrast, the predominant species found was *E. avium* (97.1%), followed by *E. faecalis* (2.9%) (Power, 2000) . In addition, Banik et al. (2018) reported 92% of meat samples were positive for *Enterococcus spp* in Bangladesh. Meanwhile, Cassenego et al., (2011) stated that the overall distribution of species isolated from fecal samples was *E. faecalis* (40%), followed by *E. casseliflavus*, *E. gallinarum* (10.8%), *E. mundtii* (10.8%), *E. faecium* (10.8%), *E. columbae* (5.8%), and *E. gallinarum* (4.2%), however this was studied in relation to dietary supplements fed to broilers in farms in Brazil. The same research cited most *Enterococcus* isolates collected from poultry in Western and North-West Germany were identified as *E. faecalis* (88%), and *E. faecium* (12%).

2.3 Veterinary significance of *Enterococcus* spp.

Enterococci possess the potential to induce severe diseases in birds, and there is a notable rise in their opportunistic tendencies to transition into a pathological state (Dolka et al., 2017). Clinical signs vary within subspecies. *Enterococcus cecorum* is the newly emerging pathogen affecting the musculoskeletal of chickens, particularly broilers. Examples of locomotor disorders are femoral necrosis, spondylitis, spinal lesions; vertebral osteomyelitis, vertebral enterococcal osteomyelitis and arthritis, enterococcal

spondylitis and, colloquially, ‘kinky-back’. Furthermore, EAD was reported at 71.5% in broilers, accounting for 9.1% of all the diseases in broiler production according to Souillard et al. (2022). *E. faecalis* and *E .faecium* predominantly inhabit the early-age chicken microbiota, potentially leading to omphalitis in chicks through fecal exposure. On the other hand, *E. durans* is associated with encephalomalacia in chicks. Common symptoms of acute enterococcosis include depression, lethargy, ruffled feathers, diarrhea, and reduced egg production. (Stępień-Pyśniak et al., 2016). In a study by Olsen et al. (2012b), the authors investigated the cause of mortality in 983 layer chicks, that was associated with bacterial infections. Pure cultures of *E. coli* and *E. faecalis* were obtained from 127 (28.7%) and 86 (19.4%) chicks, respectively, and mixed cultures of these organisms were obtained from 110 chicks (24.8%). There is a lack of study of the case-morbidity rate of enterococcosis in poultry. However, a study was done on embryo fatality rate, the overall mortality of broiler embryos inoculated with outbreak strains from knee joint/thoracic vertebrae, caeca, and with strains from healthy broilers ranged from 20% to 56%, 12% to 64% and 4% to 8%, respectively (Maasjost et al., 2015).

2.4 Zoonotic risks of *Enterococcus* spp.

Enterococcus spp. is an opportunistic zoonotic pathogen that poses a health risk to humans. The mode of transmission for zoonotic bacteria includes direct contact with feces, urine, saliva, mucus, and other bodily fluids of infected animals. Poulsen et al. (2012) proposed in a Vietnamese study that *E. faecalis,* responsible for UTIs in humans, shares close genetic ties with *E. faecalis* present in poultry, particularly in individuals residing closely with chickens. Another worrisome transmission pathway involves the consumption of poultry me. According to statistics from 1990 to 2021, poultry meat was the most consumed worldwide, followed by pork. Around 132.3 million tons of poultry meat were consumed worldwide, making it the most consumed type of meat globally in 2021 (Shahbandeh, 2023). In humans, Enterococcosis causes urinary tract infections, wound infections, bacteremia, and infective endocarditis as mentioned by Hammerum, (2012).

2.5 Antimicrobial resistance of *Enterococcus* spp.

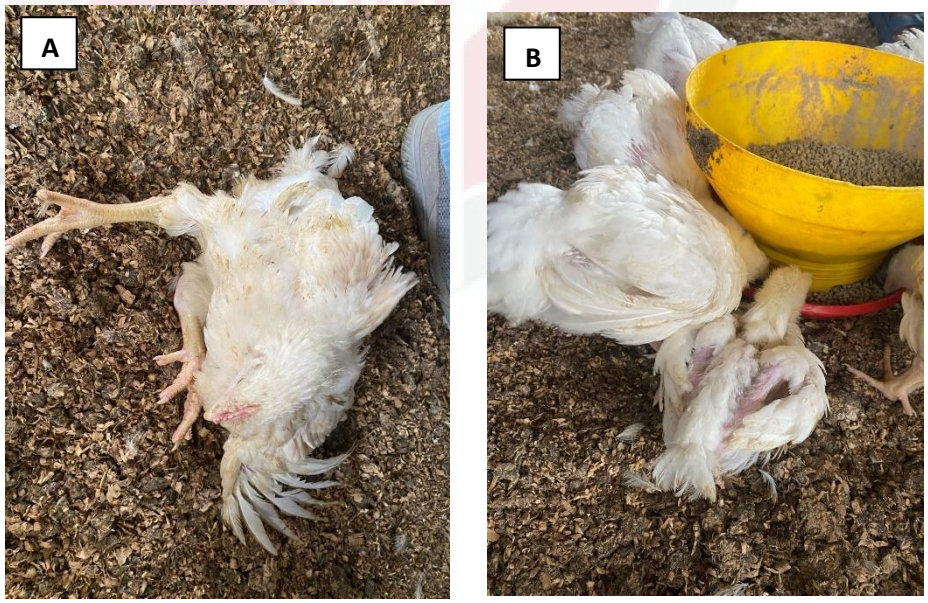
Enterococcal infections in poultry can result in reduced growth rates, less efficient feed utilization, and increased mortality. The Malaysian poultry industry, a key player in the livestock sector, is statistically important in fulfilling the growing demand for poultry meat. Thus, antibiotic has been generally used both for therapeutic and prophylaxis usage. According to Malaysia Good Agriculture Practices (MYGap), from 2015-2017, 100% of antibiotics were used for disease prevention, 25% as chemotherapy, and 0.4% as growth

promoters. More than half of the antimicrobials used were categorised under human and veterinary critically important antimicrobials according to OIE 2018. Enrofloxacin, amoxicillin, and erythromycin were among the common antibiotics used in broiler, layer farms (Rachel et al., 2020). On the other hand, Marzura et al. (2018) stated the occurrences of antimicrobial residue in chicken meat collected in peninsular Malaysia were studied and tetracyclines and quinolones residues were detected. Antibiotic residues come from exposure of bacteria to subtherapeutic doses can lead to bacterial adaptation. If consistently exposed under low doses, bacteria will be virulent and resistant. The issue of antimicrobial resistance is alarming for public health, posing a risk to both food safety and the well-being of the public as resistant bacteria can transfer from animals to humans through the food chain. (Wada et al., 2022). Currently, AMR accounts for almost 7 million deaths per year, so antibiotics residues found in the poultry industry are undeniably concerning (Habiba et al., 2023). Most of *E. faecalis* and *E. faecium* were found to be resistant to tetracycline, ampicillin and erythromycin and present a potential for multiple drug-resistant enterococci, which could be transmitted to humans, especially considering these two give rise to zoonotic risks as cited by Mwikuma et al. (2023) . Another worrying issue is the presence of vancomycin-resistant-enterococcus (VRE) which has been reported in Malaysia. Additionally, it has been documented that VRE has been identified in Malaysia among health workers, animals, hospital patients, and farmworkers, suggesting a possible origin. (Wada et al., 2022).

3.0 Methods

3.1 Sampling

A broiler farm was chosen within the vicinity of Selangor. Random sampling was carried out. A total of 30 chickens were selected, with priorities given to birds with clinical signs like depression, ruffled feathers, locomotor disorders, respiratory distress, and swollen eyes. Figure 1 depicts the clinical signs of sick birds observed. Birds were properly restrained after which sterile cotton swabs were inserted into the cloacas. Each swab was placed in the transport media, and placed inside an ice box and transported back to the Bacteriology Laboratory at the Faculty of Veterinary Medicine, UPM immediately for processing.



Bird A: Locomotor disorder as unable to stand
Bird B : Depression, ruffled feathers

Figure 1 : Clinical signs of sick birds

3. 3 Isolation of enterococci

The swab samples were streaked onto vancomycin-resistant-enterococcus (VRE) agar base as primary culture and incubated aerobically for 24 hours at 37 °C. The VRE agar base was used as presumptive identification for *Enterococcus spp.* Colony growth was detected on VRE agar. Next, colony morphology on VRE agar was described (Figure 4). Up to three colonies were described on each plate and proceeded with gram staining. Colonies with the following characteristics i.e. for gram stain (gram-positive cocci in pairs, short chains) which were presumed as Enterococci species were selected and subcultured onto Brain-Heart-Infusion (BHI) agar as secondary culture to obtain pure culture.

Gram staining of the subcultured bacterial colonies was carried out prior to the biochemical test for species identification. Identification of Enteroccocci was carried out according to A Diagnostic Manual of Veterinary Clinical Bacteriology and Mycology. All presumptive enterococci were then tested with a catalase test. This was done by dropping a drop of hydrogen peroxide onto a clean glass slide and mixing it with a single colony picked from subculture using an inoculating loop. The formation of bubbles indicated a positive test. Only colonies exhibiting negative catalase results were selected for further biochemical testing.

3.3 Biochemical test for species identification

For further confirmation of *Enterococcus* spp. , a single colony was picked from the presumptive subculture and tested in 6.5% NaCl and bile esculin. Growth on these two tests confirmed *Enterococcus* spp. This is because only enterococci can tolerate 6.5% NaCl and hydrolyse esculin in the presence of bile that turns into a dark brown complex. Culture is then identified to species level using additional tests listed in Appendix 2. Table 1 shows the identification scheme for Enterococci species.

Table 1: Identification Scheme of Enterococci

Species/ Tests	Catalase	Arabinose	Mannitol	Sorbitol	Lactose	Trehalose	Sorbose	Raffinose	ADH
E.faecalis	-	-	+	+	+	+	-	-	+
E.faecium	-	+	+	-	+	+	-	d-	+
E.avium	-	+	+	d+	d+	d+	+	-	-
E.durans	-	-	-	d-	d+	d+	-	-	+
E.gallinarum	-	-	d+	d-	d+	d+	-	+	-
E.cecorum	-	-	+	d-	d+	d+	-	+	-

3.4 Antimicrobial susceptibility test

The antimicrobial susceptibility tests were done using the Kirby-Bauer method. Sterile saline was dispensed into a test tube. A loopful of the colony from each subculture was taken and suspended in the saline. The turbidity of the suspension was standardized using the 0.5 McFarland standard. A sterile swab was dipped into the standardized suspension and then streaked onto the surface of blood agar. Blood agar was used as appearance of enterococci appears with better contrast (García-Solache & Rice, 2019). Five antibiotics were selected for tests were based on the commonly used antibiotics in broiler farms in Malaysia. The antibiotics were enrofloxacin, erythromycin, amoxicillin, vancomycin, and tetracycline. The antibiotics were dispensed using a disc dispenser and pressed lightly using forceps. The plates were incubated for 24 hours, 37°C. Zone of inhibition was measured and categorised into sensitive, intermediate, and resistant based on literature review and CLSI standard (2015).

4.0 Results

A total of 25 presumptive Enterococcus isolates were obtained from the total samples cultured on the VRE agar base media. Among these, 16% (4/25) of colonies tested positive for catalase, eliminating them as *Enterococcus* spp. Among the remaining 25 isolates, 60% (15/25) were identified as *Enterococcus* spp. based on negative results for soluble hemolysin, positive for 6.5% NaCl, and positive for bile esculin. Of these, 33.3% (5/15) were *E. faecalis*, 20% (3/15) were E*. gallinarum*, 13.3% (2/15) were *E. faecium*, another 13.3% (2/15) were *E. cecorum*, and the remaining isolates were not be able to be identified beyond the Enterococcus spp. level. Overall, *Enterococcus* spp. were found in 11 out of 30 birds, resulting in an overall occurrence rate of 36.7% of *Enterococcus* spp. isolated from broiler fecal samples on this farm.

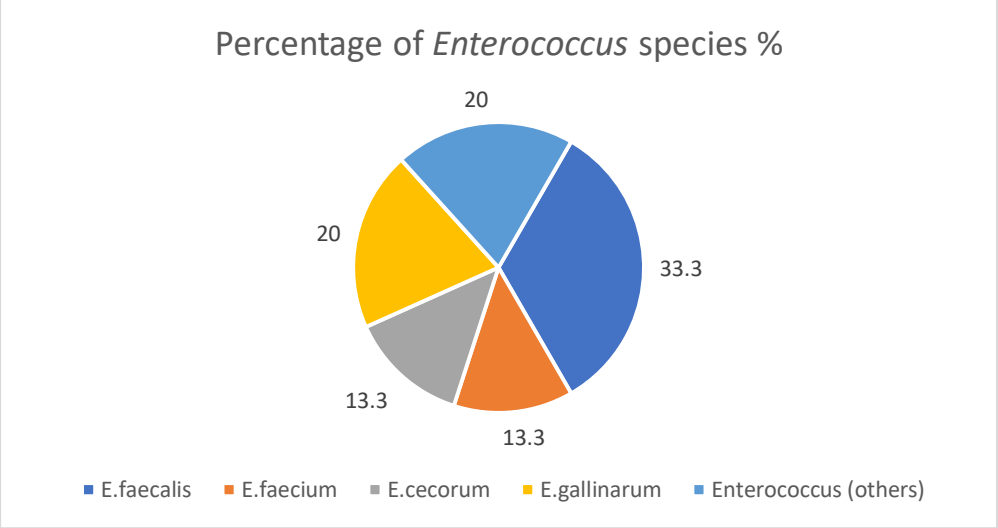


Figure 2 : Breakdown percentages of *Enterococcus* species

The results of the antimicrobial susceptibility tests showed that all isolates are resistant to erythromycin (5µg) and tetracycline (30µg). Among the identified *Enterococcus* spp., 60% exhibited resistance to enrofloxacin (5µg), while 87% (13/15) were sensitive to amoxicillin (10µg). As for vancomycin (5µg), 33.3% (5/15) of isolates were resistant, and 60% (9/15) were sensitive. The antimicrobial resistance profile is detailed in Table 2, and Figure 2 presents a bar chart illustrating the percentage of resistance against each antibiotic. Notably, all *E. faecalis*, *E. faecium*, and *E. cecorum* were classified as multiple-drug-resistant (MDR), while only 33.3% of *E. gallinarum* exhibited MDR. In summary, 86.7% of all isolates demonstrated MDR, which is a noteworthy finding in this study.

Table 2 : Antimicrobial Susceptibility Pattern of the isolated *Enterococcus* spp.

Plates	Antimicrobial agent / Species	Enrofloxacin (Fluroquinolones)	Erythromycin (Macrolides)	Amoxicillin (Penicillin)	Tetracycline	Vancomycin (Glycopeptide)
2A	*E.faecalis*	Intermediate	Resistant	Sensitive	Resistant	Resistant
3A	*E.gallinarum*	Resistant	Resistant	Sensitive	Resistant	Sensitive
3B	*E.faecalis*	Sensitive	Resistant	Sensitive	Resistant	Resistant
6A	*E.gallinarum*	Sensitive	Resistant	Sensitive	Resistant	Sensitive
6E	*E.faecium*	Resistant	Resistant	Sensitive	Resistant	Sensitive
7A	*E.faecium*	Resistant	Resistant	Sensitive	Resistant	Sensitive
8A	*E.faecalis*	Resistant	Resistant	Resistant	Resistant	Sensitive
8D	*E.cecorum*	Resistant	Resistant	Sensitive	Resistant	Sensitive
15A	*E.faecalis*	Resistant	Resistant	Sensitive	Resistant	Sensitive
17A	*Enterococcus* spp.	Sensitive	Resistant	Sensitive	Resistant	Sensitive
19C	*Enterococcus* spp.	Sensitive	Resistant	Sensitive	Resistant	Resistant
22A	*E.faecalis*	Sensitive	Resistant	Resistant	Resistant	Resistant
26C	*Enterococcus* spp	Resistant	Resistant	Sensitive	Resistant	Intermediate
26D	*E.cecorum*	Resistant	Resistant	Sensitive	Resistant	Sensitive
27A	*E.gallinarum*	Resistant	Resistant	Sensitive	Resistant	Sensitive

Sensitive Intermediate Resistant

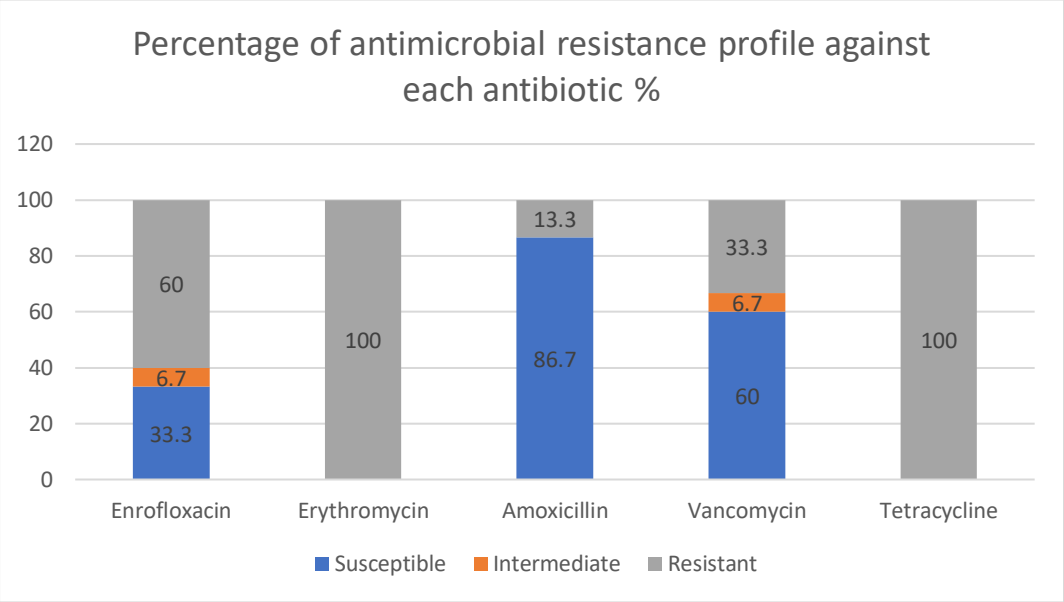
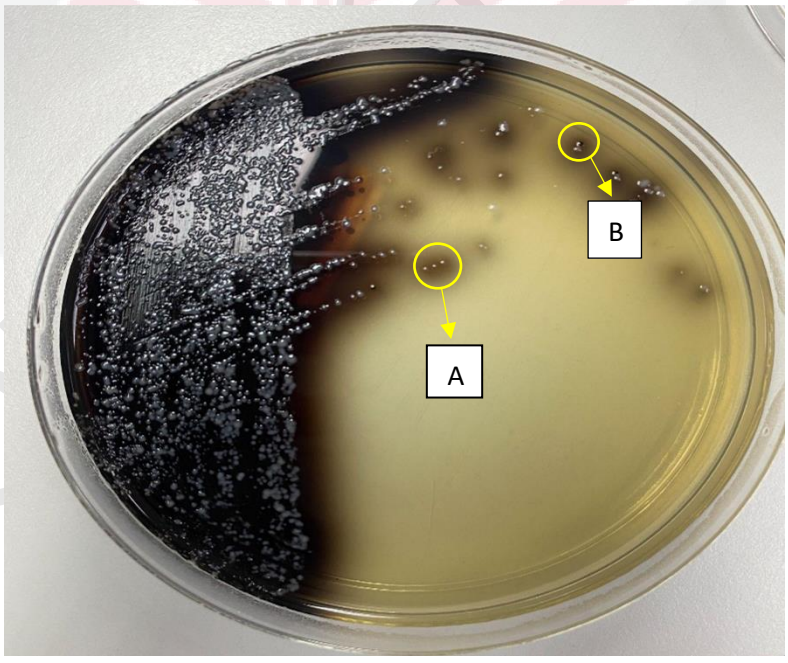
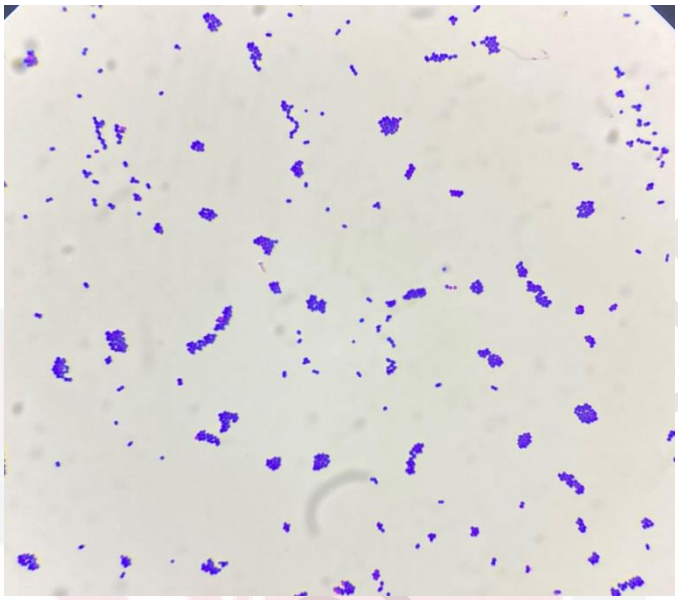


Figure 3 : Percentage of antimicrobial resistance profile against each antibiotic %



Colony A :Round, shiny, <1mm, gray (slightly darker) with irregular edges and flat surface colony
Colony B : Round, shiny, <1mm, blackish centre, smooth edges, slightly raised colony

Figure 4 : Presumptive *Enterococcus* spp. on VRE agar base



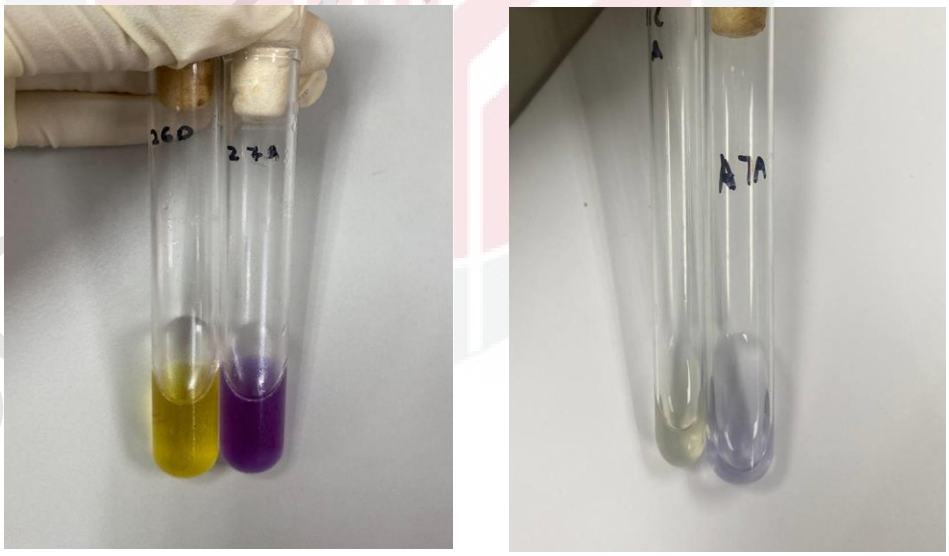
Gram positive cocci and diplococci

Figure 5 : Gram staining of presumptive *Enterococcus* spp.

Left : Positive bile esculin test (dark brown complex)

Right : Negative bile esculin test

Figure 6 : Bile esculin test



Left : Positive growth turns ADH solution into purple colour

Right : Positive growth turns sugar tests into yellow colour

Figure 7 : Color changes in sugar test with *Enterococcus* spp.

Zone of inhibition of isolated *Enterococcus* spp. in blood agar

Figure 8 : Antimicrobial susceptibility test (AST)

5.0 Discussion

This study sums up that *Enterococcus* spp. was present in fecal samples isolated from broilers in Selangor, which constitutes 36.7% of total birds. In a data collection by Dolka *et al*. (2017), from 2014-2015 in Poland, 73.7% of occurrences of *Enterococcus* spp. strains originated from chickens. Meanwhile, the incidences of *Enterococcus* spp. have shown an increasing pattern in France from 2006-2020, with an increase from 0.4% t0 12.9% respectively (Souillard *et al*., 2022). The most predominant species isolated was *E. faecalis* (33.3%), followed by *E. gallinarum, E. faecium, E. cecorum* and others. This is supported by Stępień-Pyśniak et al. (2016) who stated the most commonly encountered species were *E. faecalis* (74.7%), *E. faecium* (10.1%), *E. gallinarum* (5.5%) and *E. cecorum* (4.1%) which were isolated from different types of samples like heart, liver, brain and oviduct. In Brazil, the overall distribution of species isolated from fecal samples was *E. faecalis* (40%), followed by *E. casseliflavus/E. gallinarum* (10.8%), *E. faecium* (10.8%), however, this was researched in relation to dietary supplements fed to broilers in farms (Cassenego *et al*., 2011). In a molecular study in Africa revealed that the primary species identified are *E. faecalis* strains (34%) followed by *E. faecium* (32%) and a small proportion of *E. gallinarum,* while the remaining were unidentified *Enterococcus* spp (Molechan *et al*., 2019). This concludes that the species identified are consistent with other poultry studies conducted in several countries, but also could vary slightly. However, the scarcity of information in Malaysia makes it difficult to include *Enterococcus* spp. in the

surveillance program. Clinical signs for each subspecies are mostly general and can further be identified through histopathology from post-mortem. *E. faecalis* and *E .faecium* are among the commonly identified *Enterococcus* spp. from previous research. These two subspecies are opportunistic zoonotic pathogen and significantly causes health impact to human beings.

In general, the identified subspecies are part of the normal intestinal flora in both chickens and humans, explaining their presence in this study. More than half of the selected animals exhibited clinical signs such as ruffled feathers, ocular edema, and lameness. When chickens experience immunocompromise, opportunistic enterococci can become pathogenic, particularly when exposed to predisposing factors. These factors, encompassing nutrition, feed quality, husbandry management, hygiene, and animal welfare (Kierończyk *et al*., 2017), are broad and multifactorial. However, this study did not collect details on these factors, emphasizing the need for further investigation.

Schreier et al. (2022) recently identified *E. cecorum* (EC) as a significant bacterial pathogen in modern broiler chicken production, emphasizing its economic and animal welfare implications. This bacterium, less prevalent in prior research, has become a rising concern, particularly in Malaysia. Its impact is notable due to the clinical signs of progressive musculoskeletal diseases, resulting in lameness and leg paresis as early as the second half of the production cycle. Figure 1 illustrates that more than half of the observed chickens displayed lameness, and all were 35 days old. It is

suggested that gut-colonisation of chickens is dominated by *E. faecalis* at an early age, but an introduction to antibiotics allows *E. faecium* to grow. At mature age, EC may displace these two species (Lebreton et al., 2014). However, pathogenic EC reaches the bloodstream by the second to the third week of life and subsequently reaches the predilection sites at thoracic vertebrae and femoral heads via blood. Several predisposing factors were indicated which are unhygienic environments, inadequate ventilation, and short dark periods during the first week of life (Schreier et al., 2022). Therefore, even though the initial discovery rate was only 13.3% (2/15), it is crucial to undertake additional research on a larger scale, specifically focusing on investigating predisposing factors.

Based on Figure 2, there are high resistance profile of *Enterococcus* spp. towards tetracycline, enrofloxacin, and erythromycin. These are most likely due to the common use of these antibiotics in broilers as treatment and prophylaxis. Based on data collected by Malaysia Good Agriculture Practices (MyGap) from 2015 to 2017 regarding the widespread of antibiotic usage in poultry farms, broiler breeder farms commonly employed enrofloxacin (98%), followed by amoxicillin (64%) and erythromycin (14%). The tetracycline class (13.4%) was observed to be in use across all poultry farms. The majority of antibiotics utilised in poultry farms fall under the categories of VCIA and HIA (Rachel *et al*., 2020). These raise concerns about the possibility of AMR to spread among livestock and farmers. In August 2020, Department of Veterinary Services (DVS) and Ministry of Health (MOH) banned six antibiotics

erythromycin, tilcomycin, tylosin, neomycin, phosphomycin and enrofloxacin as prohibited in feed and feed additives for the purposes of prophylaxis and growth promoters to food-producing animals as an effort to manage AMR. Even though it has been banned for almost 2 years, and for vancomycin 10 years, multiple-drug-resistant (MDR) enterococci are still evident. These antibiotics are still used as treatments, thus might be one of the reasons behind evidence of resistance although the antibiotics have been based. Misuse, overuse, and ineffective treatment could lead to AMR. Failure of farmers to comply with the right dose, frequency and duration of treatment can cause multiplication of AMR-bacteria. In addition, accidental exposure of AMR in feces or blood could also risk transmission to the flocks as well as the farmers. The overall prevalence of antibiotic resistance between broilers and broiler farmers and poultry slaughterers indicates that contact with broilers is a risk factor for colonization of humans with resistant bacteria (Molechan *et al*., 2019).

In this study, *E .faecalis* was 100% resistant to erythromycin and tetracycline, and 40% resistant to the other antibiotics. This correlates with a study done by Mwikuma et al. (2023) where most of the *E. faecalis* and *E. faecium* isolates were resistant to tetracycline (66/74, 89.2%) and ampicillin and erythromycin (51/74, 68.9%). The findings indicate that poultry can serve as a potential reservoir for multidrug-resistant strains of *E. faecalis* and *E. faecium*, and these strains have demonstrated their ability to be transmitted to humans. Additionally, resistance genes within *Enterococcus* species may be

transferred to pathogenic bacteria if they coexist in the same poultry. This study provides evidence, as two isolates of *E. faecalis* exhibited resistance to four out of five antibiotics used.

According to Global Antibiotic Research & Development Partnership (GARDP), multiple-drug-resistant (MDR) can be defined as lack of susceptibility to at least one agent in three or more chemical classes of antibiotics. In this study, five classes of antibiotics were used include fluoroquinolones, macrolides, penicillin, tetracycline, and glycopeptide. Overall, 87% of the total isolates were MDR, which is significantly higher than data collected Molechan (2019) in South Africa with only 24% MDR Enterococci was found. Meanwhile in Malaysia, it is also slightly plowed with 56.3% of the total isolates were MDR. Higher MDR towards *E. faecium* followed by *E. faecalis* (Mitsuaki-Nishibuchi & Radu, 2001). Thus, the increasing percentage of MDR should raise an alarm, especially in the public health sector.

6.0 Conclusion

It is concluded that the occurrences of *Enterococcus* spp. is at 36.7% from total samples taken in this farm. *Enterococcus faecalis* is the most common species found followed by *E. gallinarum*. In addition, the finding of *E. cecorum* was a rising concern as it is recently emerged as pathogenic strain of *Enterococcus* spp that affects the poultry industry economically. The results for AST showed the presence of AMR enterococci with the majority of the strains were classified as MDR, with higher resistance found towards tetracycline, enrofloxacin and erythromycin. Meanwhile, the rest of the isolated were mostly susceptible to amoxicillin and vancomycin. . Therefore, further study is relevant to be done to understand the predisposing factors of EAD as well as ways to reduce transmission of AMR and zoonotic transmission of Enteroccocosis to humans, which both affect the poultry industry's economy and public health sectors.

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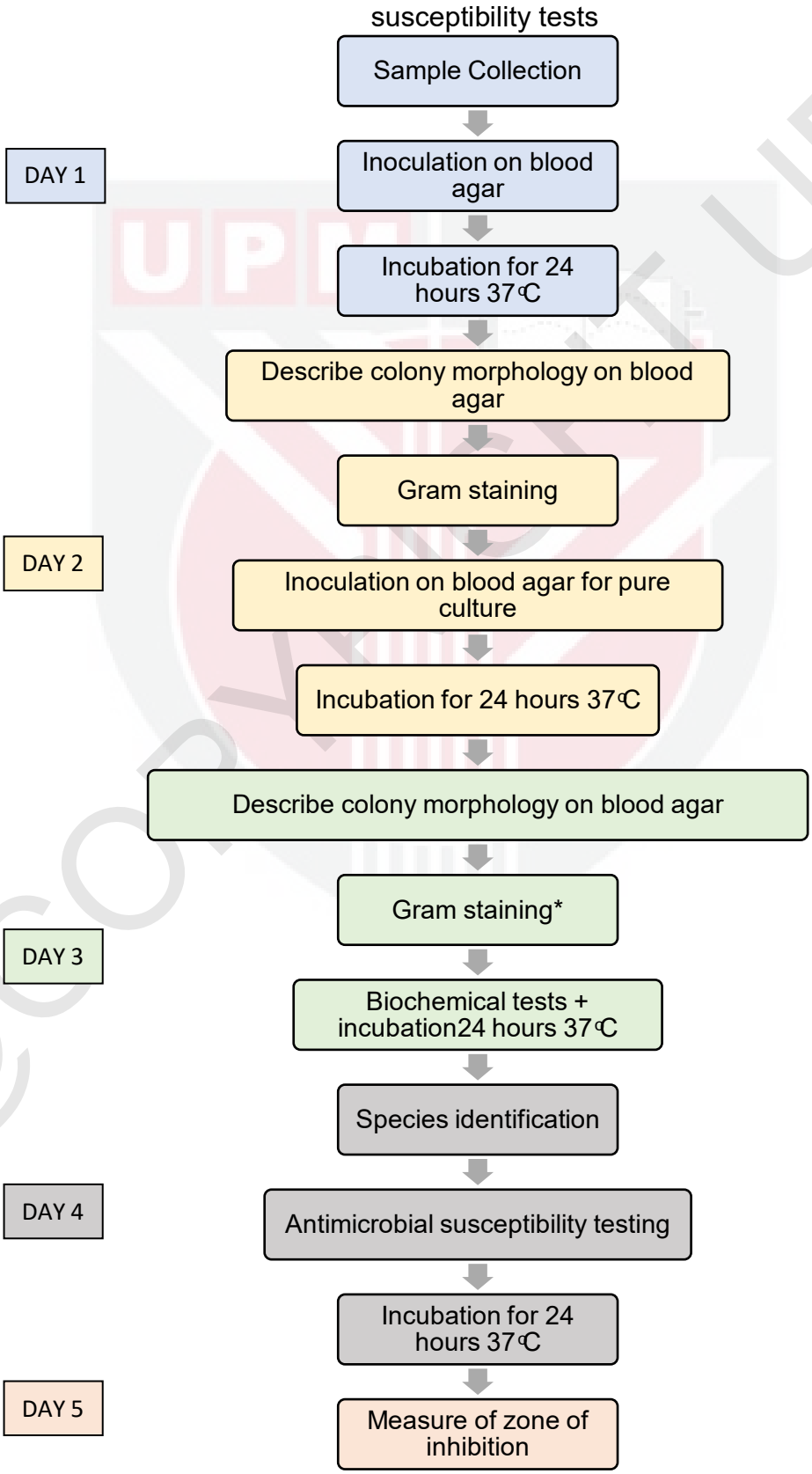
8.0 APPENDICES

APPENDIX 1 : The flow chart for isolation and identification of enterococci and susceptibility tests



APPENDIX 2 : The flow chart for isolation and identification of enterococci and susceptibility tests

TESTS	INSTRUCTIONS
Soluble hemolysin	A loop of isolated colonies from subculture was picked and mixed into blood broth. It was then incubated for 24 hours at 37°C.
6.5% NaCl	A loop of isolated colonies from subculture was picked and mixed into broth. It was then incubated for 24 hours at 37°C.
Bile esculin test	A loop of isolated colonies from subculture was picked and streaked on the slant of agar. It was then incubated for 24 hours at 37°C.
Arabinose	A loop of isolated colonies from subculture was picked and mixed into sugar broth. It was then incubated for 24 hours at 37°C.
Mannitol	
Sorbitol	
Lactose	
Trehalose	
Raffinose	
Sorbose	
Arginine ADH	A loop of isolated colonies from subculture was picked and mixed into broth. Mineral oil was added to cover the broth and incubated for 24 hours at 37°C.

APPENDIX 3 : Identification of Enterococci isolates

Plates/ catalase	Soluble hemolysin	6.5% Nacl	Bile esculin	Arabinose	Mannitol	Sorbitol	Lactose	Trehalose	Sorbose	Raffinose	ADH	Species
1A	+	+	+	-	+	+	+	+	+	+	+	-
1C	+	+	+	+	+	+	+	+	-	+	+	-
2A	-	+	+	-	+	+	+	+	-	-	+	*E.faecalis*
3A	-	+	+	-	+	-	+	+	-	+	+	*E.gallinarum*
3B	-	+	+	-	+	+	+	+	-	-	+	*E.faecalis*
3E	+	-	+	-	+	+	+	+	-	+	-	-
6A	-	+	+	-	+	-	+	+	-	+	+	*E.gallinarum*
6E	-	+	+	-	+	-	+	+	-	-	+	*E.faecium*
7A	-	+	+	-	+	-	+	+	-	-	+	*E.faecium*
8A	-	+	+	-	+	+	+	+	-	-	+	*E.faecalis*
8D	-	+	+	-	+	+	+	+	-	+	-	*E.cecorum*

15A	-	+	+	-	+	+	+	+	-	-	+	*E.faecalis*
17A	-	+	+	-	-	-	+	+	-	+	+	*Enterococcus* spp.
19C	-	+	+	+	+	+	+	+	+	-	+	*Enterococcus* spp.
22A	-	+	+	-	+	+	+	+	-	-	+	*E.faecalis*
22D	-	-	+	-	+	-	+	+	+	+	-	-
26C	-	+	+	+	+	+	+	+	+	-	+	*Enterococcus* spp.
26D	-	+	+	-	+	+	-	+	-	+	-	*E.cecorum*
27A	-	+	+	-	+	+	+	+	-	+	+	*E.gallinarum*
30A	-	-	+	-	+	+	+	-	-	+	-	-