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Research Article

Comparative Analysis of Virulence Factors and Antibiotic Resistance in Enterococci Isolated from Fruits and Vegetables

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Abstract

Enterococci are a variety of Gram-positive cocci that live in a variety of environments, such as food, plants, and gastrointestinal tracts (GI). Hospital-acquired multidrug resistance is on an upward trajectory. The increasing number of reports of *Enterococcus* strains linked to bloodstream infections, endocarditis, and urinary tract infections highlights the critical need for surveillance and research on antimicrobial resistance (AMR). While multidrug resistance refers to resistance to various antibiotic classes, which complicates therapy and raises the chance of clinical failure, antibiotic resistance is the ability of bacteria to resist the toxic effects of drugs that previously killed them. With a focus on virulence factors and AMR profiles, this study examines the occurrence and characteristics of *Enterococcus* strains in various environments. As 48 samples of raw vegetables and fruits were examined, 46 of them tested positive for Enterococci (93%), indicating a high tolerance to salt and variation in gelatinase activity (32.6%). Each isolate had the ability to hydrolyze esculin, and the tube adherence test method verified that biofilm development was seen in 56.5% samples. The surface morphology of biofilm-positive cells which are adhered to solid surfaces and form multicellular aggregates packed into extracellular matrix material and biofilm-negative cells which lack matrix material, were shown by scanning electron microscopy (SEM). Antibiotic resistance patterns differed; vegetable-associated Enterococci showed significant resistance to Ampicillin (20.7%) and Erythromycin (17.2%), while fruit-associated Enterococci showed concerning resistance to Linezolid and Vancomycin (35.3%). In order to assure food safety and public health, this study emphasizes the various characteristics of *Enterococcus* isolates and stresses the significance of surveillance and treatment with antibiotics for environmental *Enterococcus* strains.

Keywords: *Enterococcus,* Antibiotic resistance, Salt tolerance, Gelatinase activity, Esculin hydrolysis, Disc diffusion method, Scanning electron microscopy, Virulence factors

1. Introduction

Gram-positive bacteria consist of a thick layer of peptidoglycan and stain purple, while Gram-negative bacteria possess a thin peptidoglycan layer with an outer membrane and stain pink (De Benedetti, Fisher, and Mobashery 2021). The genus *Enterococcus* belongs to a group of microorganisms known as lactic acid bacteria (LAB). Enterococci are Gram-positive, catalase-negative, non-spore forming, oxidase-negative, facultative anaerobic cocci (Ferdous, Khan, and Kabir 2020). The *Enterococcus* genus is a varied and ecologically relevant group whose members are widely spread in nature (Ghattargi et al. 2018). Gram-positive cocci

known as *Enterococcus* species belong to the *Enterococcaceae* family and can be found alone, in pairs, short in chains, or in clusters (Holzapfel and Wood 2014). *Enterococcus* species primarily inhabit the GI tract, with numerous species documented to colonize the GI tracts of humans, animals, and plants (Zhong et al. 2017, Ghattargi et al. 2018). *Enterococcus* faecalis, *E. faecium* and *E. casseliflavus* species are most frequently found in plant food microbiota (Chajęcka-Wierzchowska, Zarzecka, and Zadernowska 2021). The use of some strains of Enterococci as probiotics suggests that these ubiquitous bacteria are beneficial to the balance of the microbial community (Olvera-García, Sanchez-Flores, and Quirasco Baruch 2018). Their metabolic adaptability and innate resilience to unfavorable environments allow them to colonize large areas. They are extremely resistant to desiccation and can survive for months on dried surfaces, resistant to oxidative and osmotic stressors, high heavy metal concentration, pH fluctuations, ionizing radiation, and antibiotics (Hazra and Durso 2022). Enterococci can grow or survive at temperatures 10 to 45°C (García-Solache and Rice 2019). These bacteria are widely used in food and as probiotics to help prevent or treat various illnesses. The last group of infections to surface on the global scene are hospital-acquired, multidrug-resistant Enterococci strains (Ramsey, Hartke, and Huycke 2014). The emergence of hospital-acquired, multidrug-resistant Enterococci (MDRE) strains, particularly vancomycin-resistant Enterococci (VRE), poses a significant threat to global health due to their high incidence and associated mortality rates. Notably, the all-cause mortality for hospital-acquired bloodstream infections (HA-BSIs) due to *Enterococcus spp.* is estimated at 21.9%, escalating to 33.5% for VRE-related cases (Brinkwirth et al. 2021). A systematic review and meta-analysis reported that VRE infections are associated with higher mortality compared to vancomycin-susceptible Enterococcus (VSE) infections, with a risk ratio of 1.46 (Eichel et al. 2023). A 10- year study in Qatar reported 263 cases of enterococcal BSIs, with *E. faecalis* (73.38%) and *E. faecium* (20.15%) as predominant species. Vancomycin resistance was seen in 10.6% of cases, and the 30-day mortality rate was high at 66.54%, especially among patients with chronic kidney disease and cancer (Ali et al. 2022).

Many studies have been conducted on clinical and environmental isolates to examine the connection between the use of antibiotics and the emergence and transmission of AR (Aarestrup et al. 2001; Macovei and Zurek 2007). Apart from their virulence traits, *Enterococcus* species exhibit resistance to numerous drug classes. Many *Enterococcus* species variation strains have been linked to antibiotic resistance in clinical environments (Lam et al. 2012; Zhong et al. 2017). As the number of viable antimicrobial drugs for the treatment of bacterial illnesses declines, a rising issue in clinical intensive care units is the formation and dissemination of antibiotic resistance determinants (Macovei and Zurek 2007). Moreover, the existence of pathogenic and probiotic strains within the same species suggests that their genomic features may differ resulting in variations in their phenotypic traits. Recent developments in genomic analysis, such as the discovery of epigenetics, have enabled greater resolution comparison and contrast of genomic traits to reveal previously unnoticed minute subtleties (Panthee et al. 2021).

They have been linked to nosocomial infections in humans and could have genes that code for virulence factors such as adhesins, invasins, and hemolysin also, certain strains of them are resistant to basic clinical practice drugs like vancomycin. *Enterococcus faecium*, *E. faecalis*, and *E. durans* are the most prevalent species of *Enterococcus* found in food products (Olvera-García, Sanchez-Flores, and Quirasco Baruch 2018). Comparatively speaking to clinical strains, it has been shown that Enterococci isolated from food and other naturally occurring substances (like water and vegetables) have a lower prevalence of virulence factors (Franz et al. 2001; Olvera-García, Sanchez-Flores, and Quirasco Baruch 2018). Two *Enterococcus* species provide significant clinical concern. *E. faecalis* and *E. faecium*, *E. faecalis* posed a significant threat to hospital-acquired enterococcal infections in the late

1970s and early 1980s because of their high inherent virulence (Arias & Murray, 2012; Zhong et al. 2017). *E. faecalis* originated in an extra-enteric environment and only recently adapted to the gastrointestinal tract of animals (Olvera-García, Sanchez-Flores, and Quirasco Baruch 2018). *Enterococcus* species have become a growing public health concern due to the rise of multidrug-resistant strains. Their adaptability, antibiotic resistance, and presence in environmental sources (shown in **Figure 1**) underscore their role in the global challenge of antimicrobial resistance.

This study aimed to isolate and characterize Enterococcus strains from fruits and vegetables, assess their virulence factors and AMR profiles, and compare resistance patterns across environmental sources to inform public health strategies.

2. Materials and Methods

2.1 Sample Collection and Preparation

A total of 48 samples (i.e., raw vegetables and grains, n=30; fruits, n=18) were purchased and collected from the local market and stored in a refrigerator with their original container until analysis. For enrichment, 25 g of each sample were added to 225 mL of Tryptic soy broth (Merck) then incubated for 24 hours at 35°C.

2.2 Culture Conditions

After enrichment, the inoculum was streaked onto the m-Enterococcus agar (Neogen) plate to isolate typical colonies (Enany et al. 2022). These plates were prepared by suspending 42,000 mg of m-Enterococcus agar powder in 1000 mL of purified water. Heated with frequent agitation and boiled for one minute to completely dissolve the medium. Cooled to 45–50°C and poured into plates to solidify. These streaked plates were incubated for the next 24 hours at 37°C. Isolates were stored at –80°C in a 16% glycerol solution.

2.3 Presumptive Identification

Presumptive Identification was done through cell morphology, colony characteristics, Gram staining, growth in the presence of 6.5% NaCl in the salt tolerance test, and esculin hydrolysis on bile- esculin (Franz et al. 2001; Olvera-García, Sanchez-Flores, and Quirasco Baruch 2018). Two *Enterococcus* species provide significant clinical concern agar. *Enterococcus* genus isolates were confirmed by oxidase and catalase tests.

2.4 6.5% NaCl Tolerance Test

The 6.5% NaCl tolerance test was performed according to the procedure outlined by (Gupta et al. 2022). Initially, 30 g of NaCl and 16 g of Brain Heart Infusion (BHI) were added to 500 mL of distilled water. The mixture was then autoclaved for 15 minutes at 121 °C. Separately, 1.6 g of bromocresol purple was dissolved in 100 mL of 95% ethanol. After autoclaving, the medium was cooled to 50°C, and 1 mL of the bromocresol purple indicator was added while maintaining the pH at 7.4. The solution was then poured into tubes and inoculated with the desired culture. The tubes were incubated at 37 °C for 24 to 48 hours. A positive reaction was observed when the indicator changed color from purple to yellow or when visible growth was visible, even if the indicator color remained the same. The *E. faecalis* ATCC 29212 strain was used as a control.

2.5 Gelatinase Activity

The gelatinase activity test was performed as per the protocol described previously (Teixeira et al. 2012; Lopes et al. 2006; Sakkaa, Zaghloul, and Ghanem 2022). Briefly, *Enterococcal* culture were inoculated on an agar plate containing 3% gelatin and incubated at 37 °C. The next day they were flooded with an ammonium sulfate saturated solution. Gelatinase activity is visible in the translucent zone surrounding the colonies.

2.6 Esculin Hydrolysis test

Esculin hydrolysis was assessed using bile - esculin agar as described by (Pillay, Zishiri, and and Adeleke, 2018). The bacterial cultures were inoculated onto the plates and incubated at 37°C for 24 hours. The color changes in the medium were observed the next day.

2.7 Tube Method

Biofilm formation was assessed using the tube adherence test method described (Mohamed et al. 2013). A loopful of culture was inoculated in 10 mL

Figure 1: Local market fruits and vegetables condition. This figure shows the condition of fruits (Mangifera indica) and vegetables (Cucurbita pepo, Lagenaria siceraria, Brassica oleracea), which are rotten and contaminated.

Figure 2: Typical pink pinpointed colonies of *Enterococcus* on m-Enterococcus media were small, round, and well-defined with a smooth texture. They appeared as pink color colonies.

of Trypticase soy broth with 1% glucose. Organisms from a nutrient agar plate with an overnight culture used, and the broth tubes were incubated at 37 °C. After decanting the cultures, the tubes were cleaned with phosphate-buffered saline (pH 7.3). The tubes were then dried and stained with 0.1% crystal violet. Any excess stain was removed using deionized water. The tubes were dried upside down and observed for the formation of biofilm. Biofilm formation was considered positive when a clear coating covered the wall and the base of the tube at the air-liquid interface (Ira et al. 2013; Mohamed et al. 2013; Neopane et al. 2018).

2.8 Scanning Electron Microscopy

According to previous descriptions, extracellular matrix formation and cell morphology were examined using scanning electron microscopy (SEM) (Mirani, et al. 2013). Slides of biofilm were cut into 4 mm slices, rinsed with distilled water to remove debris, and stained for 30 seconds with 0.2% uranyl acetate. Direct inspection with an

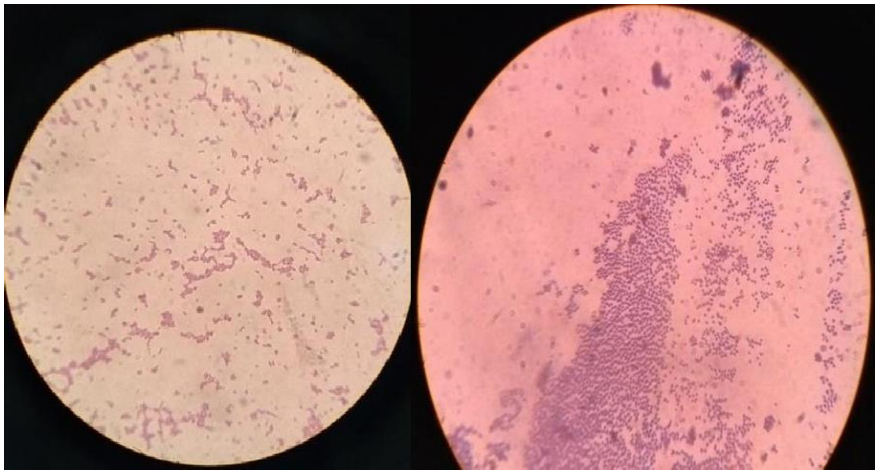

Figure 3: Gram-positive *Enterococcus* bacterium that appeared as spherical cells (cocci) under a microscope. It was in pairs (diplococci) and short chains.

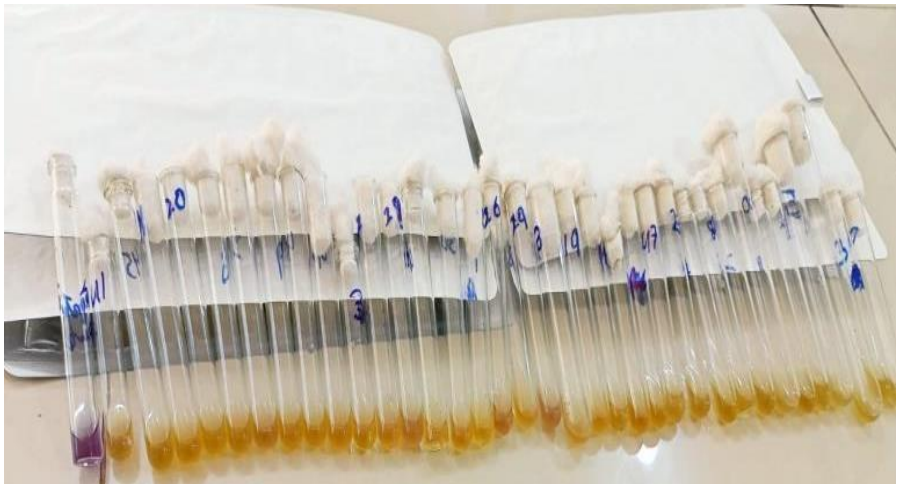

Figure 4: Positive tubes of 6.5% NaCl tolerance test, which change the indicator color from purple to yellow with visible growth, with a negative control.

electron microscope revealed the presence of biofilm material on these 4 mm slide sections.

2.9 Antibiotic Sensitivity Profiling

The disk diffusion method was employed to assess the antibiotic resistance profiles of various *Enterococcus* bacterial isolates towards many antibiotics, such as ampicillin (10 µg), vancomycin (30 µg), chloramphenicol (30 µg), linezolid (30 µg), teicoplanin (30 µg), nitrofurantoin (300 µg), erythromycin (15 µg), tigecycline (15 µg), (30 µg), and levofloxacin (5 µg). Physiological saline (0.85%) was used to prepare bacterial suspensions and ensure a close approximation to the McFarland standard of 0.5. These suspensions were then evenly spread on Mueller-Hinton (MH) agar plates using sterile cotton swabs. Subsequently, antibiotic discs were carefully positioned on the plates, followed by an incubation period of 18 hours at 37 °C. The results were recorded and interpreted based on the Clinical and Laboratory Standards Institute (CLSI) guidelines (Lewis et al. 2023) breakpoints (Alduhaidhawi et al. 2022). *Staphylococcus aureus* ATCC 25923 was used as control strain to ensure the accuracy of disc diffusion method, as per the CLSI guidelines (Lewis et al. 2023) for quality control in antimicrobial susceptibility testing.

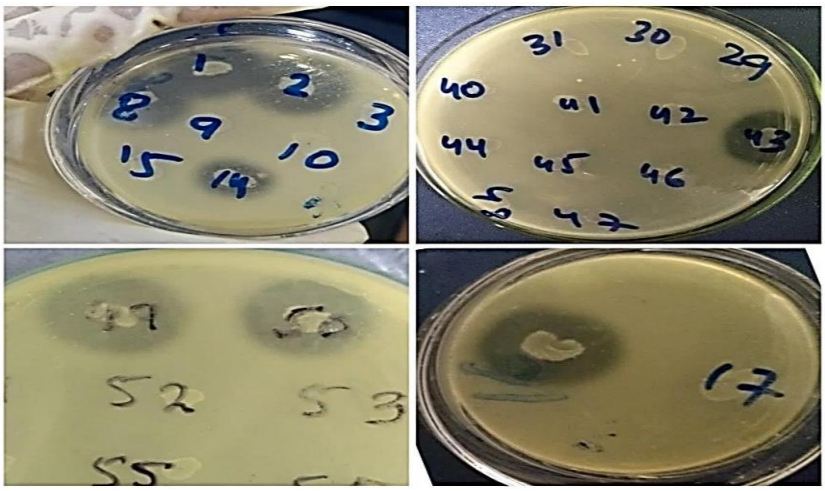

Figure 5: Images from gelatinase-positive and negative results, *Enterococcus* bacteria-inoculated gelatin agar plates, and the next day, saturated with ammonium sulfate. Positive (+): A clear whole zone around the colonies. Negative (-): No zone around the colonies.

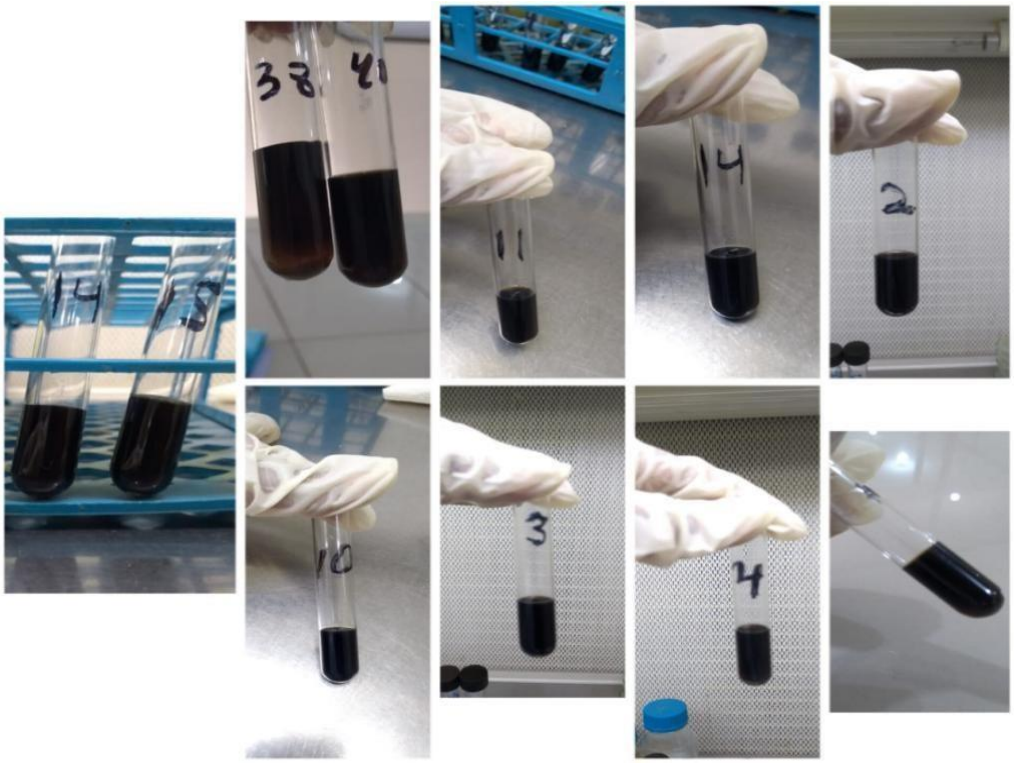

Figure 6: Positive bile esculin hydrolysis tubes. The blackening of the medium indicates the organism can hydrolyze esculin in the presence of bile salts.

3. Results

3.1 Morphology

A total of 48 samples (raw vegetables and grains, n= 30; fruits, n= 18) were analyzed, and 29 vegetables and 17 fruit samples tested positive for *Enterococcus*. The positive isolates on m-*Enterococcus* agar appeared as smooth, red, or pink colonies after incubation (**Figure 2)**. All *Enterococcus* genus isolates were confirmed by oxidase- and catalase-negative tests. **Figure 3**

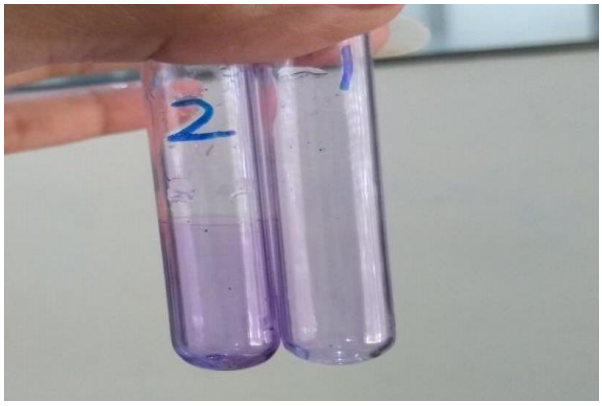

Figure 7: Biofilm positive and negative tubes. The tube labeled as 1 is negative for biofilm, which does not form a visible biofilm layer, suggesting a lack of strong adherence and biofilm-forming capability. The tube labeled as 2 is positive for biofilm and demonstrates the ability to form a visible biofilm layer on the inner surface, indicating its capacity for adhering and creating a protective community.

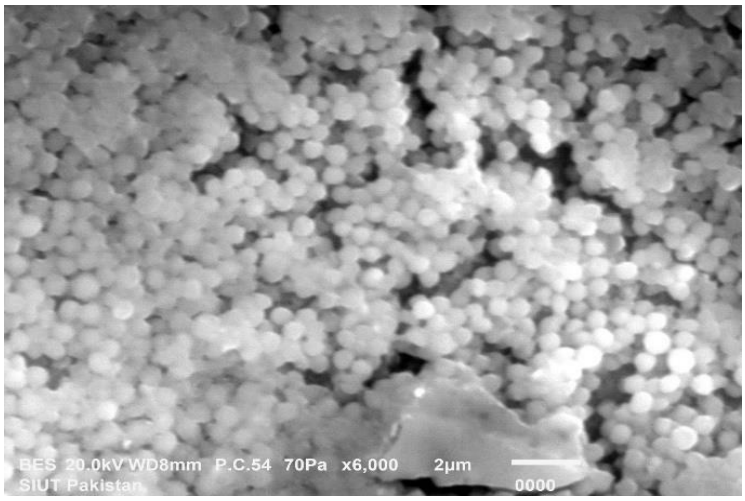

Figure 8: Scanning electron microscopy of *Enterococcus*. Biofilm-positive cells attach to solid surfaces and make multicellular aggregates packed into extracellular matrix material.

shows Gram-stained *Enterococcus* cells, appearing as Gram-positive cocci arranged in pairs (diplococci) or short chains under the microscope.

3.2 Salt Tolerance

Out of the 46 Enterococci isolates tested, 43 (93%) exhibited salt tolerance at a 6.5% NaCl concentration **(Table 1)**. The positive tubes of 6.5% NaCl test showed the colour change from purple to yellow, as illustrated in **Figure 4**. This observation indicates a high prevalence of salt-tolerant Enterococci in the environment.

3.3 Gelatinase Test

Approximately 32.6% of the *Enterococcus* isolates possessed gelatinase activity, as shown in **Table 1**. Gelatinase-positive isolates degraded gelatin and (formed a clear halo zone surrounding the growth, indicating gelatin degradation (**Figure 5**). In contrast, gelatinase-negative isolates lacked this enzymatic activity.

3.4 Esculin Hydrolysis

Among the 46 isolates of *Enterococcus* species tested, All showed positive results for esculin hydrolysis

Table 1: Overall presumptive test result, Positive = +, Negative = -

Sr.#	Samples	Enterococcus media	Biofilm by the tube method	Gelatinase	Bile esculin	Salt tolerant
1	Coriander	+	+	+	+	+
2	Sapodilla	+	+	+	+	+
3	Bottle gourd	+	+	-	+	+
4	Guava	+	+	-	+	+
5	Potato	+	-	-	+	+
6	Tomato	+	+	-	+	+
7	Onion	+	-	-	+	+
8	Strawberry	+	+	-	+	+
9	Turnip	+	-	-	+	+
10	Rice	-	no culture	no culture	no culture	no culture
11	Pickle	-	no culture	no culture	no culture	no culture
12	Cucumber	+	-	+	+	+
13	Melon	+	+	-	+	+
14	Papaya	+	+	-	+	+
15	Wheat grain	+	-	-	+	+
16	Green onion	+	-	+	+	+
17	Cauliflower	+	+	+	+	+
18	Bell pepper	+	-	+	+	+
19	Carrot	+	+	-	+	+
20	Mix vegetable	+	+	-	+	+
21	Banana	+	-	+	+	-
22	Potato	+	-	+	+	+
23	Oranges	+	+	+	+	+
24	Mix fruits	+	+	+	+	+
25	Lemon	+	-	-	+	+
26	Sapodilla	+	+	-	+	+
27	Guava	+	+	-	+	+
28	Cucumber	+	-	+	+	+
29	Taro root	+	-	-	+	-
30	Lady's finger	+	+	-	+	+
31	Peppermint	+	-	-	+	+
32	Cabbage	+	+	-	+	+
33	Carrot	+	+	-	+	+
34	Zucchini	+	-	+	+	+
35	Peas	+	+	+	+	+
36	Curry leaf	+	+	-	+	+
37	Green chilli	+	-	-	+	+
38	Zucchini	+	-	-	+	+
39	Sapodilla	+	+	-	+	+
40	Chilli	+	-	-	+	-
41	Tomato	+	-	-	+	+
42	Bell pepper	+	+	-	+	+
43	Cauliflower	+	+	-	+	+
44	Lady's finger	+	+	+	+	+
45	Melon	+	+	+	+	+
46	Carrot	+	+	-	+	+
47	Red chilli	+	-	-	+	+
48	Mix spice	+	-	-	+	+

(Table 1). The bile esculin agar medium exhibited a clear distinction between positive and negative reactions. Positive results were characterized by the development of dark brown or black pigmentation surrounding the bacterial colonies **(Figure 6)**, indicating the formation of the phenolic iron complex. In contrast, negative results were characterized by the absence of pigmentation or the presence of light brown coloration.

3.5 Biofilm Formation

Out of the 46 samples tested, 26 samples were found to be positive for biofilm formation using the tube adherence test method, indicating a prevalence rate of approximately 56.5% (26/46). The positive samples exhibited visible biofilm formation on the walls and bottom of the test tubes **(Figure 7)**, confirming the presence of biofilm-forming *Enterococcus* isolates**.** However, the method could not differentiate between moderate and weak biofilm-producing isolates**.**

3.6 Scanning Electron Microscopy

Biofilm- positive cells **(Figure 8**) were observed attached to solid surfaces, forming multicellular aggregates embedded in extracellular matrix material. This indicated the presence of a biofilm formed by cells that had attached to a solid surface and organized into structured multicellular clusters.

Additionally, the presence of an extracellular matrix material suggested that the biofilm was surrounded by a protective matrix secreted by the bacterial cells. Virulence factor percentages and

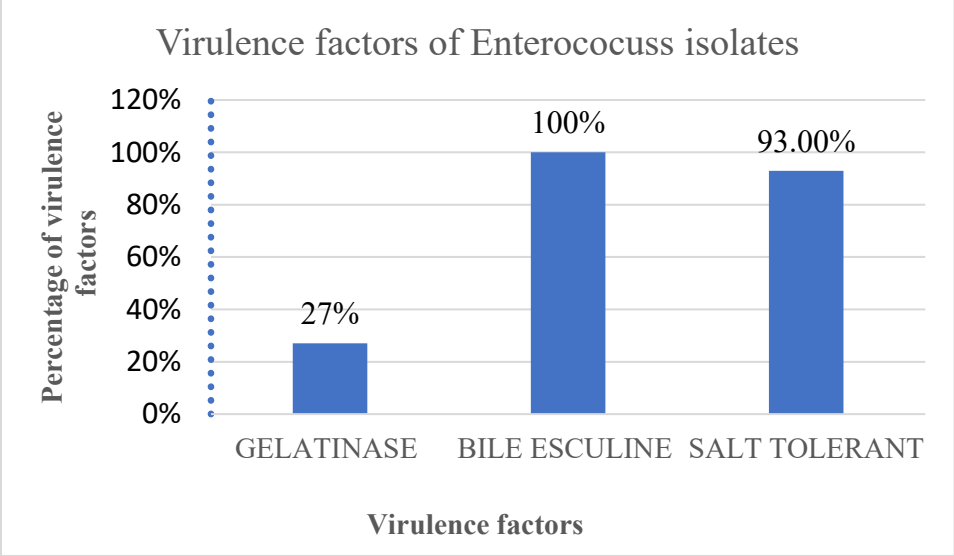

Figure 9: A total of 30 vegetable samples were tested, out of which 29 were positive for *Enterococcus*. On positive isolates, 27% of isolates show gelatinase activity, 100% of isolates show bile esculin activity, and 93% show salt tolerance.

antibiotic resistance patterns of *Enterococcus* isolates are shown in **Figures 9-11**.

3.7 Antibiotic Sensitivity Profiling

The presumptive *Enterococcus* isolates of vegetables and fruit samples were subjected to antimicrobial susceptibility testing against a panel of antibiotics. *Staphylococcus aureus* ATCC 25923 was used as a control strain. The CLSI breakpoints (CLSI, 2023) were used to analyze the results. The cutoff values for resistance were as follows: Ampicillin (≤16 mm), Vancomycin (≤ 14 mm), Chloramphenicol (≤ 12 mm), Linezolid (≤ 20 mm), Teicoplanin (≤ 10 mm), Nitrofurantoin (≤ 14 mm), Erythromycin (≤ 13 mm), Levofloxacin (≤ 13 mm), and Tigecycline (< 20 mm). The breakpoint of Tigecycline was interpreted based on the European Committee on Antimicrobial Susceptibility Testing (Committee et al. 2015). Antibiogram of Enterococcus isolates from vegetables and fruits are shown in **Table 2**. The resistance profiles of the isolates were as follows: Among the vegetables-associated Enterococci, the highest resistance was observed against Ampicillin (20.7%), followed by Erythromycin (17.2%). Resistance to Linezolid and Vancomycin was relatively low, with both antibiotics showing resistance in only 3.4% of isolates. No resistance was observed against Chloramphenicol, Levofloxacin, Teicoplanin, Tigecycline, and Nitrofurantoin in vegetable-associated Enterococci.

Among fruit Enterococci, Linezolid and Vancomycin showed the highest resistance rates at 35.3%. Erythromycin resistance was observed in 17.6% of isolates. Teicoplanin and Nitrofurantoin had relatively low resistance rates at 5.9%. No Resistance was observed against Chloramphenicol, Levofloxacin, and Tigecycline among fruit isolates (**Figure 12**).

Based on the susceptibility results (**Figure 13**) for Enterococci isolates from vegetables and fruit, as

- Nitrofurantoin: Vegetables: 96.6%, Fruits: 82.4%.
- Tigecycline: Fruits: 94.1%, Vegetables: 89.7%.
- Teicoplanin: Vegetables: 93.1%, Fruits: 82.4%.
- Levofloxacin: Vegetables: 82.8%, Fruits: 82.4%.
- Ampicillin: Fruits: 88.2%, Vegetables: 75.9%.
- Linezolid: Vegetables: 82.8%, Fruits: 52.9%.
- Chloramphenicol: Vegetables: 68.9%

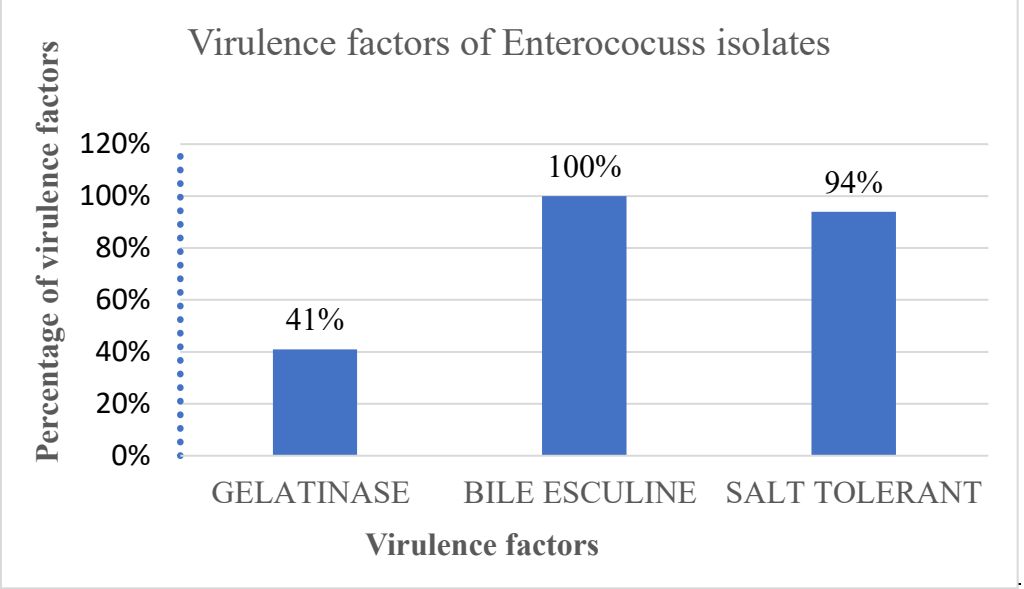

Figure 10: A total 18 fruit samples were tested, of which 17 were positive for *Enterococcus*. On positive isolates, 41% show gelatinase activity, 100% show bile esculin activity, and 94% show salt tolerance.

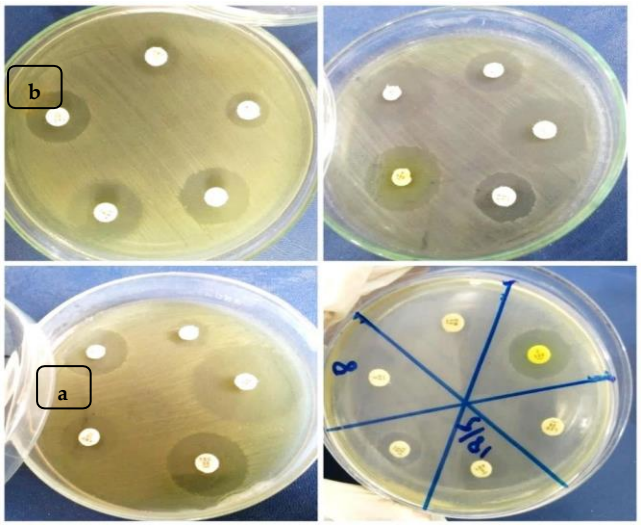

Figure 11: Antibiotic resistance through the disc diffusion method. Point "a" shows the zone of inhibition, whereas point "b" shows the resistance.

Fruits: 58.8%.

- Vancomycin: Vegetables: 68.9%, Fruits: 47%.
- Erythromycin: Vegetables: 20.6%, Fruits: 11.7 %.

4. Discussion

Outside environments such as sediments, soil, plants that grow in water, and ambient waters (rivers) all supported the survival of Enterococci. In contrast to the digestive system of warm-blooded animals, where their temperature is roughly constant, they can also be thought of as heterothermic (temperature-changing) inhabitants. Additionally, they mostly colonized the digestive system and made up 1% of the gut microflora (Mansour et al. 2023). It was observed that the overall isolation rate of Enterococci from

Table 2: Antibiogram of *Enterococcus* isolates from vegetables and fruits *CLSI 2023 breakpoints for *Enterococcus spp.*; S = Susceptible, R = Resistant, ZOI =Zone of Inhibition.

Antibiotic (Disc Potency)	CLSI Breakpoints (mm)*	Control ZOI (S. Aureus ATCC 25923)	ZOI Interpretation Range	Resistance (%) in Vegetable Isolates	Resistance (%) in Fruit Isolates
Ampicillin (10 μg)	S ≥ 17, R ≤ 16	18mm	12–16	20.7%	0%
Vancomycin (30 μg)	S ≥ 17, R ≤ 14	18mm	11–14	3.4%	35.3%
Linezolid (30 μg)	S ≥ 23, R ≤ 20	25mm	10–20	3.4%	35.3%
Teicoplanin (30 μg)	S ≥ 14, R ≤ 10	16mm	10	0%	5.9%
Nitrofurantoin (300 μg)	S ≥ 17, R ≤ 14	19mm	14	0%	5.9%
Erythromycin (15 μg)	S ≥ 23, R ≤ 13	24mm	10–13	17.2%	17.6%

the samples examined was vegetable (96%) than in fruit (94%). Also, all Enterococcus genus isolates were confirmed by oxidase -and catalase-negative tests (Pandey et al. 2023; Enany et al. 2022). We found that out of the 46 Enterococci isolates tested, , 43 isolates (93% in vegetables and 94% from fruits) exhibited salt tolerance when exposed to a 6.5% NaCl concentration. These results are inconsistent with the findings of (Lim and Hammer 2015), who reported 83% growth of *E. faecalis* isolates in 6.5% salt concentration. The high prevalence of salt-tolerant Enterococci aligned with (Enany et al. 2022), who suggested environmental adaptability and investigated the salt tolerance of Enterococci and reported that *Enterococcus* can grow in 4% bile esculin, tolerates 6.5% NaCl and heat 45 °C**.**

The high prevalence of salt-tolerant Enterococci isolates in the environment has significant implications for food safety, environmental health, and public health. These findings enhanced our understanding of the adaptive capabilities of Enterococci and their potential to thrive in high-salt conditions. Previous research has suggested various mechanisms through which these bacteria develop salt tolerance. These mechanisms involve the expression of specific genes or proteins that help them adapt to high-salt environments.

The presence of gelatinase activity in *Enterococcus* strains has been linked to their virulence potential and pathogenicity. Gelatinase enzymes facilitate tissue invasion by degrading extracellular matrix components, such as collagen and gelatin, which can aid in the dissemination of the bacteria within the host. Therefore, understanding the prevalence of gelatinase activity among *Enterococcus* isolates is crucial for assessing their pathogenicity and associated risks.

The identification of 27% of isolates from vegetables and 41% from fruit isolates exhibiting gelatinase activity suggests that a significant proportion of *Enterococcus* strains in this study possess the potential to cause tissue damage and invasive infections. These findings highlight the variability in gelatinase production among *Enterococcus* species. Our results were similar to the findings of (Ira, Sujatha, and Chandra 2013), that 31% of the isolates were positive for gelatinase activity. Gelatinase-positive strains may be more capable of colonizing and surviving in the host environment, thereby increasing their virulence and likelihood of causing severe infections. These findings are consistent with previous studies that have reported a positive association between gelatinase production and the severity of Enterococcal infections. It is worth noting that the absence of gelatinase activity in certain *Enterococcus* isolates does not necessarily imply their non-virulence, as other virulence factors,

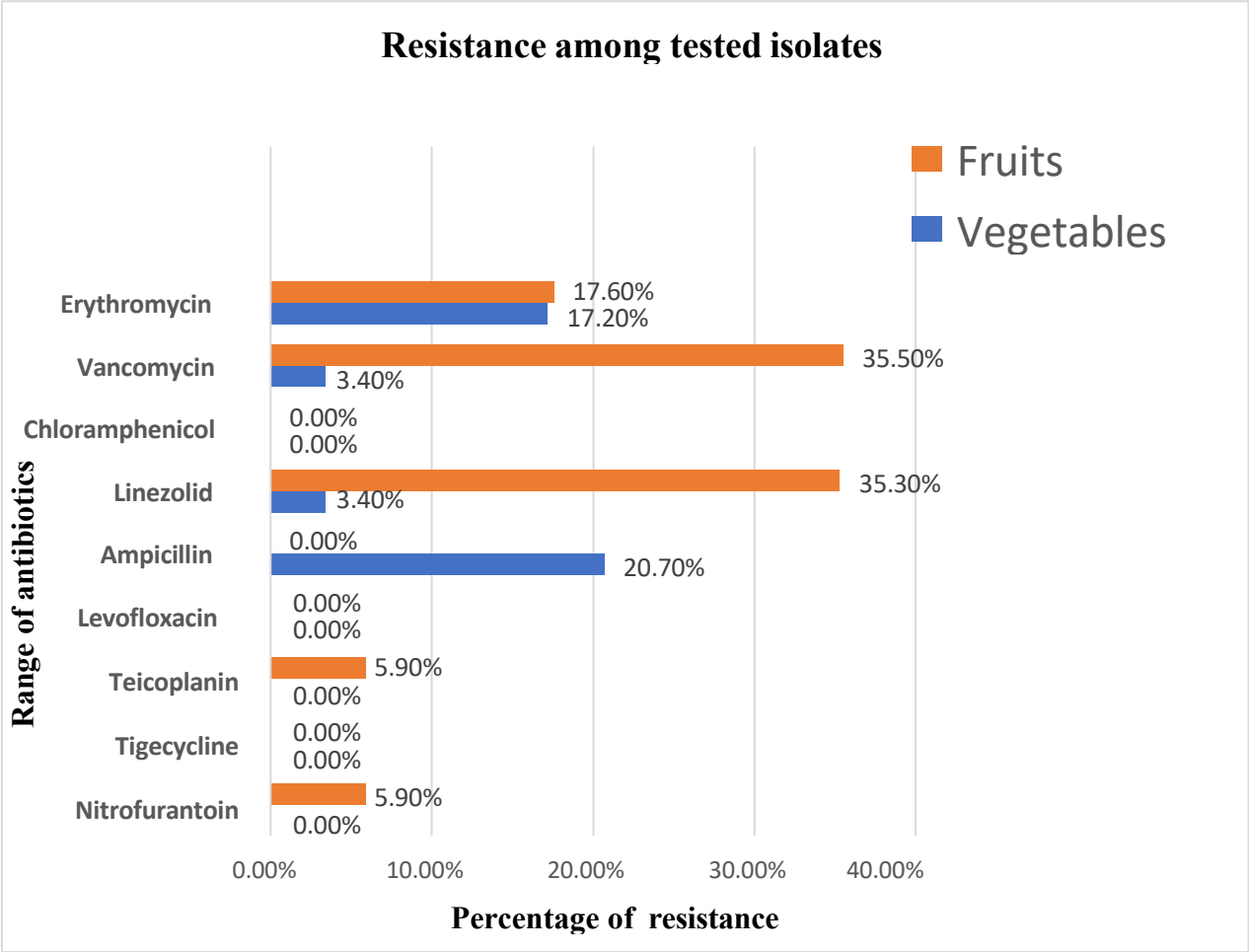

Figure 12: Incidence of antibiotic resistance in *Enterococcus* species isolated from vegetables and fruit samples.

such as adhesion molecules and toxin production, may play significant roles in the pathogenesis of Enterococcal infections.

The high prevalence of gelatinase-negative isolates (approximately 67%) observed in this study suggests that these strains may possess alternative mechanisms for invasion and tissue damage or be less virulent**.** Characterizing the factors responsible for the absence of gelatinase activity in these strains may provide valuable insight into the mechanisms of Enterococcal pathogenicity and aid in the development of targeted therapeutic interventions.

The uniform positive results for esculin hydrolysis in all 29 vegetables and 17 fruit isolates of *Enterococcus* species confirm the presence of esculin hydrolase enzyme in these strains. The ability to hydrolyze esculin suggests that these strains possess the enzymatic machinery necessary for the breakdown of esculin into glucose and esculetin. Moreover, the subsequent reaction of esculetin with ferric iron salt further supports the presence of this enzymatic activity, resulting in the formation of the phenolic iron complex responsible for the observed color change.

The detection of esculin hydrolysis in *Enterococcus* species is consistent with their well- known capability to produce esculin hydrolase, an enzyme encoded by the Esc gene. This enzyme is important for the survival and adaptation of *Enterococcus* in various environments, as it enables

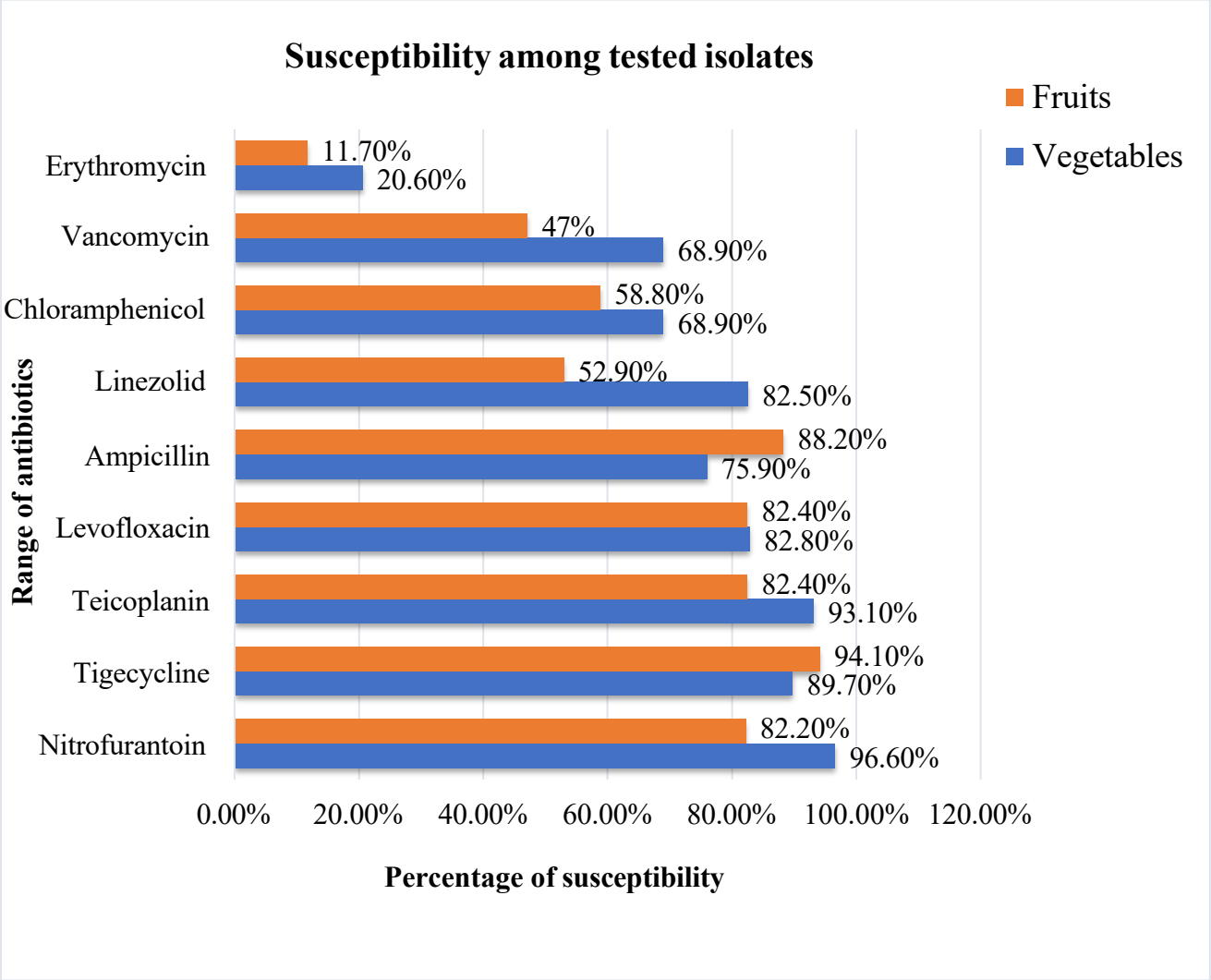

Figure 13: Incidence of antibiotic susceptibility in *Enterococcus* species isolated from vegetables and fruit samples.

the utilization of esculin as a carbon source. The ability to hydrolyze esculin is a characteristic feature of Enterococcus species and can aid in their differentiation from other Gram- positive bacteria. The bile–esculin agar medium used in this study provided a reliable and easily interpretable method for detecting esculin hydrolysis. The distinct color change from light brown to dark brown or black facilitated the differentiation of positive and negative results, allowing for rapid identification of *Enterococcus* species. However, it is important to note that the detection of esculin hydrolysis alone does not confirm the pathogenic potential or clinical significance of the *Enterococcus* isolates.

The findings of this study reveal a substantial prevalence of biofilm-forming *Enterococcus* strains among the tested samples. The observed prevalence rate of 56.5% suggests that more than half of the samples harbor *Enterococcus* strains capable of biofilm formation, which correlates with the results of (Oli et al. 2012) who reported that 55% were detected to have moderate capability of forming biofilms. This highlights the importance of considering biofilm-related issues when dealing with *Enterococcus* infections and implementing appropriate preventive and control measures.

The tube adherence test method employed in this study has been widely used to assess biofilm formation due to its simplicity and cost-effectiveness. However, it is important to acknowledge that this method is a qualitative assessment and does not provide quantitative data regarding the extent or thickness of the biofilms formed.

The high prevalence of biofilm-forming *Enterococcus* strains found in this study aligns with previous research highlighting the ability of *Enterococcus* species to develop biofilms. The formation of biofilms not only enhances the survival and persistence of *Enterococcus* strains but also contributes to their pathogenic potential. Biofilms can act as a protective barrier against host immune responses, making infections more difficult to treat and eradicate them.

Bacteria in natural environments tend to come together and form cohesive microbial communities known as biofilms. These biofilms can be found on any surface that comes into contact with natural liquids. The process of biofilm formation consists of a series of stages that remain consistent (Svensäter and Bergenholtz 2004).

After initial attachment, this occurrence occurs, and the quantity of bacteria released is correlated with the quantity of bacteria involved in the formation of the biofilm. Additionally, the biofilm stage serves as a protective barrier for the microbial community, shielding it from host defenses and antimicrobial substances (Mah and O'Toole 2001).

The biofilm formation process begins with the development of a conditioning film on the surface, to which planktonic bacteria adhere. This initial attachment can be reinforced through the production of extracellular polymer substances and the unfolding of cell-surface structures. As the biofilm matures, bacteria continuously detach from it and enter the planktonic phase. These released cells act as a consistent reservoir for persistent infections, enabling the continuous spread and survival of pathogenic populations (Costerton 2001).

The resistance of bacteria within a biofilm is significantly higher, ranging from 2 to 1,000 times greater, compared to the same bacteria in their planktonic state (Stewart and Costerton 2001).

The clinical implications of biofilm-forming *Enterococcus* strains are significant, as they are often associated with hospital-acquired infections, particularly in immunocompromised individuals or those with indwelling medical devices. The increased resistance of biofilm-associated bacteria to antimicrobial agents further complicates treatment strategies. Therefore, understanding the prevalence and characteristics of biofilm-forming Enterococcus strains is crucial for developing effective prevention and control measures.

The findings of this study highlight a significant presence of biofilm in the randomly selected samples. The prevalence rate of 53.6% indicates that biofilm formation is relatively common in the tested samples (Necidová et al. 2009). It is proposed that *Enterococcus* spp. have a proven biofilm formation capability, with 28% of the strains assessed as biofilm positive.

Enterococci have been linked to biofilm formation on a variety of implanted medical devices, including artificial hip prostheses, urine catheters, and prosthetic heart valves. This ability to generate biofilms has been seen as a key pathogenic feature for these organisms. The capacity of bacteria to create biofilms has been investigated using a variety of techniques, including the microscopic biofilm formation assay and epifluorescence microscopy (Ira, Sujatha, and Chandra 2013).

In SEM, biofilm-positive cells were observed to be attached to a solid surface and formed multi-cellular aggregates, as shown in **Figure 8**. These aggregates were tightly embedded into a structure known as the extracellular material (ECM). The presence of biofilm-positive cells attached to a surface and organized in this manner indicates their ability to form biofilms, which are complex communities of bacteria embedded

within a self- produced matrix. This ECM provides structural support and protection to the bacterial community. It consists of a combination of polysaccharides, proteins, and DNA, which contribute to the stability and adhesion of the biofilm. Vegetable results demonstrate the presence of antibiotic resistance among vegetable-associated Enterococci. Ampicillin and Erythromycin associated showed the highest rates of resistance, with 6 and 5 resistant isolates Ampicillin (20.7%), (Similar result showed in (Korajkic et al. 2021) 24.2% for ampicillin), followed by Erythromycin (17.2%), respectively. This indicates a concerning level of resistance to commonly used antibiotics in the treatment of Enterococcal infections. The emergence of resistance to Ampicillin and Erythromycin raises concerns about the potential spread of these resistant strains to humans through the consumption of contaminated vegetables.

The presence of resistance to Linezolid and Vancomycin, showed resistance in only 3.4% of isolates similar findings were found in (Korajkic et al. 2021) which showed 2.4% for vancomycin these antibiotics are reserved for the treatment of multidrug-resistant infections, is particularly alarming. The identification of resistant isolates to these last-resort antibiotics suggests a need for enhanced surveillance and strict infection control measures to prevent the dissemination of these resistant strains. the absence of resistance to Tigecycline, Chloramphenicol, Nitrofurantoin, and Levofloxacin indicates that these antibiotics remain effective against vegetable-associated Enterococci. This provides potential treatment options in case of infections caused by susceptible strains.

Fruit results indicate the presence of antibiotic resistance among the presumptive isolates of fruit Enterococci. Notably, resistance to Linezolid, Vancomycin (35.3%) and Erythromycin was observed (17.6%), highlighting the potential risk associated with these isolates. Linezolid and Vancomycin are critically important antibiotics used in the treatment of severe infections caused by multi-drug-resistant bacteria, including *Enterococci.* The emergence of resistance to these antibiotics among fruit-associated *Enterococci* raises concerns about the possible transmission of resistance genes to human pathogens.

The presence of resistance 5.9% of Teicoplanin and Nitrofurantoin similarly (Korajkic et al. 2021) showed 3.68% for teicoplanin, which is commonly used for urinary tract infections, suggests that fruit *Enterococci* may have acquired resistance mechanisms from other environmental sources. However, the resistance rates for Ampicillin, Tigecycline, Chloramphenicol and Levofloxacin were relatively low or absent, indicating that these antibiotics may still be effective against fruit-associated *Enterococci.* It is noted that the absence of resistance to some antibiotics, such as Teicoplanin, Tigecycline, Chloramphenicol, Vancomycin, and Nitrofurantoin among the tested isolates is encouraging.

Overall, the comparative analysis reveals variations in resistance patterns among *Enterococci* isolated from different environmental sources. It highlights the importance of considering these variations when formulating strategies for antibiotic stewardship, food safety, and environmental management to mitigate the spread of antibiotic resistance.

5. Conclusion

The study reveals a high level of salt tolerance in Enterococci, indicating their adaptability to saline environments. Gelatinase-positive strains suggest virulence potential, necessitating investigation into their pathogenic mechanisms. Bile-esculin hydrolysis is widespread among *Enterococcus* isolates, aiding in their identification. Biofilm formation is prevalent in *Enterococcus* strains, posing challenges for infection control, especially in healthcare settings. The study underscores the importance of combating antibiotic resistance in vegetable-associated Enterococci for food safety.

The study also identifies varying levels of

antibiotic resistance in vegetable and fruit-associated Enterococci.

Concerningly, Ampicillin, Linezolid, and Vancomycin resistance were observed, highlighting the urgent need for surveillance and prudent antibiotic use. However, antibiotics like Tigecycline and Chloramphenicol show effectiveness. In fruits, Teicoplanin resistance is low, while Linezolid and Vancomycin resistance are notable. Vigilant monitoring and research are essential to address resistance and safeguard public health. Future research can explore the genetic mechanisms behind antibiotic resistance, biofilm formation, and salt tolerance in *Enterococcus* strains. Additionally, long-term surveillance using genomic tools can help track the spread of resistance in food-related environments.

Conflict of Interest

The authors have no financial, personal, or professional relationships that could be perceived to influence the results or interpretation of this work.

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Ethical Approval

Not applicable, since the work does not involve any study involving human participants or animals.

Consent Forms

NA

Author Contributions

Main idea and conceptualization, initial draft by AI and LB, analysis and proofreading by LB, review editing and final draft by AI.

All authors read and approved the final manuscript.

Data Availability

All the data related to this manuscript, including research articles that were analyzed for this study, are available from the authors.

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