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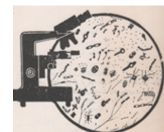

Tetracycline Resistance Genes Prevalence in *Enterococcus* spp. from Dairy Products in Egypt

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THE FREQUENCY and dispersion of tetracycline resistance and virulence traits were investigated in *Enterococcus* spp. isolated from milk and traditional cheeses in Egypt. Forty-seven presumptive enterococci were isolated, whereas thirty-four *Enterococcus* spp. isolates were examined for tetracycline antimicrobial sensitivity and antibiotic resistance genes (TET). The findings displayed that *Enterococcus* spp. isolates were highly resistant to amoxicillin /clavulanate, followed by oxytetracycline, ampicillin, tetracycline, doxytetracycline and ciprofloxacin. The frequency for tetracycline resistance genes*(tet* K *, tet*M *, tet*O and *tet* S) was estimated at 85.29% (29/34), 67.65% (23/34), 50 % (17/34) and 29.4 % (10/34) respectively. Six isolates out of seventeen were identified using 16SrRNA as *Enterococcus faecalis*. Seventeen isolates of *Enterococcus* spp. and *Enterococcus facials* isolates were tested for virulence traits. Findings displayed that all the tested Enterococci isolates produced biofilm and gelatinase enzyme except one isolate, which was negative for gelatinase production. On the other hand, all the tested isolates (17) were negative for hemolysin production. It could be concluded that *Enterococcus* spp. and *E. faecalis* have continually been recognized as adaptable pathogens, an indicator of faecal pollution in food, and documented for their aptitude to remove genetic material through portable genetic materials and distribute antibiotic resistance amongst extra bacteria. Therefore, faecal contamination of milk and its products must be prevented by applying strict hygienic measures and control of antibiotic use.

Keywords *Enterococcus* spp., Tetracycline-resistance genes, *tet*K*, tet*M*, tet*O*, tet*S genes, Virulence traits.

Introduction

Enterococcus spp. are among the lactic acid bacteria assemblage, whereas several different characteristics have been stated. Demirci et al. (2021) and Rhoades et al. (2021) defined *Enterococcus* spp. as the subdominant microbiota of numerous artisanal cheese kinds that augment to evolving of a specific taste and aroma through the maturing of certain cheeses (Hanifian, 2020). While, Haghi et al. (2019) and Heidari et al. (2016) reported that *Enterococcus* spp. has continually been recognized as an adaptable pathogen and stated as an important cause of nosocomial contagions, comprising severe urinary apparatus contagions, bacteremia, operating wound contagions, then bacterial endocarditis. It was reported that the Enterococci genus is catalase-negative, gram stain-positive cocci that commonly occurs in couples or small chain, non-spore-forming, facultative anaerobic bacteria, resist to the adverse condition such as low pH (4.5-10.0), high salinity and high temperature (5-65ºC) (Bondi et al., 2020). Two species, *E. faecalis* and *E. faecium,* of Enterococci were the most common and are accountable for a growing ratio of nosocomial contagions (Conde-Estevez et al., 2011; Van Tyne & Gilmore, 2014). It was indicated that *E. faecalis* strains have higher potential pathogenicity than *E. faecium* strains (Yuksel et al., 2015).

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Enterococcus spp. are a sign of faecal pollution in food and can be employed as a bacteriological method of sanitization principles for food and drinking water (Halkman & Halkman, 2014; Hanifian & Khani, 2016). *Enterococcus* spp. are also documented for its aptitude to transfer genetic material by portable genetic materials and distribute antibiotic resistance amongst extra bacteria (Haubert et al., 2018). The creation of biogenic amines (e.g., histamine and tyramine) and biofilm production are among the additional chief virulence characteristics of *Enterococcus* spp. (Linares et al. 2011; Shridhar & Dhanashree, 2019).

Enterococci may present in large numbers in dairy products (up to 10^8 CFU/mL) (Giraffa, 2003). Gelsomino et al. (2002) demonstrated that the milking machine and the bulk tank are important sources of enterococci in milk and dairy products. Mastitis can also be a source of multidrug-resistant enterococci (Wu et al., 2016). Raw milk may serve as a source of enterococci for dairy products, even when pasteurization is applied while processing a posteriori. There are indeed reports of enterococci surviving pasteurization temperatures (Giraffa et al., 1997). *Enterococcus faecalis, E. faecium, E. durans, E. hirae,* as well as *E. saccharominimum* and *E. italicus*, have been isolated from the raw milk of cows, goats and sheep (Callon et al., 2007; Kakgli et al., 2007; Perin et al., 2014; McAuley et al., 2012, 2015; Wu et al., 2016; Bouymajane et al., 2018).

Enterococcus faecalis bacteria are important microbes in human and farm animals' gastrointestinal tracts (Vu & Carvalho, 2011). *E. faecalis* strains can live in hot, saline, or acidic environments (Byappanahalli et al., 2012). They are mainly found in the soil, water, and the environment and can easily be adapted to the gastrointestinal tract conditions of their hosts (Daniel et al., 2017). *E. faecalis* contagions are mainly spread from one person to another through poor hygiene. Therefore, these bacteria are present in the stool. The bacteria can be transferred to foods through insufficient sanitation and food handling. Foods, especially ready-to-eat food samples and those with animal origins, may represent the sources of microbes transmission (Hammerum, 2012; Hanchi et al., 2018; Fiore et al., 2019). Previous research has shown that due to the prevalence of *E. faecalis* in nosocomial infections, studies have suggested that the hospital setting is a source of antibiotic-resistant bacteria (Ruiz-Garbajosa et al., 2006). Other studies have suggested environmental sources such as animals and water that could serve as an essential sources for antibiotic-resistant *Enterococcus faecalis* strains (Mallon et al., 2002)

Due to their antimicrobial properties, tetracycline's antimicrobial has a wide-spectrum effect, and it is commonly utilized as therapy in humans and animals (Chopra & Roberts, 2001). Roberts (2012) reported that tetracycline resistance universally seems as developed antimicrobial resistance (AMR) in the genus of *Enterococcus*. In various countries, tetracycline is consumed in veterinary and watery cultures comparable to an animal progression supporter. The coexistence of bacteria may act as reservoirs of antibiotic resistance genes dispersal these resistance genes to microorganisms, humans and the environment. Consequently, tetracycline resistance genes have been perceived in medical and ecological strains (Chopra & Roberts, 2001; De Leener et al., 2004; Poeta et al., 2005). But, countless revisions have told that AMR has continued because of horizontal transmission of antimicrobial resistance genes (Hegstad et al., 2010; Palmer et al., 2010; Cho et al., 2019) and linkage to other classes of antibiotics (Huys et al., 2004, Rizzotti et al., 2009). The principal collection comprises *tet*M, *tet*O and *tet*S genes coding ribosomal protection proteins, and the subsequent collection encodes tetracycline efflux pumps proteins by the *tet*K and *tet*L genes (Chopra & Roberts, 2001; Huys et al., 2004; Poeta et al., 2005; Kobashi et al., 2007).

Enterococci pheromone encouraged collecting, gelatinase, enterococcal surface protein and biofilm creation (Aslam et al., 2012; Jahan & Holley, 2014). This investigation aimed to discover the tetracycline resistance patterns, the dispersal of *tet*K, *tet*M, *tet*O and *tet*S genes and assess virulence traits in enterococci strains isolated from milk and traditional cheese in Egypt.

Materials and Methods

Sample collection

A total of forty-one random samples of milk and four types of milk products, including karish cheese, Romy cheese, bramely cheese and mish. The samples were collected in Cairo, Egypt, between September 2017 and April 2018. The

samples were collected and kept at +4 to +6°C before analysis.

Isolation and identification of Enterococci

Growth characteristics were tested in de Man-Rogosa-Sharpe agar (MRS) and Citrate Azide agar (CA) (Domig, 2003; Naceur & Boudjemâa, 2016). Based on catalase-negative and gram-positive, the suspected Enterococci were isolated and identified as presumptive Enterococci. The isolates of *Enterococcus* spp. were stored at -70°C in MRS broth containing 20% (v/v) glycerol for further analysis. Before use, the strains were cultivated twice for 24h at 37°C in MRS broth (Todorov et al., 1999).

Antimicrobial susceptibility testing

The antimicrobial resistance profile of thirty-four *Enterococcus* spp. isolates were determined using the standard disk diffusion method with an interpretation of breakpoint criteria established according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2011). The antibiotics used in this study; oxytetracycline (OXY, 10μg), doxytetracycline (DOX, 10μg), tetracycline (TET, 30μg), amoxicillin/clavulanate (AMC, 10/10μg), ampicillin/ (AMP, 10), imipenem (IPM, 10μg), ciprofloxacin (CIP, 5μg), and colistin sulphate (10μg). All disks were purchased from Oxoid, USA. Mueller Hinton Agar (MHA) plates (Merck, Germany) were inoculated with a bacterial suspension equal to 0.5 McFarland (1.5×10^5 CFU/mL) and incubated at 37°C for 18–24h.

PCR identification

DNA extraction

In this study, PCR assay was applied to investigate resistance genes among 34 *Enterococci* spp. isolates according to Ng et al. (2001) (Table 1) and PCR identification of *Enterococcus faecalis* isolates according to Zoletti et al. (2006) (Table 2). DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200µL of the sample suspension was incubated with 20µL of proteinase K and 200µL of lysis buffer at 56OC for 10min. After incubation, 200µL of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with the kit's 100µL of elution buffer. Oligonucleotide primers used were supplied from Metabion (Germany).

PCR amplification

Primers were utilized in a 50µL reaction containing 12.5µL of Emerald Amp Max PCR Master Mix (Takara, Japan), 1µL of each primer of 20 pmol concentration, 5.5µL of water, and 5µL of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler.

Analysis of the PCR products

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20µL of the products were loaded in each gel slot. A gene ruler 100bp ladder (Fermentas, Thermo, Germany) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra), and the data was analyzed through computer software.

TABLE 1. The genes encoding tetracycline, including *tet*K, *tet*M, *tet*O, *tet*S were detected by PCR amplification

Gene	Sequence	pb	Reference
*tet*K,	TCG ATA GGA ACA GCA GTA CAG CAG ATC CTA CTC CTT	169	Ng et al. (2001)
*tet*O	AAC TTA GGC ATT CTG GCT CAC TCC CAC TGT TCC ATA TCG TCA	515	
*tet*S	CAT AGA CAA GCC GTT GAC C ATG TTT TTG GAA CGC CAG AG	667	
*tet*M	GTG GAC AAA GGT ACA ACG AG CGG TAA AGT TCG TCA CAC AC	406	

TABLE 2. Primers sequences, target genes, amplicon sizes and cycling conditions

Target agent	Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
					Secondary denaturation	Annealing	Extension		
E. faecalis	*16S rRNA*	GTT TAT GCC GCA TGG CAT AAG AG CCG TCA GGG GAC GTT CAG	310	94˚C 5min	94˚C 30sec	50˚C 40sec	72˚C 45sec	72˚C 10min	Zoletti et al. (2006)

Quantitative biofilm assay

Antimicrobial Biofilm formation was assessed according to the methods described by Stepanovic et al. (2007). Enterococci from an overnight culture were cultivated in trypticase soy broth (TSB) supplemented with 1% glucose and incubated for 24h at 37°C. The culture density was adjusted to an approximate 0.5 McFarland standard. Each culture was diluted in sterile TSB (1:100), and 200µL from each was transferred to three wells of sterile 96-well polystyrene microtiter plates (Sigma-Aldrich, St. Louis, MO USA). A sterile TSB was used as a negative control, and *E. faecalis* (ATCC, 29212) was used as a positive control. The plates were incubated at 37°C for 48h, washed with sterile phosphate-buffered solution, air-dried, and stained with 2% crystal violet for 30 min. Subsequently, the wells were gently washed with sterile deionized water and air-dried. The dye bound to the adherent cells was re-solubilized with absolute ethanol (150µL). Each well's optical density (OD) was measured at 570nm in a plate reader (BioTek-800 ST, St. Louis, MO, USA). The experiment was performed in triplicate on three different days. Each *Enterococcus* isolate was classified as a negative, weak, moderate, or strong biofilm producer following the criteria described by Stepanovic et al. (2007). Any optical density value >0 after the correction was considered positive. Strains were classified as 1-biofilm non-producers (OD595 ≤0), 2-weak (OD595 <0.2), 3-medium (OD595 ≥0.2 to <0.5), 4- strong (OD595 ≥0.5).

Gelatinase assay

Liquefaction was characterized as described elsewhere by Marra et al. (2007). Briefly, samples were inoculated into tubes containing 4mL of MRS broth with 4% gelatin. After incubation at 35-37ºC for 24h, tubes were cooled at 4ºC for 30min, and the liquefaction of the medium was observed.

Cytolysin activity

Enterococcus isolates were streaked on MRS agar supplemented with 5% horse blood and incubated at 37°C for 24h to investigate hemolysin production. A clear (ß or complete -hemolysis) or green (alpha or partial hemolysis) zone around the colonies was defined as positive, whereas the game hemolysis was defined as negative activity (De Vuyst et al., 2003).

Statistical analysis

The standard deviation has been calculated for the studied samples. In addition, the obtained data were treated statistically using analysis of variance as described by Snedecor & Cockran (1969).

Results and Discussion

Enterococci are not only potential pathogens but also a reservoir of genes encoding antimicrobial resistance genes that can be transferred to other microbes (Pesavento et al., 2014). It was reported that tetracycline resistance universally seems as a developed antimicrobial resistance (AMR) in the genus of *Enterococcus*. So this study is one of the recent and important studies designed to highlight the presence of enterococci in dairy products, the extent of its resistance to tetracycline antibiotics and the expected different mechanisms of bacterial resistance to antibiotics shown.

Prevalence of Enterococci in milk and dairy products

Forty-seven bacterial isolates were obtained from the forty-one Egyptian milk and traditional cheese samples. The bacterial isolates were found to be gram-positive and catalase-negative cocci and were identified as presumptive enterococci.

Enterococci isolates were distributed as follows; 5 isolates from milk, 18 isolates from kareish, 2 isolates from baramely, 7 isolates from Romy, and 15 isolates from mish samples (Table 3). These findings are similar to Abouzaid et al. (2022), who isolated 30 suspected enterococci isolates (10 from milk samples, 15 from kareish cheese samples and 5 from feta cheese samples).

Seventeen suspected enterococci isolates (8 and 9 isolates that harbor 4 and 3 tetracycline resistance genes, respectively) were selected to be identified by using 16SrRNA for *E. faecalis* (Table 3). Results revealed that 6 (35.29%) isolates out of 17 were *E. faecalis*, distributed as follows: one isolate from each milk, Romy cheese and karish cheese samples and three isolates from mish samples. Also, Abouzaid et al. (2022) isolated 15 (50%) isolates as *E. faecalis* from milk and dairy products. In the same line, by utilizing biochemical analysis and 16SrRNA gene sequencing (64.7%) were identified as *E. faecalis* in raw cow's milk (Bouymajane et al., 2018). Similarly, Amidi-Fazli & Hanifian (2022) found that *E. faecalis* represented the highest percentage of 48.55% (168) out of 346 isolates in artisanal dairy product samples in Iran.

Antibacterial susceptibility testing

Enterococcus spp. were resistant to several antimicrobial agents commonly used in clinics, such as tetracycline, vancomycin, B-lactams, erythromycin glycopeptides, and linezolid (Abbo et al., 2019). The highest prevalence of resistance amongst enterococci of dairy was tetracycline which was distinguished in 30.8% of the isolates. This could be recognized by the wide distribution of these antimicrobials in veterinary applications (Pieniz et al., 2015). Huys et al. (2004) exhibited that a significant percentage of tetracycline-resistant isolates exhibited co-resistance to erythromycin and/or chloramphenicol, advising that the choice of tetracycline genotypes may deliver an appropriate molecular basis for the more choice of numerous resistances.

Out of 47 isolates, 34 Enterococci isolates (28 *Enterococcus* spp. and 6 *E. faecalis*) were selected and tested for their sensitivity to different antibacterial agents. A very different prevalence of antibiotic resistance in contrast to the antibiotics tested was observed amongst enterococci isolates (Table 3). Generally, *Enterococcus* spp. isolates were highly resistant to amoxicillin /clavulanate (75%), followed by ampicillin (57.1%), oxytetracycline (53.57%), tetracycline (46.4%), doxytetracycline (28.57), ciprofloxacin,(25 %) and Imipenem (3.57%). All the *Enterococcus* spp. isolates were sensitive to colistin. While for 6 *E. faecalis* isolates, the highest resistance rate was doxytetracycline (100%), amoxicillin/clavulanate and oxytetracycline (83.3%), tetracycline (66.7%), ampicillin (33.3%), and ciprofloxacin,(16.7%).

TABLE 3 . Sources of Enterococcus spp. and E.faecalis and distribution of tetracycline genes

Sources	Total isolates number	Tested isolates no.		tet. (K, M, O, & S)Antibiotic resistant genes				
				One gene	Two genes	Three genes	Four genes	-ve gene
Milk	5	*E*. spp. *E.faecalis*	4 1	-	1(*tet*(K&M)	-	3	-
Baramely Cheese	2	*E*. spp. *E. faecalis*	2 -	1 (*tet* K)	-	1,(*tet*(K,M,O)	-	-
Romy Cheese	7	*E*. spp. *E. faecalis*	3 1	-	1(*tet*,K&M)	1(*tet*,K,M,S)	-	2
Karish Cheese	18	*E*. spp. *E. faecalis*	13 1	2 (*tet*K)	5{4(*tet*(K,M)& 1(*tet*(K,O) }	3{(1(*tet*(K,O,S)& 2(*tet*(K,M,O)}	2	2
Mish	15	*E*. spp. *E. faecalis*	7 3	1 (*tet*(K)	1(*tet*(K,M),,	4{ (*tet*(K,,M,O)}	3	1
Total	47	*E*. spp. *E. faecalis*	28 6	4	8	9	8	5
%				11.76	23.53	26.47	23.53	14.71

E. spp. = *Enterococcus* spp.

From the current results, it appears that the resistance rate to ampicillin (57.1%) and (33.3%), tetracycline (46.4%) and (66.7%), and ciprofloxacin (25 %) and (16.7 %) of *Enterococcus* spp. and *E. faecalis* respectively. In the same line, Bouymajane et al. (2018) found that the resistance rate of *Enterococcus* spp. was very high for ampicillin, tetracycline and ciprofloxacin. Also, Abouzaid et al. (2022) recorded that out of 30 *Enterococcus* spp. isolates from milk and dairy products in Egypt, the resistance rate was against ampicillin (56.67%) and tetracycline (36.67%). On the other hand, Tuncer et al. (2013) and Naceur & Boudjemaa (2019) mentioned that all *Enterococcus* spp. (23 isolates) were sensitive to ampicillin. Jin Kim et al. (2022) found that *E. faecalis* were susceptible to ciprofloxacin. Our results showed that (75%) of the tested *Enterococcus* spp. isolates were resistant to amoxicillin /clavulanate. Abouzaid et al. (2022) found that *Enterococcus* spp. were resistant to amoxicillin (60%). Our results showed doxytetracycline resistance (100%) and (28.57%) for *E. faecalis* and *Enterococcus* spp. isolates respectively. At the same time, Abd El-Tawab et al. (2019) found 14% of the tested Enterococci. Jin Kim et al. (2022) found that 3.4% of *E. faecalis* were resistant to doxycycline. Also, Abouzaid et al. (2022) and Jin Kim et al. (2022) reported that Enterococci isolates were susceptible to doxycycline (70%). On the other hand, Jin Kim et al. (2022) found that 20 *E. faecalis* isolates in bovine normal raw milk (NRM) were sensitive to doxycycline, while 4.3% out of 70 *E. faecalis* isolates in bovine mastitis milk(BMM) were resistant to the same doxycycline antibiotic.

Frequency of tetK, tetM, tetO and tetS genes

The prevalence of antibiotic-resistant enterococci in food-of-animal origin has become a material of worry, as these unsusceptible bacteria can be diffused to humans by the food cycle (McEwen, 2011). Accordingly, infections by these microbes are considered a serious health hazard. This is because they can't be treated with known antibiotics (Pesavento et al*.,* 2014). Due to virulence genes which can be supported on movable genetic materials, milk Enterococci can donate to the prevalence of these genes in humans (Yoon & Lee, 2021).

The technique of tetracycline resistance has been designated as the outcome of efflux pumps (*tet*K and *tet*L) and the proteins responsible for ribosomal protection (*tet*M, *tet*O, *tet*S, *tet*T, and *tet*W) (Roberts, 2005). In previous research, resistance mediated by *tet*M was stated to be the most common in the isolates from food of animal origin (Aarestrup, 2000). The expression of the highest degree of tetracycline resistance might help to explain the complementary technique of the efflux pump and proteins of the ribosome (Ammo et al., 2008).

The 34 isolates were tested for four tetracycline resistance genes (*tet*K, *tet*M, *tet*O and *tet*S). Data in Table 1 showed the PCR amplification of the *tet*K, *tet*M, *tet*O and *tet*S genes. Primers for *tet*K, *tet*M, *tet*O and *tet*S genes generated an amplicon of 169, 406, 515 and 667 base pairs (bp), respectively. The *tet*K in 4 (11.76%) and 7 (20.56%) of isolates harbor the *tet*K and *tet*M genes, whereas 1(2.94%) of isolates harbor the *tet*K and *tet*O genes. Meanwhile, 7 (20.56%) isolates harbor the *tet*K, *tet*M and *tet*O genes, and 1 (2.94%) of isolates harbor the *tet*K, *tet*O and *tet*S genes. On the other hand, 1 (2.94%) of isolates have the *tet*K, *tet*M and *tet*S genes and 8 (23.53%) of the isolates harbor the *tet*K, *tet*M, *tet*O and *tet*S genes (Table 3).

The four tetracycline resistance genes *tet*K, *tet*M, *tet*O and *tet* S were found in 85.29% of the 34 tetracycline-resistant isolates. This data are in the same line with Cho et al. (2020), who detected (*tet*(K), *tet*(L), *tet*(M), *tet*(O) and *tet*(S) in 93.9% (31/33) of the tetracycline-resistant Enterococci isolates from fresh water. Also, the current results showed that the tested genes *tet*K, *tet*M, *tet*O and *tet*S are present in 34 isolates by (82.1%) and (100%), (64.29%) and (83.3%), (42.86 %) and (83.3%) and (17.86 %) and (83.3%) out of 28 *Enterococcus* spp. and 6 *E. faecalis* isolates respectively (Table 4). These results showed that *tet*K, which encodes for efflux pumps, was the most commonly detected gene. These results are contrary to the previous readings or research, which showed that *tet*M is the most prevalent in enterococci from humans, animals, food, environment and freshwater (Aarestrup et al., 2000; Cauwerts et al., 2007; Jackson et al., 2010; Sadowy & Luczkiewicz, 2014; Cho et al., 2020). On the other hand, Abd El-Tawab et al. (2019) did not find *tet*M in 120 enterococcus isolates from milk and dairy products in Egypt. Both gram-negative and gram-positive bacteria showed the presence of the ribosomal protection gene, *tet*(M) gene, which is typically chromosomally located

(Chopra & Roberts, 2001; Roberts, 2005). The *tet*S was found in (29.4 %) of the tested isolates. This is the same line as Aarestrup et al. (2000), Huys et al. (2004), and Sadowy & Luczkiewicz (2014), who stated that *tet*S appeared in *E. faecalis* and *E. faecium* only. Alternatively, *tet*S was not detected in *E. faecalis* or *Enterococcus* spp. (Cho et al., 2020). In our results *tet*O gene was detected in (42.86 %) and (83.3%) out of 28 *Enterococcus* spp. and 6 *E. faecalis* isolates, respectively. On the other hand, Diarra et al. (2010) did not detect *tet*O genes in *E. faecalis* or *Enterococcus* spp.

Phenotypic detection of virulence factors

Previous research showed that enterococci do not produce potent toxins like some other bacteria, but they have virulence factors in the form of aggregation substrate, enterococcal surface protein, gelatinase and antibiotic resistance genes (Sava et al., 2010). The seventeen isolates that revealed the largest number of antibiotic resistance genes (8 and 9 isolates) that displayed 4 and 3 genes, respectively, were selected for PCR identification and virulence factors detection. Results showed that 6 out of 17 isolates were *E. faecalis*. These findings support the importance of monitoring *E. faecalis* in dairy products. The appearance of exact virulence factors and toxins increases the severity and pathogenicity of illness caused by *E. faecalis* (Goh et al., 2017). Several virulence factors add to the ability of Enterococci to cause several diseases (Abd El-Tawab et al., 2019).

Quantitative biofilm assay

The culture media composition significantly affects the performance of phenotypic estimation of biofilm production in terms of the presence/absence of biofilm precursors and the temperature and time of incubation. Therefore, in several readings, part of the discrepancy between biofilm production and output in phenotypic and genotypic estimations may be due to differences in incubation environments and culture media (Stepien-Pysniak et al., 2019). Sharma et al. (2019) reported that strong biofilms protect microorganisms from osmolality, nutrient deficiencies, extreme pH, mechanical, and shear forces, as well as host immune cells and antimicrobial agents. It was noted that there is a significant relationship between biofilm creation and antimicrobial resistance (Abouzaid et al., 2022). Biofilm structure provides an ideal microenvironment for development and facilitates the diffusion of portable genetic elements amongst bacteria (Sieńko et al., 2015).

TABLE 4. The correlation between phenotypic ad genotypic % of antibiotic resistance amongst Enterococcus isolates.

Antibiotic disc		**Resistant isolate no. %**	**Resistant genes %** *tet* K	*tet* M	*tet*O	*tet*S
1-Amoxycillin/ Clavulanate	*E.* spp.	21 (75)	23(82.1)	18(64.29)	12(42.86)	5(17.86)
	E.faecalis	5 (83.3)	6 (100%)	5(83.3%)	5(83.3%)	5(83.3%)
2-Ampicillin	*E*. spp.	16 (57.1)				
	E.faecalis	2 (33.3)				
3-Oxytetracycline	*E*. spp.	15 (53.57)				
	E.faecalis	5 (83.3)				
4-Tetracycline	*E*. spp.	13 (46.4)				
	E.faecalis	4 (66.7)				
5-Doxytetracycline	*E*. spp.	8 (28.57)				
	E.faecalis	6 (100)				
6-Ciprofloxacin	*E*. spp.	7 (25)				
	E.faecalis	1 (16.7)				
7-Imipenem	*E*. spp.	1 (3.57)				
	E.faecalis	0				
8-Colistin	*E*. spp.	0				
	E.faecalis	0				

- *E*. spp = *Enterococcus* spp.

The current results of biofilm formation of the 17 isolates (6 *E. faecalis* and 11 *Enterococcus* spp.) are shown in Table 5. Biofilm formation criteria were determined as follows: (OD595 ≤0), = non-biofilm formation, (OD595 <0.2), = weak biofilm formation, (OD595 ≥0.2 to <0.5), = moderate biofilm formation, and (OD595 ≥0.5)= strong biofilm formation. All 17 isolates produced biofilm with biofilm OD values ranging from 0.200±0.08 to 0.590±0.28. The isolates (6 *E. faecalis* and 11 *Enterococcus* spp.) were positive for biofilm formation; one isolate of *E. faecalis* gave a strong biofilm, and the other 16 isolates gave a moderate biofilm (Table 5). These data align with Furumura et al. (2006), who noticed that all thirty-two isolates of clinical *E. faecalis* displayed an aptitude for biofilms production. Jin Kim et al. (2022) noticed that the isolates from bovine mastitis milk (BMM) proved a significantly greater ratio of biofilm creation than bacteria from bovine normal raw milk (NRM). Also, Amidi-Fazli & Hanifian (2022) recorded that 75.55% of the *Enterococcus* isolates can produce biofilm. However, Diarra et al. (2010) recorded that none of the 69 isolates of different *Enterococcus* spp. from Broiler Chickens was found to produce biofilm.

TABLE 5. Virulence properties of *E. faecalis* and *Enterococcus* spp.

Isolate no	*Enterococcus* spp.	Antibiotic resistant genes tet (K, M, O & S)		Gelatin liquifaction	Biofilm strength OD	Biofilm formation activity	Hemolysis
		3genes	4genes				
2	*Enterococcus* spp.	*tet*(K, M&,O)	-	+	0.371±0.12d	medium	-
6	*Enterococcus* spp.	*tet*(K, M&,O)	-	+	0.496±0.25b	medium	-
14	*E.faecalis*	-	+	+	0.200±0.08f	medium	-
17	*Enterococcus* spp.	-	+	+	0.345±0.14e	medium	-
19	*E.faecalis*	-	+	+	0.307±0.10e	medium	-
21	*Enterococcus* spp.	-	+	+	0.363±0.09d	medium	-
25	*Enterococcus* spp.	-	+	+	0.367±0.21d	medium	-
26	*E.faecalis*	*tet(* K, M, &S)	-	+	0.212±0.15f	medium	-
30	*Enterococcus* spp.	-	+	-	0.474±0.22b	medium	-
32	*Enterococcus* spp.	*tet*(K, M&,O	-	+	0.406±0.20c	medium	-
33	*Enterococcus* spp.	*tet*(K, M&,O	-	+	0.378±0.16d	medium	-
34	*E.faecalis*	*tet*(K, M&,O	-	+	0.590±0.28a	Strong	-
36	*E.faecalis*	-	+	+	0.297±0.11e	medium	-
37	*E.faecalis*	*tet*(K, O, &S)	-	+	0.414±0.21c	medium	-
38	*Enterococcus* spp.	-	+	+	0.318±0.15e	medium	-
40	*Enterococcus* spp.	*tet*(K, M&,O	-	+	0.381±0.18d	medium	-
45	*Enterococcus* spp.	*tet*(K, M&,O	-	+	0.371±0.12d	medium	-
Total	17	9	8	16		17	
F-value					18.65**		

**Significance deference at P<0.01

Gelatinase secretion

Gelatinase enzyme, an extracellular metalloprotease produced by *Enterococcus faecalis*, decomposes gelatin, casein, and collagen and has been implicated as a virulence factor in models of the animal. The aptitude recommends its sharing in the initiation and dissemination of inflammatory manners linking *E. faecalis* (Waters et al., 2003). Hancock & Gilmore (2006) reported that the degradation of host extracellular medium proteins by gelatinase enzyme is significant in *E. faecalis* pathogenesis, showing the potential virulence of the isolated bacteria. Our results indicated 100% and 90.91% of the 6 *E. faecalis* and 11 *Enterococcus* spp. isolates, respectively, can produce gelatinase enzymes (Table 5). In the same direction, Amidi-Fazli & Hanifian (2022) found that 168 (30.4%) *E. faecalis* strains can produce gelatinase enzymes. Anderson et al. (2016) recorded gelatinase activity in 13% of 15 *E. faecalis* isolates from raw milk. Similarly, Jin Kim et al. (2022) found that the bacteria from bovine mastitis milk (BMM) revealed a significantly greater positivity average for gelatinase creation than bacteria from normal raw milk (NRM), with 18 (22.2%) out of 81 and 2 (4.9%) out 41, respectively. On the other hand, it was reported that all the thirty-two isolates of clinical *E. faecalis* could not secrete gelatinase enzyme (Furumura et al., 2006). Jin Kim et al. (2022) demonstrated that the presence of enterococcal surface protein (esp) and gelatinase (gelE) in isolates from BMM and NRM was significantly associated with strong biofilm formation.

Cytolysin activity

Hemolysins, also known as cytolysins, are a group of pore-forming proteins capable of disrupting the eukaryotic cell membrane. Hemolysin production depends on the microbial species and composition of the media. So Furumura et al. (2006) found that hemolysin was produced on Mueller-Hinton agar dishes by blood with twenty-four (75%) isolates. On the other hand, the supernatant heat-stable cytolysin was revealed in (100%) of *E. faecalis* when inoculated in BHI-GA (brain heart infusion medium) enhanced with 1% and 0.03% of glucose and L-arginine respectively, but not in brain heart infusion liquid medium alone. Our investigation showed that none of the tested 17 (6 and 11 *E. faecalis* and *Enterococcus* spp. isolates, respectively) was positive for cytolysin production in MRS media (Table 5). In the same line as our results in the case of all isolates from normal raw milk (NRM) that were negative for hemolysin creation (Jin Kim et al. 2022), but on the other hand, in the case of isolates from bovine mastitis milk (BMM), 2/81 (2.5%) were observed. Meanwhile, Diarra et al. (2010) found that cytolysin activity was detected in 2 isolates of *E. faecalis* bacteria. In the opposite direction, Amidi-Fazli & Hanifian (2022) found hemolysin production in 8.9% of 168 *E. faecalis* isolates. Conversely, Anderson et al. (2016) found that cytolysins were secreted from 13.3% out of 15 isolates from raw milk.

Conclusion

Enterococci were resistant to several antimicrobial agents and carried potential virulence genes that could participate in bacterial colonization and pathogenesis of enterococci**.** The current study indicates the occurrence of *tet*K, *tet*M, *tet*O and *tet*S genes in strains of *Enterococcus* spp. isolates from milk and dairy products in Egypt. Food's prominent role in the transmission and distribution of antibiotic-resistant enterococci remains indistinct. However, some evidence observed here agrees with the suggestion that commensal bacteria isolated from food may act as a reservoir for resistant enterococci in food and as a potential route for the transmission of antibiotic resistance genes. Strategies of antibiotics paired with greater adherence to contagion control methods should be obligatory to prevent the appearance and spread of multidrug-resistant enterococci.

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انتشار جينات التتراسيكلين المقاومة في عزلات بكتريا الانتيرو كوكس من منتجات الالبان في مصر

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تم دراسة مدي انتشار مقاومة المضاد الحيوي التتراسيكلين في عزلات انتيرو كوكس التي تم عزلها من اللبن وبعض انواع الجبن التقليدية في مصر .تم عزل 47 عزلة من جنس الانتيروكوكس وتمت الدراسة علي 34 عزلة لمدي مقاومتها لمجموعة مضادات الحيوي التتراسيكلين الظاهري والجيني. و قد اظهرت النتاءج ان اعلي مقاومة ظاهرية للمضادات الحيوية كما يلي:

Amoxicillin /clavulanat (76.47%), Oxytetracycline (58.82%), ampicillin (52.94%), tetracycline (50%), Doxytetracycline (41.18) and ciprofloxacin,(20.59 %).

وقد اظهرت النتاءج أن اعلي الجينات المقاومة تواجد كما يلي: *tet*(K), *tet*(M), *tet*(O) and *tet*(S) genes بالنسب التالية : علي التوالي. 85.29% (29/34) , 67.65%(23/34) , 50% (17/34) and (10/34) 29.4 %). وقد تم تعريف17 عزلة التي اظهرت اربعة وثلاثة جينات مقاومة للمضادات الحيوية باستخدام 16SrRNA. واوضحت النتاءج أن 6 عزلات بنسب (35.29%) *Enterococcus faecalis* .*Enterococcus faecalis*

وقد تم اختبار وجود خصاءص الضراوة في 17 عزلة واوضحت النتاءج التالي: أن كل العزلات ليس لها قدرة علي انتاج الهيموليسين المحلل للدم بينما كل العزلات لها القدرة علي انتاج الغشاء البكتيري. ايضا كل العزلات لها القدرة على انتاج الانزيم المحلل للجبلاتين (الجيلاتينيز) عدا عزلة واحدة.