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Multidrug Resistance and Virulence Factors of Enterococci Isolated from Milk and Some Dairy Desserts

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HIGHLIGHTS

- Prevalence of *Enterococcus faecalis* in raw milk, ice cream, mehallabia, and milk rice were 64, 0, 0, and 8%, respectively.
- Prevalence of *Enterococcus faecium* in raw milk, ice cream, mehallabia, and milk rice were 12, 44, 20, and 24%, respectively.
- All Multi Drug Resistant (MDR) *E. faecalis* and *E. faecium* isolates had 16S rRNA and sodA genes, respectively.

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Acronyms and abbreviations
CFU=Colony Forming Unit
MDR=Multi Drug Resistant
PCR=Polymerase Chain Reaction
RI=Resistant Index

ABSTRACT

Background: Enterococci spp. bacteria especially *Enterococcus faecalis* and *E. faecium* have the ability to acquire antibiotic-resistance pattern and causing life-threatening hospital-acquired infections. So, the aim of this study was to count and isolate of *E. faecalis* and *E. faecium* from milk and dairy desserts consumed in Assiut city, Egypt.

Methods: A total of 100 raw milk, ice cream, mehallabia, and milk rice samples were collected from dairies shop in Assiut city, Egypt and were bacteriologically examined for the presence and count of *Enterococcus* spp. Then, identification of enterococci isolates by conventional and Polymerase Chain Reaction (PCR) methods, performance of antibiotic sensitivity assay, and some virulence genes in the Multi Drug Resistant (MDR) isolates were identified.

Results: The prevalence of counted *Enterococcus* spp. in raw milk, ice cream, mehallabia, and milk rice samples were 76, 44, 20, and 32%, respectively. The prevalence of *E. faecalis* in raw milk, ice cream, mehallabia, and milk rice samples were 64, 0, 0, and 8%, while for *E. faecium* were 12, 44, 20, and 24%, respectively. *E. faecalis* isolates were resistant to vancomycin, ciprofloxacin, gentamicin, erythromycin, and tetracycline with the rate of 72.2, 88.9, 88.9, 94.4, and 77.8%, respectively, while for the resistance rates of *E. faecium* were 16, 40, 16, 84, and 20%, respectively. *E. faecalis* and *E. faecium* were MDR in rate of 88.9 and 32%, respectively.

Conclusion: This study revealed that milk, ice cream, mehallabia, and milk rice could be a source of enterococci to consumers in Assiut, Egypt. Moreover, *E. faecalis* had higher MDR and Resistant Index (RI) than *E. faecium*.

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Introduction

Enterococci bacteria, especially *Enterococcus faecalis* and *E. faecium*, have become increasingly important pathogens worldwide. These microorganisms have the ability to acquire antibiotic-resistance pattern and causing

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life-threatening hospital-acquired infections (nosocomial infections). Hepatobiliary sepsis, infective endocarditis, meningitis, bacteremia, urinary tract infections, surgical wound infection, dental surgical infection, and recently a case of early-onset sepsis with *E. faecalis* in a neonate born to a COVID-positive mother have been reported (Poh et al., 2006; Torres et al., 2018; Williams et al., 2022).

The food isolates of *E. faecalis* strains have the ability to transmit some antibiotic resistant genes to human and animal microbiota and can cause multiple antibiotic resistance. The glycopeptide antibiotic, vancomycin, is the last resort for the treatment of severe *Staphylococcus aureus* and enterococcal infections. The resistance to such type of antibiotic is worrisome and the risk of transmission of vancomycin resistance gene from enterococci to other pathogenic bacteria such as methicillin-resistant *S. aureus* is a concern for public health (Courvalin, 2006; Fisher and Phillips, 2009; Sparo et al., 2012).

The putative virulence factors as Enterococcal Surface Protein (Esp), which is encoded by the *esp* gene, increases adherence and colonization of enterococci to biotic and abiotic surfaces. The zinc-dependent metalloendopeptidase Gelatinase (GelE) encoded by the *gel*E gene is able to hydrolyze gelatin, elastin, collagen, hemoglobin, and others bioactive compounds. In addition, these genes contributed to the bacterial adherence and biofilm formation (Franz et al., 2003; Toledo-Arana et al., 2001).

Enterococci, caused 25% of all catheter-associated urinary tract infections, are frequently isolated in wounds and are increasingly found in infective endocarditis, and in all of these infections, they are associated with biofilm formation. Enterococcal biofilms are intrinsically tolerant to antimicrobials and thus are a serious impediment for treating infections. Multidrug resistance is a growing public health concern, mainly due to the possible failure of therapeutic treatment for enterococcal infections, particularly in immunocompromised individuals, which may develop into severe urinary tract infection, endocarditis, or bacteremia (Ch'ng et al., 2019; Kayser, 2003).

Milk and some dairy desserts as ice cream (small scale produced), mehallabia (a traditional dessert in Egypt), and milk rice are considered a good vehicle for various types of microorganisms including enterococci, and sometimes these microorganisms may be antibiotic resistant and have potential public health hazards. Therefore, the aim of this study was to determine the prevalence of *Enterococcus* spp. in raw milk, ice cream, mehallabia, and milk rice samples sold in Assiut city, Egypt and testing the recovered isolates for antimicrobial susceptibility assay. In addition, *Enterococcus* spp. and virulence genes were determined by Polymerase Chain Reaction (PCR) assay. Moreover, proteolytic and lipolytic activities of isolated organisms were tested. Finally, a trial was done for finding a relationship between the presence of some virulence genes in the recovered organisms and antibiotic resistance properties of these organisms.

Materials and methods

Sample collection

A total of 100 samples were collected including raw milk, ice cream, mehallabia, and milk rice samples (25 samples, each) from dairies shop in Assiut city, Egypt. Sample collection was done during the period from February to May 2022. The samples were collected in its container as sold to the public and transported as soon as possible to the laboratory for bacteriological examination.

Preparation of samples

The apparently normal raw milk samples were mixed thoroughly and tested for heat treatment by Storch test according to Lampert (1975) before being subjected to examination. Ten ml from liquid samples and 10 g from solid samples were added individually to 90 ml of 0.1% sterile peptone water. Ten-fold serial dilutions from each sample were done in order to count up to 10^6 Colony Forming Unit (CFU)/ml (Downes and Ito, 2001).

Identification of enterococci

Enterococci counted by spreading method, using Kenner-Faecal (KF) agar medium (Himedia, India), according to Hartman et al. (2001). Isolates were identified to the species level based on colony morphology, catalase test, growth in brain heart infusion broth (Himedia, India) at 6.5% sodium chloride and at 45 ^{o}C, growth in 0.04% tellurite, positive for esculin hydrolysis, Pyrrolidonyl (PYR) aminopeptidase, acid from lactose, arabinose, mannitol, sorbose, sorbitol, sucrose, raffinose, rhamnose, and raffinose, hydrolysis of arginine and pyruvate fermentation, according to Teixeira et al. (2007).

Antimicrobial susceptibility

Antibiotic susceptibility testing was performed by Kirby Bauer disk diffusion method on Mueller Hinton agar plate media (TM Media, Titan Biotech Ltd., India) according to the Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2018). The *Enterococcus* isolate was standardized using colony suspension method and strain's suspension diluted with sterile saline and adjusted to 0.5 McFarland standards (99.5 ml of 1%

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sulfuric acid and 0.5 ml of 1.175% barium chloride) to give a resultant concentration of 1.5×10^8 CFU/ml then swabbed onto Mueller Hinton agar plate. Six types of antibiotic discs were used including amoxicillin 10 µg, vancomycin 30 µg, ciprofloxacin 5 µg, gentamicin 10 µg, erythromycin 15 µg, and tetracycline 30 µg (Bioanalyse, Turkey) which representing six groups of antibiotic families including, β-lactam, glycopeptide, quinolones, aminoglycosides, macrolide, and tetracyclines, respectively. The plates containing the discs were incubated at 35±2 ºC for 24 h. The diameter of the inhibition zone produced by each antibiotic disc was measured and interpreted using the CLSI zone diameter interpretative standards (CLSI, 2018). Isolates with intermediate levels of susceptibility were classified as resistant in this study. *E. faecalis* ATCC® 29212 standard strain was used for control.

Antibiotic resistance index was calculated as a/b, where "a" represents the number of antibiotics to which the isolates were resistant and "b" represents the total number of antibiotics to which the isolate was exposed (Krumperman, 1983). Multidrug resistance is antimicrobial resistance shown by a species of microorganism to at least one antimicrobial drug in three or more antimicrobial categories (Magiorakos et al., 2012).

Molecular confirmation

All the Multi Drug Resistant (MDR) *E. faecalis* (16 isolates) and *E. faecium* (8 isolates) isolates obtained from this study were confirmed by detection of 16S rRNA gene for *E. faecalis* and *sod*A genes for *E. faecium* isolates by application PCR assay which performed in Reference Lab., Animal Health Research Institute (AHRI), Egypt. In addition, detection of both *esp* and *gel*E virulence genes in the confirmed *E. faecalis* and *E. faecium* isolates by the following molecular procedure.

DNA was extacted from samples using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 20 µl of proteinase K and 200 µl of lysis buffer at 56 ºC for 10 min. After incubation, 200 µl 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 100 µl of elution buffer provided in the kit. Primers used were supplied from Metabion (Germany) are listed in Table 1.

Primers were utilized in a 25 µl reaction containing 12.5 µl of EmeraldAmp Max PCR master mix (Takara, Japan), 1 µl of each primer of 20 pmol concentrations, 5.5 µl of water, and 5 µl of DNA template. The reaction was performed in an applied biosystem 2,720 thermal cycler (Germany).

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1×Tris-Borate-EDTA (TBE) buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the products were loaded in each gel slot. A gene ruler 100 bp ladder (Fermentas, Thermo, Germany) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra, USA) and the data was analyzed through computer software (Automatic Image Capture Proteinsimple Formerly Cell Bioscience, USA). *E. faecalis* ATCC® 29212 and *E. faecium* BAA-2317™ strains were used as positive controls in the PCR assays.

Proteolytic activity

By using a loop, spotted inoculations of each bacterial species was done in the areas of the 10% of skim milk agar (Himedia, India) plate then incubated the plate in an inverted position at 37 ºC for 24 to 48 h. The presence of caseinases was detected by observing a clearing in the agar around the bacterial growth, which indicated that the caseins have been broken down into transparent end products (amino acids and peptides), which were then taken up by the cells (Harely, 2016).

Lipolytic activity

The strains were subcultured in tributyrin agar (plate count agar supplemented with 1% tributyrin; Hi Media, India) and then incubated at 37 °C for 48 h. The colonies were considered positive when a precipitation halo formed around the colony, indicating the release of enzymes into the growth medium (Harrigan, 1998).

Biofilm forming ability

Congo red agar was prepared by mixing brain heart infusion broth (37 g/L), sucrose (50 g/L), agar No. 1 (10 g/L), and Congo red dye (0.8 g/L) in 1 L distilled water. Congo red stain was prepared as a concentrated aqueous solution and autoclaved (121 °C for 15 min) separately from the other medium constituents and was then added when the agar had cooled to 55 °C. The organisms were plated on Congo red agar medium and incubated aerobically at 37 ºC for 24 h. The observation of black colonies with a dry crystalline consistency was considered as biofilm positive and pink colored colony as negative (Freeman et al., 1989).

Statistical analysis

Statistical analysis was done with GraphPad Prism software packaged for windows version 9.3.1 (GraphPad-Software, LLC, USA).

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Results

The prevalence of counted *Enterococcus* spp. in raw milk, ice cream, mehallabia, and milk rice samples were 76, 44, 20, and 32%, respectively (Table 2). The highest frequency distribution of positive *Enterococcus* spp. was 48% and with a range of 10^3-$<10^4$ CFU/ml in raw milk samples (Table 3). The prevalence rates of *E. faecalis* in raw milk, ice cream, mehallabia, and milk rice samples were 64, 0, 0, and 8%, while *of E. faecium* were 12, 44, 20, and 24%, respectively.

The proteolytic activity of isolated *E. faecalis* and *E. faecium* was 94.4 and 96%, respectively. In addition, *E. faecalis* and *E. faecium* isolates had no lipolytic activity.

The isolated *E. faecalis* in this study were resistant to vancomycin, ciprofloxacin, gentamicin, erythromycin, and tetracycline in the rates of 72.2, 88.9, 88.9, 94.4, and 77.8%, respectively, while the resistant rates of *E. faecium* were 16, 40, 16, 84, and 20%, respectively. In addition, 100% of *E. faecalis* and *E. faecium* isolates were sensitive to amoxicillin. *E. faecalis* and *E. faecium* isolates were MDR in the rates of 88.9 and 32%, respectively. Furthermore, 88.9% of the tested *E. faecalis* were MDR and with average RI of 0.704. The prevalence of MDR in tested *E. faecium* isolates was 32% with average RI of 0.293.

The species-specific 16S rRNA gene was present in all the MDR *E. faecalis* isolates (Figure 1). In addition, species-specific *sodA* gene was present all tested MDR *E. faecium* isolates (Figure 2). The virulence genes of *esp* and *gel*E were present in all MDR *E. faecalis* and MDR *E. faecium* isolates (Figures 3 and 4). The *E. faecalis* (18 isolates) and *E. faecium* (25 isolates) were biofilm producer in the rates of 88.9 and 100%, respectively.

Table 1: Primers sequences and amplification cycles used in Polymerase Chain Reaction (PCR) assay

Target Agent	Target gene	Primers sequences (5`-3`)	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
					Secondary denaturation	Annealing	Extension		
Enterococcus fecalis	16S rRNA	GTT TAT GCC GCA TGG CAT AAG AG CCG TCA GGG GAC GTT CAG	310	94 ˚C 5 min.	94 ˚C 30 s	50 ˚C 40 s	72 ˚C 45 s	72 ˚C 10 min	*
Enterococcus faecium	*sodA*	GAAAAAACAATAGAAGAATTAT TGCTTTTTTGAATTCTTCTTTA	215						**
E. fecalis and *E. faecium*	*gelE*	TATGACAATGCTTTTTGGGAT AGATGCACCCGAAATAATATA	213	94 ˚C 5 min	94 ˚C 30 s	50 ˚C 30 s	72 ˚C 30 s	72 ˚C 7 min	***
	esp	AGATTTCATCTTTGATTCTTGG AATTGATTCTTTAGCATCTGG	510	94 ˚C 5 min	94 ˚C 30 s	50 ˚C 40 s	72 ˚C 45 s	72 ˚C 10 min	

* Zoletti et al. (2006)
** Jackson et al. (2004)
*** Vankerckhoven et al. (2004)

Table 2: *Enterococcus* spp. counts (CFU/ ml or CFU/g) in milk and some dairy desserts samples (n=25)

Type of sample	Positive countable samples		Negative countable samples		Min.	Max.	Average±SE
	No.	%	No.	%			
Milk	19	76	6	24	*$<10^2$	4×10^4	$6.24\times10^3\pm1.78\times10^3$
Ice cream	11	44	14	56	*$<10^2$	6×10^4	$1.32\times10^4\pm4.56\times10^3$
Mehallabia	5	20	20	80	*$<10^2$	2.5×10^3	$2.56\times10^2\pm1.29\times10^2$
Milk rice	8	32	17	68	*$<10^2$	1.6×10^4	$1.1\times10^3\pm6.71\times10^2$

No colonies could be detected on the plates*
CFU=Colony Forming Unit

Table 3: Frequency distribution of *Enterococcus* spp. counts in milk and some dairy desserts samples (n=25)

Intervals	Milk		Ice cream		Mehallabia		Milk rice	
	No.	%	No.	%	No.	%	No.	%
*$<10^2$	6	24	14	56	20	80	17	68
10^2-$<10^3$	1	4	2	8	2	8	3	12
10^3-$<10^4$	12	48	2	8	3	12	4	16
10^4-$<10^5$	6	24	7	28	0	0.0	1	4
Total	25	100	25	100	25	100	25	100

*No colonies could be detected on the plates.

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Table 4: Antibiotic resistance profiles among *Enterococcus faecalis* and *Enterococcus faecium* organisms isolated from milk and some dairy desserts samples

Antimicrobial agents	*E. faecalis*				*E. faecium*			
	Sensitive		Resistant		Sensitive		Resistant	
	No./18	%	No./18	%	No./25	%	No./25	%
Amoxicillin	18	100	0	0.0	25	100	0	0.0
Vancomycin	5	27.8	13	72.2	21	84	4	16
Ciprofloxacin	2	11.1	16	88.9	15	60	10	40
Gentamicin	2	11.1	16	88.9	21	84	4	16
Erythromycin	1	5.6	17	94.4	4	16	21	84
Tetracycline	4	22.2	14	77.8	20	80	5	20

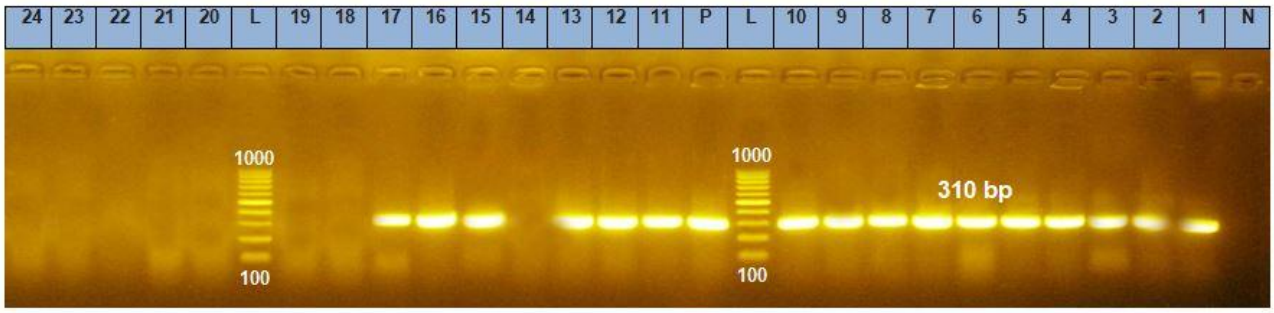


Figure 1: Agarose gel electrophoresis patterns of uniplex Polymerase Chain Reaction (PCR) amplification products for *Enterococcus faecalis* species-specific 16S rRNA gene. Lane L: 100 bp DNA ladder; lane P: positive control *E. faecalis* species-specific 16S rRNA gene (310 bp); lane N: negative control; lanes 1-10, 11-13, and 15-17: positive *E. faecalis* isolate from samples

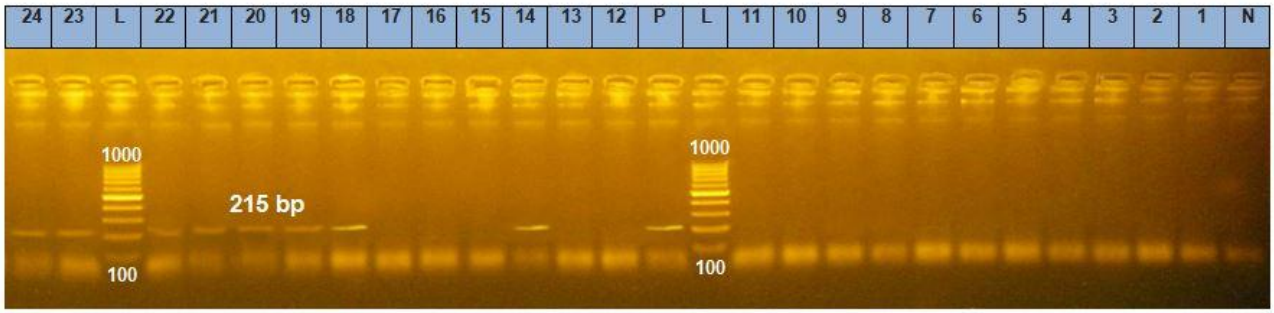


Figure 2: Agarose gel electrophoresis patterns of uniplex Polymerase Chain Reaction (PCR) amplification products for *Enterococcus faecium* species-specific *sodA* gene. Lane L: 100 bp DNA ladder; lane P: positive control *E. faecium* species-specific *sodA* gene (215 bp); lane N: negative control; lanes 14, 18-22, and 23-24: positive *E. faecium* isolates from samples

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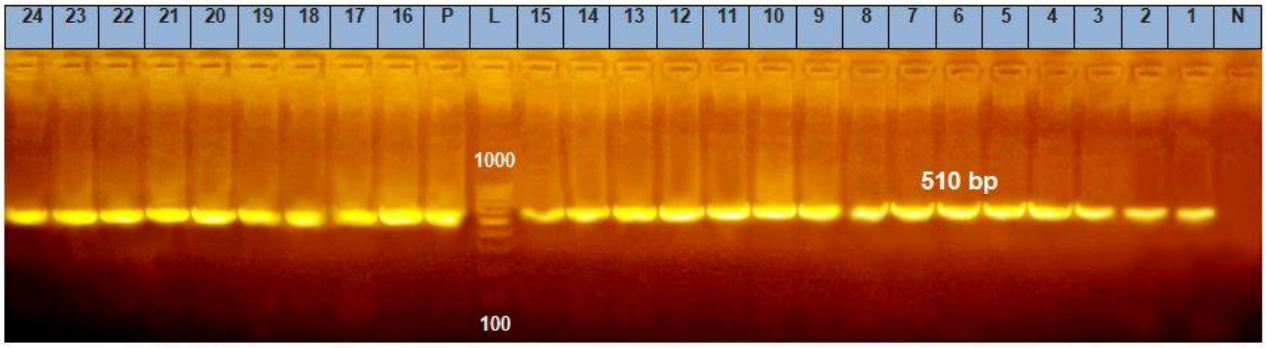


Figure 3: Agarose gel electrophoresis patterns of uniplex Polymerase Chain Reaction (PCR) amplification products for *Enterococcus faecalis* and *Enterococcus faecium esp* virulence gene. Lane L: 100 bp DNA ladder; Lane P: positive control for *esp* virulence gene (510 bp); lane N: negative control; lanes 1-15 and 16-24: positive *E. faecalis* (16 isolates) and *E. faecium* (8 isolates) for *esp* virulence gene

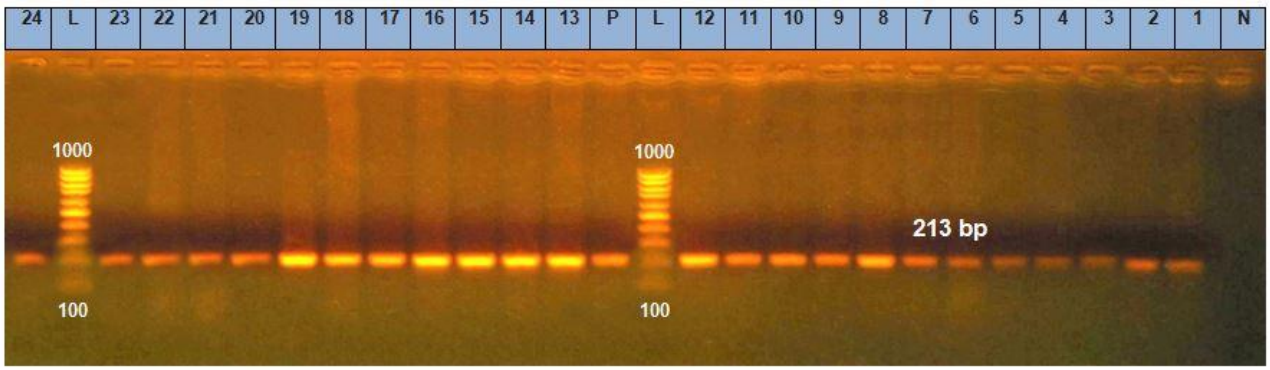


Figure 4: Agarose gel electrophoresis patterns of uniplex Polymerase Chain Reaction (PCR) amplification products for *Enterococcus faecalis* and *Enterococcus faecium gel*E virulence gene. Lane L: 100 bp DNA ladder; lane P: positive control for *gel*E virulence gene (213 bp); lane N: negative control; lanes 1-12, 13-23, and 24: positive *E. faecalis* (16 isolates) and *E. faecium* (8 isolates) for for *gel*E virulence gene

Discussion

Our results showed that the prevalence of counted *Enterococcus* spp. in the examined raw milk samples was 76% with counts ranging from $<10^2$ to 4×10^4 and with an average count of 6.24×10^3 CFU/ml. Lower prevalence (22 and 30%) was found by Gorgy et al. (2016) in El-Behera governorate, Egypt and Hamzah and Kadium (2018) in Iraq. On the other hand, Hammad (2015) revealed higher result of 86.66% by examining of 27 raw milk samples collected from different supermarkets, retail, and dairy shops in El-Menofia governorate, Egypt. The difference between our results and the previous studies would be due to variation in geographical location, timing of the study, or hygienic precautions applied during production.

We found that the highest frequency distribution of positive *Enterococcus* spp. count in raw milk samples was 48% and with a range of 10^3-$<10^4$ CFU/ml. The presence of *Enterococcus* spp. in raw milk in this study indicated a faecal contamination and unhygienic handling and distribution of milk. In addition, improper handling and distribution could play a role in milk contamination with such type of microorganisms. The prevalence of counted *Enterococcus* spp. in the examined ice cream samples was 44%, with a count ranging from $<10^2$ to 6×10^4 and with an average count of 1.32×10^4 CFU/ml (Table 2). The same result was obtained by Abd El-

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Tawab et al. (2019) with examining 25 ice cream samples collected from El-Gharbia governorate, Egypt. While, lower prevalence of 16% was reported by Shafeek et al. (2018) when they tested 25 ice cream samples collected from in Qena city, Egypt. On the contrast, El-Malt et al. (2013) recorded higher result of 62%. The discrepancies between our result and the results of previous studies could be attributed to the hygienic status of the used ingredients. The highest frequencies distribution of positive *Enterococcus* spp. in ice cream samples was 28% and in the range 10^4-$<10^5$ CFU/ml (Table 3). The presence of *Enterococcus* spp. in ice cream samples in this study could be attributed to either insufficient heat treatment or due to using contaminated utensils and equipment during production.

Concerning mehallabia samples, 20% of tested samples revealed countable *Enterococcus* spp. with a count ranging from $<10^2$ to 2.5×10^3 and with an average count of 2.56×10^2 CFU/g (Table 2). Higher result (48%) was found by Hassan and Afifi (2016) where they examined 25 mehallabis samples from different localities in Beni-Suef city, Egypt. The possible reasons for difference between our data and the previous study may be attributed to the hygienic status of the used utensils and equipment's. The highest frequencies distribution of positive *Enterococcus* spp. in mehallabia samples was 12% and in the range 10^3-$<10^4$ CFU/g (Table 3). The presence of *Enterococcus* spp. in mehallabia samples in this study indicated bad hygienic measures during production. The *Enterococcus* spp. in milk rice samples was 32%, with a count ranging from $<10^2$ to 1.6×10^4 and with an average count of 1.1×10^3 CFU/g (Table 2). Higher result of 40% was revealed by Hassan and Afifi (2016). The highest frequencies distribution of positive *Enterococcus* spp. in milk rice samples was 16% and in the range 10^3-$<10^4$ CFU/g (Table 3). To our knowledge, there is a paucity of literatures about incidence of *Enterococcus* spp. in mehallabia and milk rice samples in Egypt.

In this research, the prevalence of 64 and 12% from *E. faecalis* and *E. faecium* in the examined raw milk samples, respectively. This result somewhat coincided with Bouymajane et al. (2018) who isolated *E. faecalis* and *E. faecium* from raw milk with incidences of 64.7 and 17.6%, respectively in Meknes city, Morocco. Fortunately, *E. faecalis* couldn't recovered from ice cream and mehallabia samples in these study.. Lower result of 20% was obtained by Gundogan et al. (2013) where the authors examined 25 ice cream samples in Ankara, Turkey. While in mehallabia samples, the prevalence of *E. faecium* was 20%. Concerning milk rice samples, the prevalence of *E. faecalis* and *E. faecium* were 8 and 24%, respectively. Due to paucity of available literature dealing with the *Enterococcus* spp. in mehallabia and milk rice at Egypt; therefore, it was hard to discuss the aforementioned result.

Interestingly, *E. faecalis* was the most prevalent species in raw milk samples in this study. Whereas, *E. faecium* was the most predominant one in ice cream, mehallabia, and milk rice samples. All the 43 strains of *E. faecalis* (18) and *E. faecium* (25) were identified by virulence properties based on proteolytic and lipolytic assay methods. The proteolytic activity of *E. faecalis* and *E. faecium* was 94.4 and 96%, respectively; while for lipolytic activity, both strains gave negative lipolytic activities. Lower proteolytic activity was found in 605 *Enterococcus* by Margalho et al. (2020). Gundogan et al. (2013) reported that *E. faecalis* (20) and *E. faecium* (15) obtained from some food of animal origin in Turkey were lipase negative that coincided with the result of our study.

All the MDR *E. faecalis* in this study had proteolytic activities indicated that there was a correlations between multidrug resistance properties of the organisms and their proteolytic activities. In addition, all the MDR *E. faecium* had proteolytic activities except one sample was MDR and without proteolytic activities. It is worth mentioning that proteolytic and lipolytic activities of *E. faecalis* and *E. faecium* could impart undesirable defects and flavours in milk and milk products as bitterness and rancidity.

We observed that none of the *E. faecalis* and *E. faecium* was resistant to amoxicillin. This result agreed with Fuka et al. (2017) who discovered that none of the enterococci isolated from raw milk and Istrian cheese in Croatia were ampicillin resistant. Chajęcka-Wierzchowska et al. (2020) found that *E. faecalis* and *E. faecium* from 320 ready-to-eat dairy samples were ampicillin sensitive. Hammad et al. (2022) found that none of *Enterococcus* obtained from 100 retail raw cow's milk samples were resistant to ampicillin. The sensitivity of all *Enterococcus* to amoxicillin in this study could be attributed to rarely use of amoxicillin for treatment of human and animal infection in Egypt that may give a chance for enterococci to be sensitive to that antibiotic (it is a personal observation).

In the current work, 72.2% of *E. faecalis* and 16% of *E. faecium* were vancomycin resistant. Nearly similar result was reported by Nasiri and Hanifian (2022) with 71.9% for *E. faeclais* and 77.6% for *E. faecium*. On the other hand, higher resistant for both species was found by Výrostková et al. (2021). This disparity in results could be attributed to differences in the amount and type of antibiotics used in the treatment of enterococci-infected humans and animals from area to area. In addition, the misuse of antibiotics may give an opportunity for the emergence of strains of bacteria that are resistant to these antibiotics. From the public health point of view, the presence of vancomycin resistant *E. faecalis* and *E.*

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faecium in milk and some dairy desserts in this study could present a potential health hazards to consumers. Therefore, good hygienic measures must be applied to give products safe for human consumption. For ciprofloxacin, 88.9 and 40% of tested *E. faecalis* and *E. faecium* were resistant, respectively. Low resistances (45.9 and 18.5%) were obtained by Gökmen and Ektik (2022). On the other hand, higher resistance of 80.2% in *E. faecium* was revealed by Nasiri and Hanifian (2022).

We found that *E. faecalis* had higher gentamicin resistance of 88.9% than that of *E. faecium* with 16%. Lower resistance (26.1%) in *E. faecalis* and higher resistant (70.7%) in *E. faecium* were found by Nasiri and Hanifian (2022). In contrast, in another study, none of *E. faecalis* and *E. faecium* was resistant to gentamicin (Bouymajane et al., 2018). However, Horiuk et al. (2018) found 64.6% resistance in *E. faecalis*, and Wajda et al. (2022) revealed 55% resistant in *E. faecium*.

Based on our finding, both *E. faecalis* and *E. faecium* had high resistance to erythromycin in percentage of 94.4 and 84%, respectively. Sattari-Maraji et al. (2019) found similar high resistant results of 98.5% in *E. faecalis* and 100% in *E. faecium* that isolated from children infections in Iran. In contrast, lower resistant of 60 and 66.7% in *E. faecalis* and *E. faecium* isolated from milk of sheep and goat with subclinical mastitis, respectively, was reported by El-Zamkan and Mohamed (2021).

E. faecalis had higher tetracycline resistant of 77.8% than that of *E. faecium*, which was 20% in this study. Lower resistant revealed by and El-Zamkan and Mohamed (2021) and Šustáčková et al. (2004). On the other hand, higher resistant of 89.1 and 93.3% in *E. faecalis* and *E. faecium*, respectively, isolated from chicken carcasses samples collected from the retail stores in São Paulo State, Brazil was found by Ristori et al. (2012).

In this research, *E. faecalis* had higher resistant rate towards vancomycin, ciprofloxacin, gentamicin, erythromycin, and tetracycline than that of *E. faecium*. This result indicated that *E. faecalis* is more virulence than *E. faecium* isolate based on their antibiotic sensitivity assy. The results revealed that 88.9% of the tested *E. faecalis* were MDR and with average RI of 0.704. Cunha et al. (2021) reported that 85.7% of *E. faecalis* obtained from 40 samples collected in 12 dairy farms in the Portuguese region were MDR. The prevalence of MDR in tested *E. faecium* isolates was 32% with average RI of 0.293. Higher results were revealed by Golob et al. (2019) in strains isolated from humans and by Slovenia and Fahmy et al. (2021) in red meat from intensive care unit, Sohag University hospital, Egypt. Furthermore, both *E. faecalis* and *E. faecium* had RI more than 0.2 which indicated that the samples are contaminated from sources where antibiotics are frequently used (Poonia et al., 2014).

It is clear that *E. faecalis* isolates had multidrug resistance and RI values more than that of *E. faecium* isolate. Moreover, contaminated milk and some dairy desserts could represent a potential hazard to consumers. All MDR *E. faecalis* (16) were confirmed on species level by detection of 16S rRNA gene using PCR that all of them were positive (Figure 1). In addition, all MDR *E. faecium* (8) were confirmed on species level by detection of *sod*A gene using PCR that all of them were positive (Figure 2).

The virulence genes of *esp* and *gel*E were detected in all MDR *E. faecalis* and *E. faecium* in rate of 100% (Figure 3 and 4). This result indicated that there was a positive correlation between multidrug resistance ability of *E. faecalis* and *E. faecium* and the presence of *esp* and *gel*E virulence genes. From the public health point of view, *esp* gene promotes biofilm production and helps the organism to adhere to epithelium, assist in immune evasion and increase their resistance to antibiotics (Donlan and Costerton, 2002; Golińska et al., 2013; Zou and Shankar, 2015). In addition, *gel*E gene play a role for degradation of the fibrin layer surrounding bacteria that allows for bacterial dissemination (Rathnayake et al., 2012). From industrial point of view, *esp* gene could assist enterococci to adhere and colonize the dairy equipment and utensils that escalate enterococci dissemination and spreading in milk and milk products. In addition, *gel*E gene could assist enterococci to degrade milk protein leading to undesirable defects in milk and milk products.

All the recovered *E. faecalis* (18) and *E. faecium* (25) were tested phenotypically to detect their ability to form biofilm by using Congo red agar method. Interestingly, 88.9 and 100% of *E. faecalis* and *E. faecium* were Congo red positive. Nasiri and Hanifian (2022) found that 81 and 69% of *E. faecalis* and *E. faecium*, respectively were biofilm producer. On the other hand, Al-Shammary (2019) revealed that 100% of *E. faecalis* isolated from 50 raw milk samples pooled directly from cows and milk containers (25 each) and 25 imported milk powders pooled from Baghdad markets, Iraq, was biofilm producer. It is worth mentioning that 100% of the MDR *E. faecium* were Congo red positive indicated a correlation between multidrug property and biofilm production. In addition, 93.75% of the MDR *E. faecalis* were Congo red positive.

Conclusion

This study revealed that milk, ice cream, mehallabia, and milk rice could be a source of enterococci to consumers in Assiut, Egypt. *E. faecalis* was the most prevalent in raw milk samples; whereas, *E. faecium* was the most predominant isolates in ice cream, mehallabia, and milk rice samples. Amoxicillin was still effectives to

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enterococci. *E. faecalis* isolates had higher resistant rate towards vancomycin, ciprofloxacin, gentamicin, erythromycin, and tetracycline than that of *E. faecium*. Moreover, *E. faecalis* had higher MDR and RI than *E. faecium*. In addition, there was a correlation between MDR properties of enterococci and presence of *esp* and *gel*E virulence genes, proteolytic activities, and Congo red utilization by the organisms.

Author contributions

Both authors equally designed the study, collected the samples, and conducted the experiments; O.A.S. did statistical analysis and wrote the manuscript. Both authors read and approved the final manuscript.

Conflicts of interest

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