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Research Article
Genotyping and Virulence Genes of *Enterococcus faecalis* Isolated Form Kareish Cheese and Minced Meat in Egypt

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Abstract

Enterococcus species are recognized as a major etiological agents of nosocomial infections which worldwide distribution, commonly isolated from different food sources as meats and milk products. So, this study was conducted for detection of *Enterococcus* spp., from (50 kareish cheese and 50 minced meat) from Menofia governorate, Egypt. The results revealed 90 and 60% of samples were positive for *Enterococcus* spp. and *Enterococcus faecalis* was the most isolated strain 37 (82.22%) and 23 (76.67%) from kareish cheese and minced meat, respectively. *Enterococcus faecalis* isolates were tested for haemolysin, gelatinase and biofilm formation. The isolates were confirmed by PCR using specific primer for *ddl* gene. Also PCR was applied for detection of virulence determinants as *E. faecalis* surface proteins (*esp*), collagen-binding protein (*ace*), EF3314 and *cylA* genes among *E. faecalis* isolates. The result concluded that, EF3314 was the most predominant virulence gene followed by *esp* gene and *ace* among isolates from minced meat and kareish cheese. In contrast, none of the isolates exhibited haemolytic phenotype encoded (*cylA*) gene.

Key words: *E. faecalis*, PCR, *ace*, *esp*, EF3314

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Enterococcus species are Gram-positive non-spore forming, catalase and oxidase-negative, facultative anaerobic cocci that arranged singly, in pairs or in chains, normal inhabitant flora in gastrointestinal tract of both humans and animals (Thurlow *et al*., 2009). It is used as a indicator for faecal contamination and poor hygienic measures during manufacture process of dairy and meat products (Franz *et al*., 1999). Enterococci can tolerate different environmental conditions, such as temperature ranging from 10-45°C and NaCl 6.5% (Gardini *et al*., 2001). Enterococci causing a variety of infections including urinary tract infections, bacteraemia and endocarditis (Zhang *et al*., 2013; Zhou *et al*., 2013). The genus Enterococcus contains 38 species, two species, *E. faecalis* and *E. faecium* are responsible for the majority of human infections (Verraes *et al*., 2013). Enterococci encoding several virulence factors such as haemolysin activator (*cylA*), *E. faecalis* surface proteins (*esp*), collagen-binding protein (*ace*) and EF3314 genes. The *cylA* is important for the expression of cytolysin an extracellular protein (Jett *et al*., 1994). Enterococcal surface protein (*esp*) gene is associated with increased virulence, colonization and persistence in the urinary tract through increasing attachment to epithelial surfaces and biofilm production (Shankar *et al*., 2001). Other cell surface protein is collagen binding protein (*ace*) that mediated the association of bacteria to host cell matrix protein. The gene (EF3314) is a newly described as surface protein that contributes to the virulence determinants of *E. faecalis* and specific for *E. faecalis* strains of food and animal origin (Creti *et al*., 2009). The significant importance of EF3314 was related to its similarity with biofilm-associated proteins (Cucarella *et al*., 2001). The importance of the presence of virulent enterococci in food due to the possibility of virulence genes transmission to human microbiota through food chain. So, this study was planned for genotyping and screening of virulence genes among *E. faecalis* isolates from kareish cheese and minced meat.

MATERIALS AND METHODS

Collection and preparation of samples: One hundred food samples (50 kareish cheese and 50 minced meat) were collected from different dairy shops and street vendors in Menofia Governorate according to APHA (2004).

Isolation and identification of enterococci isolates

Phenotypic characterization: Isolates were identified based on its morphological characterization on KF Streptococcus agar medium at 37°C for 48 h. The colonies of pink or red color indicated enterococci and staining with Gram's stain which appear Gram-positive, oval cocci, in pairs or short chains (Debel and Hartman, 1976).

Biochemical characterization: Catalase test, growth at 45 and 10°C, growth at 6.5% sodium chloride (Sherman, 1937) and esculin hydrolysis.

Phenotypic assay of virulence factors: Slime production (biofilm) on congo red agar (Freeman *et al*., 1989), haemolysis on blood agar and gelatinase activity (Cowan and Steel, 1974).

Genotyping and detection of virulence genes of *Enterococcus faecalis*

DNA extraction: Genomic DNA of the isolates were extracted according to (Sambrook and Russell, 2002) using DNA Purification Kit QIAamp DNA Mini Kit (Cat. No. 51304-Qiagen) according to the instructions of the manufacturer. The DNA concentration was determined spectrophotometrically at 260/230 nm.

The PCR reaction was performed in an Gradient Thermal cycler (1000 S Thermal cycler Bio-RAD USA). The reaction mixture (total volume of 50 µL) was 25 µL Dream green PCR Mix (DreamTaq Green PCR Master Mix (2X) Fermentas Company, Cat., No. K1080, USA), 5 µL target DNA, 2 µL of each primers (containing 10 pmol μL^{-1}) and the mixture was completed by sterile dry weight to 50 µL (Table 1).

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^{-1}. For gel analysis, 15 µL of the products was loaded in each gel slot. A 100 bp and 100 bp plus DNA Ladders (Qiagen, Germany and GmbH) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (α Innotech, Biometra) and the data was analyzed through computer software.

RESULT

Prevalence of *Enterococcus* species isolated from kareish cheese and minced meat: Out of examination of 100 samples from kareish cheese and minced meat, 90 and 60%, respectively were positive for *Enterococcus* spp., *Enterococcus faecalis* was the most frequently isolates 82.22 and 76.67%, while, *E. faecium* isolates were 17.78 and

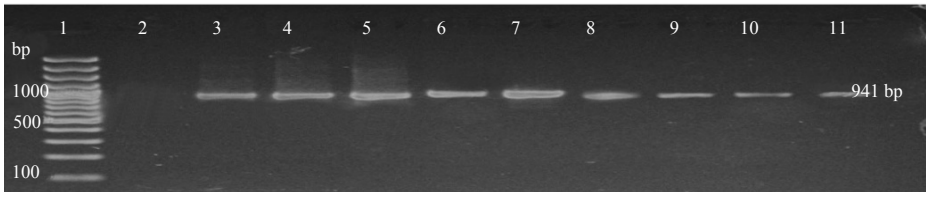

Fig. 1: Gel electrophoresis of PCR product of amplification of *ddl* gene for confirmation of *E. faecalis*, Lane 1: 100 bp DNA ladder, Lane 2: Control negative, Lane 3: Control positive and Lane 4-11: Samples (lane 4, 5, 6 and 7 from minced meat origin and lane 8, 9, 10, 11 from kareish cheese originl)

Table 1: Oligonucleotide primers for detection of *E. faecalis* and virulence determinants genes

			Amplification (35 cycles)					
Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Secondary denaturation	Annealing	Extension	Final extension	References
ddl E. faecalis F	5′-ATCAAGTACAGTTAGTCTT-3′	941	94°C, 5 min	94°C, 1 min	54°C, 1 min	72°C, 1 min	72°C, 7 min	Dutka-Malen *et al.* (1995)
ddl E. faecalis R	5′-ACGATTCAAAGCTAACTG-3′							
esp	AGATTTCATCTTTGATTCTTGG AATTGATTCTTTAGCATCTGG	510	94°C, 10 min	94°C, 45 sec	50°C, 45 sec	72°C, 45 sec	72°C, 10 min	Vankerckhoven *et al.* (2004)
cylA	ACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTT	688	94°C, 10 min	94°C, 45 sec	50°C, 45 sec	72°C, 45 sec	72°C, 10 min	
ace	GGAATGACCGAGAACGATGGC GCTTGATGTTGGCCTGCTTCCG	616	94°C, 10 min	94°C, 45 sec	58°C, 45 sec	72°C, 45 sec	72°C, 10 min	Creti *et al.* (2004)
EF3314	AGAGGGACGATCAGATGAAAAA ATTCCAATTGACGATTCACTTC CCGCCATCCTCCTGCAAAAAA	566	94°C, 10 min	94°C, 45 sec	55°C, 45 sec	72°C, 45 sec	72°C, 10 min	

Table 2: Prevalence of *Enterococcus* species isolated from kareish cheese and minced meat

	Kareish cheese		Minced meat	
Species	No.	%	No.	%
Enterococci spp.	45	90.0	30	60.0
E. faecalis	37	82.22	23	76.67
E. faecium	8	17.78	7	23.3

N = 50 samples for each kariesh cheese and minced meat, percentage of *Enterococcus* spp., according to the number of each product, n = 50, percentage of species according to positive isolates (45 in cheese and 30 in minced meat)

Table 3: Prevalence of virulence activities of *E. faecalis* isolated from examined samples

	E. faecalis			
	Kareish cheese		Minced meat	
Virulence activities	No.	%	No.	%
Biofilm production	12	32.43	7	30.43
Gelatinase production	4	10.8	3	13.04
Haemolysis on blood agar	7	18.9	2	8.69

Percentage was estimated according to total number of *E. faecalis* (37 in cheese and 23 in minced meat)

23.3% from kareish cheese and minced meat, respectively as shown in Table 2.

Prevalence of virulence activities among *E. faecalis* isolates: The phenotypic assay of virulence activities showed that, biofilm formation was the most predominant activities among *E. faecalis* isolates 12 (32.43%) and 7 (30.43%) from kareish cheese and minced meat, respectively. Gelatinase production was 4 (10.8) and 3 (13.04%), respectively. Haemolytic activity was 7 (18.9%) and 2 (8.69%) of isolates from kareish cheese and minced meat exhibit haemolysis on blood agar as shown Table 3.

Molecular identification of *E. faecalis* by using specific primer for (*ddl*) gene as shown in Fig. 1.

Molecular detection of virulence genes among *E. faecalis* isolates: The PCR was preformed to detect virulence genes of *E. faecalis* (*ace*, EF3314, *esp* and *cylA* genes). The result illustrated, EF3314 was the most predominant gene in tested isolates followed by *esp* gene detected in 5 isolates as in Fig. 2 (*ace*) gene detected in 4 isolates and non of isolates carried *cylA* gene as in Fig. 3.

DISCUSSION

Enterococci are natural inhabitants of the intestinal tract of many animals and are released in large amounts with faeces and may become the predominant contaminant microbiota in many foods (Giraffa, 2002). In the present finding, enterococci were recovered by 90 and

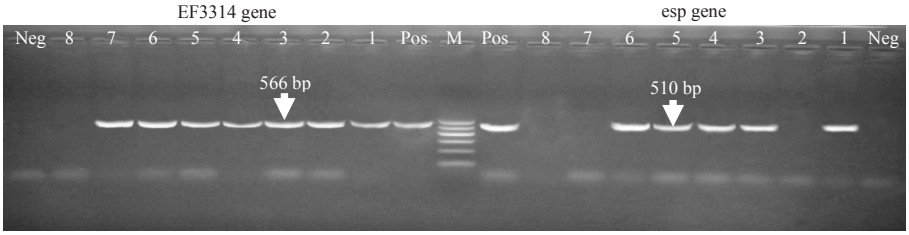

Fig. 2: Gel electrophoresis of PCR product of amplification of EF3314 and *esp* genes at 566 and 510 bp, respectively. Illustrated, EF3314 gene (Lane1-4 positive isolates from kareish cheese and lane 5-7 positive isolates from minced meat), (*esp*) gene (Lane 1, 3 and 4 positive isolates from kareish cheese and lane 5, 6 positive isolates from minced meat)

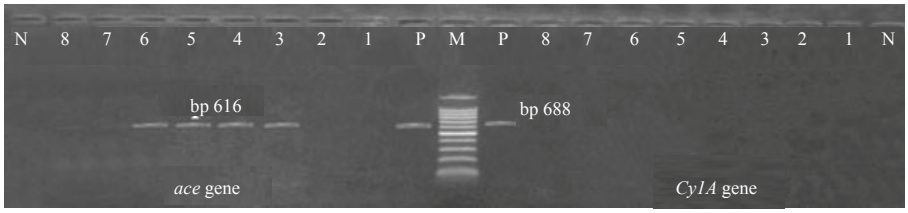

Fig. 3: Gel electrophoresis of PCR product of amplification of *ace* and *cylA* genes at 616 and 688 bp, respectively. Illustrated, *ace* gene (Lane 3 and 4 positive isolates from kareish cheese and lane 5 and 6 positive isolates from minced meat). *cylA* gene (non of isolates from kareish cheese and minced meat were detected)

60% from the examined kareish cheese and minced meat samples, respectively. The same result obtained by (Chajecka-Wierzchowska *et al*., 2012) revealed the incidence of *Enterococcus* spp., was (89.9%) from cheese. The lower prevalence (34.5%) of enterococci was recorded by (Pesavento *et al*., 2014) in retail cheese. In comparison with minced meat (60%) of samples were positive for enterococci. This nearly similar to (Chajecka-Wierzchowska *et al*., 2012) isolated *Enterococcus* spp., (69.8%). Our results revealed that, *E. faecalis* was the most common isolate in kareish cheese and minecd meat 82.22 and 76.67% in comparison to *E. faecium* 17.78 and 23.3%, respectively. This supported by Chajecka-Wierzchowska *et al*. (2012) and McGowan *et al*. (2006), *E. faecalis* was a higher frequently than *E. faecium* in kareish cheese and minced meat. In contrast previous studies (Furlaneto-Maia *et al*., 2014; Pesavento *et al*., 2014) *E. faecium* was a higher frequently than *E. faecalis*. Biofilm formation play a significant role in colonization during infection (Stewart *et al*., 2004). Phenotypic assay illustrated, 12 (32.43%) and 7 (30.43%) of *E. faecalis* from kareish cheese and minced meat, respectively were positive for biofilm formation. This is supported by Creti *et al*. (2004) that most of *E. faecalis* isolates have ability to form biofilm. Haemolytic activity, 7 (18.9%) and 2 (8.69%) isolates from kareish cheese and minced meat, respectively exhibited haemolysis on blood agar. Figure 1 shows that in PCR technique was applied for detection of specific gene (*ddl*) of *E. faecalis* for confirmatory identification (Harwood *et al*., 2004). Pathogenic *E. faecalis* can produce several virulence properties encoded by various genes *ace*, *cylA*, *esp* and EF3314 (Vankerckhoven *et al*., 2004; Harada *et al*., 2005; Dupont *et al*., 2008). These virulence determinants play an important role in mediating disease severity and pathogenicity (Leavis *et al*., 2004) through, the colonization and invasion of host tissues (Martin-Platero *et al*., 2009). Molecular detection of *cylA*, EF3314, *esp* and *ace* genes cleared that, EF3314 was the most predominant virulence gene followed by (*esp*) gene and (*ace*) among isolates from minced meat and kareish cheese as shown in Fig. 2 and 3. Previous investigations reported, the presence of the *cylA* gene did not correlate completely with its phenotypic expression (Creti *et al*., 2004). In the present finding, PCR couldn't detect *cylA* gene in positive haemolytic phenotype *E. faecalis*. This is similar to Gulhan *et al*. (2015) that all isolates were positive for β haemolysis were negative for *cylA* gene. This may attributed to phenotypic expression of cytolysin is encoded by the cytolsin operon (Medeiros *et al*., 2014). While, the present study screened a single gene (*cylA*) inside an operon. This is confirmed by Jurkovic *et al*. (2006), who detected other genes than *cylA* as $cylL_L$, $cylL_S$, *cylM*, *cylB* in cytolysin operon which may be responsible for cytolysin expression. So further investigation should be focused on roles of these and others virulence genes in pathogenesis of *E. faecalis* and possibility to disseminate these genes to human microbiota through food chain.

CONCLUSION

Our data recorded that *E. faecalis* was the most predominant *Enterococcus* species from kareish cheese and minced meat that possesse different virulence genes as EF3314, *esp* and *ace*) genes. Additional researches are needed to investigate the hazard effect of these factors on human health.

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