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Prevalence and Characterization of Some Pathogenic Bacteria in Fermented Milk Products and Mish Cheese in Dakahalia Governorate, Egypt

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Abstract

Bacterial contamination of fermented dairy products has serious implications for both safeties of the final products, and the transmission of foodborne pathogens to the consumers. In this regard, 175 samples of fermented dairy products including 50 each of plain yoghurt, fruit yoghurt, laban rayeb, and 25 from mish cheese were randomly collected from different supermarkets and retail shops in Dakahlia Governorate, Egypt. Samples were examined bacteriologically for the prevalence of *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), particularly, *E. coli* O157. The recovered isolates were examined for the detection of toxin, and virulence-associated genes using PCR. The obtained results showed that the average counts (CFU/g) of total bacterial, psychrotrophic, coliform, and *S. aureus* were $1.72x10^5$, $9.9 x10^3$, $5.08x10^2$, and $3.07 x10^2$ for the plain yoghurt, $7.1 x 10^4$, $2.3 x 10^3$, 9.5 x10, and 3.3 x10 for fruit yoghurt, $6.07x10^4$, $6.1 x 10^3$, 9.8 x10, and $1.35x10^2$ for laban rayeb, and $1.2 x 10^6$, $2.5 x10^4$, 8.3 x10, and $7.2 x10^3$ for Mish cheese, respectively. *E. coli* and *E. coli* O157 were detected in 13 out of 175 (7.43%) and 4 out of 175 (2.3%) samples, respectively. Using PCR for 9 selected *E. coli* isolates showed that 7 out of 9 *E. coli* isolates were positive for the *stx1* gene, 5 out of 9 were positive for *stx2*, and 3 out of 9 were positive for *eaeA*, and 4 out of 9 were positive for *hylA*. *S. aureus* showed that 55.6% of the recovered isolates were coagulase positive. Ten randomly selected *S. aureus* isolates tested positive for *nuc* (thermo*nuc*lease genes), while *mecA* (methicillin-resistant *S. aureus* "MRSA") gene was detected in 20% of the examined isolates. Therefore, strict hygienic measures should be adopted during all steps of the manufacture of such dairy products.

KEYWORDS
Fermented milk, Mish cheese, *E. coli, E. coli* O157, *S. aureus,* Virulence genes

INTRODUCTION

Fermented dairy products are the main constituents of regular diets all over the world due to their long shelf life, high nutritional value and desirable organoleptic properties. Probiotics are often incorporated into these products for their technological and health benefits. Pathogenic microorganisms and toxins in fermented dairy products from different sources cause outbreaks and toxicity cases. Therefore, it is critical to identify and detect the related microbiota. Psychrotrophic bacteria are abundant bacteria capable of growing at refrigerated temperature, causing spoilage of dairy products, and affecting the quality through the production of proteases and lipase enzymes (Huck *et al*., 2008; Machado *et al*., 2017; Rabelo *et al*., 2021). Psychrotrophic bacteria have a significant role in food poisoning (Samaržija *et al*., 2012).

Coliforms are usually used as indicators to detect the safety of the milk and fermented dairy products as many members of coliforms play a part in the objectionable taints interpreting inferior quality or unmarketable in milk and its dairy products (Yabaya and Idris., 2012).

Staphylococcus aureus is considered a unique of the most imperative foodborne pathogens. Contamination by *S. aureus* can be found in raw milk got from mastitis-suffering cows or food handlers who are carriers of *S. aureus* as a consequence of unhygienic practices (Bingol *et al*., 2012). Its ability for producing extensive varieties of heat-stable enterotoxins (Ahmed *et al*., 2019) causes food poisoning if they present extremely in foods (Pereira *et al*., 2009).

Escherichia coli is a Gram-negative, short rod bacillus, flagellated, nonsporulating facultative anaerobic bacteria and a member of the Enterobacteriaceae family (Bavaro, 2012). It is considered an indicator of faecal contamination in foods due to its presence in the gut. *E. coli* is a matter of alarm in foods because some strains may be pathogenic (Thaker *et al*., 2012). Some serotypes of *E. coli* are grouped as enterohaemorrhagic *E. coli* (EHEC), and verotoxin-producing *E. coli* (VTEC), such as *E. coli* O157:H7 which are documented as the main cause of haemorrhagic colitis (HC) and haemolytic-uremic syndrome (HUS) (Rahimi *et al*. 2011). Fermented dairy products made with raw milk or unpasteurised milk are considered a prospective vehicle of *E. coli* O157:H7 for transmission and causing infection to consumers. It is revealed

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that if the pathogen is found in raw milk, the health risk is high because it is associated with sporadic occurrences of diarrhoea in infants and adults and a low dose-response relative of *E. coli* is sufficient to cause infection (Yang and Yoon, 2022).

In sight of the previous facts, this study aimed to investigate the bacterial quality, and the prevalence of *S. aureus*, and *E. coli*, particularly *E. coli* O157:H7 in four fermented dairy products including plain yoghurt, fruit yoghurt, laban rayeb, and mish cheese retailed in Dakahlia Governorate, Egypt. Moreover, detection of the toxin and virulence-associated genes in the recovered isolates was done using PCR.

MATERIALS AND METHODS

Collection of samples

A total of 175 samples of fermented dairy products including plain yoghurt (50 samples), fruit yoghurt (50 samples), laban rayeb (50 samples), and mish cheese (25 samples) were collected randomly from different shops and supermarkets in Dakahlia Governorate, Egypt. The collected samples were transferred to the laboratory in an ice-packed container under aseptic conditions. Samples were kept at refrigeration temperature.

Microbiological examination

Sample preparation and microbiological examinations including total bacterial count (TBC) was done according to Roberts *et al*. (1995). Total psychrotrophic count (TPsC) was done according to Griffiths and Phillips (1988). Total coliform count (TCC) was done according to Roberts *et al*. (1995) and total *S. aureus* count (TSC) was done according to previous methods (Roberts and Greenwood, 2003; Michaylova *et al*., 2007).

Isolation and identification of S. aureus

It was carried out according to Roberts and Greenwood (2003). Identification of *S. aureus* isolates was done based on microscopic examination, and biochemical tests, specifically catalase and coagulase tests (Silva *et al*., 2019). Detection and typing of enterotoxins A, B, C, and D were done serologically using Reverse Passive Latex Agglutination kits (SET-RPLA, Denka Sekeu LTD, Japan) (Shingaki *et al*., 1981).

Molecular confirmation of S. aureus isolates

Detection of the species-specific thermo*nuc*lease (*nuc*), and Methicillin Resistant *S. aureus* "MRSA" (*mecA*) genes was done using multiplex PCR. DNA extraction from the bacterial isolates was done using QIAamp® DNA Mini Kit (Catalogue no.51304) (Cho *et al*., 2007). PCR was done following the method of Sambrook *et al*. (1989). PCR products were visualized on 1.5% agarose gel.

Isolation of E. coli and E. coli O157

It was done according to the American Public Health Association (APHA, 2004) guidelines. Identification of *E. coli* was done through biochemical examination (MacFaddin,., 2000; Silva *et al*. 2019), followed by serological identification using specific kits (DENKA SEIKEN CO., LTD) (Kok *et al*., 1996).

Detection of Shiga toxin and virulence-associated genes

Multiplex PCR was carried out for the identification of Shiga toxins (*stx1* & *stx2*), intimin (*eaeA*) and haemolysin (*hylA*) virulence-associated genes of *E. coli* using specific primers (Pharmacia Biotech) (Table 1). Same to *S. aureus*, PCR was done following the method of Sambrook *et al*. (1989). PCR products were visualized on 1.5% agarose gel.

Data analysis

Data were presented as mean ± standard error using Statistical Package for Social Science (SPSS) software version 16.

RESULTS AND DISCUSSION

The obtained results indicated that the average TBC was $1.72x10^5$, $7.1x10^4$, $6.07x10^4$, and $1.2x10^6$, TPsC was $9.9x10^3$, $2.3x10^3$, $6.1x10^3$, and 2.5×10^4, TCC was 5.08×10^2, 9.5x10, 9.8 x 10, and 8.3x10, and TSC was 3.07×10^2, 3.3x10, $1.35x10^2$, and 7.2×10^3 in plain yoghurt, fruit yoghurt, laban rayeb, and mish cheeses, respectively (Table 2). The recorded TBC in the current investigation agreed with that reported by El-Diasty and El-Kaseh (2009), and Mohammed (2020) who found that TBC in yoghurt samples ranged from $1.36x\ 10^3$ to $9.6x10^4$ CFU/g. The recorded TBC was considered high when compared with the upper limit

Table 1. Primer sequences of *E. coli* and *S. aureus* used for the application of PCR

Primer	Oligo*nuc*leotide sequence (5′ → 3′)	Product size (bp)	References
stx1 (F)	5′ ACACTGGATGATCTCAGTGG ′3	614	Dhanashree and Mallya (2008)
stx1 (R)	5′ CTGAATCCCCCTCCATTATG ′3		
stx2 (F)	5′ CCATGACAACGGACAGCAGTT ′3	779	
stx2 (R)	5′ CCTGTCAACTGAGCAGCACTTTG ′3		
eaeA (F)	5′ GTGGCGAATACTGGCGAGACT ′3	890	Mazaheri *et al*. (2014)
eaeA (R)	5′ CCCCATTCTTTTTCACCGTCG ′3		
hylA (F)	5′ ACGATGTGGTTTATTCTGGA ′3	165	Fratamico *et al*. (1995)
hylA (R)	5′ CTTCACGTGACCATACATAT ′3		
nuc (F)	5′ GCGATTGATGGTGATACGGTT ′3	*270*	*Brakstad et al. (1992)*
nuc (R)	5′ AGCCAAGCCTTGACGAACTAAAGC ′3		
mecA (F)	5′ TAGAAATGACTGAAC GTCCG ′3	*533*	*Jukes et al. (2010)*
mecA (R)	5′ TTGCGATCA ATGTTACCGTAG ′3		

Stx1: Shiga toxins 1; *stx2*: Shiga toxins 2; *eaeA*: intimin; *hylA*: haemolysin; *nuc*: thermo*nuc*lease; *mecA*: Methicillin-Resistant *S. aureus* "MRSA"

(10,000/g) set by the Egyptian Standards (ES, 2005). Results of TPsC in this study were nearly similar to Ali *et al*. (2004), and El-Malt *et al*. (2013) who found that 70% and 92% of the examined large and small-scale yoghurt samples contained average TPsC of 6.8 x 10^3 and 3.9 x 10^4, respectively. Higher counts were recorded by Zeinhom (2007), and Gamal *et al*. (2019), while lower counts were reported by Abou El- Makarem (2013). The high TPsC in this study might be due to unsatisfactory handling or post-pasteurization contamination.

Coliforms in the present study were similar to Osman (2015) who found that TCC was $5.02x10^2 \pm 0.57x10^2$ CFU/g in plain yoghurt (Aman *et al*., 2021) with a prevalence rate of 13%, and 5.4x10±1.04x10 cfu/g in the fruit yoghurt. Higher coliforms count ($5.6\times10^4 \pm 3.68\times10^3$) in the plain yoghurt was recorded by El-Ansary (2014) and (El-Leboudy *et al*. (2017) who stated that the coliforms count in rayeb samples was $1.65x10^3 \pm 2.69x10^2$ CFU/g. The results of the present study were higher than the upper limit (less than 10/g) set by the Egyptian Standards (ES, 2005).

The high bacterial contamination of the examined samples in the present study might be due to the use of raw milk or insufficient preheating (Hussain., 2010), and indicates unhygienic conditions during production or processing or post-processing contamination of such dairy products (El-Malt *et al*., 2013). *S. aureus* count was in agreement with AL-Ashmawy (2016) who reported that the mean count of *S. aureus* was $1.7x10^3$ CFU/g in yoghurt, but lower than El-Leboudy *et al*. (2015), who reported that the mean *S. aureus* count was $9.2x10^4 \pm 2.9x10^4$. The recorded results far exceeded the upper limit set by the Egyptian Standards (ES, 2005), which recommend the absence of *S. aureus*.

The extreme high occurrence of *S. aureus* in the fermented dairy product samples might be due to contamination from food handlers (Hussain, 2010), or the unhygienic conditions during processing.

The recorded results in Table 3 indicated the occurrence of *E. coli* and *E. coli* O157 at 16%, and 4% in the plain yoghurt, 8% in mish cheese samples, 4%, and 0% in laban rayeb, and 2%, and 0% in the fruit yoghurt samples, respectively. These results are higher than that recorded before (Hegab *et al*., 2020), and the Egyptian Standards (ES, 2005), but lower than that obtained by Dawoud *et al*. (2018) in mish samples, and Atef *et al*. (2017) in yoghurt (40%), and laban rayeb (55%), respectively. Serological identification of the recovered isolates of *E. coli* revealed detection of the following serotypes: O103:H2, O91:H21, O128:H2, O11:H8, O55:H7, O26:H11, O146:H21, O119:H6, and O157:H7 that coincided with Mohammed *et al*. (2021) who reported that O26 is the most predominant serovar of *E. coli* isolates at 28%, and 27.77% in kariesh

Table 2. Microbiological results of the examined fermented dairy product samples

Microbiological results		Type of sample			
		Plain yoghurt No=50	Fruit yoghurt No=50	Laban rayeb No=50	Mish cheese No=25
Total bacterial count	No. positive samples (%)	50 (100%)	50 (100%)	50 (100%)	25 (100%)
	Mean ± SE (CFU/g)	$1.72x10^5 \pm 3.2\ x10^4$	$7.1\ x10^4 \pm 1.2\ x10^4$	$6.07x10^4 \pm 1.47\ x10^4$	$1.2\ x10^6 \pm 1.7\ x10^5$
Psychrotrophic count	No. positive samples (%)	42 (84%)	36 (72%)	40 (80%)	1 6 (64%)
	Mean ± SE (CFU/g)	$9.9\ x\ 10^3 \pm 2.1\ x10^3$	$2.3x10^3 \pm 4.9\ x10^2$	$6.1x10^3 \pm 1.7\ x10^3$	$2.5x10^4 \pm 6.9x10^3$
S. aureus	No. positive samples (%)	36 (72%)	24 (48%)	24 (48%)	18 (72%)
	Mean ± SE (CFU/g)	$3.07x10^2 \pm 5.5x10$	$3.3x10 \pm 3.5$	$1.35x10^2 \pm 3.0x10$	$7.2\ x10^3 \pm 1.2x10^3$
Coliforms count	No. positive samples (%)	26 (52%)	14 (28%)	19 (38%)	10 (40%)
	Mean ± SE (CFU/g)	$5.08x10^2 \pm 6.4x10$	$9.5x10 \pm 1.5\ x10$	$9.8x10 \pm 1.3\ x10$	$8.3x10 \pm 1.8x10$

Table 3. Prevalence and Serotypes of *E. coli* and *E. coli* O157 in the examined fermented dairy product samples

Type of sample	Positive samples of *E. coli*		No. isolates of *E. coli*	Positive amples of *E. coli* O157 (%)	Identified serotype
	No.	%			
Plain yoghurt No.=50	8	16%	11	2 (4%)	O157:H7, O26:H11, O11:H8, O157:H7, O91:H21, O157:H7, O146:H21, O128:H2, O146:H21, O91:H21, O128:H2
Fruit yoghurt No.=50	1	2%	2	0	O55:H7, O55:H7
Laban rayeb No.=50	2	4%	2	0	O119:H6, O103:H2
Mish cheese No.=25	2	8%	3	2 (8%)	O157:H7, O157:H7, O128:H2

Table 4. Occurrence of virulence genes in the identified *E. coli* serotypes in the present study

Serovars \ Virulence genes	*Stx1*	*Stx2*	*EaeA*	*hylA*
O11: H8	-	+	-	-
O91: H21	+	+	-	+
O55: H7	+	-	-	-
O26: H11	+	-	+	+
O103: H2	+	-	+	+
O119: H6	+	+	-	-
O128: H2	+	-	-	-
O146: H21	-	+	-	-
O157: H7	+	+	+	+

Table 5. Prevalence of Coagulase-positive *S. aureus* in the examined fermented dairy product samples

Sample type	No. of the positive sample (%)	Number of Coagulase +ve isolates (%)	Number of Coagulases –ve isolates	Total isolates
Plain yoghurt N=50	36(72)	35(58.3)	25(41.7)	60
Fruit yoghurt N=50	24(48)	20(48.8)	21(51.2)	41
Laban rayeb N=50	24(48)	10(40)	15(60)	25
Mish cheese N=25	18(72)	35(64.8)	19(35.2)	54
Total =175	102(58.3)	100(55.6)	80(44.4)	180

Table 6. Enterotoxin production and occurrence of *nuc* and *mecA* genes of some *S. aureus* isolates recovered from the examined fermented dairy product samples

Target gene	N. of the examined isolates	No.	%
Nuc gene	10	10	100
MecA gene	10	2	20
Enterotoxin production Type A&D	10	2	20

cheese, and yoghurt samples, respectively. and Megawer *et al.* (2021) isolated most of these serotypes from small-scale yoghurt and kariesh cheese.

Molecular characterization of 9 selected *E. coli* isolates showed that 7 out of 9 *E. coli* isolates were positive for the *stx1* gene, 5 out of 9 were positive for *stx2*, 3 out of 9 were positive for *eaeA*, and 4 out of 9 were positive for *hylA* (Table 4, Fig. 1). Likely, Mohammed *et al.* (2021) reported that *stx1*was the most frequently detected between the target genes at 81.82%.

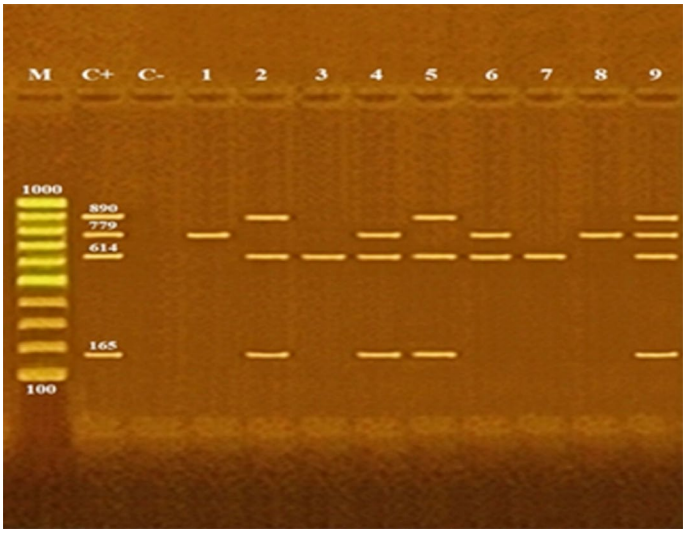

Figure 1. Multiplex PCR of *stx1* (614 bp), *stx2* (779 bp), *eaeA* (890 bp) and hlyA (165 bp) virulence genes for characterization of different *E. coli* serovars. Lane M: 100 bp ladder as a molecular size DNA marker. Lane C+: Control positive *E. coli* for *stx1*, *stx2*, *eaeA*, and hlyA genes, Lane C-: Control negative, Lanes 1 (O11) & 8 (O146): Positive *E. coli* for *stx2* gene, Lanes 2 (O26) & 5 (O103): Positive *E. coli* for *stx1*, eae, and hlyA genes Lanes 3 (O55) & 7 (O128): Positive *E. coli* for *stx1* gene, Lanes 4 (O91): Positive *E. coli* for *stx1*, *stx2*, and hlyA genes, Lane 6 (O119): Positive *E. coli* for *stx1* and *stx2* genes, and Lanes 9 (O157): Positive *E. coli* for *stx1*, *stx2*, *eaeA*, and hlyA genes.

S. aureus was isolated at72%, 48%, 48%, and 72% from plain yoghurt, fruit yoghurt, laban rayeb, and mish samples, respectively. 100 out of 180 (55.6%) *S. aureus* isolates were coagulase positive (Table 5). These results go in agreement with Abdulrahman and Sanmi (2021) who recovered 88 *S. aureus* isolates with coagulase-positive reaction.

The obtained results in Table 6 indicated that 2 out of 10 selected *S. aureus* (20%) isolates were positive for enterotoxins A and D production which was similar to Meshref *et al.* (2019) who found that 30% of the examined *S. aureus* isolates could produce enterotoxins.

The recorded results in Figure 2 indicated that the molecular identification of 10 isolates of *S. aureus* by using multiplex PCR revealed positive detection of the *nuc* gene, while 2 /10 (20%) of the tested isolates were positive for the *mecA* gene. The results stated in this study were lower than Monecke *et al.* (2011) but higher than those stated by Aydin *et al.* (2011) as the *mecA* gene was detected at 11.4%. This variation might be due to the differences in the characteristics of *S. aureus* among different studies.

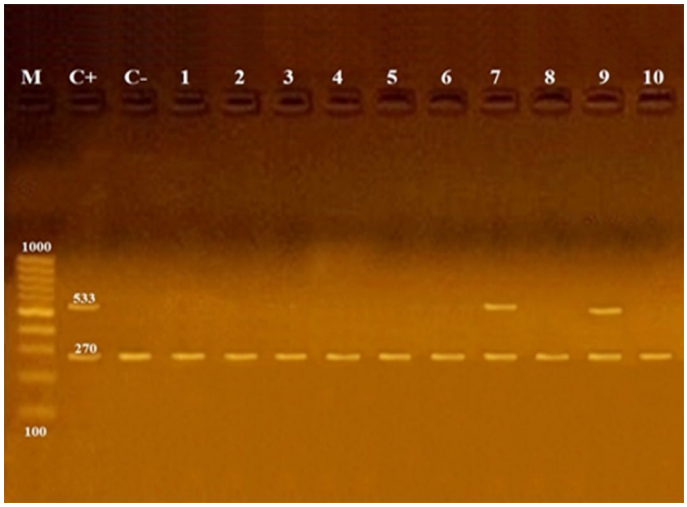

Figure 2. Multiplex PCR of *nuc* (270bp), and *mecA* (533bp) virulence genes of *S. aureus*. Lane M: 100 bp ladder as a molecular size DNA marker, Lane C+: Control positive for a *nuc*, and *mecA* genes, Lane C-: Control negative. Lanes from 1 to 10: Positive strains of *S. aureus* for *nuc* gene and Lanes 7 & 9: Positive strains of *S. aureus* for *nuc* and *mecA* genes.

CONCLUSION

Fermented dairy product samples in the present study showed contamination with foodborne pathogens such as *E. coli*, and *S. aureus*. Therefore, strict hygienic measures should be adopted during the manufacture of such products. In addition, application of the HACCP, and GMP principles are highly recommended.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest related.

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