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MOLECULAR CHARACTERIZATION AND VIRULENCE GENES PROFILING OF SHIGA TOXIGENIC *E. COLI* AND *BACILLUS CEREUS SENSU LATO* ISOLATED FROM RAW MILK AND SOME DAIRY PRODUCTS

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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) and *Bacillus cereus sensu lato* have been recognized as major foodborne pathogens that not only poses a significant risk to human health but also result in economic losses in dairy sectors. Therefore, this study was conducted to assess the presence of STEC and *B. cereus s.l.* in 90 samples of raw milk, baladi yoghurt and white soft cheese (Tallaga) (30 each) according to biochemical identification and molecular characterization of their virulence and toxigenic genes. The obtained results revealed that STEC was detected in 10% of the baladi yoghurt and 6.6% of Tallaga samples, but in contrast, it was not detected in any of the raw milk samples. Meanwhile, *B. cereus s.l.* was prevalent in 26.6% of the raw milk and 30% of both baladi yoghurt and Tallaga samples. The molecular characterization of toxigenic genes revealed that 80 % of the identified STEC isolates harboured stx_{2d}, while only 20% carried stx_{2e}; nonetheless, the virulence *hlyA* and *fliCH7* genes were prevalent in 40 & 60% of isolates, respectively. Noteworthy, stx_1, stx_{2f}, stx_2, stx_{2c} and *eae* genes were not detected. *B. cereus s.l.* isolates, were found to possess toxigenic genes, such as *bceT* by 96.1% and both of *nhe*, and *cytK* by 76.9%. These findings indicated that raw milk and dairy products may be a potential source of STEC and *B. cereus* as foodborne pathogens as well as, their virulence and toxigenic genes, necessitating strict hygiene measures, along with periodic authority inspections in Egypt's dairy sector.

Key Words: Cheese, Yoghurt, Shiga toxins, *E. coli*, and *Bacillus cereus.*

INTRODUCTION

Food-borne pathogens have been linked to numerous outbreaks reported around the world. The high nutritional content of milk and dairy products provides ideal conditions for the growth of many pathogenic bacteria, contributing to outbreaks associated with their consumption (Dash *et al*., 2022). *Escherichia coli* has been recognized as one of the primary pathogens responsible for many food borne outbreaks, particularly from raw milk and dairy products (Madani *et al*., 2022). Although *E. coli* is a natural flora of both animals and human gastric tract, certain strains of *E. coli* such as *Enterohemorrhagic E. coli* (EHEC), also known as Shiga toxin-producing *E. coli* (STEC) or Verotoxin-producing *E. coli* (VTEC), can cause serious health issues,

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such as bloody diarrhea, haemorrhagic colitis, haemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpure (Gökmen *et al*., 2024; Ullah *et al*., 2024). Furthermore, it poses a major challenge to the dairy sector authorities, as it can not only survive in raw milk, but also in stressful conditions like cheese's high salt content and yoghurt's acidity. Its ability to withstand such conditions is attributed to its capacity to alter its metabolic rate or physiological functions as a microbial stress response (Paswan and Park, 2020; Fahim *et al*., 2023).

Between 2010 and 2017, a total of 466 reported outbreaks of STEC affected 4,769 individuals, with 3,353 cases classified as foodborne in the United States (Tack *et al*., 2021). STEC outbreaks have been linked to raw milk consumption in several regions, including Wisconsin, Washington, and Oregon in the United States, as well as in Canada and Finland. However, the extent of STEC food poisoning goes beyond raw milk; outbreaks have also been associated with yoghurt in the UK and cheese consumption in both Wisconsin and France (Ullah *et al*., 2024). Furthermore, in the first four months of 2024, the European Rapid Alert System for Food and Feed (RASFF) issued four alerts regarding the detection of STEC in raw milk cheese (Morandi *et al*., 2024).

STEC encodes different virulence factors, yet its pathogenicity is primarily determined by the Shiga toxins; *stx$_1$ and stx$_2$*. Although both toxins are regarded as similar, strains that produce *stx$_1$ (stx$_{1a}$, stx$_{1c}$, and stx$_{1d}$)* are often less dangerous than those that produce *stx$_2$*, which has seven subtypes of *stx$_2$ (stx$_{2a}$, stx$_{2b}$, stx$_{2c}$, stx$_{2d}$, stx$_{2e}$, stx$_{2f}$, and stx$_{2g}$)* (Rosario *et al*., 2021). Other virulence factors, such as intimin, encoded by the *eae* gene and entero-hemolysin encoded by the *hlyA* gene facilitate the attachment of the bacteria to the epithelial cells of the intestinal tract, promoting their colonization, which plays a crucial role in the pathogenicity of STEC, contributing to its ability to cause severe infections (Zarei *et al*., 2021).

B. cereus is regarded as the most significant species from the food safety and quality perspective (Bianco *et al*., 2023). Given its heat tolerance, spoiling ability, and pathogenic potential, this spore-forming bacterium is one that continuously challenges the dairy business and could jeopardize sustainable dairy production (Finton *et al*., 2024). Furthermore, their resistance to harsh chemicals (such as disinfectants and biocides) and physical treatments (such as pasteurization, desiccation, freezing, and UV irradiation) makes them particularly difficult to eliminate from the dairy production chain (Setlow, 2016; André *et al*., 2017; Taher *et al*., 2023)

B. cereus sensu lato is responsible for around 500 confirmed instances of foodborne illnesses in European Union each year (Meng *et al*., 2022). A total of 619 outbreaks of *Bacillus*-related poisoning were reported by the Centre for Disease Control (CDC) from 1998 to 2015. The majority of which were diarrheal episodes caused by *B. cereus* in various foods, including milk (Tretyak, 2019). According to recent studies, *B. cereus* has been detected in pasteurized milk in a number of Chinese cities, including Hong Kong, Guangzhou, Shenzhen, Harbin, Ningxia, Beihai, and Hai Kou (Zhao *et al*., 2020).

B. cereus sensu lato. is associated with 2 types of food poisoning: emetic and diarrheal. The emetic type is always triggered by a heat-stable *cerulide (ces)* toxin, a cyclic peptide toxin produced by *cesA (cer)* and *cesB*, causing emesis. The diarrheal type is always caused by enterotoxins: the haemolytic enterotoxin *BL (HBL),* encoded by *hblA, hblC,* and *hblD*, the non-haemolytic enterotoxin *(NHE),* encoded by *nheA, nheB*, and *nheC* and the cytotoxin K *(CytK),* which are invariably associated with milk and dairy products (David *et al*., 2024).

Moreover, *B. cereus s.l.* has been reported to cause serious extra-intestinal infections, including severe eye infections, osteomyelitis, hepatitis, and inflammatory reactions (Gao *et al*., 2018).

Hence, this study aimed to isolate both Shiga toxigenic *E. coli* and *B. cereus s.l.* from raw milk, baladi yoghurt and Tallaga cheeses samples, along with their biochemical identification and molecular characterization of their virulence and toxigenic genes, to evaluate the potential health risks associated with their consumption.

MATERIALS AND METHODS

1 Samples collection

Ninety samples of raw milk, baladi yoghurt and Tallaga cheeses samples (30 each) were purchased randomly from local markets, dairy stores, and supermarkets in Cairo and Giza governorates, Egypt in the period between September 2023 and May 2024. The samples were aseptically collected in sterile Whirl-Pak's and promptly transferred in an insulated ice box to the Food Hygiene and Control department, PC2 lab at Faculty of Veterinary Medicine, Cairo University for further microbiological and molecular analysis.

2. Serial dilution preparation of the examined samples

Initially, Guaiac test, as described by Schonberg (1956), was performed to identify and eliminate all raw milk samples that had been heat treated. Food homogenate and decimal dilutions of the examined samples were performed in accordance with APHA (2015). Briefly, well-mixed milk, baladi yoghurt, and Tallaga cheeses samples were added to either sterile peptone water 0.1% (99 mL) or sterile sodium citrate solution 2% for cheese, and then thoroughly homogenized in a stomacher bag for 2-4 min with a Lab-blender 400 (Stomacher; Inter Science, France). Subsequently, the samples were subjected to tenfold serial dilutions.

3 Isolation of shiga toxin-producing *E. coli* (STEC) *and B. cereus sensu lato (s.l).*

Shiga toxigenic *E. coli* (STEC) was isolated using Sorbitol MacConkey agar (SMA; OXOID, USA) medium supplemented with MUG (4-methylumbelliferyl-beta-d-glucuronide) according to Oxoid manual (2010) and BAM (2013). Confirmed non-sorbitol fermenting colonies were picked and further streaked on Eosin Methylene Blue (EMB) and incubated at 37°C for 24 h according to ISO 7251 (2005). After 24 h of incubation, colonies with greenish metallic sheen were subsequently identified biochemically.

B. cereus s.l. was isolated by lab pasteurization of the homogenate in an 80°C water bath for 12 min and then cooling immediately. Following that, 0.1 mL of food homogenate was plated on Mannitol-Yolk Polymyxin Agar Plate (OXOID, USA) and incubated at 30°C for 24 h. Pink colonies were picked for further biochemical identification (APHA, 2015).

4 Biochemical identification of the isolates

The isolates (10 colonies from each positive sample) were further biochemically identified. Briefly, microscopical examination, indole, methyl red, Voges Proskauer and citrate tests were utilized for *E. coli* identification, as described by De Vos *et al*. (2009). While *B. cereus s.l.* was identified using several tests, including egg yolk reaction, starch hydrolysis, glucose fermentation, Voges Proskauer, nitrate reduction, citrate, growth at 50°C and growth in different salt concentrations (2 and 7%) according to Whitman *et al*. (2015), where all tests were done in triplicates. All isolates were stored in glycerol 30% at -20 ºC for subsequent molecular characterization.

5 Molecular characterization of the isolates and their virulence and toxigenic genes

5.1 DNA extraction of the isolates

Crude DNA was extracted from bacterial isolates using the boiling method (Darwish and Asfour 2013). In brief, 1 mL of broth from each isolate was placed in a 1.5 mL Eppendorf tube and centrifuged at 6000 rpm to pellet the bacteria. Subsequently, the resulting pellet was washed twice with Tris-EDTA buffer consisting of 10 mM Tris and 1 mM EDTA (pH 8). After washing, the pellet was resuspended in 200 µL of lysis buffer, which contained 1% Triton X-100, 0.5% Tween 20, 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. The suspension was then boiled for 10 min before being centrifuged at 8000 rpm for 10 min. Finally, the supernatant was carefully siphoned and transferred to a new tube where it was stored at -20°C for future PCR amplification.

5.2. Molecular characterization of *E. coli* and *B. cereus s.l.*

The *phoA* gene was amplified to confirm *E.coli* isolates according to Hu *et al*. (2011). The gene encoding the flagellar motor protein (*motB* gene) to confirm *B. cereus s.l.* group species (Oliwa-Stasiak *et al*. 2010). The PCR amplification was conducted following a previously adopted protocol of Asfour *et al*. (2024). Briefly, a 25 µL total volume of 5 µL of the extracted DNA template, 1 µL (20 pmol) of each forward and reverse primer, 12.5 µl of PCR master mix (Dream Taq Green PCR Master Mix, Fermentas Life Science) and 5.5 µL of nuclease free water. STEC (ATCC-93111) and *B. cereus* (ATCC-33018) reference strains were utilized as control positive strains. Amplification was performed in 35 cycles using a SimpliAmp Thermal Cycler (ThermoFisher Scientific). The cycling conditions included an initial denaturation at 94°C for 4 min, followed denaturation at 94°C for 30 seconds, annealing at the temperatures specified (Table 1) for 30 seconds, and extension at 72°C for 30 seconds and final extension at 72°C for 10 min. The amplification products were resolved by electrophoresis in 1.5% agarose gel prepared with 0.5X TBE operated at 70 volts until the bands achieved complete separation. Following this, the gel was visualized under ultraviolet light.

5.2. Molecular characterization of virulence and toxigenic genes profile

A single and multiplexes PCR were performed on the confirmed *E. coli* and *B. cereus s.l.* isolates to characterize their virulence and toxigenic genes, as described in Table (1). Single PCRs were conducted in a 20 µL reaction volume, while multiplex PCR was performed in a 40 µL volume. For the multiplex reactions, 30 pmol of each primer was combined with 1X DreamTaq Green PCR Master Mix, while the reaction conditions were maintained (Asfour *et al*. 2024). All PCR were performed in duplicates. The screening of toxigenic and virulence genes was conducted only on isolates that demonstrated good amplification signals with genus-specific primers to reduce false negatives.

6 Statistical analysis

All tests were carried out in triplicate, and the results were calculated and expressed in the form of mean %, using IBM SPSS Statistics (version 27.0) for Windows.

RESULTS

E. coli was detected in 30% of the examined Tallaga cheese samples. However, the baladi yoghurt and the raw milk samples showed lower incidences of 23.3 and 20%, respectively, on Sorbitol MacConkey agar (SMAC) (Table 2). A confirmatory MUG test revealed that the incidence of STEC was 10 and 6.6% in the baladi yoghurt and the Tallaga cheese samples, respectively. Quite unexpectedly, no *E. coli* (STEC) was isolated from the examined raw milk samples (Figure 1). The biochemical identification of the isolated strains (n=50) demonstrated that 3/32 isolates from the baladi yoghurt samples and 2/18 isolates from the Tallaga cheese samples were confirmed as *E. coli* (Table 3).

Table 1: Oligonucleotide PCR primers sequence for detection of species-specific and virulence/toxigenic genes in the examined isolates

Primer name		Target gene	Oligonucleotide primer sequences (5′-3′)	Product size (bp)	Annealing temperature	References
***E. coli* identification**		***phoA***	**F:** CGA TTC TGG AAA TGG CAA AAG **R**: CGT GAT CAG CGG TGA CTA TGAC	720	55℃	(Hu *et al.,* 2011)
Shiga toxigenic producing *E. coli* toxigenic genes	**Multiplex assay 1**	***stx1* gene**	**F:** TCTCAGTGGGCGTTCTTATG **R:** TACCCCCTCAACTGCTAATA	338	58 °C	(Wang *et al.,* 2002)
		***stx2f* gene**	**F:** TGTCTTCAGCATCTTATGCAG **R:** CATGATTAATTACTGAAACAGAAAC	150		
		***stx2* gene**	**F:** GCGGTTTTATTTGCATTAGC **R:** TCCCGTCAACCTTCACTGTA	115		
	Multiplex assay 2	***stx2c* gene**	**F:** GCGGTTTTATTTGCATTAGT **R:** AGTACTCTTTTCCGGCCACT	124		
		***stx2e* gene**	**F:** ATGAAGTGTATATTGTTAAAGTGGA **R:** AGCCACATATAAATTATTTCGT	303		
		***eaeA* gene**	**F:** ATGCTTAGTGCTGGTTTAGG **R:** GCCTTCATCATTTCGCTTTC	248		
	Multiplex assay 3	***stx2d* gene**	**F:** GGTAAAATTGAGTTCTCTAAGTAT **R:** CAGCAAATCCTGAACCTGACG	175		
		***hlyA* gene**	**F:** AGCTGCAAGTGCGGGTCTG **R:** TACGGGTTATGCCTGCAAGTTCAC	569		
		***fliCH7* gene**	**F:** TACCATCGCAAAAGCAACTCC **R:** GTCGGCAACGTTAGTGATACC	247		
***B. cereus sensu lato* group Identification**		***(motB gene)***	**F:** ATCGCCTCGTTGGATGACGA **R:** CTGCATATCCTACCGCAGCTA	575	54.5 °C	(Oliwa-Stasiak *et al.,* 2010)
Virulence genes of *B. cereus s.l.* group	**Quadraplex assay 4**	***hbl* gene**	**F:** GTAAATTGATGAICAATTTC **R:** AGAATAGGCATTCATAGATT	1091	49 °C	(Ehling-Schulz *et al.,* 2006)
		***nhe-* gene**	**F:** AAGCIGCTCTTCGIATTC **R:** ITIGTTGAAATAAGCTGTGG	766		
		***cytK* gene**	**F:** ACAGATATCGGICAAAATGC **R:** CAAGTIACTTGACCIGTTGC	421		
		***ces* gene**	**F:** GGTGACACATTATCATATAAGGTG **R:** GTAAGCGAACCTGTCTGTAACAACA	1271		(Ehling-Schulz *et al.,* 2005)
	bceT gene		**F:** TTACATTACCAGGACGTGCTT **R:** TGTTTGTGATTGTAATTCAGG	428	58°C	(Agata *et al.,* 1995)

The molecular characterization confirmed that the phoA gene (*E. coli* specific gene) is presented in all biochemically identified *E. coli* isolates. Furthermore, molecular characterization of shiga toxigenic genes (*stx1* and *stx2* groups) in the confirmed *E. coli* isolates demonstrated that 80% (4/5) of the isolates harboured *stx2d*, which was detected in all *E. coli* isolates from the Tallaga cheese but only detected in 66.6% (2/3 isolates) from the baladi yoghurt samples, *stx2e* was only detected in 20 % (1/5) of the *E. coli* isolates, originating from the Tallaga cheese. Notably, none of the *E. coli* isolates harboured the *stx1, stx2f, stx2* and *stx2c* genes (Table 4 & Figure 2).

Table 2: Incidence (%) of the isolated shiga toxigenic *E. coli* (STEC) on Sorbitol MacConkey agar (SMAC) and MUG test from the examined samples.

Products	No. of samples	*E. coli* positive samples on SMAC		*E. coli* positive samples using MUG	
		No.	Percentage%	No.	Percentage%
Raw milk	30	6	20	-	-
Baladi yoghurt	30	7	23.3	3	10
Tallaga cheese	30	9	30	2	6.6

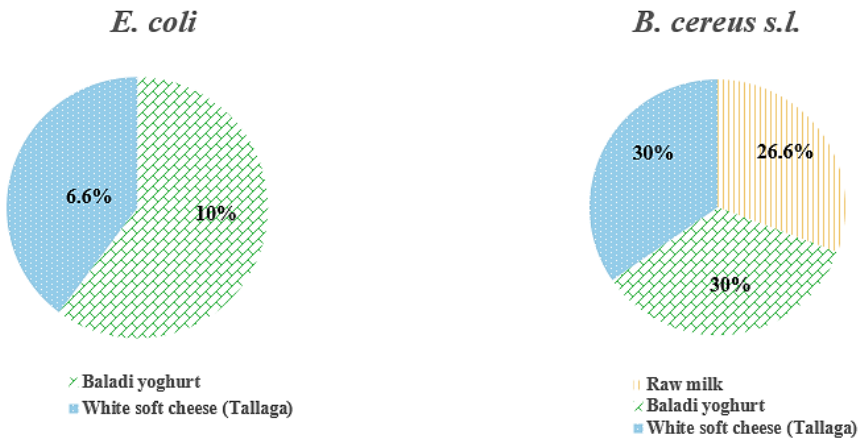

Figure 1: Incidence of biochemical identified *E. coli* and *B. cereus s.l.* in the raw milk, baladi yoghurt and Tallaga cheese examined samples (Mean%).

The virulence genes findings indicated that *fliCH7and hlyA* genes were prevalent in 60% (3/5) & 40% (2/5) of the *E. coli* isolates respectively. Interestingly, both *hlyA* and *FliCH7* genes were detected in all *E. coli* isolated from the Tallaga cheese samples. *eae* gene was not detected in any of the *E. coli* isolates (Table 4 & Figure 2).

Table 3: Biochemical and molecular characterization of *E. coli* isolates from the examined samples

Sample	Total isolates No. (%)	Biochemical identified *E. coli* No. (%)	Detection of *phoA* gene No. (%)
Baladi Yoghurt	32(64%)	3(9.3%)	3(9.3%)
Tallaga cheeses	18(36%)	2(11.1%)	2(11.1%)

The results demonstrated in Figure (1) revealed that *B. cereus s.l.* was detected in 26.6% of the raw milk samples and 30% in both the baladi yoghurt and Tallaga cheese samples. The biochemical identification of the isolated strains (n=130) confirmed that 26 isolates; (8/40 isolates) from the raw milk samples, (9/45 isolates) from the baladi yoghurt samples and (9/45 isolates) from the Tallaga cheese samples were *B. cereus s.l.*

Subsequent molecular characterization of the biochemical identified strains (n=26) revealed that *motB* (*B. cereus s.l.* specific gene) had been identified in all *B. cereus s.l.*

isolates (Figure 3). Furthermore, the molecular characterization of toxigenic genes (diarrheal enterotoxins) demonstrated that *bceT* enterotoxin gene was detected in the majority of the *B. cereus s.l.* isolates (96.1%; 25/26 isolates). In particular, it was detected in all isolates from raw milk (n=8) and Tallaga cheese (n=9), however, its incidence in the isolates from baladi yoghurt was slightly lower 88.8% (8/9 isolates) (Table 5 & Figure 3). The non-haemolytic enterotoxin gene *(nhe)* and the cytotoxin k gene *(cytK)* were the second most prevalent enterotoxigenic genes in the *B. cereus s.l.* isolated strains, with prevalences 76.9% (20/26 isolates) for both (Table 5 & Figure 3). According to the findings given in Table 5, the *B. cereus s.l* isolates from the raw milk exhibited the highest occurrences of the *nhe* gene with 87.5% (7/8 isolates), whereas (*cytK*) was present in all isolates.

Haemolytic enterotoxin gene (*hbl*) was detected only in 7.6% (2/26) of the isolated *B. cereus s.l* strains that is originally from the baladi yoghurt samples. Nevertheless, no emetic enterotoxins (*ces* gene) were detected in any of the isolated strains (Table 5 & Figure 3).

Table 4: Molecular characterization of toxigenic and virulence genes in *E. coli* (n=5) isolated from the examined samples.

Products	No. of isolates	Shiga toxigenic genes No. (%)									
		phoA	***stx$_1$***	***stx$_{2f}$***	***stx$_2$***	***stx$_{2c}$***	***stx$_{2e}$***	***stx$_{2d}$***	***eae***	***hlyA***	***fliCH7***
Raw milk	-	-	-	-	-	-	-	-	-	-	-
Baladi yoghurt	3	3(100)	-	-	-	-	-	2(66.6)	-	-	1(33.3)
Tallaga cheeses	2	2(100)	-	-	-	-	1(50)	2(100)	-	2(100)	2(100)
Total	5	5(100)	-	-	-	-	1(20)	4(80)	-	2(40)	3(60)

(-) means not detected

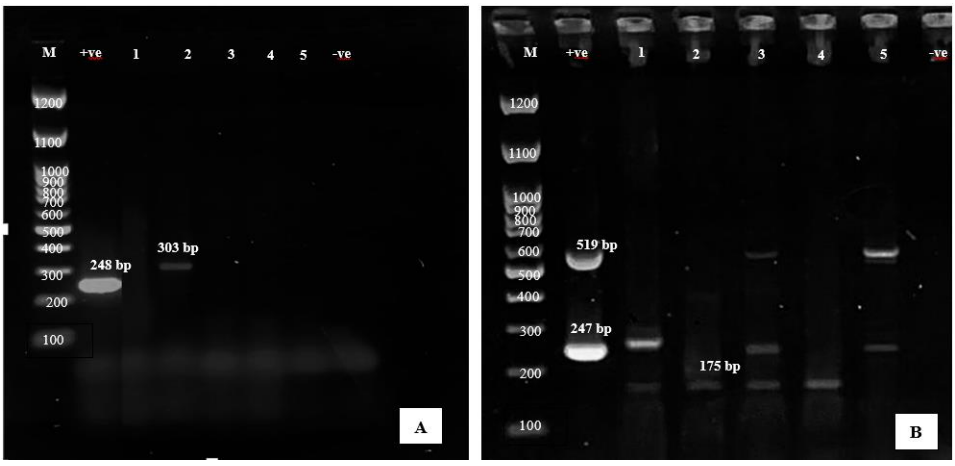

Figure 2. Molecular characterization of toxigenic and virulence genes of *E. coli.* Isolates (1,2,3) from Baladi yoghurt and (4,5) from white soft cheese (Tallaga) (**A**) *stx$_{2c}$* (124bp), *eae* (248bp) and *stx$_{2e}$* (303bp) genes, lane 2: positive *stx$_{2e}$* isolates (**B**)*stx$_{2d}$* (175bp), *fliCH7* (247bp) and *hlyA* (519bp) genes, Lanes 1,3,5: *fliCH7* positive isolates, lane 1,2,3,4: *stx$_{2d}$* positive isolates and lane 3,5: *hlyA* positive isolates.**M**: 100 bp ladder DNA size marker, Lane +ve: Positive control, Lane -ve: Negative control

Table 5: Molecular characterization of toxigenic genes of *B. cereus sl.* strains (n=26) isolated from the examined samples.

Products	*Bacillus cereus sensu lato group* toxigenic genes No. (%)						
	No. of isolates (%)	*motB*	*bcet*	*cytK*	*nhe*	*hbl*	*ces*
Raw milk	8(30.7)	8(100)	8 (100)	8(100)	7(87.5)	-	-
Baladi yoghurt	9(34.6)	9(100)	8(88.8)	4(44.4)	6(66.6)	2(22.2)	-
Tallaga cheeses	9(34.6)	9(100)	9(100)	8(88.8)	7(77.7)	-	-
Total	26(100)	26(100)	25(96.1)	20(76.9)	20(76.9)	2(7.6)	-

(-) means not detected

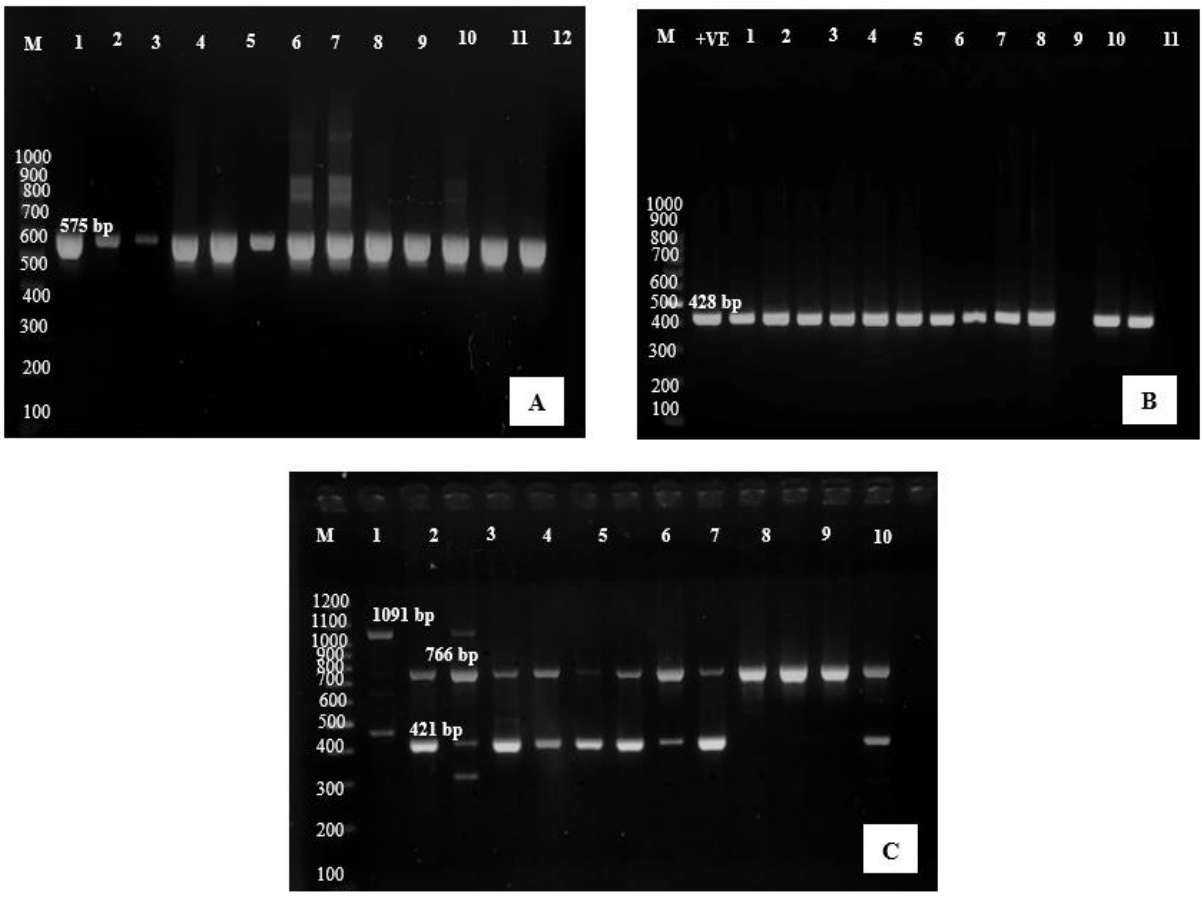

Figure 3: Molecular characterization of *B. cereus s.l. group* and their toxigenic genes, isolates from (1-5) from raw milk, (6-9) from baladi yoghurt, (10-13) from Tallaga cheese
(A) *motB* gene (575 bp), lanes 1-12: positive *motB* gene isolates
(B) *bcet* gene (428) bp, Lanes 1-10 and 12,13: positive *bcet* gene isolates
(C) *cytK* gene (421bp), *nhe* gene (766bp), *hbl* gene (1091bp) and *ces* gene (1271bp), lanes 2-9: *cytK* positive isolates, lanes 2-12: *nhe* positive isolates and lanes 1,3: *hbl* positive isolates, **M**: 100bp ladder DNA size marker, Lane +ve: Positive control genes, Lane -ve: Negative control.

DISCUSSION

Shiga toxin-producing *E. coli* poses significant public health and economic challenges worldwide, causing a wide range of illnesses ranging from minor gastrointestinal discomfort to severe conditions such as diarrhea, dysentery, and haemorrhagic colitis (HC). Furthermore, in high-risk people, infections may progress to haemolytic uremic syndrome (HUS), a potentially life-threatening complication (Augustin *et al*., 2021; Ullah *et al*., 2024).
In this study, the STEC was detected in both the baladi yoghurt (10%) and the Tallaga cheese samples (6.6%), and it was not detected in any of the examined raw milk samples (Table 1). These comparatively higher incidences in dairy products compared to the raw milk could be attributed to inadequate sanitation, improper handling, storage, and packaging in dairy premises that may play a major role in the introduction of *E. coli* (STEC) in the subsequently produced dairy products (Fetouh *et al*., 2022). These results were consistent with earlier findings where STEC was detected in 10% of fresh soft cheese samples (Baz *et al*. 2019). Contrarily, Shiga toxigenic *E. coli* genes could not be detect any in yoghurt samples (Abushaala *et al*. 2022).

It is noteworthy that, the examined yoghurt and cheese samples failed to meet the Egyptian standards ES (2005/1000) and ES (2005/1-1008) which stipulated that the *E. coli* should be totally absent in milk and all dairy products. This non-compliance raises a serious public health concern, as the likelihood of developing STEC infection is influenced by the number (CFU) of cells consumed, which is remarkably low. For instance, outbreaks linked to cheese have been reported with 5–10 CFU/g of STEC, highlighting that even minimal exposure can lead to STEC infection (Koutsoumanis *et al.*, 2020).

STEC's pathogenicity is typically associated with their ability to produce Shiga toxins. According to the results demonstrated in (Table 4 & Figure 2), stx_{2d} and stx_{2e} were the most prevalent toxigenic genes in the isolated *E. coli s*trains, with 80% and 20%, respectively; however, the other toxigenic genes; stx_1, stx_{2f}, stx_2 and stx_{2c} were not detected. These findings represent a significant concern, as the stx_2 group is widely recognized for its association with more severe clinical instances compared to stx_1 (Okuno *et al*., 2021). Specifically, stx_{2d} is significantly linked to severe clinical manifestations such as haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS), while the other subtypes are often associated with milder symptoms (Yang *et al*., 2021). These results were in accordance with the findings of Rosario *et al*. (2021) and Zhang *et al*. (2024) who identified 6 subtypes including stx_{1a} (53.1%), stx_{2g} (15.6%), stx_{2a} (3.1%) and 6.3% for each of (stx_{2d}, stx_{2a}, stx_{2d}, stx_{1a} & stx_{2a}) in milk samples. Conversely to the results obtained by Fetouh *et al*. (2022) who only detected stx_1 gene in yoghurt samples. The virulence genes (*hlyA, FlicH7 &eae*) profiling showed that *FlicH7* gene was detected in more than 50% of the *E. coli* isolates, which is usually linked with watery diarrhea and hemolytic-uremic syndrome STEC intoxication cases (Pang *et al*., 2022). Those results were comparatively similar to the percentages reported by Elsherif and Ali, (2020), while it was relatively higher compared to a study that found the *FlicH7* gene in only 20% of *E.coli* isolates (Calvopiña Montenegro *et al*. 2024). Furthermore, the *hlyA* gene was the second prevalent virulence gene in the *E. coli* isolates with 40%, similar to Hussien *et al.* (2019) and Abd El Latif *et al*. (2022) findings (44.4%). *hlyA* plays a crucial role in the STEC virulence. Remarkably, *eaeA* gene was not detected in any of the *E. coli* isolates (Chaleshtori *et al*. 2017).

Those high incidences of both toxigenic and virulence genes in the isolated *E. coli* strains from dairy products, *i.e.*, yoghurt and

cheese, despite their high acidity and salt content, raises concerns about their potential presence with higher frequencies in less processed dairy products in the Egyptian market, highlighting a current major health concern.

Another major hurdle in the dairy sector is *B. cereus s.l.* group particularly, *B. cereus* spp., which are frequently found in both raw and pasteurized milk. As spore-forming bacteria, they can survive pasteurization, remaining dormant until exposed to favourable environmental conditions, when they germinate and produce toxins causing foodborne illnesses (Ledina *et al*., 2021; Porcellato *et al*., 2021)

The incidence of *B. cereus s.l.* in both baladi yoghurt and Tallaga cheese was 30%, which is relatively higher (26.6%) of the raw milk samples (Figure 1). These results were consistent with those reported by Bianco *et al*. (2023). However, it was relatively lower than the percentages obtained by Maktabdar *et al*. (2024) which was 42% in the examined cheese samples. Furthermore, these findings signifies that the majority of the tested raw milk samples did not comply the Egyptian standards (ES 154/2005), which state that raw milk should not have any *B. cereus* or any pathogenic bacteria. Such presence mostly due to spores' ability to withstand extreme environmental conditions such as: low nutrients, osmotic pressure in high salt products (cheese), high acidity of yoghurt, and temperature fluctuations (Trunet *et al*., 2017). A molecular characterization confirmed that all biochemically identified isolates had the *motB* gene, which is a species-specific gene for the *B. cereus s.l.* Group (Figure 3).

The pathogenicity of *B. cereus s.l.* is largely attributed to its ability to produce a range of enterotoxins, including both diarrheal and emetic toxins. The results of toxigenic genes profiling (Table 5 & Figure 3) showed that the most abundant enterotoxigenic (diarrheal) genes in the isolated *B. cereus s.l.* spp. was *bcet* (96.1%), *nhe* (76.9%) and *cytK* (76.9%). Nevertheless, the *hbl* gene was only present in 7.6% of the isolates. These results corresponded with Gao *et al*. (2018) and Chang *et al*. (2021) findings, who similarly reported significant prevalences of nhe, *cytK*, and *bceT* genes in more than 50% of milk, while were opposed to Radmehr's (2024) findings, who identified *hbl* in 70% of the raw milk isolates.

Fortunately, none of the *B. cereus s.l.* isolates harboured the emetic toxigenic gene (*ces*) (Table 5& Figure 3), these results lined up with those of Hamidpour and Mahdavi, (2020), while were significantly lower compared with the research conducted by Abouelhag *et al.* (2021) which detected *ces* gene in 18.18% of the raw milk isolates.

As the contamination during and after processing is not completely prevented, it is evident that proper sanitary and strict hygienic practices are critical in preventing and managing the potential circulation of *B. cereus s.l.* spp. throughout the dairy supply chain. Hence, future research on the synergic deployment of several mild technologies to inactivate both STEC and *B. cereus* spores across Egyptian dairy facilities is still required.

CONCLUSION

This study investigated the prevalence of Shiga toxigenic *Escherichia coli* (STEC) and *Bacillus cereus s.l.* group in raw milk, baladi yoghurt, and Tallaga cheese, indicating high incidences of both with a significant degree of non-compliance with the Egyptian standards. Moreover, the molecular characterization of the isolates revealed the presence of Shiga toxigenic genes (stx_{2d} and stx_{2e}), as well as virulence-associated genes, including *hlyA* and *FlicH7*. While the genetic profile of *B. cereus s.l.* isolates revealed that all isolates harboured various diarrheal toxigenic genes, including *bcet*, *nhe*, *cytK*, and *hbl*. These findings provide valuable insights into the levels of the microbial

contamination in the Egyptian dairy sector and highlights a current potential health risk to the dairy consumers; emphasizing the importance of ongoing surveillance and regular monitoring of those foodborne pathogens with molecular characterization of their virulence genes to assess and mitigate their potential health risks. Further research is still essential to expand the understanding of the genetic diversity of such pathogenic strains in milk and dairy products.

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Author contribution
RA carried out the laboratory experiments, data analysis and writing manuscript. SD contributed with technical support, molecular work and corrected the draft of the manuscript. HE contributed to project conceptualisation & corrected the draft of the manuscript. ET contributed to project conceptualisation, statistical analysis, resources & funding acquisition, writing, review & editing.

Competing Interests
The authors declare no competing interest.

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التوصيف الجزيئي وتحديد الجينات المسئولة عن الضراوة لبكتيريا الإشيرشيا كولاي المفرزة لسموم الشيجا ومجموعة الباسيليس سيريس سينسو لاتو المعزولة من اللبن وبعض منتجات الألبان

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تعد بكتيريا الإشيرشيا كولاي المفرزة لسموم الشيجا ومجموعة الباسيليس سيريس سينسو لاتو من مسببات الامراض التي تشكل خطراً كبيراً على صحة الإنسان، بالإضافة إلى ما تسببه من خسائر اقتصادية كبيرة في قطاع الألبان ومنتجاتها. لذلك تم إجراء هذه الدراسة لتقييم انتشار بكتيريا الإشيرشيا كولاي المفرزة لسموم الشيجا ومجموعة الباسيليس سيريس سينسو لاتو في ٩٠ عينه من اللبن الخام، والزبادي البلدي، والجبن الثلاجه (٣٠ عينة من كل)، بالإضافة الي التعرف البيوكيميائي لهم والتوصيف الجزيئي لجينات الضراوه والسمية. وهذا وقد تم الكشف عن الإشيرشيا كولاي المفرزة لسموم الشيجا في ١٠٪ من عينات الزبادي البلدي و٦.٦٪ من عينات الجبن الثلاجه؛ في حين لم يتم الكشف عنها في أي من عينات اللبن الخام. في المقابل كانت مجموعة الباسيليس سيريس سينسو لاتو منتشرة في ٣٠٪ من عينات اللبن الخام و٢٦.٦٪ من عينات الزبادي البلدي والجبن الثلاجه. وقد اوضح التوصيف الجزيئي للجينات المسببة للسمية أن ٨٠٪ من بكتيريا الإشيرشيا كولاي تحتوي على جين stx_{2d}. بينما ٢٠٪ فقط تحتوي على جين stx_{2e}. ومع ذلك، كانت الجينات المسئولة عن الضراوة; $hlyA$ و $fliCH7$موجوده في ٤٠٪ و ٦٠٪ من المعزولات علي التوالي. من الجدير بالذكر أن جينات stx_1, stx_{2f}, stx_2, لم يتم الكشف عنها. من الناحية الأخرى مجموعة الباسيليس سيريس سينسو لاتو تحتوي على جينات مسببة stx_{2c}و eae للسمية مثل $bceT$ بنسبة ١٠٠٪ وكلا من جيني nheو $cytK$ بنسبة ٧٦.٩٪. تشير هذه النتائج إلى أن اللبن الخام ومنتجات الألبان قد تكون مصدرًا محتملاً لمسببات الأمراض مثل الإشيرشيا كولاي المفرزة لسموم الشيجا ومجموعة الباسيليس

سيريس سينسو لاتو، فضلاً عن جيناتها المسببة للسمية والجينات المسئولة عن الضراوة، مما يستدعي اتخاذ تدابير صحية صارمة بالإضافة إلى الفحوصات الدورية من قبل الجهات المختصة في قطاع الألبان في مصر.