International Journal of Food Science +Technology

Institute of Food Science +Technology ifst

Original article

Molecular characterisation and stress tolerance of *Escherichia coli* isolated from dairy and dried milk-related products

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(Received 10 May 2014; Accepted in revised form 21 July 2014)

Summary This study characterised the phylogenetic groups, pathotypes, antibiotic resistance and stress response of *Escherichia coli* isolates recovered from dairy and dried milk-related products. A total of 54 isolates of *E. coli* were recovered from thirty-three of 180 samples (18%) of these products. Representatives from all the four phylogenetic groups of the organism were identified, although groups A and B1 dominated groups B2 and D. Enterotoxigenic *E. coli* (ETEC) strains producing heat-labile toxin (LT) could be detected among the isolates and were found to be associated with the Kariesh cheese. *Escherichia coli* isolates showed single-drug and multiple-drug resistance to ß-lactams and other antibiotic classes. The $bla_{\text{TEM-1}}$ gene was found to be associated with resistance to ampicillin but not to cephalothin. *Escherichia coli* survived heating at 50 and 55 °C for 60 min, but was undetectable after only 10 min of exposure to 60 °C. It also survived in the presence of 6% and 8% salt for 96 h and in pH 4.0 for 72 h.

Keywords Antibiotic resistance, *Escherichia coli*, multiplex PCR, phylogeny, stress tolerance.

Introduction

Dairy products are very popular foodstuffs that have been regularly consumed by humans over the times. This is due to their high nutritional value, being a rich source of energy and various macro- and micronutrients (Visioli & Strata, 2014). Dairy foods, particularly fermented milks are also known for their therapeutic effects (Nagpal *et al*., 2012). However, dairy and milk-related products have been shown to be associated with different spoilage and pathogenic bacteria. Examples include the presence of *Listeria monocytogenes* and *Staphylococcus aureus* in cheese (El-Sharoud & Spano, 2008; Almeida *et al*., 2013; Barancelli *et al*., 2014), pathogenic *E. coli* strains in different dairy products (Farrokh *et al*., 2013), and *Cronobacter* in dried milks (El-Sharoud *et al*., 2009a).

Escherichia coli is a frequent component of the gut microbiome of most warm-blooded organisms including humans (Bettelheim, 1997). It has been also repeatedly isolated from various food products including milk, dairy products, meat products, vegetables and fruits (Bell & Kyriakides, 1998). The presence of *E. coli* in these products has been thus taken as an indicator of potential faecal contamination introduced by contaminated raw materials, the application of unhygienic manufacturing practices or unsanitary food handlers (Bell & Kyriakides, 1998). However, strains of diarrheagenic *E. coli* (DEC) are known to be associated with foodborne outbreaks, so the organism is also potentially hazardous and not just an indicator of contamination. DEC are divided into five pathotypes including enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC) and enterotoxigenic *E. coli* (ETEC) (Kaper *et al*., 2004). In dairy products, *E. coli* has been also associated with undesirable fermentations leading to quality defects such as early gas formation and off-flavours in cheese (Sheehan, 2011). Taken together, the contamination of dairy products with *E. coli* poses both safety and quality hazards to consumers.

Previous studies have characterised different pathotypes, phylogenetic groups and antibiotic resistance of *E. coli* isolates recovered from clinical, environmental and food product sources (Duriez *et al*., 2001; Saleh *et al*., 2009; Shaheen *et al*., 2009; Rúgeles *et al*., 2010; Ali *et al*., 2012). However, little is known about the phylogenetic groups of *E. coli* isolated from dairy products and their association with the different pathotypes and antibiotic resistance patterns. In this study, *E. coli* was isolated from a number of dairy and dried milk-related products and subjected to PCR analyses to determine

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doi:10.1111/ijfs.12646

the phylogenetic group, the presence of virulence genes and the mechanism of ß-lactam-antibiotic resistance. *Escherichia coli* isolates were also assessed for their tolerance to various stress conditions.

Materials and methods

Isolation and identification of *E. coli*

One hundred and eighty samples of dairy and dried milk-related products were aseptically collected from local markets in the Nile Delta region, Egypt and examined for the presence of *E. coli*. These samples included twenty samples of each of fresh Domiati cheese (moderately salted soft cheese), pickled Domiati cheese (highly salted soft cheese), Kariesh cheese (acid curd, slightly salted soft cheese), Ras cheese (a hard cheese variety), yoghurt, dried skim milk, dried whole milk, ice cream powder and infant formula milk powder. To ensure the recovery of both the intact and sublethal injured cells of *E. coli*, a detection method consisting of three successive stages of pre-enrichment, enrichment and plating was employed (Mackey, 2000). Samples were pre-enriched by mixing 25 g with 225-mL tryptone soy broth (TSB) (Oxoid, Basingstoke, UK) followed by incubation at 37 °C for 24 h. Ten mL of each pre-enriched sample was diluted with 90 mL of the *Enterobacteriaceae* Enrichment (EE) broth (Oxoid) followed by incubation at 37 °C for 24 h. Finally, 2–3 loopfuls from each enriched sample were streaked onto plates of MacConkey agar medium (Oxoid) followed by incubation at 37 °C for 24 h. Three to five suspected colonies were picked up from each sample and identified using Gram staining and the API 20E miniaturised kits (bioMerieux, Marcy l'Etoile, France).

Cell lysis and PCR reactions

Escherichia coli cells were lysed for the extraction of DNA using the QIAamp DNA Mini Kit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). Extracted DNA was examined for the presence of a range of genes encoding phylogenetic, pathotype and antibiotic resistance markers using PCR. PCR reaction composition and conditions were applied as described by relevant protocols indicated below using a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). PCR products were electrophoresed through 2% agarose in Tris–acetate–EDTA buffer, stained with ethidium bromide and visualised using a UV transilluminator.

Phylogenetic typing of *E. coli* isolates

Escherichia coli isolates were phylogenetically characterised using a triplex PCR method targeting the *chuA* and *yjaA* genes and the DNA fragment TSPE4.C2 (Clermont *et al*., 2000).

Pathotyping of *E. coli* isolates

Escherichia coli isolates were screened for different DEC pathotypes by PCR detection of characteristic virulence genes. These involved the *ipaH* gene for EIEC (Sethabuter *et al.,* 1993), the *stx1* and *stx2* genes for EHEC (Pal *et al*., 1999), genes on the pCVD432 plasmid for EAEC (Schmidt *et al*., 1995), the *eaeA* gene for EPEC (Yu & Kaper, 1992) and *estA1*, *estA2-4* and *eltBI* for ETEC (Nada *et al*., 2010). Isolates identified as ETEC were further examined for the presence of genes encoding the colonisation factors CFA/I, CS6, CS4, CS14, CS3, CS5, CS7, CS15, CS18, CS12, CS2, CS17, CS19, CS8, CS21, CS1, PCFO71, CS22, CS13 and CS20 using multiplex PCR assays as described by Nada *et al*. (2010).

Antibiotic resistance testing of *E. coli* isolates

Escherichia coli isolates were examined for their sensitivity to ampicillin (10 μg), cephalothin (30 μg), chloramphenicol (30 μg), nalidixic acid (30 μg), streptomycin (10 μg), tetracycline (30 μg), sulfamethoxazole (23.75 μg) + trimethoprim (1.25 μg), ciprofloxacin (5 μg), amoxicillin/clavulanic acid (20 μg 10 μg^{-1}), cefotaxime (30 μg) and cefotaxime/clavulanic acid (30 μg 10 μg^{-1}). Antibiotic sensitivity was assessed using the Kirby-Bauer disc-diffusion method, and results were interpreted using the criteria of the Clinical & Laboratory Standards Institute (CLSI) (2011).

Detection of ß-lactamase genes

Escherichia coli isolates showing resistance to ß-lactam antibiotics were further examined for the presence of genes encoding 7 ß-lactamases using the PCR protocol described by Chen *et al*. (2004). These genes involved $bla_{\text{TEM-1}}$, $bla_{\text{OXA-1}}$, $bla_{\text{OXA-2}}$, $bla_{\text{OXA-7}}$, $bla_{\text{SHV-1}}$, $bla_{\text{CTX-M1}}$ and $bla_{\text{CTX-M2}}$.

Assessment of stress tolerance of *E. coli*

To assess the heat tolerance of *E. coli*, a 24-h culture of the organism was inoculated in reconstituted skim milk (RSM; 10% total solids), preheated to 50, 55 or 60 °C, followed by incubation at the same relevant temperature for 1 h. To examine salt tolerance, a 24-h culture of *E. coli* was inoculated in RSM salted at 6%, 8%, 10% or 12% w/w, followed by incubation at 37 °C for 96 h. *Escherichia coli* was also assessed for acid tolerance by inoculating a 24-h culture of the organism into TSB adjusted to pH 3.0, 3.5 and 4.0,

followed by incubation at 37 °C for 72 h. Samples were taken at relevant intervals during exposure to heat, salt or acidic pH to assess the viable numbers of *E. coli* by plating onto tryptone soy agar (TSA) (Oxoid), followed by incubation at 37 °C for 24–48 h.

Statistical analysis

Each experiment was replicated at least three times. Categorical data were generally compared using the chi-squared test. Fisher's exact test was, however, used for small numbers (Steel *et al*., 1996). Statistical significance was considered at $P < 0.05$.

Results

Incidence of *E. coli* in dairy and dried milk-related products

A total of thirty-three out of 180 samples (18%) from various dairy and dried milk-related products were found to be contaminated with *E. coli* (Table 1). The bacterium was most prevalent in Kariesh cheese, where it was detected in 40% of the cheese samples. This was followed by Ras cheese and yoghurt (each 30%), fresh Domiati cheese (20%), and dried skim milk, ice cream powder and infant formula milk powder (each 15%). *Escherichia coli* could not be, however, recovered from pickled Domiati cheese or dried whole milk samples.

Phylogenetic groups of *E. coli* isolates

Fifty-four confirmed isolates of *E. coli* recovered from dairy and dried milk-related products were examined for the presence of the *chuA*, *yjaA* and TSPE4.C2 genetic markers (Clermont *et al*., 2000). All the four phylogenetic groups of *E. coli* were identified in these isolates (Table 1 and Fig. 1). Phylogenetic group A was the most abundant (55.5%), followed by group B1 (37%) (Table 1). The remaining two groups, B2 and D, each contained 3.7% of the isolates.

Pathotypes of *E. coli* isolates

Only three of fifty-four confirmed *E. coli* isolates (5.5%) could be identified as DEC; all three contained *eltBI*, identifying them as heat-labile toxin (LT)-producing ETEC (Table 1). These isolates were recovered

Table 1 Isolation, phylogenetic groups and pathotypes of *Escherichia coli* associated with dairy and dried milk-related products

Product	No. of samples	No. of positive samples (%)	No. of *E. coli* isolates	Phylogenetic group [No. of isolates]				Pathotype [No. of isolates (phylogenetic group)]				
				A	B1	B2	D	EIEC	EHEC	EAEC	EPEC	ETEC
Fresh Domiati cheese	20	4 (20)	6	4	2	0	0	0	0	0	0	0
Pickled Domiati cheese	20	0 (0)	0	0	0	0	0	0	0	0	0	0
Kariesh cheese	20	8 (40)	15	10	3	0	2	0	0	0	0	3 (A)
Ras cheese	20	6 (30)	8	5	3	0	0	0	0	0	0	0
Yoghurt	20	6 (30)	9	5	4	0	0	0	0	0	0	0
Dried skim milk	20	3 (15)	5	1	4	0	0	0	0	0	0	0
Dried whole milk	20	0 (0)	0	0	0	0	0	0	0	0	0	0
Ice cream powder	20	3 (15)	5	2	2	1	0	0	0	0	0	0
Infant formula milk powder	20	3 (15)	6	3	2	1	0	0	0	0	0	0
Total (%)	180	33 (18)	54	30 (55.5)	20 (37)	2 (3.7)	2 (3.7)	0	0	0	0	3 (5.5)

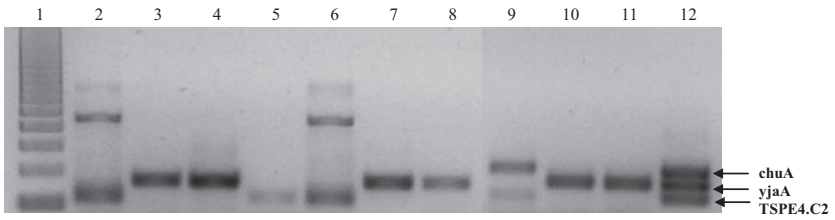

Figure 1 A representative gel of a triplex PCR analysis of the *chuA*, and *yjaA* genes and TSPE4.C2 fragment for detecting the phylogenetic group of *Escherichia coli* isolates recovered from dairy and dried milk-related products. Lane 1: DNA ladder, Lanes 3, 4, 7, 8, 10 and 11: phylogenetic group A, Lanes 2, 5 and 6: phylogenetic group B1, Lane 12: phylogenetic group B2 and Lane 9: phylogenetic D.

from three Kariesh cheese samples and belonged to the phylogenetic group A (Table 1). None of the possible twenty colonisation factors could be detected by PCR in these isolates.

Antibiotic resistance of *E. coli* isolates

Escherichia coli isolates showed resistance to five antibiotics including ampicillin, cephalothin, chloramphenicol, streptomycin and tetracycline (*data not shown*). The highest incidence of antibiotic resistance was found to cephalothin, and ampicillin that were resisted by 61% and 27% of the isolates, respectively. Lower resistance rates of 2–10% were observed with the other three antibiotics. Both single-drug resistance (SDR) and multidrug resistance (MDR) (resistance to two or more antibiotics) were demonstrated by *E. coli* isolates. SDR could be detected in *E. coli* strains within the four phylogenetic groups, whereas MDR was only identified in isolates belonging to groups A and B1 (Table 2). Both SDR and MDR were observed in nonpathogenic *E. coli* isolates, with the LT-producing ETEC strains showing only SDR to cephalothin.

Given the generally higher resistance of the *E. coli* isolates to ß-lactam antibiotics including cephalothin and ampicillin, compared to other drugs, the mechanism(s) of resistance were further assessed for specific ß-lactamase encoding genes. This involved PCR detection of $bla_{\text{TEM-1}}$, $bla_{\text{OXA-1}}$, $bla_{\text{OXA-2}}$, $bla_{\text{OXA-7}}$, $bla_{\text{SHV-1}}$, $bla_{\text{CTX-M1}}$ and $bla_{\text{CTX-M2}}$. Among these genes, only $bla_{\text{TEM-1}}$ could be detected in ampicillin-resistant *E. coli* isolates that showed MDR to other antibiotics including cephalothin, streptomycin and tetracycline. However, isolates with SDR to cephalothin did not harbour the $bla_{\text{TEM-1}}$ or any of the other examined antibiotic-resistant genes. This indicated that $bla_{\text{TEM-1}}$ contributed to *E. coli* resistance to ampicillin rather than cephalothin.

Stress tolerance of *E. coli*

Escherichia coli isolates showed typical survival patterns under stress conditions relevant to those used during the preparation of dairy products (Fig. 2). Exposure to temperatures of 50 and 55 °C resulted in a progressive decline in the viability of *E. coli*, but the organism was still able to survive to detectable numbers of approximately 10^6 and 10^4 CFU mL^{-1}, respectively, after 60 min (Fig. 2a). Increasing the temperature to 60 °C had a more detrimental effect, resulting in cell death after only 10 min (Fig. 2a). Elevated salt concentrations ranging from 6% to 12% (w/w%) in RSM caused declines in the viability of *E. coli* over 96 h (Fig. 2b). While the organism could not be detected by the end of this incubation time in

Table 2 Distribution of single-drug and multidrug resistance in *Escherichia coli* isolates

Percentage of Isolates (Phylogenetic group)	SDR					Percentage of Isolates (Phylogenetic group)	MDR				
	Ampicillin	Cephalothin	Chloramphenicol	Streptomycin	Tetracyclin		Ampicillin	Cephalothin	Chloramphenicol	Streptomycin	Tetracyclin
16.0 (A)		+*				17.0 (A)	+	+			
11.0 (B1)		+				7.0 (A)	+	+		+	+
2.0 (B1)			+			3.0 (B1)	+	+		+	+
5.0 (B2)		+									
2.0 (D)		+									

*LT-producing ETEC isolates were among these cephalothin-resistant organisms.

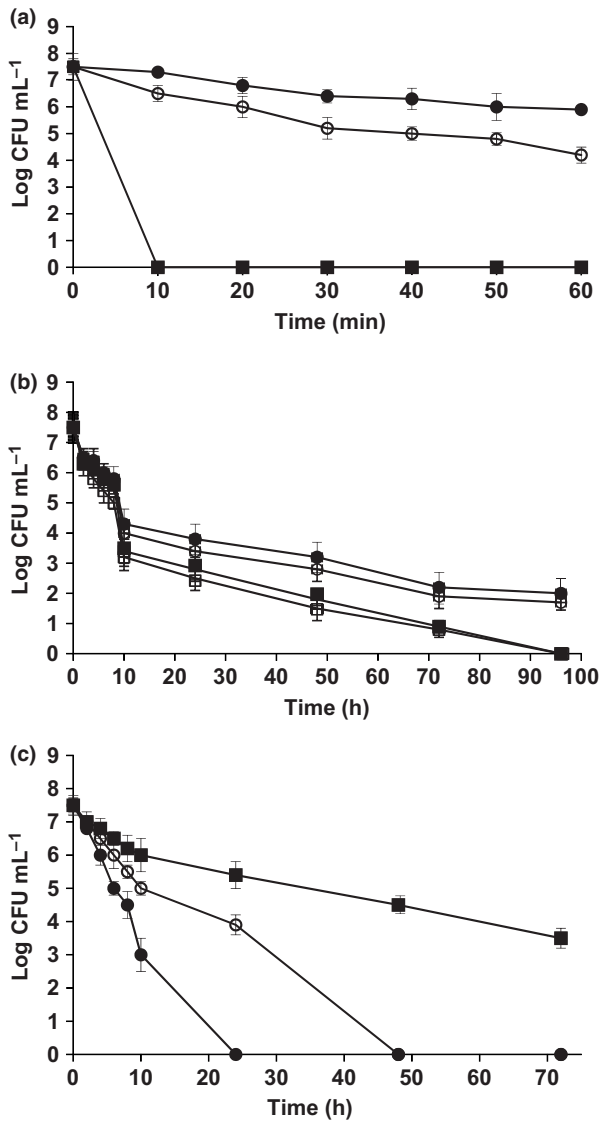

Figure 2 Survival of *Escherichia coli* recovered from dairy and dried milk-related products during exposure to: (a) heating temperatures of 50 °C (●), 55 °C (○) and 60 °C (■) in RSM, (b) salt levels of 6% (●), 8% (○), 10% (■) and 12% (□) (w/w%) in RSM and (c) acidic pH of 3.0 (●), 3.5 (○) and 4.0 (■) in TSB. Presented results are means of at least three replicate determinations and the error bars represent ± 1SD.

RSM containing 10% or 12% salt, it survived in the presence of 6% and 8% salt with viable numbers of approximately 10^2 CFU mL^{-1}. *Escherichia coli* also decreased in numbers on exposure to acidic pH of 4.0, 3.5 and 3.0, but could remain viable with approximately 10^4 CFU mL^{-1} for up to 72 h in pH 4.0 (Fig. 2c). However, the organism was undetectable in lower pH of 3.0 and 3.5 after 24 and 48 h, respectively.

Discussion

The present results show that *E. coli* could be detected in most dairy and dried milk-related products examined in this study, with the exception of pickled Domiati cheese and dried whole milk. Kariesh cheese was associated with the highest incidence rate of *E. coli*, and from which all the ETEC isolates were recovered. This could be due to the use of raw milk in the preparation of this soft cheese combined with human handling during its preparation (El-Sharoud *et al.*, 2009b). Other dairy and dried milk-related samples that were found contaminated with *E. coli* were produced by large or medium-sized production facilities, where milk is pasteurised. This suggests that the presence of *E. coli* in these samples was due to postpasteurisation and/or postprocessing contamination. Assuming that pasteurisation processes were working adequately, this could be supported by the observation that *E. coli* isolates were inactivated within 10 min of exposure to 60 °C (Fig. 2a), which is a less severe heating compared with pasteurisation at 63 °C for 30 min.

The ability of *E. coli* isolates to maintain their viability for 96 h in the presence of 6% and 8% salt in milk could allow them to survive in fresh Domiati cheese prepared from milk salted to these levels and consumed within few weeks of production. Pickled Domiati cheese is prepared from milk salted at higher concentrations of 10–16% NaCl and consumed within 3–6 months of preparation. Taken together with the poor survivability of *Escherichia coli* isolates for even 96 h in milk salted to 10% or 12% NaCl, this could explain the absence of *E. coli* from pickled Domiati cheese samples. *Escherichia coli* isolates could survive for up to 72 h in pH 4.0 and this could aid their survival in fermented dairy products including Kariesh cheese, Ras cheese and yoghurt.

The contamination of dairy and dried milk-related products examined in this study with *E. coli* could generally reflect the lack of an integrated food safety approach combining good manufacturing practices (GMPs), sanitation standard operating procedures, training of the food handlers and the hazard analysis critical control point (HACCP) system (Cusato *et al.*, 2013). The implementation of the GMPs and an integrated food safety system involving HACCP was reported to reduce contamination with various microorganisms and eliminate pathogens from dairy products (Costa Dias *et al.*, 2012; Cusato *et al.*, 2013; Domenech *et al.*, 2013). While the implementation of a food safety system adds to the total production cost, it presents an effective cost-benefit relationship (Cusato *et al.*, 2014). Improving the safety of dairy products reduces the health and economic losses due to the consumption of contaminated products, which reflects

positively on the dairy producers' reputation and enhances their marketing potential.

Escherichia coli isolates recovered from dairy and dried milk-related products showed diversity in their phylogenetic groups, with isolates of phylogenetic groups A and B1 being the most frequently detected. This is consistent with a previous study showing a very similar distribution of *E. coli* isolates from cow milk into the four phylogenetic groups (Higgins *et al.*, 2007). *Escherichia coli* strains of groups A and B1 have been considered as commensal flora in the intestines due to their frequent occurrence and lack of virulence factors (Duriez *et al.*, 2001; Skurnik *et al.*, 2009). Whereas, group B2 and, to a lesser extent, D expressing virulence factors were described as potentially pathogenic extraintestinal strains. Commensal *E. coli* strains could, however, evolve into pathogenic organisms by the acquisition of virulent genetic elements through horizontal gene transfers, genomic deletions and random functional point mutations (Duriez *et al.*, 2001). For instance, pathogenic EAEC, ETEC and EPEC strains belonging to phylogenetic groups A and B1 could be isolated from various food, clinical and water samples (Rúgeles *et al.*, 2010; Ali *et al.*, 2012). This agrees with the present study, in which three *E. coli* isolates belonging to the phylogenetic group A were found to be ETEC.

Enterotoxigenic *E. coli* strains are a significant aetiological agent of diarrhoeal disease worldwide (Fleckenstein *et al.*, 2013). The present study presents dairy products as a potential transmission vehicle of these pathogenic strains. The lack of colonisation factors (CFs) in the LT-producing ETEC isolates described in the present work was not unexpected since this has been observed before in ETEC isolates producing only an LT (Rao *et al.*, 2003; Steinsland *et al.*, 2004). Recent studies showed that LTs could enhance ETEC adherence to intestinal epithelial cells conferring colonisation advantage to these organisms (Johnson *et al.*, 2009; Fekete *et al.*, 2013). This could compensate for the absence of colonisation factors in LT-producing ETEC isolates.

Both SDR and MDR phenotypes could be observed in *E. coli* isolates cultured from dairy and dried milk-related products. Resistance to ß-lactams including cephalothin and ampicillin was more frequent in these isolates compared to other drugs. Among 7 ß-lactamase encoding genes, only $bla_{\text{TEM-1}}$ was found to be associated with resistance to ampicillin but not to cephalothin. This agrees with previous studies describing TEM-1 ß-lactamase as a widely common plasmid-mediated enzyme in ampicillin-resistant strains of Enterobacteriaceae (Philippon *et al.*, 1989; Chen *et al.*, 2004). TEM-1 ß-lactamase was also reported to hydrolyse ampicillin at a greater rate compared to cephalothin (Philippon *et al.*, 1989). However, the present study shows that MDR was particularly associated with commensal nonpathogenic *E. coli* strains of the phylogenetic groups A and B1, with LT-producing ETEC isolates demonstrating only SDR to cephalothin. Still, this poses a health risk given the ability of the commensal nonpathogenic *E. coli* strains to evolve into pathogenic organisms. On their ingestion with contaminated dairy and dried milk-related products, these strains may also serve as a reservoir spreading MDR genetic elements to other bacteria in the human gut.

It could be concluded that *E. coli* could exist in different dairy and dried milk-related products due to postpasteurisation and/or postprocessing contamination. *Escherichia coli* isolates from these products belonged to diverse phylogenetic groups, and some of which were enterotoxigenic producing heat-labile toxins. The isolates showed both single-drug and multidrug resistance, presenting them as a reservoir spreading antibiotic resistance genetic elements to other pathogenic and nonpathogenic bacteria in foods or the human gut. Together, these results raise the need for devising more effective measures to eliminate this micro-organism from dairy products.

Acknowledgments

This work was supported by Mansoura University, EGYPT. The authors have no conflict of interest to declare.

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