Frequency of extended-spectrum β -lactamase (ESBL)– and AmpC β -lactamase–producing *Enterobacteriaceae* in a cheese production process

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ABSTRACT

The aim of this study was to investigate the extended-spectrum β -lactamase (ESBL) and AmpC β -lactamase activities of *Enterobacteriaceae* isolated from raw milk and cheese production line and to determine the probability of transmitting these bacteria to consumers. One hundred seventy-three samples from raw milk and cheese production lines were analyzed; 64 isolates were confirmed as *Enterobacteriaceae*. Sixteen of 64 isolates (25%) were resistant to at least one cephalosporin according to European Committee on Antimicrobial Susceptibility Testing Standards (EUCAST) and Clinical and Laboratory Standards Institute criteria. Seven of the 16 resistant isolates (43.75%) had confirmed ESBL activity. Additionally, phenotypic AmpC β -lactamase activity was observed in 31 (48.44%) of 64 *Enterobacteriaceae* isolates and confirmed in 27 of the 31 strains (89.1%). Overall, 3 isolates showed both ESBL activity and AmpC resistance, 28 isolates were only AmpC resistant, and 5 isolates had ESBL activity alone. Of the 173 samples, the proportions of samples that contained ESBL- and AmpC-producing isolates were 4.64 and 15.6%, respectively. Five of the ESBL-positive isolates (62.5%) and 11 of the AmpC-positive isolates (40.7%) were obtained from bulk milk tanks; therefore, the bulk tank plays a very important role in the spread of antibiotic-resistant bacteria. Periodic cleaning and maintenance of bulk tanks should be performed and recorded. Effective food safety and hygiene practices should significantly reduce cross-contamination in dairy plants.

Key words: antibiotic resistance, *Enterobacteriaceae*, cheese, raw milk

INTRODUCTION

Bacterial antimicrobial resistance is a major public threat to public health worldwide (EFSA, 2011; WHO, 2014). Antibiotics of the β -lactam group are the most commonly used antibiotics globally for treating infections caused by gram-negative bacteria because of their limited side effects and strong bactericidal properties (de Oca et al., 2015). Penicillins, cephalosporins, monobactams, and carbapenems are the major β -lactam antibiotics for treating many infectious diseases (Bush and Jacoby, 2010). Gram-negative bacteria can develop resistance to β -lactam-group antibiotics by several mechanisms (Thomson, 2010). β -Lactamase enzyme synthesis is the most important resistance mechanism (Bush and Jacoby, 2010; Schmid et al., 2013). The production of β -lactamase enzymes is encoded by genes on the chromosomes, plasmids, or transposons of microorganisms (EFSA, 2011), and these plasmids and mobile elements are easily transferred to other bacteria. Extended-spectrum β -lactamases (ESBL) are enzymes that can hydrolyze third-generation cephalosporins (oxymino-cephalosporins), such as ceftriaxone, ceftiofame, and cefepime. They are also partially susceptible to cephamycin and carbapenem. The ESBL are usually inhibited by β -lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam (Thomson, 2010; Schmid et al., 2013; Stefani et al., 2014). The AmpC β -lactamases confer resistance to cephalosporins, including β -lactam inhibitors, monobactams, cephamycins, and some carbapenems, depending on the type of β -lactamases. They are inhibited by cloxacillin and aztreonam (Jacoby, 2009). Although researchers have reported the existence of AmpC β -lactamases less frequently than ESBL, AmpC β -lactamases exhibit resistance to a broader spectrum of β -lactam antibiotics than ESBL (Jacoby, 2009; Ibrahim et al., 2016). Community- and hospital-acquired infections with AmpC β -lactamase-producing bacteria are increasing worldwide (Liebana et al., 2013). In Turkey, the unnecessary use of antibiotics in clinical practice is restricted by government regulations; however, reports from the

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National Surveillance Network of Antimicrobial Resistance by the Ministry of Health in Turkey (Şimşek, 2017) show an increasing prevalence of ESBL-producing invasive *Escherichia coli* (33.2% in 2008 and 51% in 2015) and ESBL-producing *Klebsiella pneumoniae* (40% in 2008 and 65% in 2015). These rates are much higher than those in European countries. Antibiotic usage in veterinary medicine and for growth promotion and disease prevention is also an important problem in antibiotic resistance (Capita and Alonso-Calleja, 2013). In recent years, many researchers have shown the emergence of *Enterobacteriaceae* producing ESBL- and AmpC β -lactamases on dairy farms in numerous countries (Stefani et al., 2014; Gonggrijp et al., 2016; Ibrahim et al., 2016; Santman-Berends et al., 2017). Recently, ESBL- and AmpC-producing *Enterobacteriaceae* in food from milk, red meat, and poultry have been identified (Liebana et al., 2013; Kurekci et al., 2016; Tark et al., 2017). Therefore, food-producing animals and foods of animal origin such as meat, milk, and cheese are under suspicion for being transmission vectors for colonization and infection of the humans with ESBL-producing *Enterobacteriaceae* (Verraes et al., 2013; Nguyen et al., 2016). Veterinary medicine is unregulated in Turkey, although several congresses, training sessions, and seminars have been produced to raise awareness.

In Turkey, in studies in farm animals, the prevalence of ESBL-positive *E. coli* is reported to range from 1.5 to 15.53% (Elmacioğlu, 2013; Pehlivanoğlu et al., 2016). Various researchers have reported the presence of antibiotic-resistant *Enterobacteriaceae* in milk and cheese samples, and the percentage of ESBL-producing *Enterobacteriaceae* strains ranges from 9.9 to 50% (Arslan and Özdemir, 2008; Gundogan and Avci, 2013; Kurekci et al., 2016; Tekiner and Özpınar, 2016). However, there are limited data about the prevalence of ESBL- and AmpC-producing *Enterobacteriaceae* in various geographical locations.

Here, we aimed to investigate ESBL and AmpC β -lactamase activity in *Enterobacteriaceae* isolated from raw milk collected on farms and in the cheese processing line, including curd and cheese, in a small artisanal dairy factory in order to determine the transmission probability for these bacteria in the final food product.

MATERIALS AND METHODS

Sampling Area

The small dairy factory used for analyzing the cheese production process has been operating since 1987 in Çanakkale, Turkey. They have 75 employees and a closed area of 1,500 m². Their capacity averages 25 t of

Table 1. Sampling plan

Sample	No. of samples
Raw milk ¹	136
Raw milk from farm bulk tanks	17
Cheese production process ²	20
Total	173

¹Raw milk from each farmer who gave milk to the sampled dairy for cheese production.

²Cheese production process sampling points: (1) raw milk from reception and weighing unit in dairy, (2) raw milk before pasteurization, (3) pasteurized milk, (4) pasteurized milk on the transfer line, (5) milk from cheese vat, (6) curd in cheese vat (before pressure), (7) curd without salt (after pressure), (8) raw cheese in brine, (9) cheese in tin (first day), (10) cheese ripened for 3 mo.

cheese per day. Twelve dairy products are produced, including cheese, yogurt, and buttermilk. Approximately 2 t of white cheese is produced daily and sold to both domestic and overseas markets.

Sample Collection

One hundred seventy-three samples were obtained under aseptic conditions during the raw milk and cheese production process. Samplings of raw milk were performed in 136 dairy farms and 17 bulk tanks in Çanakkale province, Turkey. A cheese processing line from the small dairy factory was also sampled (Table 1). Milk samples (250 mL each) and cheese and curd samples (approximately 250 g each) were collected aseptically using a sterile ladle into sterile plastic containers. We transported the samples to the laboratory in an insulated container at 4°C within 2 to 4 h after collection. All samples were analyzed the same day. Experiments were done in duplicate. Samples were collected twice between October 2015 and June 2016.

Reference Cultures

Reference cultures were obtained from the microbiology laboratory culture collection of the Food Engineering Department of Çanakkale Onsekiz Mart University. An ESBL-positive strain (*Klebsiella pneumoniae* ATCC 700603) and an ESBL-negative strain (*Escherichia coli* ATCC 25922) were used as positive and negative controls, respectively.

Isolation and Identification

Milk samples (1 mL) were diluted with 9 mL of 0.1% sterile peptone water (Merck, Darmstadt, Germany), and 0.1-mL aliquots from undiluted milk and its dilutions was spread onto plates containing MacConkey agar (Merck) containing cefotaxime (2 µg/

mL) and eosin methylene blue agar (Merck) containing cefotaxime (2 µg/mL), and Fluorocult violet red bile (Merck) agar. Curd and cheese samples (10 g) were weighed into sterile stomacher bags and diluted with 90 mL of 0.1% sterile peptone water. Then, 1 mL of the first dilution was spread onto 2 plates (0.5 mL on each plate). Plates were incubated at 37°C for 24 to 48 h. After incubation, colony morphologies (pink or colorless, mucoid, purple, greenish-metallic sheen by reflected light and light-blue fluorescing colonies with a UV lamp depending on medium) were assessed on each plate and selected as suspected *Enterobacteriaceae* (up to 2 colonies per plate). To isolate ESBL-susceptible *E. coli* O157, 25 mL or 25 g of sample was enriched in modified tryptic soy broth (**mTSB**) containing novobiocin (Merck) at 37°C for 24 to 48 h and then 0.1 mL of mTSB was streaked onto plates of sorbitol-MacConkey (SMAC) agar with selective supplement (Merck). Colonies from both sorbitol-fermenting and non-sorbitol-fermenting strains were selected. In samples in which *Enterobacteriaceae* were not detected (<1 log cfu/g or cfu/mL), 0.1 mL of mTSB was streaked onto plates of MacConkey agar containing cefotaxime (2 µg/mL), and colonies from both lactose-fermenting and non-lactose-fermenting strains were selected. These colonies were streaked onto nutrient agar (Merck) to obtain pure cultures and HiCrome ESBL agar with HiCrome ESBL Selective Supplement (HiMedia, Mumbai, India) to separate resistant colonies from nonresistant colonies. Both pink-to-purple and bluish-green colonies were selected from HiCrome ESBL agar for identification. Sorbitol-negative colonies obtained from SMAC agar were serologically screened for the presence of the serogroup O157 antigen using a latex agglutination test (*E. coli* O157 Latex Test Kit, Oxoid, Basingstoke, UK) according to the manufacturer's instructions.

Gram-negative, coccobacillus, rod-shaped, and oxidase-negative isolates were identified at genus level by using indole, methyl red, Voges-Proskauer, citrate, motility, hydrogen sulfide production, and carbohydrate fermentation tests according to the *Bergey's Manual of Determinative Bacteriology* (Holt et al., 1994). The ESBL production confirmed isolates were also identified by API 20E (bioMérieux, Florence, Italy). Isolates were stored in TSB (Merck) containing 16% glycerol at -20°C until use.

Antimicrobial Susceptibility Test

The disk diffusion antibiotic susceptibility tests were carried out according to the agar disk diffusion method (CLSI, 2013). One or 2 bacterial colonies were taken from blood agar (5%), which had been previously inoculated and incubated overnight at 37°C. The colonies were

suspended in 5 mL of sterile saline (0.85% NaCl) until it reached 0.5 McFarland ($1-2 \times 10^8$ cfu/mL) standard concentration. Then, 100 µL of culture was distributed onto a Mueller-Hinton agar plate (Merck). The antibiotic disks were placed onto the plate's surface after the plate was dried at room temperature (≤ 15 min). The plates were then incubated at 37°C for 24 h, and the results were recorded by measuring the inhibition zone diameter across the disk and interpreted according to the guidelines recommended by the clinical breakpoint tables of the European Committee on Antimicrobial Susceptibility Testing Standards (EUCAST, 2013) and the Clinical and Laboratory Standards Institute (CLSI, 2013). Production of ESBL was confirmed using the combination disk test according to EUCAST (2013).

To detect inducible AmpC activity, the disk approximation test was used according to the methods of Tan et al. (2009) and Gupta et al. (2014). All isolates were also tested using a double-disk synergy test. Briefly, 500 µg of cloxacillin (CX500, Bioanalyse, Ankara, Turkey) was used to inhibit AmpC enzymes (CLSI, 2013). After incubation at 37°C for 24 h, an enhanced zone of inhibition between any cephalosporin disk and the CX500 disk was interpreted as evidence of AmpC production (Mirelis et al., 2006).

The boronic acid (**BA**)-clavulanic acid (**CA**) method (BA-CA) was used to remove false-negatives for ESBL that resulted from AmpC or false-negatives for AmpC related to ESBL. The methods described by Coudron (2005) and Song et al. (2007) were used with some modifications. The antibiotic disks used in this test were 30 µg of cefotaxime (**CTX**); 30 µg of CTX and 400 µg of BA; 30 µg of CTX and 10 µg of CA; and 30 µg of CTX, 400 µg of BA, and 10 µg of CA. Combinations of antibiotics and inhibitors were used in this test. After incubation at 37°C for 24 h, an increase in zone diameters ≥ 5 mm for CTX-CA-BA antibiotics versus CTX-CA antibiotics was considered positive for AmpC. Furthermore, an increase ≥ 5 mm for CTX-CA-BA versus CTX-BA was considered positive for ESBL.

RESULTS

The *Enterobacteriaceae* load of the raw milk samples ranged between 3.0 and 5.0 log cfu/mL and between 4.0 and 7.0 log cfu/mL in farmer and bulk milk, respectively. In the cheese processing line, the *Enterobacteriaceae* load ranged from <1.0 to 5.79 log cfu per mL for milk and per g for curd and cheese. Pasteurization decreased the load to undetectable levels depending on the milk load; however, curd and cheese samples were contaminated with *Enterobacteriaceae* (data not shown). We obtained 490 isolates from 173 samples (Table 2) using cefotaxime (2 mg/mL) containing media (MacConkey

Table 2. The distribution of obtained isolates according to sampling points and their properties

Sampling point	No. of isolates from all media	HiCrome ESBL agar ¹		Gram-negative isolates	Oxidase-negative isolates
		Including weak-growing isolates ²	Only well-growing isolates ²		
Raw milk from farmer	350	244	88	41	26
Raw milk from bulk tanks	64	35	35	26	23
Cheese production process	76	68	24	22	15
Total	490	347	147	89	64

¹Hi-Media (Mumbai, India); ESBL = extended spectrum β-lactamase.

²Weak growing: only a few small colonies; well growing: showing good growth and typical color mentioned by producer.

and eosin methylene blue agar), and Fluorocult violet red bile agar and SMAC agar. Of these, 347 isolates harbored ESBL after growth on HiCrome ESBL agar (including weak-growing isolates). Furthermore, on the HiCrome ESBL agar, 147 of 347 isolates displayed colony morphologies typical of ESBL, and these 147 isolates were analyzed further. We found 89 of 147 isolates to be gram-negative coccobacillus or rod-shaped bacteria. Additionally, 64 of 89 isolates were gram-negative, oxidase-negative, and fermented glucose, which confirmed them to be ESBL *Enterobacteriaceae* (Table 2). We confirm genus identity with biochemical and morphological tests. Of the 64 isolates, 41% derived from farm raw milk samples, 36% came from bulk milk tanks, and 23% originated from the cheese production line samples. Isolates included *Enterobacter* spp. (50.00%), *Klebsiella* spp. (20.31%), *Citrobacter* spp. (17.19%), *E. coli* (10.94%), and *Shigella* spp. (1.56%; Figure 1). In addition, *E. coli* O157 was isolated from farm milk (0.73%).

Production of ESBL by isolates was detected using 2 methods. According to EUCAST and CLSI breakpoints, the number of presumptive ESBL producers was very high in *E. coli* isolates (71.43%). These isolates showed resistance to all cephalosporins except ceftazidime (30 µg). *Enterobacter* spp. (18.75%) showed more resistance to cefpodoxime (10 µg) and cefpodoxime (5 µg) compared with other cephalosporins. *Citrobacter* sp. (9.09%) and *Klebsiella* sp. (7.69%) showed resistance to all cephalosporins and *Shigella* spp. did not show resistance to any cephalosporins according to EUCAST and CLSI (Table 3). In addition, one *E. coli* O157 isolate was susceptible to all antibiotics used in ESBL detection.

Overall, 25% (16 isolates) of the presumptive ESBL-producing *Enterobacteriaceae* tested positive for at least one of the antibiotics (Table 4). We confirmed ESBL activity of 7 of 16 isolates using the combination disk test method. The frequency of ESBL *Enterobacteriaceae* in the cheese production line was 10.9%. Five

Figure 1. The distribution of *Enterobacteriaceae* isolates at genus level according to sampling points.

Table 3. Resistance (R) and susceptibility (S; number with % in parentheses) to antibiotics of 64 *Enterobacteriaceae* strains according to EUCAST (2013) and CLSI (2013)

Standard	Antibiotic ¹	<i>Enterobacter</i> spp.		<i>Citrobacter</i> spp.		<i>Klebsiella</i> spp.		<i>Escherichia coli</i>		<i>Shigella</i> sp.	
		R	S	R	S	R	S	R	S	R	S
EUCAST (2013)	CPD10	6 (18.75)	26 (81.25)	1 (9.09)	10 (90.91)	1 (7.69)	12 (92.31)	5 (71.43)	2 (28.57)	0 (0)	1 (100.00)
	CAZ10	4 (12.50)	28 (87.5)	1 (9.09)	10 (90.91)	1 (7.69)	12 (92.31)	5 (71.43)	2 (28.57)	0 (0)	1 (100.00)
	CTX5	6 (18.75)	26 (81.25)	1 (9.09)	10 (90.91)	1 (7.69)	12 (92.31)	5 (71.43)	2 (28.57)	0 (0)	1 (100.00)
	CRO30	4 (12.50)	28 (87.5)	1 (9.09)	10 (90.91)	1 (7.69)	12 (92.31)	5 (71.43)	2 (28.57)	0 (0)	1 (100.00)
CLSI (2013)	CPD10	6 (18.75)	26 (81.25)	1 (9.09)	10 (90.91)	1 (7.69)	12 (92.31)	5 (71.43)	2 (28.57)	0 (0)	1 (100.00)
	CTX30	5 (15.63)	27 (84.37)	1 (9.09)	10 (90.91)	1 (7.69)	12 (92.31)	5 (71.43)	2 (28.57)	0 (0)	1 (100.00)
	CAZ30	2 (6.25)	30 (93.75)	1 (9.09)	10 (90.91)	1 (7.69)	12 (92.31)	3 (42.86)	4 (57.14)	0 (0)	1 (100.00)
	ATM30	3 (9.38)	29 (90.62)	1 (9.09)	10 (90.91)	1 (7.69)	12 (92.31)	5 (71.43)	2 (28.57)	0 (0)	1 (100.00)
	CRO30	4 (12.50)	28 (87.5)	1 (9.09)	10 (90.91)	1 (7.69)	12 (92.31)	5 (71.43)	2 (28.57)	0 (0)	1 (100.00)
Total isolates		32		11		13		7		1	

¹CPD10 = cefpodoxime 10 µg; CAZ10 and CAZ30 = ceftazidime 10 and 30 µg, respectively; CTX5 and CTX30 = cefotaxime 5 and 30 µg, respectively; CRO30 = ceftriaxone 30 µg; ATM30 = aztreonam 30 µg.

and 2 of the confirmed ESBL-positive isolates belonged to samples from the bulk tank and cheese production line, respectively. According to API 20E results, we identified the 7 confirmed ESBL-positive isolates as *E. coli* (5; 71.43%), *Enterobacter cloacae* (1; 14.29%), and *Klebsiella pneumoniae* ssp. *ozonae* (1; 14.29%; Table 5)

We observed phenotypic AmpC β-lactamase activity in 31 (48.44%) of the 64 *Enterobacteriaceae* isolates us-

ing the disk approximation test method and confirmed it in 27 strains using the double-disk synergy test. Moreover, one isolate showed a positive ESBL result in the CTX-BA disk test, and 3 isolates showed AmpC β-lactamase activity in the CTX-CA test (Table 6). The *E. coli* O157 isolate was suspected to be an AmpC producer by the disk approximation test, but this was not confirmed by double-disk synergy test.

Table 4. Resistance profile (R = resistant; S = susceptible) of *Enterobacteriaceae* isolates resistant to at least one cephalosporin¹ according to EUCAST (2013) and CLSI (2013)

Species	Strain ²	EUCAST (2013)				CLSI (2013)				
		CPD10 <21 mm	CAZ10 <22 mm	CTX5 <21 mm	CRO30 <23 mm	CPD10 ≤17 mm	CTX30 ≤27 mm	CAZ30 ≤22 mm	ATM30 ≤27 mm	CRO30 ≤25 mm
<i>Enterobacter</i> sp.	M17	S	R	S	S	S	S	S	S	S
<i>Enterobacter</i> sp.	M26	R	S	R	S	R	R	S	S	R
<i>Enterobacter</i> sp.	T5	R	S	R	S	R	S	S	S	S
<i>Enterobacter</i> sp.	T6	R	S	S	S	R	S	S	S	S
<i>Enterobacter</i> sp.	T11	S	S	R	S	S	S	S	S	S
<i>Enterobacter</i> sp.	I2	R	R	R	R	R	R	R	R	R
<i>Enterobacter</i> sp.	I4	R	R	R	R	R	R	R	R	R
<i>Enterobacter</i> sp.	I8	S	S	R	S	S	S	S	S	S
<i>Enterobacter</i> sp.	I11	R	R	R	R	S	R	S	R	R
Rate of resistance (%)		66.7	44.4	66.7	33.3	55.6	55.6	22.2	33.3	44.4
<i>Escherichia coli</i>	T17	R	R	R	R	R	R	S	R	R
<i>E. coli</i>	T21	R	R	R	R	R	R	R	R	R
<i>E. coli</i>	T22	R	R	R	R	R	R	S	R	R
<i>E. coli</i>	T23	R	R	R	R	R	R	R	R	R
<i>E. coli</i>	I6	R	R	R	R	R	R	R	R	R
Rate of resistance (%)		100	100	100	100	100	100	60	100	100
<i>Citrobacter</i> sp.	T9	R	R	R	R	R	R	R	R	R
Rate of resistance (%)		100	100	100	100	100	100	100	100	100
<i>Klebsiella</i> sp.	T16	R	R	R	R	R	R	R	R	R
Rate of resistance (%)		100	100	100	100	100	100	100	100	100

¹CPD10 = cefpodoxime 10 µg; CAZ10 and CAZ30 = ceftazidime 10 and 30 µg, respectively; CTX5 and CTX30 = cefotaxime 5 and 30 µg, respectively; CRO30 = ceftriaxone 30 µg; ATM30 = aztreonam 30 µg. The breakpoint zone diameter (in mm) is given after each antibiotic name and dose.

²M = isolate from farmer raw milk sample, T = isolate from bulk milk tank, I = isolate from the cheese production line.

Table 5. Screening results of the samples for extended-spectrum β -lactamase (ESBL)-producing *Enterobacteriaceae*

Origin	No. (%) of ESBL isolates	No. (%) of confirmed ESBL isolates	Genus-based distribution, no. (%)		
			<i>Enterobacter</i> spp.	<i>Escherichia coli</i>	<i>Klebsiella</i> spp.
Raw milk from farmer	2 (12.50)	— ¹	—	—	—
Bulk tank milk	9 (56.25)	5 (71.43)	—	4 (80.0)	1 (100.0)
Cheese production line	5 (31.25)	2 (28.57)	1 (100.0)	1 (20.0)	—
Total (%)	16 (100.0)	7 (100.0)	1 (100.0)	5 (100.0)	1 (100.0)

¹Not determined.

Overall, 3 isolates displayed both ESBL and AmpC activity, 28 isolates showed AmpC resistance, and 5 isolates showed ESBL resistance.

DISCUSSION

In our study, 136 dairy farms with 17 bulk tanks and a cheese processing line were sampled to determine the frequency of ESBL- and AmpC-producing *Enterobacteriaceae* and to explore the risk factors associated with their presence. The frequency of ESBL-producing *Enterobacteriaceae* in raw milk from dairy farms reported in this study (0.73%) is consistent with other studies that reported the frequencies between 0 and 9.5% (Geser et al., 2012; Skočková et al., 2015; Odenthal et al., 2016). Ntuli et al. (2017) reported that bulk milk tanks were contaminated with a wide diversity of *Enterobacteriaceae*. In this study, the frequency of ESBL-producing *Enterobacteriaceae* in bulk tank milk was 29.41%. In contrast, 9.5% of bulk tanks from German dairy farms yielded ESBL-producing *Enterobacteriaceae* (Odenhal et al., 2016). In previous studies, researchers emphasized the importance of controlling the bulk tank (Sudarwanto et al., 2015; Odenthal et al., 2016; Azevedo et al., 2016; Ntuli et al., 2017). In Turkey, no further information has been published regarding ESBL-producers in bulk milk tanks except Tekiner and Özpınar (2016). However, they reported a total rate for ESBL-producing *Enterobacteriaceae* (43.6%) obtained from raw cow milk from containers and holding tanks in markets and dairy farms.

In our study, 21.74% of the strains isolated from the bulk tank produced ESBL. This result is higher than that of Odenthal et al. (2016). Improving general hygiene in bulk tanks and in all stages of production would reduce the microbial load and antimicrobial resistance. The reason for differences between the frequencies of ESBL isolates obtained from farm (0.78%) and bulk tank (21.74%) milks might be unsatisfactory hygiene application in bulk tanks. In addition, antibiotic-resistant isolates might have come from other farms adding milk to the bulk tanks that we did not sample. This is a significant finding and increased farmer awareness about bulk tank hygiene—besides antibiotic usage—is needed. Another important finding is that, although more presumptive ESBL isolates were obtained from farmer milks, ESBL activity was confirmed in only one such isolate during screening for AmpC activity with the BA-CA method. Thus, in antibiotic resistance investigations, it is important to screen for additional β -lactamase activity such as AmpC. In addition, molecular studies on the detection of ESBL strains have shown that up to 33% of ESBL strains remain undetected with phenotypic methods; therefore, genotypic confirmation is needed (Thomson, 2010; Liebana et al., 2013; Verraes et al., 2013).

In this study, 13% of *Enterobacteriaceae* isolates obtained from the cheese production stages possessed ESBL activity. In a previous study, Gundogan and Avci (2013) showed that 23% of *Enterobacteriaceae* isolates obtained from milk and cheese samples were ESBL-positive. Ahmed and Shimamoto (2015) reported that

Table 6. Screening results of the samples for AmpC β -lactamase-producing *Enterobacteriaceae*

Origin	No. (%) of AmpC isolates	No. (%) of confirmed AmpC	Genus-based distribution, no. (%)			
			<i>Enterobacter</i> spp.	<i>Citrobacter</i> spp.	<i>Escherichia coli</i>	<i>Klebsiella</i> spp.
Raw milk from farmer	9 (29.03)	8 (29.6)	5 (25.0)	2 (40.0)	— ¹	1 (50.0)
Bulk tank milk	13 (41.94)	11 (40.7)	9 (45.0)	1 (20.0)	—	1 (50.0)
Cheese production line	9 (29.03)	8 (29.6)	6 (30.0)	2 (40.0)	—	—
Total	31 (100.0)	27 (100.0)	20 (100.0)	5 (100.0)	—	2 (100.0)

¹Not determined.

65.5% of isolates obtained from milk and dairy products in Egypt were ESBL-positive Shiga toxin-producing *E. coli* (STEC) O157:H7. We confirmed that 10.9% of the 64 *Enterobacteriaceae* isolates were positive for ESBL, lower than that observed in previous studies. Gundogan and Yakar (2007) determined that 34.9% of the *Klebsiella* spp. isolated from raw milk and cheese samples were ESBL-positive. However, in our study, 15.38% of all *Klebsiella* spp. isolates tested positive for ESBL production. Dias et al. (2012) observed that *E. coli* strains from Minas soft cheese displayed 100% susceptibility to ceftazidime and cefotaxime antibiotics and concluded that these isolates were ESBL negative. Vrabec et al. (2015) also determined that 42.2% of the *E. coli* present in typical fresh Slovak cheese produced ESBL. However, we determined that 83.3% of the 6 *E. coli* isolates in the current study were ESBL positive, similar to results reported by Özadam and Özpınar (2016), Aidara-Kane et al. (2013), and Amador et al. (2009). However, lower resistance rates have been reported in Germany (Odenthal et al., 2016) and Slovenia (Krizman et al., 2017). Kurekci et al. (2016) also determined that 13.5% of all 87 Sürk cheese samples in Turkey contained ESBL-producing *E. coli*. Additionally, Tekiner and Özpınar (2016) reported that 17% of all *Enterobacteriaceae* isolates from 150 raw milk and cheeses samples were ESBL positive. In this study, we examined 173 samples (farm raw milk, bulk milk, curd, and cheese) and 4.64% contained ESBL-producing isolates, most of which were obtained from bulk tank milk samples. The lower level of ESBL producers in the current study (compared with other studies) may be the result of increased awareness regarding the incorrect usage of antibiotics in animals. In addition, in previous studies of ESBL-producing *Enterobacteriaceae*, researchers obtained samples under different market conditions (market survey) and contamination may have occurred during post-production storage or transport, or at the time of sale. Thus, most of the studies cited above reported a higher prevalence of ESBL-producing isolates. However, in our study, samples were taken from one cheese production line (from producers' bulk milk to cheese), which could explain the lower incidence because if cheese samples are taken from markets, cheese may be contaminated from different sources and the rate may be higher.

The co-existence of ESBL and AmpC β -lactamases is also a significant problem (Thomson, 2010; EFSA, 2011). Although AmpC β -lactamases are less common than ESBL, the former exhibit a broader resistance profile (Black et al., 2005). Detection of AmpC is important because it could cause a failure in treatment (Gupta et al., 2014). AmpC β -lactamases may appear to be mistakenly susceptible to broad-spectrum cephalosporins such as ceftazidime or cefotaxime. As a result

of this treatment, derepressed mutants are formed and clinical failure is detected in the patients. *Enterobacteriaceae* producing ESBL and AmpC have been reported in clinical settings and in food (Jacoby, 2009; Liebana et al., 2013; Gupta et al., 2014). Furthermore, 32.8% of 598 *E. coli* isolates obtained from milk and meat farms in Germany produced ESBL, and 23.5% isolates carried the *ampC* gene (Schmid et al., 2013). In a study carried out in Switzerland, AmpC β -lactamase-producing *E. coli* were isolated from dairy milk (Endimiani et al., 2012). Özadam and Özpınar (2016) reported that 50% of 83 cheese samples collected from public bazaars and markets in İstanbul contained AmpC-producing *Enterobacteriaceae*. Ibrahim et al. (2016) showed that *E. coli* is phenotypically AmpC β -lactamase resistant rather than ESBL-resistant on most dairy farms. In the current study, 15.6% of samples contained AmpC-producing *Enterobacteriaceae*, and 42.2% of *Enterobacteriaceae* strains possessed AmpC β -lactamase activity. Although none of the *E. coli* strains isolated in this study had confirmed AmpC β -lactamase activity, the amount of AmpC-producing *Enterobacteriaceae* isolates was higher than ESBL-producing isolates. Geser et al. (2012) reported that *Enterobacteriaceae* function as reservoirs of antibiotic resistance, such as ESBL and AmpC. Previous researchers showed that food-producing animals and the food chain have played an important role in the transmission of antibiotic-resistant bacteria to humans (Liebana et al., 2013; Stefani, et al., 2014; Ibrahim et al., 2016; Ntuli et al., 2017).

In our study, we obtained 5 ESBL-positive and 11 AmpC-positive isolates from bulk milk tanks. These isolates could be derived from many potential sources, such as waste, environment, equipment, and the farmer. We also obtained 2 ESBL-positive and 8 AmpC-positive isolates from the cheese production line. We obtained one ESBL-positive isolate from raw milk before pasteurization and one from pasteurized milk on the transfer line. In addition, 5 and 3 of the AmpC-positive isolates were obtained from pre-pasteurization stages and in fresh cheese. Inadequate heating (low temperature and time), heating method (boiler or heat exchanger), a contaminated pipeline or piece of equipment, and poor personal hygiene are all possible reasons for contamination. In addition, these isolates could have been heat-resistant. The dairy factory used a boiler for pasteurization. After the results of this study were shared with them, they changed to a plate heat exchanger for pasteurization. In the second sampling after implementing use of the heat exchanger, no ESBL- or AmpC-producing isolates were detected in post-pasteurization stages. Genotypic confirmation of ESBL- and AmpC-positive strains and determination of pulsed-field gel electrophoresis (PFGE) types should

be studied to determine whether strains are from the same source.

Antibiotic resistance in the dairy industry is important and preventative measures should be taken for prevention. First, the development and spread of bacterial resistance should be prevented or reduced by careful use of antibiotics. Antibiotics should be used only for the treatment of animal diseases caused by pathogenic bacteria. Biosecurity in animal trade should be increased to prevent the spread of resistant bacteria (Liebana et al., 2013). Good waste management on farms is necessary to avoid contamination in humans and food animals from wastes that contain antibiotics and antibiotic-resistant microorganisms (Finley et al., 2013). Contamination of raw milk by resistant bacteria from animals may be prevented by good milking hygiene. The cheese production company should raise awareness about the use of antibiotics and milking hygiene. Award programs could be applied according to SCC and California Mastitis Test score of raw milk. Because bulk tanks play a very important role in the spread of antibiotic-resistant bacteria, periodic cleaning and maintenance of bulk tanks should be performed and recorded. Dairy producers must adhere to pasteurization standards (a minimum of 63°C for 30 min) to prevent contamination in post-pasteurization stages with antibiotic-resistant bacteria. Finally, cross-contamination should be avoided by applying effective food safety and hygiene standards in dairy plants.

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