



Short communication: Molecular characterization and antimicrobial resistance of pathogenic *Escherichia coli* isolated from raw milk and Minas Frescal cheeses in Brazil

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ABSTRACT

The aim of this study was to quantify, identify, evaluate antimicrobial resistance, and characterize the virulence factors of enteropathogenic (EPEC), Shiga-toxigenic (STEC), and enterohemorrhagic (EHEC) *Escherichia coli* in raw milk (RM) and legal (LMFC) and illegal (IMFC) Minas Frescal cheeses in southern and northeast Brazil. Illegal cheeses are those made without official inspection service or sanitary surveillance. We evaluated samples of RM produced in Paraná (southern) and Maranhão (northeast) States, LMFC produced using pasteurized milk in inspected industries, and IMFC potentially produced with raw milk. Mean total coliform counts were 8.4×10^4 cfu/mL for RM, 1.4×10^7 cfu/mL for LMFC, and 2.9×10^7 cfu/mL for IMFC. Mean *E. coli* counts were 2.4×10^3 cfu/mL for RM, 1.9×10^2 cfu/mL for LMFC, and 1.1×10^5 cfu/mL for IMFC. Among the 205 *E. coli* isolates from RM, 9.75% were identified as EPEC, mainly (90%) in samples from Paraná. Of the total isolates from the cheese samples, 97.4% ($n = 111$) came from IMFC, of which 1.8 and 2.7% were identified as EPEC and STEC, respectively; no EHEC was detected. The phylogenetic group A (60%) and typical EPEC (68%) predominated, which confirms the possible human origin of pathogenic isolates in RM and IMFC. Of these, 50% were resistant to at least one antibiotic, and streptomycin was the antimicrobial with the highest number (8) of EPEC and STEC resistant isolates. This study reports the first isolation of serogroup O28ac in Brazilian milk. We found no predominance of a specific serogroup of EPEC or STEC in milk or cheese or clonal isolates in the same

sample, indicating different origins of the contamination in these products, presumably mostly related to poor hygienic handling.

Key words: coliforms, enteropathogenic *Escherichia coli* (EPEC), food safety, Shiga-toxigenic *Escherichia coli* (STEC)

Short Communication

Brazil is a large country and significant differences exist in milk quality in different regions of the country (Nero et al., 2005; Ribeiro Júnior et al., 2015). Brazilian small producers, to increase their profitability, often produce dairy products such as Minas Frescal cheese, mostly made with raw milk, and market it illegally. The illegality of these products is associated with the lack of official inspection service and sanitary surveillance. Minas Frescal is one of the most consumed in Brazil and, because it is produced and marketed fresh without maturation and is a very high moisture cheese (46 to 55%), it offers ideal conditions for the multiplication and maintenance of pathogenic microorganisms (de Campos et al., 2018).

In contrast to the production conditions of small Brazilian producers, some dairy production areas have a smaller number of producers that produce large volumes of milk using high quality, improved animals and advanced technology (Ribeiro Júnior et al., 2015, 2018).

Among the thermotolerant coliforms (*Citrobacter*, *Enterobacter*, *Klebsiella*, and *Escherichia*), *Escherichia coli* is the only microorganism that indicates fecal contamination when present in milk or milk products (Altalhi and Hassan, 2009). Strains of this species may be commensal or pathogenic to the consumer (Kaper et al., 2004; Farrokh et al., 2013; Gomes et al., 2016; Douëllou et al., 2017), and their presence in foods can indicate the presence of other enteropathogens. The

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consumption of raw milk or raw dairy products is still common in Brazil. Thus, pathogenic microorganisms, such as some strains of *E. coli*, in raw milk may pose a risk to public health. These isolates may also be resistant to antibiotics, which hinders hospital treatments (Sáenz et al., 2004).

Considering differences in the quality and conditions of production of raw milk among Brazilian regions, the consumption habits of raw milk or raw milk products, and the potential pathogenicity of strains of *E. coli* in milk and dairy products, we aimed to quantify total coliforms and *E. coli*, identify the presence of serotypes with pathogenic potential (i.e., those carrying the *eaeA*, *stx1*, *stx2*, and *bfpA* genes), and determine the phylogenetic groups and antimicrobial resistance patterns of *E. coli* isolates from refrigerated raw milk produced in 2 regions of Brazil (northeast and south) and Minas Frescal cheeses sold legally and illegally.

In Paraná State in southern Brazil, 70 raw milk (RM) samples were collected from large ($n = 20$) and small producers ($n = 50$). In Maranhão State, in northeastern Brazil, 10 samples of refrigerated RM were collected from small farms. All of these dairy farms were previously characterized (Ribeiro Júnior et al., 2015, 2018).

The samples were collected aseptically from storage tanks of raw milk and transported under refrigeration to the National Institute of Science and Technology for the Milk Productive Chain at the State University of Londrina (UEL; Londrina, Paraná, Brazil) or sent to the Microbiology Laboratory of the Federal Institute of Maranhão, Caxias campus (Maranhão, Brazil), where they were immediately processed.

Serial dilutions of milk samples were performed in saline (0.9%) peptone (0.001%) and with selected samples plated on Petrifilm EC (3M Microbiology, St. Paul, MN) and incubated at 35°C for counting total coliforms (TC; 24 h) and *E. coli* (48 h) according to the manufacturer's recommendations. The results of the counts were submitted to nonparametric statistical analyses by χ^2 test using SAS software (v. 9.0; SAS Institute Inc., Cary, NC).

Ten illegal Minas Frescal cheeses (IMFC), potentially produced with raw milk, were collected from markets in the city of Londrina, Paraná State. Another 10 samples of legally produced Minas Frescal cheese (LMFC) were collected from supermarkets; these cheeses were produced with pasteurized milk, refrigerated, and inspected.

Aliquots (25 ± 0.2 g) of each cheese sample were diluted in 225 mL of 0.1% buffered saline (Oxoid, Basingstoke, UK), followed by homogenization and serial decimal dilution. The counts of TC and *E. coli* of the cheese samples were performed in the same way as the milk samples.

All isolates identified as *E. coli* in Petrifilm EC plates were recovered in brain heart infusion broth (Acumedia, Baltimore, MD) and submitted to genomic DNA extraction by simple boiling (Ribeiro Júnior et al., 2016). The extracts (~50 ng) were subjected to amplification of the *eaeA* gene to identify enteropathogenic *E. coli* (EPEC) and of the *stx1* and *stx2* genes to verify the production potential of Shiga-like toxins (STEC) according to the primers and reaction conditions described by Paton and Paton (1998), using individual assays for each gene. The presence of enterohemorrhagic *E. coli* (EHEC) was therefore investigated by the simultaneous presence of *eaeA* and *stx1* or *stx2* genes.

Isolates identified as EPEC were subjected to new PCR to verify the presence of the *bfpA* gene (localized adherence) using primers and reaction conditions described by Aranda et al. (2004) for differentiation typical enteropathogenic *E. coli* (t-EPEC) and atypical (a-EPEC).

The phylogenetic groups of the EPEC or STEC isolates were determined using multiplex PCR described by Clermont et al. (2013). The serotypes of the isolates were identified using the VITEK system (bioMérieux Inc., Durham, NC) and typed with rabbit sera against 56 somatic and flagellar *E. coli* antigens (Orskov and Orskov, 1984; Scheutz et al., 2004) obtained from the Department of Public Health, Faculty of Medicine, National Autonomous University of Mexico (Ciudad Universitaria, Mexico City).

Antimicrobial resistance was determined using the agar disk diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2016) for *E. coli*. The following antimicrobial agents were used on *E. coli* samples: amoxicillin-clavulanic acid (30 μ g), aztreonam (30 μ g), tetracycline (30 μ g), cefotaxime (30 μ g), cefoxitin (30 μ g), nalidixic acid (30 μ g), gentamicin (10 μ g), chloramphenicol (30 μ g), ampicillin (10 μ g), ciprofloxacin (5 μ g), and streptomycin (10 μ g). Enrofloxacin (5 μ g) was also tested because this antimicrobial is commonly used in Brazilian veterinary practice. *Escherichia coli* strain ATCC 25922 was used for quality control.

Extended-spectrum β -lactamase (ESBL)-producing *E. coli* were confirmed by using the double disk diffusion testing for amoxicillin-clavulanic acid and cefotaxime or ceftazidime, or by using a combination disk test with cefotaxime, cefotaxime-clavulanic acid (Becton Dickinson, Sparks, MD), ceftazidime, and ceftazidime-clavulanic acid (Becton Dickinson), according to Clinical and Laboratory Standards Institute (CLSI) recommendations.

The TC and *E. coli* counts for RM, IMFC, and LMFC samples are described in Table 1. Considering the TC and *E. coli* counts of the RM samples evalu-

Table 1. Total coliform (TC) and *Escherichia coli* (EC) counts in refrigerated raw milk produced in the states of Paraná and Maranhão and in legal (LMFC) and illegal (IMFC) Minas Frescal cheeses sold in the city of Londrina, Paraná, Brazil

Sample	n	TC (cfu/mL)	EC (cfu/mL)
Raw milk	80		
Maximum		2.4×10^6	1.5×10^5
Minimum		<1	<1
Mean (SD)		$8.4 (\pm 3.3) \times 10^4$	$2.4 (\pm 16) \times 10^3$
IMFC	10		
Maximum		1.4×10^8	4×10^5
Minimum		$<10^3$	$<10^2$
Mean (SD)		$2.9 (\pm 4.6) \times 10^7$	$1.1 (\pm 1.6) \times 10^5$
LMFC	10		
Maximum		7.8×10^7	10^3
Minimum		10^2	$<10^2$
Mean (SD)		$1.4 (\pm 2.3) \times 10^7$	$1.9 (\pm 2.8) \times 10^2$

ated, only 2.9% of the total coliforms were *E. coli*. Of the 80 RM samples, 30 had *E. coli* isolates, indicating direct or indirect fecal contamination in 37.5% of the total samples.

Regarding cheese samples, no significant variations ($P > 0.05$) were observed between TC and *E. coli* counts between LMFC and IMFC, although the counts were considerably lower in LMFC samples. We observed that the average *E. coli* count corresponded to 0.37% of the mean observed for TC for IMFC and 0.0013% for LMFC.

From the Petrifilm EC plates used in the counts, 205 *E. coli* strains were isolated from the milk samples: 34 (16.6%) from RM samples of the large producers, 60 (29.3%) from RM samples of small producers of Paraná, and 111 (51.1%) strains from milk samples of Maranhão producers. From the cheese samples, colony recovery was proportional to the *E. coli* counts, with 3 isolates from 10 LMFC and 111 from 10 IMFC, totaling 114 isolates.

All 319 *E. coli* isolates were submitted to uniplex PCR to detect the *stx1*, *stx2*, and *eaeA* genes, totaling 957 reactions. The results are shown in Table 2. No single strain from RM samples was positive for the presence of the *stx1* or *stx2* genes; therefore, no milk sample evaluated by the present study contained STEC. However, 20 (9.75%) of the total RM isolates were positive for EPEC. Of these, 18 (90%) were isolated from producers in Paraná State and 2 (10%) from milk sampled in Maranhão State (Table 2). Considering the total of *E. coli* isolates in each production region, EPEC strains represented 19.15% of the *E. coli* in milk from producers in Paraná and 1.8% of the isolates of the milk in Maranhão. Thus, regardless of the Brazilian region of production, raw milk is at risk of being contaminated with EPEC, and milk produced in southern Brazil is more likely to be contaminated.

Regarding cheese samples, 2 (1.8%) EPEC strains (strains EC11 and EC12) originated from 2 distinct samples of IMFC and 3 other STEC strains (strains EC13^{a-c}) were isolated from the same sample of IMFC. The cheeses legally produced and marketed did not carry any strains of EPEC or STEC, mainly because they were produced with pasteurized milk. Of the strains that tested positive for the *eaeA* or *stx2* genes, none were positive for the 2 genes simultaneously, so no isolates were characterized as EHEC (Table 2).

The *bfpA* gene was detected (t-EPEC) in 14 (70%) and 1 (50%) of the EPEC isolates from raw milk and cheese samples, respectively (Table 2). Typical and atypical EPEC strains differ primarily in relation to their natural hosts. Humans are the only reservoir of t-EPEC, which are more involved in outbreaks of diarrhea in developing countries, whereas a-EPEC have animal and human reservoirs and are more involved in outbreaks in developed countries (Nataro and Kaper, 1998; Chen and Frankel, 2005; Gomes et al., 2016).

Of the 22 EPEC isolates from RM or IMFC, 9 (40.1%) were resistant to at least one of the antibiotics tested. Among them, streptomycin had the most resistant strains ($n = 8$) originating from samples and belonging to serogroups and distinct phylogenetic groups (Table 2); ESBL strains were not identified among the 24 EPEC and STEC isolates.

Among the 3 STEC isolates from an IMFC sample, the EC13^a isolate was resistant to 3 antibiotics (ampicillin, tetracycline, and streptomycin). The EC13^c strain was a STEC of the serogroup OR:H- that was resistant to tetracycline. These 2 strains, however, belong to distinct phylogenetic groups (B2 and A, respectively), indicating that they were not clones within the same cheese sample, as well as the third STEC isolate from the same cheese sample (EC13^b), of serogroup O1:H8 and phylogenetic group B1, which was sensitive to all the antibiotics tested.

Tamaki et al. (2005) correlated serogroup O28ac with enteroinvasive *Escherichia coli* (EIEC); however, the *ipaH* gene was not tested in this study. This study is the first to report the O28ac serogroup in Brazilian milk samples.

As large producers control and manage cases of mastitis more closely in their dairy herds, there is greater pressure for the selection of resistant strains on these farms. This study demonstrated that 60% of the EPEC isolates in milk from large producers were resistant to at least one antibiotic, whereas only 26.6% of the EPEC isolates from the milk from small producers were resistant.

Phylogenetic group A represented 63.6% (14/22) of EPEC isolates from milk and cheese samples. Carlos et al. (2010) concluded that phylogenetic group A is more

Table 2. Serotypes, virulence factors, antimicrobial resistance, and phylogenetic groups of pathogenic strains of *Escherichia coli* isolated from raw milk samples from large and small producers in 2 regions of Brazil and illegal Minas Frescal cheeses

Origin	Strain ¹	Brazilian State	Serotype	Virulence genes	Antimicrobial resistance ²	Phylogenetic group ³
Raw milk, large producers	EC1	Paraná	O76:H39	<i>eaeA</i> , <i>bfpA</i>	AMP, TET, STR	A
	EC2	Paraná	O59:H21	<i>eaeA</i> , <i>bfpA</i>	NAL, TET, STR	A
	EC3	Paraná	O51:H49	<i>eaeA</i>	STR	E
	EC4	Paraná	O49:H21	<i>eaeA</i> , <i>bfpA</i>	Susceptible	B1
	EC5	Paraná	O28ac:H37	<i>eaeA</i> , <i>bfpA</i>	Susceptible	A
Raw milk, small producers	EC6	Paraná	O59:H21	<i>eaeA</i> , <i>bfpA</i>	Susceptible	B1
	EC7 ^a	Paraná	O164:H4	<i>eaeA</i> , <i>bfpA</i>	TET, STR	A
	EC7 ^b	Paraná	O73:H39	<i>eaeA</i> , <i>bfpA</i>	Susceptible	A
	EC8 ^a	Paraná	O53:H4	<i>eaeA</i> , <i>bfpA</i>	Susceptible	A
	EC8 ^b	Paraná	O85:H16	<i>eaeA</i>	Susceptible	A
	EC8 ^c	Paraná	O76:H4	<i>eaeA</i>	Susceptible	A
	EC8 ^d	Paraná	O53:H16	<i>eaeA</i>	Susceptible	Clade I or II
	EC8 ^e	Paraná	O53:H-	<i>eaeA</i> , <i>bfpA</i>	Susceptible	A
	EC8 ^f	Paraná	O76:H4	<i>eaeA</i> , <i>bfpA</i>	Susceptible	A
	EC8 ^g	Paraná	O85:H16	<i>eaeA</i> , <i>bfpA</i>	Susceptible	A
	EC9 ^a	Paraná	O70:H19	<i>eaeA</i> , <i>bfpA</i>	Susceptible	A
	EC9 ^b	Paraná	O85:H16	<i>eaeA</i> , <i>bfpA</i>	STR	Clade I or II
	EC9 ^c	Paraná	O187:H8	<i>eaeA</i> , <i>bfpA</i>	Susceptible	B1
	EC10 ^a	Maranhão	ONT:H37	<i>eaeA</i>	FOX, STR	Unknown
	EC10 ^b	Maranhão	O184:H10	<i>eaeA</i>	AMP, STR, FOX	Unknown
Illegal Minas Frescal cheese	EC11	Paraná	O179:H8	<i>eaeA</i> , <i>bfpA</i>	STR	A
	EC12	Paraná	O101:HNT	<i>eaeA</i>	FOX, AMP	A
	EC13 ^a	Paraná	OR:H-	<i>stx2</i>	AMP, TET, STR	B2
	EC13 ^b	Paraná	O1:H8	<i>stx2</i>	Susceptible	B1
	EC13 ^c	Paraná	OR:H-	<i>stx2</i>	TET	A

¹Superscript letters denote strains obtained from the same sample.

²AMP = ampicillin; TET = tetracycline; STR = streptomycin; NAL = nalidixic acid; FOX = ceftiofur.

³According to Clermont et al. (2013).

related to humans, which agrees with the predominance of t-EPEC in the present study (Table 2).

The STEC found in the IFMC samples (EC13^{a-c}) belonged to the phylogenetic groups B1, B2, and A, whose hosts are more related to bovines (B1) and humans (B2 and A; Carlos et al., 2010). Thus, the contamination of milk or cheeses by STEC, as well as by t-EPEC, probably originated in the cheese makers due to hygiene deficiencies.

Brazilian raw milk, regardless of the conditions and region of its production, contains pathogenic *E. coli* strains, some of which may have antimicrobial resistance. Minas Frescal-type cheeses produced from raw milk pose the same risk, whereas cheeses made with pasteurized milk do not present any risk from pathogenic *E. coli* isolates. Consumption of Brazilian raw milk or dairy products should be avoided.

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