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Original Article

Virulence Genes and Antimicrobial Resistance in *Escherichia coli* from Cheese Made from Unpasteurized Milk in Brazil

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Abstract

Cow raw milk cheese is widely eaten in Brazil. These products may be contaminated with pathogenic bacteria. In this work, we investigated the presence of *Escherichia coli* in raw milk cheese from different States in Brazil. From 147 ''Minas'' cheese samples, 28 cheeses were positive for *E. coli*. Among 39 *E. coli* isolates of the cheeses, one was positive for *eae* and negative for *bpf*A and *efa*1/*lif*A using PCR, and so was classified as atypical Enteropathogenic *E. coli* (aEPEC). Two other isolates were positive for extraintestinal pathogenic *E. coli* (ExPEC) genes. The aEPEC isolate belongs to serogroup O127 and was classified in A phylogenetic group, and ExPEC isolates were found in O73:H12 (EC-2 strain) and O64474:H8 (EC-9 strain) serotype. This ExPEC belongs to A and C phylogenetic group, respectively. Most of *E. coli* strains belonged to Clermont phylogenetic groups A (28.2%), C, and E (23.1%). Six strains (15.4%) of *E. coli* were positive for group B1 and two (5.1%) for B2. *E. coli* isolates presented an aggregative (46.0%) and diffuse (12.6%) adherence pattern to HeLa cells, and the other isolates did not show adhesion (41.4%). Four *E. coli* isolates (10.3%) were shown to produce moderate biofilm. The antimicrobial resistance rate was tetracycline (25.6%), followed by ampicillin (17.9%), cefoxitin (7.7%), nalidixic acid (5.1%), and amoxicillin–clavulanic acid (2.6%). One strain was resistant to three antimicrobials (tetracycline, ampicillin, and nalidixic acid). The presence of these microorganisms, the O127 strain, and a new serogroup in Brazil is a potential risk for public health.

Keywords: diarrheagenic *E. coli*, extraintestinal pathogenic *E. coli*, raw milk cheese, antimicrobial resistance

Introduction

''Minas cheese'' is one of the most popular cheeses consumed in Brazil. It can be made with raw or pasteurized milk through enzymatic coagulation. This category of cheese is characterized by high pH and moisture (>55%), with low salt percentage. This cheese becomes susceptible to contamination due to its high moisture, associated with poor hygiene conditions during its production, use of unpasteurized milk, and long transportation time (Cunha *et al.*, 2006; Moraes *et al.*, 2009)

In Brazil, there are rules for the production and commercialization of dairy products, but they are not commonly enforced. *Escherichia coli* has been used as a marker for contamination and hygiene conditions, during processing or postprocessing of food. Its presence may indicate the presence of enteric pathogens (Moraes *et al.*, 2009; Okura and Marin, 2014). Moreover, some *E. coli* strains can acquire virulence genes and become pathogenic.

Urinary tract infections or enteric diseases are caused by specific *E. coli* categories, termed ExPEC (extraintestinal pathogenic *E. coli*) or DEC (diarrheagenic *E. coli*), respectively. DECs are separated into six pathotpyes, which are separated by pathogenicity patterns: enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC), and Shiga toxigenic *E. coli* (STEC). STEC has a subcategory named enterohemorrhagic *E. coli* (EHEC), which includes O157:H7 strains. Some studies suggest that food contamination by *E. coli* comes from food handling (Kaper *et al.*, 2004; Rapini *et al.*, 2005; Vincent *et al.*, 2010).

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Besides *E. coli* pathotypes, other pathogens, such as *Listeria monocytogenes*, *Salmonella* sp., and *Staphylococcus aureus*, are transmitted through dairy products (Kadariya *et al.*, 2014; Ferreira *et al.*, 2016). Another important aspect is bacterial resistance to antimicrobials. Extended-spectrum beta-lactamase (ESBL)-producing *E. coli* is among the most resistant pathogens. These resistant strains are predominantly found in clinical samples. However, they have also been found in food (Carattoli, 2013; Nagy *et al.*, 2015).

In this context, products of animal origin require special attention. They can be vehicles for propagation of resistance determinants. However, the role of raw milk cheeses as reservoirs for resistance determinants is not well known in Brazil (Szmolka *et al.*, 2012; Carattoli, 2013; Ribeiro *et al.*, 2016). Therefore, the objective of this study was to investigate the presence of *E. coli* potentially pathogenic in raw milk cheese commercialized in Brazil.

Materials and Methods

Samples and bacterial strains

A total of 147 unpasteurized cow milk cheese samples were collected, between July 2014 to January 2016, from streets vendors and at markets from five Brazilian States: Paraná (64), São Paulo (49), Minas Gerais (24), MatoGrosso do Sul (8), and Bahia (2).

Approximately 25 g of each cheese was diluted into 225 mL of buffered peptone water, plated onto MacConkey agar (Difco®, Sparks, MD) and incubated at 37°C for 24 h. Two or three colonies from each plate were selected and tested by using biochemical assays (Enterokit - Probac®) to identify *E. coli*. All strains of *E. coli* were stored in brain heart infusion (Difco) plus 25% glycerol (Sigma®, St. Louis, MO) at −80°C.

Positive and negative controls used in this work are from the Bacterial Collection of the Laboratory of Basic and Applied Bacteriology, Universidade Estadual de Londrina (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/fpd).

DNA preparation

E. coli DNA template preparation was performed by thermal lysis. *E. coli* strains were grown on TSA agar (Difco) at 37°C for 24 h. DNA was extracted by suspending seven colonies, from the same pure culture, in 200 μL of sterile water. The mixture was heated at 100°C for 10 min and centrifuged at 10,000 × *g* for 6 min. The supernatant was used as the template in PCR assays.

PCR for detection of DEC and ExPEC virulence genes with E. coli *isolates*

The presence of virulence genes was established by using three Multiplex PCR techniques. The following virulence markers were used to detect DEC: *eae*A (structural gene for intimin of EPEC and EHEC), *bfp*A (structural gene for the BFP of typical EPEC), *efa*1/*lif*A (EHEC factor for adherence [*efa*1]/lymphocyte inhibitory factor A [*lif*A]), *agg*R (transcriptional activator of typical EAEC), *elt*, *est* (enterotoxins of ETEC), *ipa*H (invasion plasmid antigen H gene, found in EIEC), *stx*1, *stx*2 (Shiga toxins of EHEC), and *ehx*A (enterohemolysin, which can be found in EHEC and EPEC). Multiplex PCR methods were tested previously (Puño-Sarmiento *et al.*, 2014). Amplicons were subjected to 2% agarose gel electrophoresis, followed by Gel Red staining (Biotium®, Hayward, CA) and visualization at UV transilluminator. A 1-kb DNA ladder (Invitrogen®) was loaded on each gel.

Furthermore, EAEC virulence genes were also tested. They are localized at the AA plasmid, and include the aggregative adherence fimbrial adhesion gene *aa*F, an antiaggregation protein (dispersin) encoded by the *aap* gene (formerly known as *asp*U); *aai*C (*agg*R-activated island); and *aat*A (antiaggregation protein transporter). For ExPEC detection, the genes *iro*N, *omp*T, *hly*F, *iss*, and *iut*A were assayed (Supplementary Table S1).

PCR for determination of phylogenetic group

Phylogenetic group was carried out for the *E. coli* isolates, using a Multiplex PCR assay as described by Clermont *et al.* (2013). Based on the presence or absence of genes (*chu*A, *yja*A, and *arp*A) and a noncoding DNA fragment (TSPE4.C2), the isolates were classified into seven *E. coli* phylogenetic groups (A, B1, B2, C, D, E, or F).

Serotyping

Isolate serotyping was identified using the VITEK system and typed with rabbit sera obtained against 187 somatic and 56 flagellar *E. coli* antigens (Orskov and Orskov, 1984; Scheutz et al., 2004) from the Departamento de Salud Pública, Facultad de Medicina, Universidad Nacional Autonoma de México, Ciudad Universitaria, Mexico City.

Biofilm formation

Biofilm production was tested as described by Wakimoto *et al.* (2004), with slight modifications. The strains were grown in Luria-Bertani broth (LB; Difco) for 24 h at 37°C with shaking. Then, 5 μL of culture was inoculated into 195 μL of Dulbecco's modified Eagle medium, containing 0.45% glucose, in 96-well flat-bottom microliter polystyrene plates (BD Falcon, Bedford, MA). The plates were covered and incubated aerobically for 24 h at 37°C. Before staining for 5 min with 0.5% crystal violet (Sigma) solution, the samples were washed twice with phosphate-buffered saline (PBS) 0.01 M, pH 7.4. After staining, they were washed five times more. The dye bound to adherent cells was solubilized with 200 μL of 95% (v/v) ethanol per well. Biofilm was quantified at 570 nm using an automated plate reader (Synergy™ HT; Bio-Tek, Winooski, VT). Strain EAEC 042 and *Escherichia coli* K12 HB101 were used as a positive and negative control, respectively. The isolates were evaluated according to Wakimoto *et al.* (2004) into three categories: group 1 ($OD_{570} > 0.2$), strong biofilm formation; group 2 ($0.1 \leq OD_{570} \leq 0.2$), moderate biofilm formation; and group 3 ($OD_{570} < 0.1$), without biofilm formation.

Adherence assays

E. coli adherence to HeLa cells was assayed as previously described (Cravioto *et al.*, 1979), with slight modifications. Cells were grown in 24-well tissue culture microplates (BD Falcon) in which sterile round cover slips (13 mm in diameter) were placed before inoculation. The growth medium in each well of the microplate contained 0.9 mL of Eagle's minimal essential medium (MEM; Invitrogen) supplemented

with 10% fetal calf serum (Invitrogen) and 1% antibiotic solution (penicillin 100,000 U, and streptomycin 100 μg/mL; Sigma). The HeLa monolayer was grown overnight at 37°C with 5% CO_2 to yield at least 70% confluence. The slides were washed thrice with sterile PBS 0.05 M, pH 7.4 (PBS). Forty microliters of the overnight bacterial culture was incubated in Luria-Bertani broth (LB; Difco) at 37°C and added to 0.96 mL of MEM containing 2% fetal bovine serum and 3% D-mannose (Sigma). After 3 h of incubation at 37°C with 5% CO_2, the monolayers were washed with sterile PBS and incubated for an additional 3 h. Next, the slides were washed five times with PBS, fixed with absolute methanol for 10 min, and stained with May-Grunwald (Sigma) and Giemsa (Sigma) stain. The slides were examined under a light microscope using an oil immersion lens. To determine the adhesion pattern, previously described criteria were used (Nataro *et al.*, 1987; Scaletsky *et al.*, 1999).

Antimicrobial resistance testing

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 (AMC, 30 μg), aztreonam (ATM, 30 μg), tetracycline (TET, 30 μg), cefotaxime (CTX, 30 μg), cefoxitin (FOX, 30 μg), nalidixic acid (NAL, 30 μg), gentamicin (GEN, 10 μg), chloramphenicol (CHL,

Table 1. Genotypic and Phenotypic Characteristics of *Escherichia coli* Isolated from Raw Milk Cheese in Brazil

Strains	*Origin*	*Serotype*	*Virulence genes*	*Antimicrobial resistance*	*Pattern of adherence to HeLa cells*	*Biofilm formation*	*Clermont phylogenetic group*
EC-1	MG	O127:HNT	*eae*A	TET	AA	None	A
EC-2	MG	O73:H12	*iro*N, *omp*T, *hly*F, *iss*	Susceptible	AA	None	A
EC-3[a]	PR	—	None	Susceptible	AA	Moderate	A
EC-3[b]	PR	—	None	TET	AA	None	E
EC-4[a]	MG	—	None	Susceptible	AA	None	A
EC-4[b]	MG	—	None	Susceptible	DA	None	A
EC-4[c]	MG	—	None	Susceptible	NA	None	A
EC-5[a]	MG	—	None	Susceptible	NA	None	C
EC-5[b]	MG	—	None	Susceptible	NA	None	C
EC-5[c]	MG	—	None	Susceptible	NA	None	C
EC-6[a]	MG	—	None	TET, AMP	NA	Moderate	E
EC-6[b]	MG	—	None	TET	AA	None	E
EC-7[a]	MG	—	None	Susceptible	AA	None	Unknown
EC-7[b]	MG	—	None	Susceptible	NA	None	E
EC-8[a]	BA	—	None	TET, NAL	NA	None	E
EC-8[b]	BA	—	None	Susceptible	AA	None	E
EC-9	MG	O64474:H8	*iro*N, *omp*T, *hly*F, *iss*, *iut*A	Susceptible	AA	None	C
EC-10	PR	—	None	Susceptible	NA	None	B1
EC-11	PR	—	None	Susceptible	NA	None	B1
EC-12	MG	—	None	Susceptible	DA	None	B1
EC-13	PR	—	None	FOX	AA	None	A
EC-14	MG	—	None	Susceptible	NA	None	A
EC-15	MG	—	None	FOX, AMP	AA	None	C
EC-16	MG	—	None	TET, AMP, NAL	NA	None	C
EC-17	SP	—	None	TET, FOX	AA	None	C
EC-18	PR	—	None	TET	NA	None	C
EC-19	MG	—	None	AMC	NA	None	Unknown
EC-20[a]	MG	—	None	TET, AMP	NA	None	B1
EC-20[b]	MG	—	None	TET, AMP	DA	None	B1
EC-21	MG	—	None	Susceptible	AA	None	E
EC-22	PR	—	None	Susceptible	DA	None	A
EC-23	SP	—	None	Susceptible	DA	None	A
EC-24	MG	—	None	Susceptible	AA	None	E
EC-25	MG	—	None	Susceptible	AA	Moderate	B2
EC-26[a]	MG	—	None	Susceptible	AA	None	C
EC-26[b]	MG	—	None	Susceptible	AA	None	B1
EC-27	MG	—	None	AMP	NA	Moderate	B2
EC-28	MG	—	None	Susceptible	NA	None	A
EC-29	MG	—	None	Susceptible	NA	None	E

[a–c]Strains obtained from the same cheese.

AA, aggregative adherence; AMC, amoxicillin–clavulanic acid; AMP, ampicillin; BA, Bahia; DA, diffuse adherence; FOX, cefoxitin; HNT, H nontypable; MG, Minas Gerais; NA, nonadherence; NAL, nalidixic acid; PR, Paraná; SP, São Paulo; TET, tetracycline.

30 μg), ampicillin (AMP, 10 μg), ciprofloxacin (CIP, 5 μg), and streptomycin (STR, 10 μg). Enrofloxacin (ENR, 5 μg) was also tested because this antimicrobial is commonly used in veterinary practice. *E. coli* strain ATCC 25922 was used as quality control. ESBL production was confirmed with 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 CLSI recommendations.

Results

From a total of 147 samples of unpasteurized cheese analyzed in this study, 28 cheeses were positive for *E. coli* and 39 isolates were further tested because some cheeses had more than one isolate.

Thirty-nine *E. coli* were isolated from the cheeses for further testing. By PCR, one *E. coli* isolate was positive for *eae* and negative for *bpf* and *efa*1/*lif*A, and so was classified as atypical EPEC (aEPEC) (Table 1).

The aEPEC isolate belongs to serogroup O127 and was classified in A phylogenetic group, and ExPEC isolates were found in O73:H12 (EC-2 strain) and O64474:H8 (EC-9 strain) serotype. This ExPEC belong to A and C phylogenetic group, respectively.

The majority of *E. coli* strains belonged to phylogenetic groups A (28.2%), C, or E (23.1%). Six strains (15.4%) of *E. coli* were positive for group B1 and two (5.1%) for B2 (Table 1). The *E. coli* isolates presented an aggregative adherence pattern (46.0%) and diffuse adherence (12.6%), and the rest did not show adhesion (41.4%) (Table 1 and Fig. 1).

Based on the absorbance, four *E. coli* isolates (10.3%) were shown to produce moderate biofilm (Table 1). The remaining *E. coli* isolates did not produce biofilm.

For *E. coli,* the antimicrobial with higher resistance rate was tetracycline (25.6%), followed by ampicillin (17.9%), cefoxitin (7.7%), nalidixic acid (5.1%), and amoxicillin–clavulanic acid (2.6%). One strain (EC-16) was resistant to three antimicrobials. Most strains were resistant to either one or two antimicrobials (Table 1). No strain was shown to produce ESBL.

Discussion

Raw milk cheese is commercialized in Brazilian markets and streets. It is consumed by a large share of the population. On the other hand, the inspection is deficient, what makes cheese a potential vehicle for zoonotic diseases.

In this study, *E. coli* strains were isolated in about 20% of the raw milk cheese, in contrast with previous works in Brazil, where this contaminant was detected in 60–70% of the cheese samples (Carvalho *et al.*, 2007; Okura and Marin, 2014 and Ribeiro *et al.*, 2016). However, while the previous works did not report the detection of DEC, in this work, we isolated an aEPEC strain. aEPEC is associated with diarrhea in children (Croxen *et al.*, 2013) and has been detected in fresh cheese before (Araujo *et al.*, 2002). One of the genes confers virulence to aEPEC, associating with cases of diarrhea is *efa*1/*lif*A, present in OI-122 pathogenicity island (Afset *et al.*, 2006), but this gene is poorly researched in cheeses and not found on aEPEC strain.

However, several studies have reported an increase in reports of aEPEC instead of typical EPEC and, currently, this pathotype is considered an important emerging diarrheagenic pathogen, mainly in children (Afset *et al.*, 2004; Hernandes *et al.*, 2009; Ochoa and Contreras *et al.*, 2011). The O127 serogroup is among the 12 recognized by the World Health Organization as potentially pathogenic, being quite isolated in several studies, including cheeses (Araujo *et al.*, 2002; Paneto *et al.*, 2007; Dias *et al.*, 2012). Our study shows the presence of O127, confirming that *E. coli* serogroup strains are being isolated and conveyed by cheeses.

Moreover, the study of Levine *et al.* (1985) showed that an O127:H6 strain without EAF plasmid was less virulent for

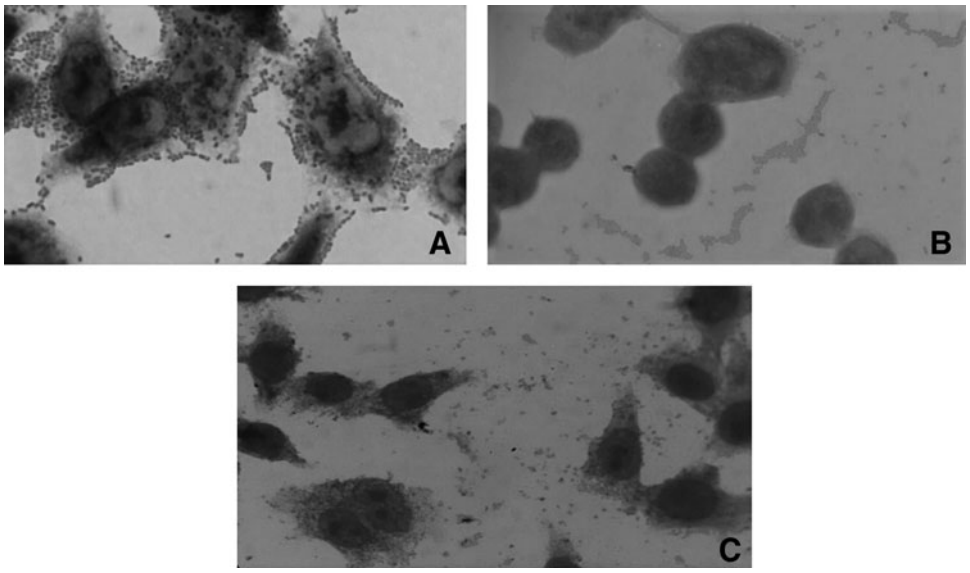

FIG. 1. **(A)** EAEC 042 strain showing aggregative adherence on HeLa cells; **(B)** EPEC EC-01 (O127 serogroup) aggregative adherence on HeLa cells; **(C)** *Escherichia coli* EC-12 strain showing diffuse adherence on HeLa cells. EAEC, enteroaggregative *E. coli*; EPEC, enteropathogenic *E. coli*.

adult volunteers than the wild-type strain. However, aEPEC strains have not been proven to be less pathogenic; besides that, these organisms may have others virulence factors to compensate the lack of the plasmid. Some O127 strains can express potential virulence factor as the production of the enteroaggregative heat-stable toxin (EAST1). aEPEC strains frequently express EAST1 and other potential virulence factors not encoded in the LEE region, such as Afa and E-hly (Trabulsi *et al.*, 2002). This EPEC EC-01 showed to have an aggregate adhesion pattern, which can be inferred to produce EAST1 toxin, since the cells were killed (Fig. 1B).

In our study, we also found serotypes of ExPEC different from Okura and Marin (2014); Guzman-Hernandez *et al.* (2016); and Ribeiro *et al.* (2016). The O64474:H8 serotype was found to share all the biochemical characteristics of *E. coli*, including lactose, motility, and lysine positive, in addition to having a defined virulence ability and sharing an O antigen with *Shigella boydii* 16 (Navarro *et al.*, 2010).

This serogroup was isolated in Egypt, Bangladesh and Mexico, between 1980 to 2007 years and was described by Navarro *et al.* (2010). However these strains belonged to the DEC group, enterotoxigenic pathotype and, until then, this serogroup was restricted to this pathotype. Our study showed that O64474 serogroup can be found in another pathogenic *E. coli* group, not being associated only with DEC.

This may be the first report of *Escherichia coli* O64474 isolated in Brazil in cheeses and in the ExPEC group.

The lack of detection of STEC, a pathogen that can be dangerous (particularly the serotype O157:H7), in this work and in previous works in Brazil (Okura and Marin, 2014; Ribeiro *et al.*, 2016) should not be overinterpreted. Most researchers test only a few *E. coli* colonies on agar plates. Then, STEC isolates may be overshadowed by abundant commensal *E. coli.* In other countries, such as Saudi Arabia, Egypt, Mexico, and Italy, STEC have been isolated from fresh, unpasteurized cheese (Altalhi and Hanssen, 2009; Guzman-Hernandez *et al.*, 2016; Marozzi *et al.*, 2016; Nobili *at al.*, 2016; Ombarak *et al.*, 2016). So, it is very unlikely that, in Brazil, the true incidence of STEC in raw milk cheese is zero.

E. coli strains with ExPEC genes were also isolated in this work, as reported previously in works with cheese (Okura and Marin, 2014; Ribeiro *et al.*, 2016). Strains of this pathotype have emerged as hypothetical foodborne pathogens. The isolates of this work possessed a virulence repertoire similar to that detected in *E. coli* associated with human meningitis (Lemaître *et al.*, 2013), highlighting their pathogenic potential.

Most *E. coli* isolates in this work belonged to phylogenetic group A, including the two ExPEC strains. A previous work also reported that A was the more prevalent phylogroup among *E. coli*, including ExPEC, from raw milk cheese, Ribeiro *et al.* (2016). Besides phylogroup A, this work also reports a high frequency of phylogroups C and E among *E. coli*. In contrast, besides phylogroup A, Ribeiro *et al.* (2016) also reported a high frequency of B1 isolates. The differences may have occurred because the cited authors did not test their strains for phylogroups A and C, since these phylogroups have been recently established (Clermont *et al.*, 2000; Clermont *et al.*, 2013). It is also possible that the difference is due to distinct sampling locations.

Biofilm production facilitates the stay of bacteria in distinct surfaces, including the bovine mammary glands (Costa *et al.*, 2014; Peixoto *et al.*, 2015). So, it is possible that the biofilm-producing isolates have originated from mastitis cases, rather than postmilking contamination.

Most *E. coli* isolates of this work displayed resistance to few antimicrobials, similar to previous works (Okura and Marin, 2014; Nagy *et al.*, 2015; Ribeiro *et al.*, 2016). The antimicrobial resistance may be associated with selective pressure due to the use of antimicrobials in dairy cows. Also, the resistant bacteria could have been supported by intensive use of antimicrobials, with after contamination of the milk or dairy products (Chambers and DeLeo, 2003; Argudín *et al.*, 2015; Jamali *et al.*, 2015). Either way, the emergence of resistant bacteria has been of concern, due to the risk of dissemination among cheese consumers.

Conclusions

Cheeses made with raw milk, collected in the various regions of Brazil, were contaminated with *E. coli*. Among the *E. coli*, it was detected known pathogens (atypicalEPEC and ExPEC). The O64474 it is a new serogroup in Brazil and of atypicalEPEC description. Resistance to antimicrobials was detected in some isolates. Because cheese made from raw milk is highly consumed, the presence of these microorganisms is a potential risk, which should be monitored frequently.

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Disclosure Statement

No competing financial interests exist.

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