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 $8.4 \times 10^4$ cfu/mL for RM, $1.4 \times 10^7$ cfu/mL for LMFC, and $2.9 \times 10^7$ cfu/mL for IMFC. Mean *E. coli* counts were $2.4 \times 10^3$ cfu/mL for RM, $1.9 \times 10^7$ cfu/mL for LMFC, and $1.1 \times 10^5$ 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
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 χ² 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 µg), aztreonam (30 µg), tetracycline (30 µg), cefotaxime (30 µg), cefoxitin (30 µg), nalidixic acid (30 µg), gentamicin (10 µg), chloramphenicol (30 µg), ampicillin (10 µg), ciprofloxacin (5 µg), and streptomycin (10 µg). Enrofloxacin (5 µg) 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-
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 vari-
ations (*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 evalu-
ated 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 produc-
ers in Paraná 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 produc-
ers 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.

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>n</th>
<th>TC (cfu/mL)</th>
<th>EC (cfu/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw milk</td>
<td>80</td>
<td>2.4 × 10⁶</td>
<td>1.5 × 10⁵</td>
</tr>
<tr>
<td>Minimum</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>8.4 (±3.3) × 10⁴</td>
<td>2.4 (±16) × 10⁴</td>
<td></td>
</tr>
<tr>
<td>IMFC</td>
<td>10</td>
<td>1.4 × 10⁷</td>
<td>4 × 10⁵</td>
</tr>
<tr>
<td>Minimum</td>
<td>&lt;10³</td>
<td>&lt;10²</td>
<td>&lt;10²</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>2.9 (±4.6) × 10⁷</td>
<td>1.1 (±1.6) × 10⁷</td>
<td></td>
</tr>
<tr>
<td>LMFC</td>
<td>10</td>
<td>7.8 × 10⁷</td>
<td>10³</td>
</tr>
<tr>
<td>Minimum</td>
<td>10²</td>
<td>10²</td>
<td>10²</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>1.4 (±2.3) × 10⁷</td>
<td>1.9 (±2.8) × 10²</td>
<td></td>
</tr>
</tbody>
</table>

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*) 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 di-
arrhea 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 IFMC, 9
(40.1%) were resistant to at least one of the antibiotics
tested. Among them, streptomycin had the most resis-
tant strains (*n = 8*) originating from samples and be-
longing 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 IFMC sample,
the EC13* isolate was resistant to 3 antibiotics (ampicil-
lin, tetracycline, and streptomycin). The EC13* 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*), 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 mast-
ititis 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
related to humans, which agrees with the predominance of t-EPEC in the present study (Table 2).

The STEC found in the IFMC samples (EC13a–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 \textit{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 \textit{E. coli} isolates. Consumption of Brazilian raw milk or dairy products should be avoided.

**ACKNOWLEDGMENTS**

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**REFERENCES**


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

<table>
<thead>
<tr>
<th>Origin</th>
<th>Strain</th>
<th>State</th>
<th>Serotype</th>
<th>Virulence genes</th>
<th>Antimicrobial resistance</th>
<th>Phylogenetic group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw milk, large producers</td>
<td>EC1</td>
<td>Paraná</td>
<td>O76:H39</td>
<td>eaeA, bfpA</td>
<td>AMP, TET, STR</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>EC2</td>
<td>Paraná</td>
<td>O59:H21</td>
<td>eaeA, bfpA</td>
<td>NAL, TET, STR</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>EC3</td>
<td>Paraná</td>
<td>O51:H49</td>
<td>eaeA</td>
<td>STR</td>
<td>E</td>
</tr>
<tr>
<td></td>
<td>EC4</td>
<td>Paraná</td>
<td>O149:H21</td>
<td>eaeA, bfpA</td>
<td>Susceptible B1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC5</td>
<td>Paraná</td>
<td>O28ac:H37</td>
<td>eaeA, bfpA</td>
<td>Susceptible A</td>
<td></td>
</tr>
<tr>
<td>Raw milk, small producers</td>
<td>EC6</td>
<td>Paraná</td>
<td>O59:H21</td>
<td>eaeA, bfpA</td>
<td>Susceptible B1</td>
<td></td>
</tr>
<tr>
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<td>EC7a</td>
<td>Paraná</td>
<td>O164:H4</td>
<td>eaeA, bfpA</td>
<td>TET, STR</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>EC7b</td>
<td>Paraná</td>
<td>O73:H39</td>
<td>eaeA, bfpA</td>
<td>Susceptible A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC8a</td>
<td>Paraná</td>
<td>O53:H4</td>
<td>eaeA, bfpA</td>
<td>Susceptible A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC8b</td>
<td>Paraná</td>
<td>O85:H16</td>
<td>eaeA</td>
<td>Susceptible A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC8c</td>
<td>Paraná</td>
<td>O76:H4</td>
<td>eaeA</td>
<td>Susceptible A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC8d</td>
<td>Paraná</td>
<td>O53:H16</td>
<td>eaeA</td>
<td>Susceptible Clade I or II</td>
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<tr>
<td></td>
<td>EC8e</td>
<td>Paraná</td>
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<td>eaeA, bfpA</td>
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<td></td>
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<td>eaeA, bfpA</td>
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</tr>
<tr>
<td></td>
<td>EC8g</td>
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</tr>
<tr>
<td></td>
<td>EC9a</td>
<td>Paraná</td>
<td>O187:H8</td>
<td>eaeA, bfpA</td>
<td>Susceptible B1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC9b</td>
<td>Paraná</td>
<td>O187:H8</td>
<td>eaeA</td>
<td>Susceptible A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC9c</td>
<td>Paraná</td>
<td>O187:H8</td>
<td>eaeA</td>
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<td></td>
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<tr>
<td></td>
<td>EC10a</td>
<td>Paraná</td>
<td>O187:H8</td>
<td>eaeA</td>
<td>FOX, STR</td>
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<tr>
<td></td>
<td>EC10b</td>
<td>Paraná</td>
<td>O184:H10</td>
<td>eaeA</td>
<td>AMP, STR, FOX</td>
<td>Unknown</td>
</tr>
<tr>
<td>Illega Minas</td>
<td>EC11</td>
<td>Paraná</td>
<td>O179:H8</td>
<td>eaeA, bfpA</td>
<td>STR</td>
<td>A</td>
</tr>
<tr>
<td>Frescal cheese</td>
<td>EC12</td>
<td>Paraná</td>
<td>O101:HNT</td>
<td>eaeA</td>
<td>FOX, AMP</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>EC13a</td>
<td>Paraná</td>
<td>OR-H</td>
<td>stx2</td>
<td>AMP, TET, STR</td>
<td>B2</td>
</tr>
<tr>
<td></td>
<td>EC13b</td>
<td>Paraná</td>
<td>OR-H</td>
<td>stx2</td>
<td>Susceptible B1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC13c</td>
<td>Paraná</td>
<td>OR-H</td>
<td>stx2</td>
<td>TET</td>
<td>A</td>
</tr>
</tbody>
</table>

1Superscript letters denote strains obtained from the same sample.

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

3According to Clermont et al. (2013).


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