



# Heat-resistant and biofilm-forming *Escherichia coli* in pasteurized milk from Brazil

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## Abstract

*Escherichia coli* harboring a transmissible locus of stress tolerance (tLST) and the ability to form biofilms represent a serious risk in dairy production. Thus, we aimed to evaluate the microbiological quality of pasteurized milk from two dairy producers in Mato Grosso, Brazil, with a focus on determining the possible presence of *E. coli* with heat resistance (60 °C/6 min), biofilm-forming potential phenotypes and genotypes, and antimicrobial susceptibility. For this, fifty pasteurized milk samples from producers named A and B were obtained for 5 weeks to investigate the presence of *Enterobacteriaceae* members, coliforms, and *E. coli*. For heat resistance, *E. coli* isolates were exposed to a water bath at 60 °C for 0 and 6 min. In antibiogram analysis, eight antibiotics belonging to six antimicrobial classes were analyzed. The potential to form biofilms was quantified at 570 nm, and curli expression by Congo Red was analyzed. To determine the genotypic profile, we performed PCR for the tLST and *rpoS* genes, and pulsed-field gel electrophoresis (PFGE) was used to investigate the clonal profile of the isolates. Thus, producer A presented unsatisfactory microbiological conditions regarding *Enterobacteriaceae* and coliforms for weeks 4 and 5, while all samples analyzed for producer B were contaminated at above-the-limit levels established by national and international legislation. These unsatisfactory conditions enabled us to isolate 31 *E. coli* from both producers (7 isolates from producer A and 24 isolates from producer B). In this way, 6 *E. coli* isolates (5 from producer A and 1 from producer B) were highly heat resistant. However, although only 6 *E. coli* showed a highly heat-resistant profile, 97% (30/31) of all *E. coli* were tLST-positive. In contrast, all isolates were sensitive to all antimicrobials tested. In addition, moderate or weak biofilm potential was verified in 51.6% (16/31), and the expression of curli and presence of *rpoS* was not always related to this biofilm potential. Therefore, the results emphasize the spreading of heat-resistant *E. coli* with tLST in both producers and indicate the biofilm as a possible source of contamination during milk pasteurization. However, the possibility of *E. coli* producing biofilm and surviving pasteurization temperatures cannot be ruled out, and this should be investigated.

**Keywords** Antimicrobial susceptibility · Heat treatment · Locus of heat resistance · *Rpos* · Transmissible locus of stress tolerance

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## Introduction

Milk is an important source of vital nutrients, especially proteins and micronutrients, for humans [1]. Milk production has increased by 67% in the last three decades worldwide [1]. In Brazil, the 2020 production reached approximately 25.53 billion liters, with the state of Mato Grosso ranking second in production volume in the Midwest region of the country [2]. Because of this consumption potential, microbiological controls are paramount to ensure food quality and safety, and the pasteurization process is required by both national and international standards for microbiological milk product safety [3–5].

Pasteurization can be categorized as low temperature and long time (LTLT), applying temperatures between 62 and 65 °C for 30 min, or high temperature and short time (HTST), applying temperatures between 72 and 75 °C for 15–20 s, followed by cooling to 4 °C [6,7]. However, temperature abuse during the handling or transport of this milk and a lack of hygiene practices can result in microbial contamination or even an outbreak involving foodborne pathogens [8–10].

On the other hand, some studies using subpasteurization temperatures (between 57 and 68 °C for 15 s or more), commonly used as raw milk thermalization, have demonstrated the survivability of some pathogenic *Escherichia coli* (*E. coli*) [11, 12]. In this context, some *E. coli* pathotypes can cause foodborne diseases, including several forms of diarrhea and even hemolytic uremic syndrome [13, 14]. Furthermore, *E. coli* is a member of the *Enterobacteriaceae* family and is the only bacterium in the coliform group that indicates possible fecal contamination [15, 16]. Therefore, the control of *Enterobacteriaceae* species and coliforms has been described in several legislations and must meet the acceptance limits for commercialized pasteurized milk and dairy products [5, 16–18].

*E. coli* has been historically classified as heat-sensitive [19]. However, the emergence of heat-resistant *E. coli* strains isolated from animal-based foods has been reported. Dlusskaya et al. [20] reported that *E. coli* AW1.7 with a survivability of approximately 5 log CFU/ml in ground beef heated to an internal temperature of 71 °C. Similarly, *E. coli* AW 1.1 presented a bacterial reduction below 1 log CFU/ml after 5 min at 60 °C [21]. In another study, *E. coli* FAM23288 showed survivability of approximately 4.9 log CFU/ml after 30 min at 55 °C [22], and *E. coli* XHR presented growth capacity in MacConkey agar after exposure to 80 °C for 15 min [23]. Strains displaying heat resistance have been associated with gene markers inserted in a mobile island named the *locus* of heat resistance (LHR), more recently termed the transmissible *locus* of stress tolerance (tLST) [24]. These genes

are responsible for protecting microorganisms against stress factors, including heat shock stress, envelope stress, and oxidative stress [25]. Other studies have associated the presence of pathogenic and nonpathogenic *E. coli* with tLST in beef produced in Canada, the USA [21, 23, 26–28], and in Brazil by our group [29] and cheese prepared with raw milk [22].

In addition to heat resistance, *E. coli* has been found to have the capacity for biofilm formation, e.g., isolates from dairy products [30]. Additionally, a considerable portion of tLST-positive *E. coli* has also been identified to harbor antimicrobial resistance genes [31]. Xu et al. [32] reported that biofilm-embedded tLST-positive *E. coli* strains are more resistant to chemical sanitizers than tLST-negative *E. coli* strains. According to a study by Boll et al. [22], the presence of *orfE* in a plasmid with tLST can encode putative di-guanyl cyclase and cyclic di-GMP (c-di-GMP) enzymes, showing an effect on biofilm formation in *E. coli*. Biofilms comprise the formation of bacterial multilayers on both biotic and abiotic surfaces, which may promote the persistence of *E. coli* in the food chain [33]. This can bring challenges to the pasteurized milk chain if the binomial time and temperature are not carefully monitored.

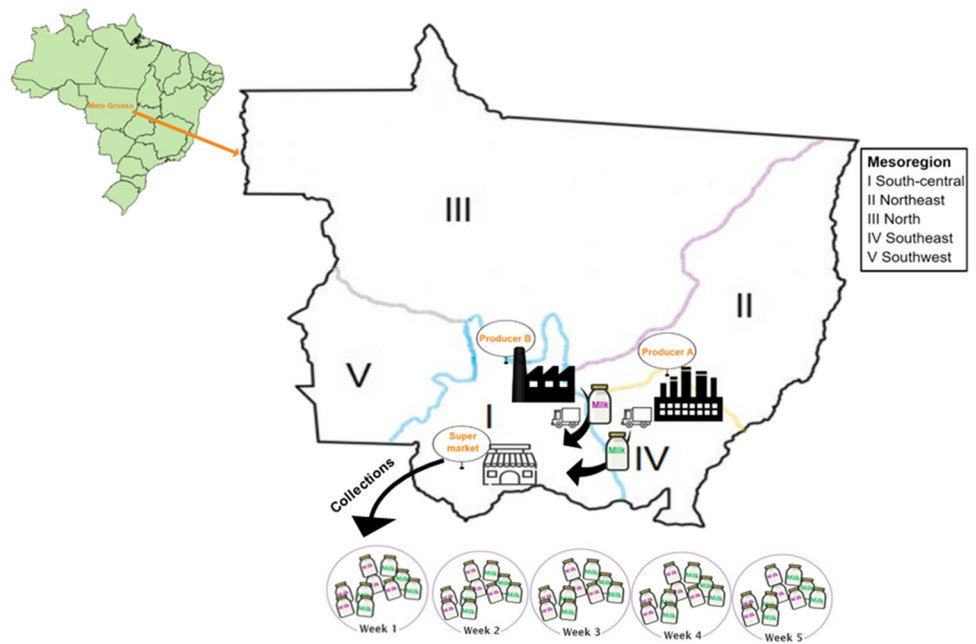
In this context, this work aimed to evaluate the quality of pasteurized milk, investigate the presence of *E. coli* isolates in this milk, and understand the factors related to persistence or postpasteurization contamination. The study consists of four steps: (i) evaluate the milk quality from two producers (A and B) taking into account the *Enterobacteriaceae* and coliform counts; (ii) isolate *E. coli* strains from pasteurized milk; (iii) perform a phenotypic analysis for heat resistance, antimicrobial susceptibility, biofilm potential, and curli expression; and (iv) perform a genotypic analysis searching for genetic similarity profiles of *E. coli* isolates and verifying the presence of gene markers for the tLST and *rpoS* genes (involved in stress conditions and curli/cellulose control, respectively).

## Materials and methods

### Milk sampling

Fifty pasteurized milk samples were obtained from two producers, named A and B (Fig. 1). These producers distribute pasteurized milk in supermarkets and grocery stores throughout the mesoregion of the state of Mato Grosso, Brazil. Sampling was performed over 5 weeks, and five individual samples from each producer were collected weekly. It is important to note that Producer A is monitored by the federal inspection system (SIF), while producer B is regulated by a local Mato Grosso state inspection system (SISE).

**Fig. 1** Collection of pasteurized milk over 5 weeks in supermarkets supplied by two milk producers located in Mato Grosso, Brazil. The green map is Brazil, and the mesoregions of the state of Mato Grosso correspond to the territorial demarcations where the cities and districts are located



## Physical–chemical analysis

Alkaline phosphatase and peroxidase tests were performed to verify whether the milk samples were pasteurized correctly by testing with reactive enzyme strips (Cap-Lab, Brazil). Both tests are regulated by Normative Instruction Nr. 76 (Brazilian legislation) [4].

## Microbiological analyses

Quantification of *Enterobacteriaceae* members, total coliforms (35 °C) (TC) and thermotolerant coliforms (45 °C) (TTC), was performed according to the American Public Health Association (APHA) with modifications [34]. Positive *E. coli* broth (EC broth) (Kasvi, Brazil) was evaluated for the presence of typical *E. coli* colonies. The mean and standard deviation of the mentioned groups of microorganisms in relation to each collection week were obtained from five experimental replicates. Furthermore, 0.1 ml of EC broth was resuspended through the spread plate technique on plates containing agar Eosin Methylene Blue (EMB) (Kasvi, Spain) with subsequent incubation at 35 °C for 18–24 h. Colonies presenting a metallic green edge color with or without a black center were considered positive (suggestive) for *E. coli*. All typical colonies were submitted to biochemical tests, such as indole, methyl red, Vogues-Proskauer, and citrate (IMViC). For subsequent analysis, a single typical colony was transferred to 10 ml of lysogenic broth (LB broth) (Kasvi, Brazil) at 35 °C with constant agitation for 18–24 h.

Concerning *E. coli* identification, each isolate was named the letter of the producer + sample number with two digits + letter and number representing the isolate. For example, B (producer) + 20 (sample) + C3 (isolate). Each week, five samples were collected from each producer: samples 1–5 = week 1, samples 6–10 = week 2, samples 11–15 = week 3, samples 16–20 = week 4, and samples 21–25 = week 5.

## Evaluation of heat resistance

*E. coli* isolates from producers A and B were evaluated through heat treatment at 60 °C in a water bath (model N1040, Centauro, Brazil). The heat treatment was performed in microtubes (2 ml) containing 1.5 ml of bacterial culture in LB broth [35]. The bacterial culture was heated two times: “time to reach T0” or come-up time (time required to reach the target temperature) and 6 min (T6). Treatments T0 and T6 were performed with three independent replicates, each containing duplicate samples (3 × 2). After each thermal exposure, the temperature was rapidly decreased through an ice bath for approximately 30 min. Temperature control was applied using microtubes containing 1.5 ml of LB broth without bacterial growth and a thermometer with an external probe (Equitherm, Brazil). The probe was inserted in the center of the microtube and sealed with adhesive tape. Initial cell concentrations were estimated by optical density (OD) at 600 nm using a spectrophotometer (Model Q8980PT, Quimis, Brazil) and confirmed by plating in plate count agar (PC agar) (Kasvi, Brazil). A high heat resistance wild-type *E. coli* C31, identified in a previous study by our group, displaying the ability to survive at high temperatures [36], was used as a positive control.

Following the heat treatment, serial dilutions (from  $10^{-1}$  to  $10^{-9}$ ) were prepared using peptone saline solution, and 1 ml of the appropriate dilutions was added through the pour plate technique on plates containing PC agar with subsequent incubation at 35 °C for 18–24 h. After that, colonies were enumerated by plate counting between 30 and 300 colony-forming units per milliliter (CFU/ml). The logarithmic reduction was calculated considering the difference in CFU/ml counts between the unheated strains in relation to those heated to 60 °C for 0 min. Concerning the 6 min exposure, logarithmic reduction was calculated by the difference between CFU/ml counts at T0 and T6. Strains were classified as sensitive (reduction > 5 log), moderate resistance (reduction between 1 and 5 log), or high resistance (reduction < 1 log) under heat treatment [21]. Furthermore,  $D_{60^{\circ}\text{C}}$  values were calculated by dividing the incubation time ( $t$ ) by the log reduction, i.e.,  $D_{60^{\circ}\text{C}} = t/\log(\text{T0}-\text{T6})$  [28]. This value measures the time required at 60 °C to kill 90% or 1 log of a microbial population [20].

### Antimicrobial susceptibility assays

Antimicrobial susceptibility assays for *E. coli* were conducted according to the Clinical and Laboratory Standards Institute (CLSI) through the disk-diffusion technique [37]. Thus, eight antibiotics were used: ampicillin (30 µg), cefepime (30 µg), ciprofloxacin (5 µg), enrofloxacin (5 µg), florfenicol (30 µg), gentamicin (10 µg), nitrofurantoin (100 µg), and sulfamethoxazole-trimetoprim (25 µg). These antibiotics were chosen since they belong to some of the principal classes of antibiotics (betalactams, fluoroquinolones, phenicals, aminoglycosides, nitrofurantoin, and folate pathway antagonists) used in human and animal therapy [37]. In the test, a typical colony of EMB agar was inoculated onto Mueller–Hinton broth (MH broth) (Sigma–Aldrich, India) and incubated at 37 °C between 2 and 4 h until achieving growth of approximately 0.5 on the MacFarland scale. Subsequently, 0.1 ml of MH broth growths were streaked for the spread plate technique on MH agar (Kasvi, Italy), and disks containing the respective antibiotic were included, followed by incubation of plates at 37 °C for 18 h. Inhibition zones were then measured in millimeters using a halo-size ruler, and the results were correlated with CLSI standards.

### Biofilm potential formation

Biofilm formation assays were performed according to the method described by Bang et al. [38] with modifications. For this, 240 µl of bacterial culture (approximately 8 log CFU/ml) was added in duplicate into 24-well cell culture plates (Corning Incorporated, USA) followed by 2760 µl of sterile LB broth and incubated at 35 °C for 48 h. Sterile LB

broth was used as a negative control. After 48 h, the broth was removed using a pipette, and the well plates were rinsed three times with 3 ml sterile phosphate-buffered saline (PBS, pH 7.4) to remove the residual attached cells. Next, the potential biofilms were fixed using 3 ml of 95% ethyl alcohol for 15 min (Química Moderna, Brazil). After that, the plates were dried overnight. Crystal violet dye (Vetec, Brazil) (0.1%; 3 ml) was used to stain the potential biofilm for 15 min. Subsequently, the crystal violet solution was removed by washing with distilled water until all free crystal violet present was completely removed. The plates were air-dried overnight, and the next day, the bound crystal violet dye was removed using 3 ml of 95% ethyl alcohol for 30 min. The OD of the solution containing the crystal violet dye was measured at 570 nm ( $A_{570\text{nm}}$ ) using a spectrophotometer (model Q8980PT, Quimis, Brazil). The results were calculated following parameters established in previous studies [39, 40]. Thus, the results are shown as the mean ± standard deviation (SD) of two independent replicates, with each plate containing duplicate samples ( $n = 4$ ). For biofilm classification, the absorbance ( $A_{570\text{nm}}$ ) of three times the standard deviation of the negative control was used as the cutoff value (ODc), which was 0.11 in this study. Based on the ODc calculation, the classification was established as follows: nonbiofilm/absence ( $\text{OD}_{\text{strain}} < \text{ODc}$ ), weak biofilm ( $\text{ODc} < \text{OD}_{\text{strain}} < 2 \times \text{ODc}$ ), moderate biofilm ( $2 \times \text{ODc} < \text{OD}_{\text{strain}} < 4 \times \text{ODc}$ ), and strong ( $4 \times \text{ODc} < \text{OD}_{\text{strain}} < 8 \times \text{ODc}$ ).

### Curli expression

*E. coli* isolates were inoculated on LB agar modified and supplemented with 0.004 g/L of Congo Red dye (Merck, Germany), according to Hassan et al. [41] and Bahri et al. [42]. Isolates were incubated at 25 °C for 4 days and were then checked for different morphotypes, as follows: saw (white and smooth colonies; curli-negative), rdar (red, dry and rough colonies; curli-positive), and intermediate (red and dry or rough colonies; medium curli expression or potentially present) [42].

### Pulsed-field gel electrophoresis (PFGE)

PFGE was performed according to the CDC PulseNet protocol [43]. Briefly, the bacterial growth from Columbia agar plates (Merck, USA) (37 °C/18 h) was encased in agarose plugs and lysed with proteinase K (Promega, USA) in a shaking water bath at 55 °C/2 h. The plugs were digested using 10 U of the *Xba*I restriction enzyme (Thermo Scientific, USA). DNA fragments were separated using CHEF-DR III equipment (Bio-Rad, USA) with 1% SeaKem® Gold Agarose (Lonza, Switzerland) and 0.5 × TBE (Tris-boric-acid/EDTA) buffer (Bio-Rad,



USA). The PFGE conditions were as follows: 6 V/cm for 18 h with pulse times ranging from 6.76 to 35.38 s. *Salmonella enterica* serovar Branderup H9812 was used as a DNA size marker. Gel images were exported and analyzed using Photo Documenter software (Vilber Lourmat, EU). The dendrogram was constructed using GelJ version 2.0 software by applying the unweighted pair-group with averages (UPGMA) method and the Dice coefficient, with a tolerance index of 2% [44]. Isolates displaying a similarity index above 90% were considered related [45].

### DNA extraction and PCR for tLST and *rpoS* genes

DNA extraction was performed according to Ma and Chui [46], with modifications through thermal lysis. Thus, 0.1 ml of bacterial suspension in LB broth was transferred to PC agar by the spread plate technique and incubated at 35 °C/18–24 h. Then, a single colony grown on the PC agar into 200 µL of ultrapure water was boiled at 100 °C for 10 min and centrifuged at 13,000 × g for 15 min. The obtained supernatants were then collected, and DNA was quantified through a fluorescence technique (QUBIT 2.0 system, Invitrogen, USA).

PCR simplex was conducted for the amplification of *orf3*-, *orf8*-, and *orf11*-targeted primer sequences of tLST described by Ma and Chui [46] and *rpoS* gene described by Uhlich et al. [47] (Table 1). The tLST primers designed by Ma and Chui [46] provide good coverage across the whole tLST. The cycling conditions for genes of tLST were set as follows: initial denaturation at 95 °C for 5 min, 40 denaturation cycles at 95 °C for 30 s, annealing at 60 °C for 30 s, an extension at 72 °C for 30 s, and a final extension at 72 °C for 10 min. The tLST-positive *E. coli* AW1.7, provided by Alberta Agriculture and Forestry, was used as a positive control. For the *rpoS* gene, the cycling

conditions comprised initial denaturation at 95 °C for 5 min, 30 denaturation cycles at 95 °C for 60 s, annealing at 59 °C for 30 s, an extension at 72 °C for 60 s and a final extension at 72 °C for 5 min. *E. coli* ATCC 11229 was used as a positive control, and ultrapure water was used as a negative control.

### Data analysis

A one-way ANOVA at 5% significance with means separated by Tukey's post hoc test was used to determine the significant differences between the *Enterobacteriaceae* results from producer B. A nonparametric analysis (Kruskal–Wallis test) was performed for TC and TTC to compare significant differences between the sampling weeks for both groups. Statistical differences were verified through pairwise comparisons using SPSS software (version 22).

## Results

### Physical–chemical tests

The enzymatic tests verified that all samples from producers A and B were efficiently pasteurized. The absence of alkaline phosphatase in all samples indicated adequate pasteurization time and temperature, and the presence of peroxidase indicated adequate temperature during the pasteurization process [4].

### Enterobacteriaceae, coliform counts, and presence of *E. coli*

The results of *Enterobacteriaceae* and coliform counts indicate that producer A only partially complied with the established legislation [5, 16–18]. Samples from weeks 1 and 2 contained *Enterobacteriaceae*, TC and TTC below the APHA method's limit of detection (< 10 CFU/ml for *Enterobacteriaceae* and < 3.6 MPN/ml for TC and TTC) [34] and below the minimum limit of legislation recommend (< 1 log CFU/ml or 7.4 MPN/ml for TC and TTC) [5, 16–18] (Table 2). On the other hand, all milk samples from producer B contained *Enterobacteriaceae*, TC and TTC above the minimum limit of legislation recommended, with week 3 displaying the greatest *Enterobacteriaceae* contamination, which was significantly different between weeks 1, 2, and 5 ( $p < 0.05$ ). The Kruskal–Wallis test indicated that weeks 2 and 3 displayed the highest counts in producer B samples regarding TC. Concerning TTC, there was no difference between the weeks (Table 2). None of the samples obtained during the 5 weeks of

**Table 1** Primers used in this study

Target/gene	Sequence	Reference
<i>orf3</i>	F: 5'—CCATTCTTATGTCGGTCC AGAG—3'	[46]
	R: 5'—CCACCTTGCTGACCTGTT—3'	
<i>orf8</i>	F: 5'—TCGGTAAAGAAAGCGGTCAAG —3'	
	R: 5'—CATCGGAAGGTTGTCGGTTT —3'	
<i>orf11</i>	F: 5'—GAAGCGATTGTCCGAGCTAAG —3'	
	R: 5'—TGCTTGCCACTTCGTTATCC—3'	
<i>rpoS</i>	F: 5'—TATCGCCTGGATTACTGGCAAC —3	[47]
	R: 5'—TAGGACGCTGACGTGTCTTATC —3	

F forward, R reverse

**Table 2** *Enterobacteriaceae*, TC, and TTC values in pasteurized milk samples obtained from producers A and B during five sampling weeks

<sup>A</sup> Producer A					<sup>B</sup> Producer B			
Week	<i>Enterobacteriaceae</i> (Log CFU/ml)	TC (MPN/ml)	TTC (MPN/ml)	*Total <i>E. coli</i> isolates	<sup>C</sup> <i>Enterobacteriaceae</i> (Log CFU/ml)	<sup>D</sup> TC (MPN/ml)	<sup>E</sup> TTC (MPN/ml)	*Total <i>E. coli</i> isolates
1	< 1	< 3.6	< 3.6	0	4.65 ± 0.94 <sup>b</sup>	1.10 × 10 <sup>3</sup> <sup>b</sup>	1.10 × 10 <sup>3</sup> <sup>a</sup>	5
2	< 1	< 3.6	< 3.6	0	5.48 ± 0.50 <sup>b</sup>	1.10 × 10 <sup>4</sup> <sup>a</sup>	7.71 × 10 <sup>3</sup> <sup>a</sup>	4
3	< 1	23	< 3.6	0	6.54 ± 0.16 <sup>a</sup>	1.10 × 10 <sup>4</sup> <sup>a</sup>	5.32 × 10 <sup>3</sup> <sup>a</sup>	5
4	3.1 ± 0.63	5.56 × 10 <sup>2</sup>	1.44	3	5.67 ± 0.25 <sup>ab</sup>	1.10 × 10 <sup>3</sup> <sup>b</sup>	1.53 × 10 <sup>3</sup> <sup>a</sup>	5
5	< 1	2.04 × 10	9.36	4	5.09 ± 0.48 <sup>b</sup>	1.10 × 10 <sup>3</sup> <sup>b</sup>	1.14 × 10 <sup>3</sup> <sup>a</sup>	5

<sup>A</sup>Samples from producer A comprise only the averages obtained each week, without undergoing any statistical analyses. <sup>B</sup>Samples from producer B were statistically evaluated concerning potential differences between sampling weeks; <sup>a,b</sup>Means ± standard deviations in the same row differ significantly ( $p < 0.05$ ) and were obtained from five experimental replicates. <sup>C</sup>Tukey's test; <sup>D</sup>Kruskal–Wallis test, chi-square = 24.000 and  $p = 0.000$ ; <sup>E</sup>Kruskal–Wallis test, chi-square = 7.334 and  $p = 0.119$ . TC total coliforms, TTC thermotolerant coliforms, MPN/ml most likely number per milliliter, CFU/ml colony forming unit per milliliter. \*Total *E. coli* isolates refer to the number of *E. coli* per sample collected, and these isolates are identified in Table 3. Minimum limits established for pasteurized milk: < 1 log CFU/ml for *Enterobacteriaceae* [17, 18], < 1 log CFU/ml or 7.4 MPN/ml for TC and TTC [5, 16]

sampling from producer B complied with the limits set by both national and international legislations [5, 16–18] and were, therefore, inadequate for consumption. Thus, the results for both producers could not be statistically compared.

Furthermore, 31 strains of *E. coli* were isolated from the positive EC broth followed by inoculation in EMB agar and confirmed by IMViC tests. Of these, 7 strains were from producer A, isolated from the only weeks with verified TTC counts (Table 2). The other 24 strains were isolated from samples obtained from producer B. The weeks with the highest numbers of *E. coli* isolates were not associated with the highest TTC loads, indicating that low TTC counts did not decrease the risk for the presence of *E. coli*.

### Heat resistance and antimicrobial susceptibility

Regarding producer A, 5/7 isolates presented high heat resistance after 6 min of exposure, and the remaining two (A20C3 and A20C6) showed moderate and sensitive profiles to resistance to heat treatment (Table 3). Concerning producer B, only one isolate (B04C1) exhibited high heat resistance, whereas all 23 were classified as displaying moderate heat resistance (Table 3).

The highest  $D_{60^\circ\text{C}}$  values in isolates displaying the high resistance phenotype were observed for *E. coli* from producer A, ranging from 13.75 to 17.62 min, while  $D_{60^\circ\text{C}}$  values ranged from 2.15 to 11 min in isolates obtained from producer B.

In relation to the antimicrobial test, the 31 *E. coli* isolates were susceptible to the eight evaluated antibiotics.

### Biofilm formation, curli expression and PFGE

The results showed that 51.6% (16/31) of the isolates showed the potential for biofilm formation (Table 4). Of these, 5 isolates of producer A showed weak biofilm potential in 4 isolates and moderate biofilm potential in one (A24C1). For producer B, 9 isolates were classified as having weak biofilm potential, and 2 isolates (B04C1 and B06C5) showed moderate biofilm potential. However, curli expression, namely as rdar colonies, was identified in 77.4% (24/31) of the isolates (Table 4). Although curli is a major component of biofilms, its absence did not prevent the formation of biofilm in some isolates.

In relation to PFGE, the 31 isolates generated nine profiles with decipherable fragments (Fig. 2). Each profile was composed of clonal isolates with 100% similarity. Three profiles were associated with the isolates from producer A, with clonal profile IV and two orphan profiles obtained from samples from different weeks (different lots). Regarding producer B, five clonal profiles and one orphan profile were identified. Profile II contained the highest number of isolates, and these were obtained from samples of different lots.

### tLST and *rpoS* genes by PCR

The tLST genes were present in 97% (30/31) of all isolates. The only exception was the B20C3 isolate (Table 3). For the *rpoS* gene, the findings indicated its presence in 64.5% (20/31) of *E. coli* isolates.

**Table 3** Phenotypic and genotypic characteristics of heat resistance in *E. coli* isolated from pasteurized milk samples obtained over 5 weeks from two producers from Mato Grosso, Brazil

Week	Isolate ID	*Heat resistance classification	D <sub>60°C</sub> (min)	tLST genes
4	<sup>A</sup> A19C6	High	13.75	+
	<sup>A</sup> A20C3	Sensitive	1.17	+
	<sup>A</sup> A20C6	Moderate	5.95	+
5	<sup>A</sup> A21C1	High	17.62	+
	<sup>A</sup> A22C1	High	16.34	+
	<sup>A</sup> A24C1	High	14.43	+
	<sup>A</sup> A25C2	High	17.11	+
1	<sup>B</sup> B01C3	Moderate	5.17	+
	<sup>B</sup> B02C1	Moderate	5.07	+
	<sup>B</sup> B03C4	Moderate	4.13	+
	<sup>B</sup> B04C1	High	8.28	+
	<sup>B</sup> B05C2	Moderate	4.11	+
2	<sup>B</sup> B06C5	Moderate	6.91	+
	<sup>B</sup> B07C2	Moderate	5.73	+
	<sup>B</sup> B08C3	Moderate	7.69	+
	<sup>B</sup> B10C2	Moderate	5.75	+
3	<sup>B</sup> B11C1	Moderate	6.88	+
	<sup>B</sup> B12C1	Moderate	5.21	+
	<sup>B</sup> B13C1	Moderate	5.37	+
	<sup>B</sup> B14C1	Moderate	4.23	+
	<sup>B</sup> B15C1	Moderate	6.40	+
4	<sup>B</sup> B16C2	Moderate	5.77	+
	<sup>B</sup> B17C1	Moderate	5.07	+
	<sup>B</sup> B18C2	Moderate	6.61	+
	<sup>B</sup> B19C1	Moderate	7.00	+
	<sup>B</sup> B20C3	Moderate	2.15	-
5	<sup>B</sup> B21C1	Moderate	6.88	+
	<sup>B</sup> B22C1	Moderate	11.00	+
	<sup>B</sup> B23C2	Moderate	3.95	+
	<sup>B</sup> B24C2	Moderate	6.00	+
	<sup>B</sup> B25C1	Moderate	4.10	+

<sup>A</sup>*E. coli* isolate from producer A; <sup>B</sup>*E. coli* isolate from producer B; presence or absence of transmissible *locus* of stress tolerance (tLST) genes. The heat resistance classification and D<sub>60°C</sub> values were obtained with three independent replicates, each containing duplicate samples (3 × 2)

## Discussion

Foodborne pathogens in milk and dairy products have been reported, and among the many causes, lack of hygiene and deficiencies in good manufacturing practices are identified as the main reasons for contamination [14, 49–51]. In this regard, *Enterobacteriaceae* and coliforms represent important quality indicators to measure a possible failure during postpasteurization contamination [52]. The failure in the postpasteurization process may indicate the presence of

pathogenic *E. coli* serogroups [53]. In this respect, small-scale milk producers may have a tendency to produce lower quality milk than large-scale producers due to several factors, such as low productivity, low capital reserve, and little access to higher qualified professionals, among others [54, 55]. Therefore, in the present study, producer B presented more contaminated milk than producer A; this reason could be because producer B has only a SISE, while producer A has SIF. In this way, we speculate that the lower production demand combined with lower investment capacity can explain the higher contamination verified in producer B.

Furthermore, the implementation and constant monitoring of good hygiene practices (GHP), good manufacturing practices (GMP), and hazard analysis and critical control points (HACCP) are important factors during food processing [56]. However, the inefficient use of these tools may impact food quality and safety [51, 56]. We highlight that the samples from the two evaluated producers did not undergo incorrect pasteurization temperatures (evaluated by phosphatase negative and peroxidase positive in all samples). Even so, high *Enterobacteriaceae* and coliform counts were above the established standards [5, 16–18] and could be caused by postpasteurization contamination. Corroborating this hypothesis, *E. coli* isolates from different weeks were noted in a single PFGE profile (Fig. 2). In this regard, notable isolates B04C1 and B06C5 with moderate potential to form biofilms were identified in cluster II, and isolate A24C1, the only isolate from producer A with moderate potential to form biofilm, was identified in cluster IV. This may indicate recurrent contamination during the entire analysis period for producer B samples and during weeks 4 and 5 for producer A. However, isolate B04C1, was the only isolate to present a high heat resistance compared to the other isolates from producer B (Table 3). One hypothesis is that selective pressure may generate different phenotypic responses even in clonal isolates [57].

Milk pasteurization is the only mechanism employed to destroy microorganisms in dairy and dairy products in Brazil [4]. However, subpasteurization temperatures, commonly used in the manufacture of some cheeses, can promote an increase in thermotolerance of *E. coli*, as has been observed in some strains isolated from raw milk [10]. Although inherent aspects of the food matrix may influence the heat resistance of microorganisms [58], the acquisition of tLST genes by *E. coli* can provide resistance to times and temperatures beyond those of pasteurization, as was observed in *E. coli* isolated from a beef processing plant [23]. Our findings reveal *E. coli* isolates displaying moderate to high heat resistance and a D<sub>60°C</sub> value exceeding 17 min (isolate A25C2; Table 3). These isolates pose a risk in milk production that applies subpasteurization as a unique microbiological method to control microbial contamination. Furthermore, the adaptive response mediated by selective pressure in *E.*

**Table 4** Biofilm formation potential, curli expression phenotype, and identification of *rpoS* gene in *E. coli* isolated from pasteurized milk obtained from two producers from Mato Grosso, Brazil

Week	Isolate ID	Biofilm formation ( $A_{570nm}$ )	*Biofilm classification	**Curli expression	<i>rpoS</i> gene
4	<sup>A</sup> A19C6	0.09 ± 0.01	Absent	Rdar	+
	<sup>A</sup> A20C3	0.17 ± 0.02	Weak	Rdar	+
	<sup>A</sup> A20C6	0.21 ± 0.05	Weak	Rdar	+
5	<sup>A</sup> A21C1	0.12 ± 0.01	Weak	Rdar	+
	<sup>A</sup> A22C1	0.14 ± 0.04	Weak	Rdar	+
	<sup>A</sup> A24C1	0.29 ± 0.06	Moderate	Rdar	-
	<sup>A</sup> A25C2	0.08 ± 0.001	Absent	Rdar	+
1	<sup>B</sup> B01C3	0.09 ± 0.01	Absent	Rdar	+
	<sup>B</sup> B02C1	0.13 ± 0.02	Weak	Rdar	-
	<sup>B</sup> B03C4	0.09 ± 0.01	Absent	Intermediate	-
	<sup>B</sup> B04C1	0.30 ± 0.02	Moderate	Saw	-
	<sup>B</sup> B05C2	0.09 ± 0.004	Absent	Rdar	-
2	<sup>B</sup> B06C5	0.22 ± 0.09	Moderate	Rdar	+
	<sup>B</sup> B07C2	0.10 ± 0.01	Absent	Rdar	+
	<sup>B</sup> B08C3	0.12 ± 0.02	Weak	Rdar	+
	<sup>B</sup> B10C2	0.10 ± 0.01	Absent	Rdar	+
3	<sup>B</sup> B11C1	0.14 ± 0.04	Weak	Intermediate	+
	<sup>B</sup> B12C1	0.11 ± 0.01	Absent	Rdar	-
	<sup>B</sup> B13C1	0.11 ± 0.02	Absent	Rdar	+
	<sup>B</sup> B14C1	0.10 ± 0.01	Absent	Rdar	-
	<sup>B</sup> B15C1	0.09 ± 0.01	Absent	Saw	+
4	<sup>B</sup> B16C2	0.11 ± 0.02	Absent	Rdar	+
	<sup>B</sup> B17C1	0.17 ± 0.03	Weak	Rdar	+
	<sup>B</sup> B18C2	0.09 ± 0.01	Absent	Intermediate	+
	<sup>B</sup> B19C1	0.09 ± 0.005	Absent	Saw	+
5	<sup>B</sup> B20C3	0.20 ± 0.06	Weak	Rdar	+
	<sup>B</sup> B21C1	0.11 ± 0.01	Absent	Rdar	-
	<sup>B</sup> B22C1	0.13 ± 0.02	Weak	Intermediate	+
	<sup>B</sup> B23C2	0.22 ± 0.03	Weak	Rdar	-
	<sup>B</sup> B24C2	0.12 ± 0.01	Weak	Rdar	-
	<sup>B</sup> B25C1	0.16 ± 0.002	Weak	Rdar	-

\*The biofilm classification was obtained based on the cutoff optical density (ODc; 0.11) of two independent replicates, with each plate containing duplicate samples: nonbiofilm ( $OD_{strain} < ODc$ ), weak biofilm ( $ODc < OD_{strain} < 2 \times ODc$ ), moderate biofilm ( $2 \times ODc < OD_{strain} < 4 \times ODc$ ), and strong biofilm ( $4 \times ODc < OD_{strain} < 8 \times ODc$ ). \*\*Colony characteristics on Congo red agar after 4 days of incubation, comprising rdar (curli-positive colonies), intermediate (medium characteristics), or observed (negative colonies). Presence or absence of *rpoS* gene (involved in curli/cellulose control in stress conditions) [48]

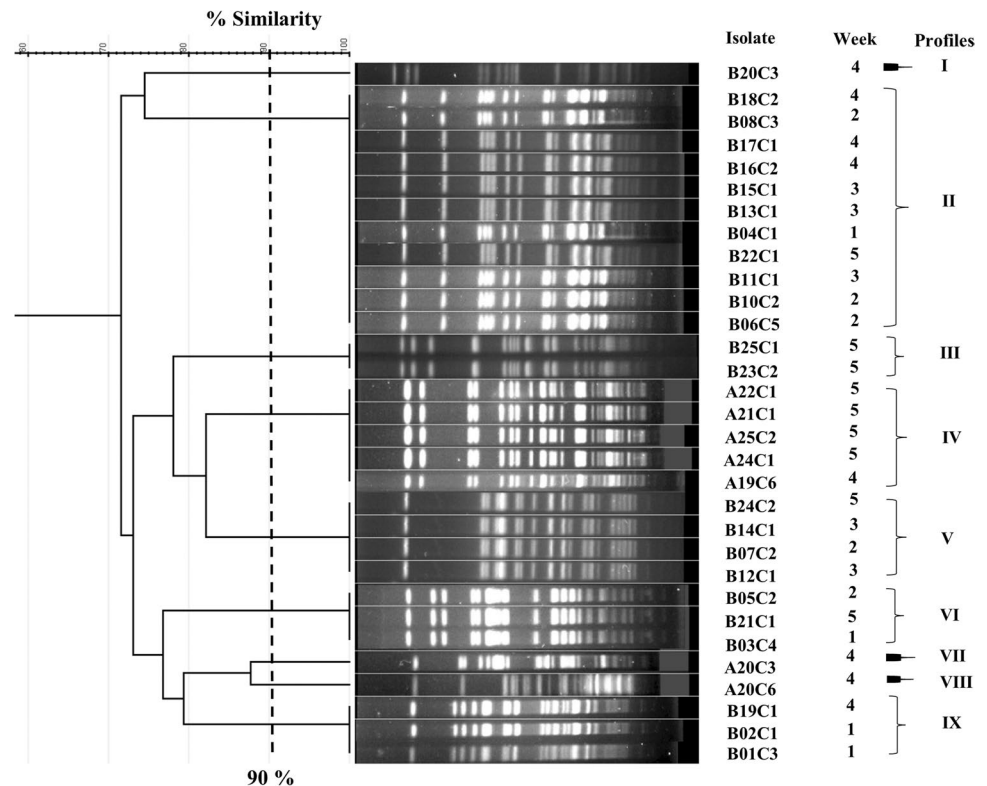
*coli* has been suggested for the emergence of tLST genes [24, 59]. In our results, 97% of the isolates presented tLST, which may have contributed to the survival of the isolates at 60 °C/6 min. In addition, even nonclonal isolates harbored tLST (A20C3 and A20C6; Fig. 2), which suggests possible gene dissemination in the milk producers analyzed in the present study. On the other hand, it is necessary to investigate whether these strains are also capable of withstanding HTST pasteurization temperatures (72–75 °C/15 at 20 s).

Another important point in our study is the number of isolates associated with potential to form biofilms (51.6%; Table 4). Biofilm formation in food processing plants can

be a vector for pathogen transmission and, in some cases, can be formed by pathogenic microbial communities, such as *E. coli* O157:H7 [60–62]. Furthermore, microbial communities in biofilms produce extracellular polymeric substances (EPS) that protect them against antimicrobial treatments [41]. The EPS of *E. coli* is formed especially by polysaccharides such as cellulose, proteins such as curli, and other matrix components [63]. The presence of these components ensures biofilm formation in the early stages, allowing for greater environmental persistence and protecting the bacteria from sanitization [42]. In our results, 77.4% of the isolates expressed curli (Table 4). However,



**Fig. 2** Dendrogram based on nine PFGE profiles (90% similarity) of *E. coli* isolated from pasteurized milk samples obtained over 5 weeks from two producers from Mato Grosso, Brazil. Isolates with the initial A were obtained from producer A samples, and isolates with the initial B were obtained from producer B samples



the absence of curli did not prevent the potential to form biofilm (isolate B04C1) under the investigated conditions.

Many other studies have reported that curli is not always crucial for biofilm formation in *E. coli* since medium conditions, temperature, time, and others need to be considered [32, 40, 61, 64, 65]. Moreover, several genes are related to the expression of biofilm proteins in *E. coli* [48, 66, 67], and under stress conditions (low nutrient, chlorine, high temperature, and others), the RNA polymerase sigma factor S (product of *rpoS* gene) can contribute to the expression of the EPS components [48, 66, 68]. Therefore, our initial hypothesis was that *rpoS* might be involved in the expression of curli as the strains were challenged with stress conditions (60 °C/6 min). Although most isolates showed curli expression characteristics, there was no correlation between curli and *rpoS* since many isolates that expressed curli did not show the gene (Table 4). In our PCR assays, *rpoS* was associated with 64.5% of *E. coli* isolates, which is curious because it is conserved gene in *E. coli*. In this regard, Stanford et al. [40] identified only 38.8% *rpoS* in the *E. coli* isolates evaluated. According to these authors, mutations in *rpoS* may interfere with PCR detection. A second hypothesis is that storage conditions of *E. coli* in the laboratory can easily inactivate *rpoS*, which rarely occurs in *E. coli* in its natural habitat [69]. However, further studies need to be performed to further investigate the non-detection of this gene in *E. coli* by PCR.

Overall, the results of this study indicated that even clonal isolates showed distinct phenotypes for heat resistance.

Nevertheless, the phenotype for moderate potential to form biofilms and high heat resistance was noted in the same strain (A24C1, B04C1), contributing to the high risk of survival of these strains in the final product. In contrast, all the antimicrobials tested were efficient in inactivating *E. coli* isolates. This fact indicates that the use of antimicrobials may be occurring correctly. However, the appropriate use of antibiotics in animal production should be considered with larger numbers of samples that can represent the range of producers in the state.

In conclusion, both dairy producers presented unsatisfactory milk contamination levels during the weeks evaluated. This unsatisfactory contamination allowed us to isolate 31 *E. coli* isolates, and surprisingly, 97% (30/31) of the isolates presented tLST genes and high heat resistance (60 °C/6 min). In addition, through PFGE analyses, a high genetic similarity was identified between the isolates in different weeks, which suggest a recurrent source of contamination present in both dairy producers. Therefore, our study determined that 51.6% of the isolates had biofilm formation potential. Thus, although postpasteurization contamination may have occurred, the heat resistance phenotype was different even for clone isolates. In this sense, the present study is the first to determine the presence of *E. coli* strains with the potential for heat resistance in milk produced from Brazil. Therefore, further studies can be carried out to determine whether these isolates are able to survive the pasteurization process or were included in the milk in a postpasteurization contamination, or both, as evidenced in isolates A24C1 and B04C1.

**Author contribution** Conceptualization: Maxsueli Aparecida Moura Machado, Eduardo Eustáquio de Souza Figueiredo, and Carlos Adam Conte-Junior; methodology: Maxsueli Aparecida Moura Machado, Vinicius Silva Castro, Adelino Cunha-Neto, Deyse Christina Vallim, Rodrigo de Castro Lisboa Pereira, Jaqueline Oliveira dos Reis, and Patricia Veiga de Almeida; formal analysis and investigation: Maxsueli Aparecida Moura Machado, Vinicius Silva Castro, Diego Galvan, and Adelino da Cunha-Neto; writing—original draft preparation: Maxsueli Aparecida Moura Machado; writing—review and editing: Maxsueli Aparecida Moura Machado, Vinicius Silva Castro, Adelino Cunha-Neto, Deyse Christina Vallim, Rodrigo de Castro Lisboa Pereira, Jaqueline Oliveira dos Reis, Patricia Veiga de Almeida, Diego Galvan, Eduardo Eustáquio de Souza Figueiredo, and Carlos Adam Conte-Junior; funding acquisition: Maxsueli Aparecida Moura Machado and Eduardo Eustáquio de Souza Figueiredo; resources: Patricia Veiga de Almeida, Deyse Christina Vallim and Carlos Adam Conte-Junior; supervision: Eduardo Eustáquio de Souza Figueiredo, and Carlos Adam Conte-Junior.

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**Data availability** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Conflict of interest** The authors declare no competing interests.

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