Análise microbiológica e de resíduos antimicrobianos presentes em leite Tipo C comercializados na cidade de Goiânia-Goiás-Brasil

Microbiological analysis and antimicrobial residues present in Type C milk sold in the city of Goiânia-Goiás-Brazil

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RESUMO
Objetivou-se avaliar a qualidade microbiológica, bem como a presença de resíduos antimicrobianos em leites comercializados na cidade de Goiânia-GO. Foram coletadas seis amostras de leite em quatro momentos. O experimento procedeu-se em duas etapas: 1) análises microbiológicas de orientação, para verificação quanto aos padrões microbiológicos, de acordo com a Resolução da Diretoria Colegiada nº12 e pelo método da American Public Health Association; e 2) detecção da presença de resíduos antimicrobianos, pelo método antibiograma por disco-difusão com controle do experimento feito pela inoculação de Escherichia coli com os antibióticos ampicilina, cefalotina, penicilina e tetraciclina. Foram observados resultados negativos para a presença de coliformes totais e resíduos antimicrobianos nas amostras investigadas. Observou-se também, uma sensibilidade total do microrganismo Escherichia coli, frente aos antibióticos ampicilina, cefalotina e tetraciclina, no experimento controle. Contudo, para a penicilina notou-se uma alteração no comportamento, sendo que inicialmente a bactéria mostrou-se sensível ao fármaco e, ao longo das análises, apontou...
resistência ao fármaco. Os resultados sugerem um bom controle de qualidade higiênico sanitária das indústrias que manuseiam as amostras de leite selecionadas para o presente estudo, revelando a preocupação com o fornecimento de um produto alimentício de qualidade. E, aliado a isto, garantia à saúde do consumidor e fluidez no processamento tecnológico dos leites beneficiados.

Palavras-chave: leite; microbiologia de alimentos; controle de qualidade.

ABSTRACT
The objective was to evaluate the microbiological quality, as well as the presence of antimicrobial residues in milk sold in the city of Goiânia-GO. Six milk samples were collected at four times. The experiment proceeded in two stages: 1) microbiological analysis of orientation, to verify the microbiological standards, according to the Resolution of the Collegiate Board nº12 and by the method of the American Public Health Association; and 2) detection of the presence of antimicrobial residues, using the disk-diffusion antibiogram method with control of the experiment carried out by inoculating Escherichia coli with the antibiotics ampicillin, cephalothin, penicillin and tetracycline. Negative results were observed for the presence of total coliforms and antimicrobial residues in the investigated samples. It was also observed, a total sensitivity of the microorganism Escherichia coli, against the antibiotics ampicillin, cephalothin and tetracycline, in the control experiment. However, for penicillin there was a change in behavior, and initially the bacteria was sensitive to the drug and, throughout the analysis, it showed resistance to the drug. The results suggest a good sanitary hygienic quality control of the industries that handle the milk samples selected for the present study, revealing the concern with the supply of a quality food product. And, coupled with this, it guarantees consumer health and fluidity in the technological processing of the processed milk.

Key Words: milk; food microbiology; quality control.

1 INTRODUCTION

Milk is a food widely consumed by humans, classified as a drink of high biological value, because it contains all essential amino acids, constituting a great source of protein and vitamins,, in addition to minerals, in its predominance of calcium (Kich et al., 2012).

In a study conducted with milk without heat treatment in the midwest region of Brazil, they verified inadequate sanitary hygienic conditions used during the milking procedure, thus implying the poor quality of the final product, facilitating the emergence of damage to the final consumer and dairy products (Gonçalves et al., 2020).

As a tropical country, a climate that favors the conditions for the management of ruminants, milk production in Brazil has always had economic importance and has grown and intensified considerably, along with the increase in the demand for dairy consumption (Motta, 2015). Due to the high consumption of this product, the monitoring of microbiological quality and the presence of contaminants in the dairy industry has been shown to be inefficient in relation to the control of biological and chemical hazards, as residues of antimicrobial substances have been verified in milk distributed to the Brazilian population (Leite et al., 2002).

When contamination of milk by antimicrobial residues is detected, the most likely hypothesis is that it is due to inadequate administration and/or excess of medicines used to treat
infections in the animal, such as mastitis (inflammation of the cow's udder). Such conduct may generate concern, since the deleterious effects of such substances on the human body is not known for sure (Silva, 2014).

Based on the assumption, the present study aimed to evaluate the microbiological quality, as well as the presence of antimicrobial residues in milks sold in the city of Goiânia-GO.

2 MATERIAL AND METHODS

Among the pasteurized milks, type C, sold in the city of Goiania-GO, samples of six distinct brands were randomly acquired in the local trade. The same brands were acquired in four moments, one each month from June to September 2019. The samples were transported to the Laboratory of Hygienic Sanitary Control of Food (Faculty of Nutrition, Federal University of Goiás - LCHSA/FANUT/UFG), within two hours. The transport was carried out in an isothermal box, hermetically sealed, with recyclable ice, in order to avoid temperature variation, contamination and/or deterioration of the product. The hygienic-sanitary control of the samples was strictly followed. The packages were opened aseptically and were subsequently submitted to preparation for analysis at LCHSA. The experiment was carried out in two stages: microbiological orientation analyses, to verify the microbiological patterns, according to RDC 12 (Brazil, 2001), by the American Public Health Association (APHA) method (APHA, 2015) and detection of the presence of antimicrobial residues, by the antibiogram method (CLSI, 2009).

For the microbiological analyses, which were performed in triplicate, 25 mL of sample were removed, later transferred to sterile homogenization bags, in which 225 mL of buffered peptone water was added. The homogenization of this liquid occurred in stomacher, for one minute. After this stage, the serial dilution technique was performed until the third dilution was obtained, using 0.1% buffered peptone water as a diluent.

To confirm the presence of total coliforms, biochemical tests of the APHA method (APHA, 2015) were followed. For which, after serial dilution, they were inoculated in Violet Bile Red Agar (VRBA), using the in-depth plating technique. After complete solidification of the medium, it was covered with a 5-8 mL overlayer of the same medium. The plates were incubated in the inverted position, at 32°C for 18-24h. After this period, plates containing between 15 and 150 typical colonies of total coliforms should be selected to determine the number of colony-forming units per mL (CFU/mL), multiplying the number of typical colonies by the inverse of dilution. The confirmatory test consisted of transferring one elevation from each suspected colony to 2% Bile Bright Green Broth (VB) tubes, with subsequent incubation at 32°C for 24-48h. All pipes with gas growth and production without surface film should be considered as total coliform (confirmed colony). If it presented gas
production and surface film, there was a need to carry out further tests, such as the confirmation test for thermotolerant coliforms.

To evaluate the antibacterial susceptibility by the disc-diffusion method, according to the recommendations of CLSI (2009), 50 mL of each milk sample was pipetted. This aliquot was submitted to heating at 80°C for 5 minutes, with subsequent cooling, until reaching room temperature for inactivation of inhibitory enzymes such as lactoferrin (glycoprotein with bacteriostatic potential). Concomitantly, an inoculum of Escherichia coli ATCC 25922 was suspended, recovered, in Brain Heart Infusion broth (BHI), at 36°C / 18 hours.

Culture suspensions were prepared, diluted in 0.85% saline solution, using the MacFarland 0.5 scale until approximately $1.5 \times 10^8$ (CFU/mL) was obtained. With the aid of a swab, the Escherichia coli ATCC 25922 strain was inoculated on the surface of sterile 90 mm plates with Mueller Hinton Agar culture medium (MH) and then inert filter paper discs were impregnated with 20 mL of milk samples to be analyzed separately in distinct plates. In each plate containing the microorganism, a paper disc impregnated with one of the milk aliquots was placed in order to verify the presence or absence of antimicrobial residue. Control plates were also prepared and antibiotic discs of ampicillin, cephalothin, penicillin and tetracycline were placed.

These analyses were done in duplicate and the results were read after incubation at 37°C for 24 hours. Microbial growth inhibition halos were measured in millimeters, with the aid of a millimeter ruler comparing the data in specific NCCLS/CLSI tables (2009). Through this assay, it is possible to determine the presence or absence of antimicrobial residue and also to analyze the resistance or sensitivity of the microorganism to the antimicrobial tested.

Descriptive analysis was performed to analyze the data collected with the presentation of the mean and standard deviation of the mean. Data normality was tested by the Shapiro-Wilk test and, due to the presence of data normality, the one-way ANOVA test was used to verify differences between the groups. When there was significance in the comparison tests between evaluations, the Bonferroni post-hoc test was performed and arranged with symbols flanked by their means and standard deviations from the mean in the table. The significance level used for all tests was 5%. Stata software was used® version 14.0 in this analysis.

3 RESULTS

Colony growth was not observed in the analyzed samples, and total coliforms were not found in milk samples and making it unnecessary to search for thermotolerant coliforms. It was also verified that there was no halo formation in any of the six samples in any of the four analyses performed, thus
evidencing the absence of antibiotic residues in significant amounts in the volume of milk tested and the method used.

When performing the antibiogram control experiment using the selected antibiotics, halo formation was investigated in all tests. However, in the third and fourth analyses, for penicillin, the presence of halo was not verified. This result suggests a possible resistance of the biological material used, in relation to the drug or, that there has been a loss of its efficiency, as shown in Table 1.

When comparing the average halo sizes for the third and fourth evaluations compared to the first and second, it was found that for ampicillin, cephalothin and tetracycline there was a smaller halo size, and therefore lower drug efficiency or increased bacterial resistance to it. In fact, a smaller halo size was also verified for penicillin, in the comparison between the first and second evaluations, as observed in Table 1.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>1st evaluation</th>
<th>2nd evaluation</th>
<th>3rd evaluation</th>
<th>4th evaluation</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>4.60±0.36</td>
<td>4.27±0.25</td>
<td>2.33±0.15†</td>
<td>2.07±0.12†</td>
<td>0.001</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>4.07±0.12</td>
<td>3.73±0.25</td>
<td>2.07±0.12†</td>
<td>2.23±0.06†</td>
<td>0.001</td>
</tr>
<tr>
<td>Penicillin</td>
<td>4.20±0.17</td>
<td>3.10±0.17†</td>
<td>Resistant</td>
<td>Resistant</td>
<td>0.001</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>3.43±0.12</td>
<td>3.27±0.25</td>
<td>2.53±0.06†</td>
<td>2.43±0.12†</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Values presented in mean ± standard deviation of the mean. p-value obtained by one-way ANOVA test and flanked symbols of their means and standard deviations obtained by the Bonferroni comparison test so that: † differs significantly from evaluation 1; ‡ differs significantly from evaluation 2.

4 DISCUSSION

This study aimed to analyze the presence of biological contamination and the presence of antimicrobial residues in samples of pasteurized milk type C, commercialized in the city of Goiânia-GO. The presence of biological contamination is due to poor processing and storage of the product, and the presence of residues is closely related to the inadequate administration of antimicrobial agents for the treatment of mastitis in ruminants and contamination of milk extracted from them.

Mastitis can be of environmental or contagious, subclinical or clinical origin, and is defined as an inflammatory response of the animal to a pathogen. The visible signs are the appearance of pus in the udders, followed by the presence of blood and, therefore, there is a need to discard milk, generating a drop in milk production. In addition to the financial commitment to the reduction in the volume of milk produced, there is an increase in production costs, since veterinary services are requested, increased labor, investments in measures to prevent the disease in the animal and the use of medicines, such as antibiotics (Callefe, 2015).

Antibiotics, or antimicrobials, can be applied to the animal or used as a dietary supplement, incorporated into its diet, and thus can reach the bloodstream and consequently to milk. Thus, it is necessary to respect the time of drug deficiency, or half-life, in order to continue with milking. If this
does not occur, the presence of antimicrobial agents in milk is verified. This can lead to public health problems such as hypersensitivities, intestinal microbiota alteration, rhinitis and wings (Silva et al., 2014; Pereira and Scussel, 2017).

In addition to these physiological hazards, there is a technological problem of milk by-products, more specifically in the production of dairy fermented, since the antimicrobial agents present in milk directly interfere in the fermentation of products, such as yogurts, cheeses and others. Due to this problem, both in the industrial and physiological technological parts of the human body, it becomes even more essential to perform analyses to attest to the quality of milk and determine its composition, in order to ensure the supply of a product with microbiological and nutritional quality, as recommended by Brazilian regulations (Souza et al., 2017; Silva, 2014).

In fact, this concern seems valid, because contrary to what we found in the present study, that is, absence of antimicrobial contamination, a study conducted in Paraná using commercial kits with the immunoenzymatically assay method found antimicrobial residues in 15 of the 79 samples analyzed (18.99%). Three of these 15 samples were contaminated by tetracyclines (Vieira et al., 2012).

In line, the presence of antimicrobial residues in fresh milk was verified in more than 50% of the municipalities in the Region of Garanhuns-PE. The drugs found more frequently were tetracycline and aminoglycosides the same as those used for the treatment of mastitis and anemia (Nunes, 2016).

In another study, a comparative analysis was performed between the immunoenzymatically and microbiological assays for the detection of antimicrobial residues in milk. And for the samples submitted to three types of tests: one of them using the immunoenzymatically methodology "SNAP® test" (Gentamicin, β-lactams and Tetracycline), another from microbiological analysis "Delvotest®" and, finally, the conventional microbiology test (diffusion in agar using paper discs). From the total samples analyzed (n=114), by the "Delvotest ®" method, 7% of positive samples were observed in the presence of antimicrobial residues. The "SNAP® test" detected a total of 37.7% positivity for antimicrobial residues and the conventional test (antibiogram) did not indicate any of the samples with positive results for the presence of these agents (Benetti et al., 2011). This data suggests fragility of the results of this last test, especially because it only brings qualitative results reporting presence or absence, but not concentration of the antimicrobial.

On the other hand, the antibiogram is a test widely used to evaluate the resistance/sensitivity of a given biological agent in the face of some type of antimicrobial. This is because this method is low cost, easy to reproduce and able to give relatively fast results. Based on this assumption, it is necessary to pay attention to the results of the antibiograms, because the amount of remnants of the
drug found in milk may not be sufficient to provide the formation of a significant inhibition halo, and therefore more sensitive tests such as liquid chromatography and mass spectrometry methodology are needed (Benetti et al., 2011).

Escherichia coli stands out as a pathogen microorganism causing mastitis in its clinical and subclinical forms. Colonies isolated from this bacterium cause breast infections and categorize E. coli as a breast pathogenic Mammary Pathogenic E. coli - (MPEC) (Guerra, 2019). For this reason, this microorganism was selected in the present study and elected as a target for antibiotics, since it is quite common in manual managements with precarious sanitary hygiene control. It is known that, in normal situations, E. coli integrates the intestinal microbiota, however, in certain immunosuppressive situations, they have virulence potential that favors infections of the gastrointestinal tract (Casale, 2019). Moreover, in the present study, the choice of antibiotics used was justified by the recurrent and indiscriminate use of them in the treatment of characteristic infections in cow udders (Silva et al., 2014).

With regard to sanitary hygiene control, no sample evaluated here was considered inadequate for human consumption, thus showing that the food industry has followed all health protocols established by Normative Instruction No. 62, of 29 December 2011 (MAPA, 2011). However, this finding is not a rule, since in other investigations contaminations were found in pasteurized milk type C. For example, in a study with 20 samples of this type of milk marketed in Salvador-Bahia-Brazil, the results showed that 13 samples showed a number of mesophilic aerobic bacteria ranging from $10^2$ to $10^6$ CFU/mL and 13 samples with total coliforms in most probable number (MPN), ranging from 4 to ≤2400/mL, among which, 11 (55%) values above the acceptable limit. For fecal coliforms, of the 20 only 7 samples presented MPN ranging from 9 to ≤ 2400/mL, and are therefore in unacceptable conditions according to the legislation. In the same study, no samples contaminated with Salmonellas and coagulase-positive staphylococci were found (Leite et al., 2002).

In another study, which aimed to evaluate microbiological parameters of cattle milk marketed in the city of Tucuruí-Pará-Brazil, analyses of total coliforms, thermotolerant coliforms were performed, Salmonella sp. and Staphylococcus spp. of seven milk samples pasteurized. It was observed that total coliforms had six samples with values of <0.3 NMP/mL and only one with a value of 20 NMP/mL; and for Staphylococcus spp., three samples were positive, with results from 2x10 CFU/mL to 8x10 CFU/mL. This indicates that the pasteurization process or the storage of the product was inadequate, requiring improvement in the standardization of this raw material and in even more effective techniques of milk handling (Pereira et al., 2019).
Therefore, it is essential to clarify to producers about the aspects that lead to milk contamination and how to avoid them, raising awareness about the importance of adequate veterinary treatment in addition to adequate handling and processing, which can grant go to consumer health and industrial production (Pereira and Scussel, 2017). In addition, it is worth noting that it is essential to invest in infrastructure for better animal confinement, thus reducing episodes of infections, such as mastitis and, consequently, the reduction in drug administration (Schneider, 2016).

5 CONCLUSIONS

It was concluded that all milk samples analyzed are in accordance with current legislation, since they showed no evidence of poor microbiological quality of the product, besides not showing contamination with antimicrobial residues. Therefore, we can conclude that, from the method used for the analyses, the antibiogram, the product offered to the final consumer is free of microbiological and chemical contaminations (antibiotics), thus presenting adequate nutritional and microbiological quality and, indicating the commitment of the milk processing industries in relation to food safety and consumer health. However, in future studies, more sensitive tests, such as chromatographic tests, are suggested, which can quantify possible antibiotics not detected in the antibiogram.

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