Climate conditions associated with the occurrence of antimicrobial and macrocyclic lactone residues in bulk tank milk

[Condições climáticas associadas à ocorrência de resíduos de antimicrobianos e lactonas macrocíclicas em amostras de leite de tanque]

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ABSTRACT

The present study aimed to identify the climate condition parameters that are associated with the occurrence of antimicrobial and macrocyclic lactone residues in bulk tank milk using a multivariate principal components analysis (PCA). A total of 132 raw milk samples were collected at dairy farms in Minas Gerais State in Brazil and analyzed for 35 analytes, comprising macrocyclic lactones and antibacterials, using liquid chromatography coupled with mass spectrometry in tandem mode spectrometry. Of the 132 samples, 34 (25.76%) bulk tank milk samples were positive for at least one analyte. PCA showed that antimicrobial residues in bulk tank milk occurred less frequently on days with a higher average temperature, maximum temperature and temperature-humidity index. In contrast, relative humidity was inversely associated with antimicrobial residues in raw milk. The PCA showed that daily milk production was also related to macrocyclic lactone residues, while rainfall showed an inverse association. Thus, some climate conditions, such as average temperature, maximum temperature and temperature-humidity index, can predict the moments with lower risk of occurrence of antimicrobial residues in bulk tank milk, in contrast to relative humidity. Furthermore, the risk of macrocyclic lactone residues in bulk tank milk was higher in months with less rainfall.

Keywords: anthelmintic, antibiotic, veterinary drug, seasonality, raw milk

O presente trabalho objetivou identificar fatores climáticos associados à ocorrência de resíduos de antimicrobianos e lactonas macrocíclicas em amostras de leite de tanque por análise multivariada de componentes principais (ACP). Para o presente trabalho, 132 amostras de leite cru foram coletadas em fazendas leiteiras localizadas no estado de Minas Gerais (Brasil) e analisadas por cromatografia líquida de alta eficiência e espectrometria de massas em tandem para detecção de 35 analitos, incluindo antimicrobianos e lactonas macrocíclicas. Das 132 amostras de leite analisadas, detectou-se pelo menos um analito em 34 (25,76%) amostras. A ACP demonstrou que a presença de resíduos de antimicrobianos no leite de tanque ocorreu menos frequentemente nos dias com maior temperatura média, temperatura máxima e índice de temperatura e umidade. Por outro lado, a umidade relativa foi inversamente associada à presença de resíduos antimicrobianos no leite. A ACP demonstrou associação entre a presença de resíduos de lactonas macrocíclicas no leite e a produção diária de leite, e a presença de resíduos de lactonas macrocíclicas ocorreu menos frequentemente nos meses com maiores índices pluviométricos. Dessa forma, conclui-se que alguns índices climáticos, como temperatura média, temperatura máxima e índice de temperatura e umidade, podem predizer períodos com maior risco de
ocorrência de resíduos de antimicrobianos, em contraste com a umidade relativa. Além disso, o risco de resíduos de lactonas macrocíclicas no leite de tanque foi maior nos meses com menores índices pluviométricos.

Palavras-chave: antelmíntico, antibiótico, medicamento veterinário, sazonalidade, leite cru

INTRODUCTION

Successful milk quality assurance programs start with farm bulk tank milk that is free of veterinary drug residues and has a low somatic cell count (SCC) and bacterial counts, resulting in better quality products with a longer shelf life (Ruegg and Tabone, 2000; Gonzalo et al., 2010). As a result, bulk tank milk parameters have been the target of payment-by-quality schemes and different legal limits proposed by many countries and regions (Gonzalo et al., 2010).

Antimicrobials and macrocyclic lactones are the most widely used veterinary drugs in dairy cattle management for treatment of disease and the control of bacterial and parasitic infections (Bilandžić et al., 2011; Toaldo et al., 2012). Extensive and improper use of these active compounds can lead to the presence of their residues in milk, causing human health risks, the development of microbial drug resistance, the spread of resistant pathogens, loss of industrial output and technological problems in dairy products (Ruegg and Tabone, 2000; Toaldo et al., 2012).

It is widely known that environmental conditions are associated with the incidence of infectious diseases such as mastitis (Nóbrega and Langoni, 2011; Arcaro et al., 2013), which is the primary cause of antimicrobial drug usage in adult dairy cows (Pol and Ruegg, 2007). Furthermore, there is a seasonal effect on the number of helminth eggs per gram of feces (Araujo and Lima, 2005) with possible implications for the control of helminth infections by anthelmintic treatment in dairy cattle (Antonello et al., 2010). Thus, we hypothesized that some climate conditions could favor the appearance of bacterial and parasitic diseases, which in turn leads to higher veterinary drug usage, and consequently results in a higher risk of the presence of veterinary drug residues in raw milk.

Most studies that have assessed the risk factors related to veterinary drug residues used rapid screening methods to detect veterinary drug residues in raw milk and analyzed a single or limited class of active compounds (Ruegg and Tabone, 2000; Saville et al., 2000; Molina et al., 2003; Gonzalo et al., 2010; Nebot et al., 2012). Furthermore, several authors have noted the problem of false-positive results in certain microbiological inhibitor tests. One possible cause of false-positive results in these screening tests is the presence of indigenous antimicrobial agents and free fatty acids (Molina et al., 2003). Although these screening methods (i.e., immunological or microbial inhibition assays) are widely used to detect the presence of veterinary drugs in foods, more accurate chromatographic methods coupled with highly specific and sensible detection systems, such as tandem mass spectrometry, are required to identify, confirm the presence of and quantify these compounds (Molina et al., 2003; Samanidou and Nisyriou, 2008; Toaldo et al., 2012) because they provide full or complementary information enabling the unequivocal identification and quantification of the analyte at levels of interest (Samanidou and Nisyriou, 2008).

To the best of our knowledge, this is the first study to associate climate conditions with the detection of several antimicrobials and macrocyclic lactones (35 analytes) in raw milk using combined analytical methods based on liquid chromatography coupled with tandem mass spectrometry. Thus, the present study aimed to identify the climate condition parameters associated with the occurrence of antimicrobial and macrocyclic lactone residues using multivariate principal component analysis.

MATERIALS AND METHODS

Raw milk samples were collected from August 2009 to February 2010 from 45 dairy farms in Minas Gerais State, Brazil. A total of 132 samples were collected from bulk milk tanks after milk homogenization. Given the extensive sampling that was required and the cost of the analyses, dairy herds were randomly selected based on a list of farms from the most important
dairy plant in the region by considering their levels of daily milk production. Twenty-seven (60%) of the dairy farms had production levels of ≤500 L/day, five (11.11%) had production levels between 501 and 1,000 L/day, and 13 (28.89%) had levels of >1,000 L/day. The daily milk production was recorded at each sampling. Thus, the milk samples that were obtained were representative of the dairy farms belonging to the region of study. Regarding the time period that was selected, this study was initiated in the dry period and finished in the rainy period, which resulted in great variability in climate conditions. This is representative of typical annual climate conditions that have been previously recorded. The milk samples were stored frozen at -18°C until the quantitative analysis of veterinary drug residues.

The relative humidity, average temperature and maximum temperature were recorded on the day the milk samples were collected. The amount of rainfall was also recorded monthly. Furthermore, the Temperature-Humidity Index (THI) was calculated with the following equation:

\[
\text{THI} = 1.8 \times \text{Ta} - (1 - \text{RH}) \times (\text{Ta} -14.3) + 32,
\]

as described by Bouraoui et al. (2002), where Ta is the average temperature in °C and RH is the average relative humidity as a fraction of unit.

The analytical standards penicillin G (PNG), penicillin V (PNV), ceftiofur (CFT), cloxacillin (CLX), dicloxacillin (DCX), oxacillin (OXA), chlorotetracycline (CTC), doxycycline (DOX), tetracycline (TC), oxytetracycline (OTC), oxolinic acid (OXO), nalidixic acid (NALIDIX), flumequine (FLU), difloxacin (DIFLO), ciprofloxacin (CIPRO), enrofloxacin (ENRO), norfloxacin (NOR), sarafloxacin (SARA), trimethoprim (TMP), sulfadimethoxine (SDMX), sulfaquinoxaline (SQX), sulfadiazine (SDZ), sulfathiazole (STZ), sulfapyridine (SPY), sulfamethoxazole (SMA), sulfamethazine (SMZ), sulfachloropyridazine (SCP), sulfisoxazole (SFX), sulfadoxine (SDX), sulfamerazine (SMR), ivermectin (IVR), eprinomectin (EPR), emamectin (EMA), doramectin (DOR), abamectin (ABA) and moxidectin (MOX) were purchased from Sigma-Aldrich (St. Louis, MO, USA) as Vetranal™ analytical-grade standards (> 95% certified purity).

For the β-lactams, stock standard solutions were prepared by dissolving all compounds in polypropylene glycol 3000 in acetate buffer (pH = 4.5) at a concentration of 0.5 to 3.75mg mL⁻¹. For the tetracyclines, sulfonamides and fluoroquinolones, stock standard solutions of each compound were prepared by dissolving 10 mg of the analytical standard in 10 mL of the appropriate solvent (acetone for tetracyclines and sulfonamides, methanol with a couple of drops (~2) of 1 M NaOH for fluoroquinolones, and methanol for TMP). Aliquots of each stock solution were diluted to obtain final concentrations of 10µg mL⁻¹ and 1 µg mL⁻¹ and were stored at -20°C.

For macrocyclic lactones, individual stock solutions of 1.0 mg mL⁻¹ were prepared by dissolving 10 mg of each standard in 10 mL of ACN. The working solutions were prepared by combining aliquots of each stock solution in ACN to obtain a final concentration of 1 µg mL⁻¹ for ABA, IVR and MOX; 1.5µg mL⁻¹ for DOR; and 2.0µg mL⁻¹ for EPR in ACN. EMA was used just as an internal standard, and its working solution was prepared at 1.0µg mL⁻¹ in ACN.

All reagents were of analytical grade unless otherwise indicated. Acetic acid, trichloroacetic acid, ACN (MeCN, MS-grade), ethanol and methanol (MeOH, MS-grade) were purchased from Merck (Darmstadt, Germany). Formic acid and triethylamine were supplied by J.T. Baker (Phillipsburg, NJ, USA). Deionized ultra-pure water (<18.2 MΩ cm resistivity) was obtained from the Milli-Q SP Water System (Millipore, Bedford, MA, US). Disodium ethylenediamine tetraacetate (Na₂EDTA) was obtained from Sigma. Ammonium acetate (analytical grade) was obtained from Mallinckrodt-Baker (Phillipsburg, NJ, USA).

Prior to the quantitative analysis, an LC-MS/MS multi-residue screening method was applied for the qualitative analysis of fluoroquinolones, tetracyclines, sulfonamides and trimethoprim in milk. The LC-MS/MS screening analysis was performed with a Waters Alliance 2795 system (Milford, USA) coupled to a Micromass Quattro Micro triple quadrupole mass spectrometer (Waters, Milford, USA) with an electrospray source. The multi-residue separation was performed in a Waters Symmetry C18 LC column (75mm x 4.6mm, 3.5µm). A Phenomenex C18 column (4.0mm x 3.0mm) was used as a guard column. A gradient elution program was used with solvent A (aqueous solution of 0.1% formic acid) and solvent B...
Chromatographic conditions and mass spectrometry parameters were performed according to Bittencourt et al. (2011). Detection was performed in multiple reaction monitoring mode, with 2 m/z transitions monitored for each analyte. The m/z transitions associated with the retention time of the analytes provide confirmatory data, fulfilling the requirements for confirmatory analysis. For positive results, quantitative methods were applied to determine the analyte concentration.

The LC system used in this study was an Agilent 1100 series LC (Santa Clara, CA, USA) with a quaternary pump, a vacuum degasser and an autosampler, coupled with an API 5000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) with an electrospray ionization interface. Chromatographic separation of the β-lactams was performed with a Phenomenex Synergy® C18 analytical column (150 x 3.0 mm, 4.0 µm), preceded by a security guard system C18 (4.0 x 3.0 mm, 5 µm) (Phenomenex). A binary mobile phase was used, with a flow of 500 µL min⁻¹, for a total run time of 12 min. Mobile phase component A was an aqueous solution of 0.1% formic acid, and component B was ACN with 0.1% formic acid. Detailed chromatographic and mass spectrometry conditions are provided in Jank et al. (2011).

For the tetracyclines, separation was performed on an Xterra C18 column (2.1mm x 100mm, 1.7µm) preceded by a security guard column C18 (4.0 x 3.0 mm, 5 µm) (Phenomenex). A binary mobile phase was used, with a flow of 500 µL min⁻¹, for a total run time of 12 min. Mobile phase component A was an aqueous solution of 0.1% formic acid, and component B was ACN with 0.1% formic acid. Detailed chromatographic and mass spectrometry conditions are provided in Jank et al. (2011).

The separation of fluoroquinolones was performed in a Waters Symmetry C18 column (75mm x 4.6 mm, 3.5µm) with a Phenomenex C18 (4.0mm x 3.0mm) as a guard column, through a gradient elution using an aqueous solution of 0.1% formic acid as solvent A and ACN with 0.1% formic acid as solvent B. For sulfonamides and trimethoprim, the separation was performed in a Zorbax® XDB C18 column (150 x 4.6mm, 5µm) (Agilent). The mobile phase was composed of 10 mM ammonium acetate acidified with 0.01% acetic acid (solvent A) and methanol (solvent B) in a gradient program. The detailed parameters are described in Hoff et al. (2009).

Separation of macrocyclic lactones was performed on a Luna C18 column (150 mm x 2.1mm, 5µm) preceded by a guard column (4mm x 3mm, 5µm) of the same packing material (Phenomenex, Torrance, CA, USA). All analytes were eluted after 4min with an isocratic mobile phase consisting of 50 mM ammonium acetate buffer (pH 5): ACN (5:95, v/v) at 0.2mL min⁻¹. The electrospray voltage and the source temperature were set at 4500 V and 500°C, respectively.

Aliquots (500µL) of raw milk samples were extracted in microcentrifuge tubes (1.5mL) by adding 20µL of 20mM EDTA and 200µL of acidified ethanol (3% acetic acid). The samples were mixed for approximately 15 s in a vortex and then centrifuged at 10,000g. An aliquot of the supernatant was diluted with water:ACN (1:1, v/v, both with 0.1% formic acid) in a high-performance liquid chromatography (HPLC) vial and subjected to multi-residue analysis. A volume of 10µL was injected into the LC-MS/MS system.

β-lactams were determined as described in Jank et al. (2011). The extraction procedure consisted of adding 1.0mL ACN to 2.0mL milk four times in sequence and mixing in a vortex between each addition. The sample was then mixed in a head-over-head shaker for 15min, 1.0g of sodium chloride was added, and the sample was further mixed for 15min in the head-over-head shaker. The samples were centrifuged for 5min at 3000g. Aliquots of the supernatant were transferred to HPLC vials, and a volume of 10µL was injected into the chromatograph.

For the quantitative analysis of tetracyclines, 500µL milk was placed in a microcentrifuge tube (2.0mL), and 5µL of 100 mM EDTA was then added to the tube. After mixing for 15 s, 200µL acidified ethanol (3% acetic acid) was added. The samples were then mixed (15s) and centrifuged for 10min at 12,000 rpm. An aliquot of the supernatant (350µL) was diluted with water (650µL) in an HPLC vial and subjected to LC-MS/MS analysis.

The quantitative analysis of fluoroquinolones was performed by applying the same procedure.
described above in “Qualitative analysis of fluoroquinolones, tetracyclines, sulfonamides and trimethoprim”, using a matrix-matched calibration curve and a specific mass spectrometry method for fluoroquinolone quantification.

For the determination of the presence of sulfonamides and trimethoprim, 500μL milk was homogenized for 15s in a vortex mixer, and the analyte extraction was performed with 200μL acidified ethanol (3% acetic acid). The extract was mixed for 15s and centrifuged for 10min at 12,000 rpm. An aliquot of the supernatant (350μL) was diluted with water (650μL) in an HPLC vial, and 10μL was injected into the chromatograph for LC-MS/MS analysis.

The extraction of the samples was performed as described in Rübensam et al. (2011). For the procedure, 5.0mL milk was extracted with four aliquots of 2.5mL ACN using liquid-liquid extraction with low temperature purification. To this mixture, 2g sodium chloride was added, followed by shaking until the salt dissolved. Then, the mixture was centrifuged. The top phase was transferred to a 15-mL polypropylene centrifuge tube and kept in a freezer for 12 h at −20°C. The remaining liquid phase was then transferred to a new 50-mL centrifuge tube and evaporated in a water bath (50-55°C) under a gentle nitrogen flow until completely dry. Finally, the dry extract was reconstituted with 1 mL ACN for further LC-MS/MS analysis.

All samples were subjected to all described methods. First, the samples were analyzed by an LC-MS/MS screening method for antibacterial residues. For each positive result, an LC-MS/MS quantitative and confirmatory method was performed. All methods were previously fully validated and are currently in routine use in the laboratory network of the Ministry of Agriculture, Livestock and Food Supply of Brazil (MAPA) in order to perform the NRCCP (Lins et al. 2012). The results were corrected by recovery. All of the applied methods met internal criteria for residue analysis.

Multidimensional principal components analysis (PCA) was performed with STATA statistical software version 12 (Stata Corp., College Station, Texas, USA). To test associations among all variables surveyed together, PCA was performed to determine inferences about the possible biological meaning of associations among the association, without pre-establishing cause/effect. Thus, this statistical technique allows us to visualize graphically the variables in the same dimensional plane and establish any relationship among them. The minimum percentage of inertia of the system that is acceptable to use the multidimensional PCA was 65.0%, regarding the percentage of variance explained by the first three axes (components).

RESULTS AND DISCUSSION

The present study was able to identify 10 veterinary drug residues (PNV, CFT, OXA, DCX, OTC, CIPRO, ENRO, ABA, DOR, and IVR) of the 35 investigated. The residues were identified in 34 (25.76%) samples with at least one veterinary drug residue detected by confirmatory tests, whereas nine (6.82%) samples showed multiple detections with at least two residues quantified for each sample.

The multidimensional PCA showed that antimicrobial residues in bulk tank milk were less frequent on days with a higher average temperature, maximum temperature and THI, although they were associated with relative humidity (Figure 1a, Table 1). We hypothesize that Brazilian dairy cattle management can influence our results because during the rainy period, the cattle were maintained mainly on pasture, which contrasts with months with lower maximum temperature and lower rainfall (dry season) in which animals remained in a confinement system. In fact, some comparison trials have shown differences in favor of pasture systems for reproductive performance, mastitis, somatic cell count, hoof health and general cow health (Goldberg et al., 1992; Washburn et al., 2002; White et al., 2002) that could thus influence antimicrobial treatment. For instance, Washburn et al. (2002) reported that cows in confinement had 1.8 times more clinical mastitis and eight times the rate of culling for mastitis than did cows on pasture. Thus, it should be considered that clinical mastitis is a major reason for antimicrobial drug usage in dairy cows and is even higher than dry cow therapy, treatment of metritis, and foot and respiratory diseases (Pol and Ruegg, 2007; Saini et al., 2012).
Table 1. The relationship among milk production, climate conditions and antimicrobial residues by confirmatory tests expressed as loading in a principal component analysis

<table>
<thead>
<tr>
<th>Variables</th>
<th>Component 1</th>
<th>Component 2</th>
<th>Component 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk Production</td>
<td>0.2268</td>
<td>-0.6364</td>
<td>-0.0325</td>
</tr>
<tr>
<td>Rainfall</td>
<td>-0.1687</td>
<td>0.6979</td>
<td>0.1154</td>
</tr>
<tr>
<td>Temperature</td>
<td>0.5314</td>
<td>0.0893</td>
<td>0.0426</td>
</tr>
<tr>
<td>Max. Temperature</td>
<td>0.453</td>
<td>0.2029</td>
<td>0.0706</td>
</tr>
<tr>
<td>THI</td>
<td>0.4867</td>
<td>0.0665</td>
<td>-0.0292</td>
</tr>
<tr>
<td>Relative Humidity</td>
<td>-0.4252</td>
<td>-0.1654</td>
<td>-0.2428</td>
</tr>
<tr>
<td>Antimicrobials</td>
<td>-0.1218</td>
<td>-0.1644</td>
<td>0.9587</td>
</tr>
</tbody>
</table>

The screen test displays the values of the first three components of the principal component analysis. Climate condition variables are indicated by the following: rainfall, average temperature, max. temperature, THI and relative humidity. Temperature: average temperature. Max. Temperature: maximum temperature. THI: temperature-humidity index.

Altogether, these facts may explain, at least in part, why the antimicrobial residues in bulk tank milk are associated with months with lower maximum and average temperatures and lower THI (dry season; Azevedo et al., 2005) found here.

The PCA showed that milk production was also related to macrocyclic lactone residues, while rainfall showed an inverse association (Figure 1b, Table 2). The association of milk production with macrocyclic lactone residues observed here was consistent with Gross et al. (1999); Sanchez et al. (2004) and Reist et al. (2011), who...
reported that anthelmintic treatments lead to higher milk production per animal due to the reduction of gastrointestinal nematode infections. Consistently with our data, Araujo and Lima (2005) have described in Minas Gerais State, Brazil, that among the helminths, the genera *Haemonchus* sp. and *Trichostrongylus* sp. were highly prevalent in dairy cows, and the largest number of eggs per gram of feces peaked in months of low rainfall (July and August). Therefore, we hypothesize that climate conditions can be associated with the susceptibility of cows to disease and/or the biology of pathogens and parasites involved in diseases, which could then be related to the use of veterinary drugs in dairy cattle and consequently the presence of their residues in food of animal origin. Additionally, as observed with antimicrobial treatment, we hypothesize that the higher animal density during the months that cattle primarily remain indoors, which occurs in the dry season, can lead to a higher impact of gastrointestinal nematode infections, thus resulting in anthelmintic treatments and their residues in bulk tank milk.

Although the present study evaluated the effect of climate conditions on the antimicrobial and macrocyclic lactone residues in raw milk in a short period, their importance should at least be regarded in terms of world climate change. The changes in temperature and rainfall patterns may lead to alterations in the dynamics of pathogens of veterinary importance (Gale et al., 2009; Van Dijk et al., 2010) and, consequently, in the veterinary drug usage and the presence of their residues in food of animal origin with important human health implications. For instance, in the last few years, lower-than-usual precipitation rates were observed over southeastern Brazil (Getirana, 2016). Thus, further studies are required considering a longer term to better determine the effect of these changes on animal health, veterinary drug usage and their residues in food of animal origin.

### Table 2. The relationship among milk production, climate conditions and macrocyclic lactone residues by confirmatory tests expressed as loading in a principal component analysis

<table>
<thead>
<tr>
<th>Variables</th>
<th>Component 1</th>
<th>Component 2</th>
<th>Component 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk Production</td>
<td>0.2327</td>
<td>-0.5619</td>
<td>-0.3865</td>
</tr>
<tr>
<td>Rainfall</td>
<td>-0.1808</td>
<td>0.6857</td>
<td>0.0922</td>
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<tr>
<td>Temperature</td>
<td>0.5299</td>
<td>0.1217</td>
<td>-0.0297</td>
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<tr>
<td>Max. Temperature</td>
<td>0.4478</td>
<td>0.2304</td>
<td>-0.0152</td>
</tr>
<tr>
<td>THI</td>
<td>0.4835</td>
<td>0.0992</td>
<td>-0.0792</td>
</tr>
<tr>
<td>Relative Humidity</td>
<td>-0.4287</td>
<td>-0.1809</td>
<td>-0.1391</td>
</tr>
<tr>
<td>Macrocyclic Lactones</td>
<td>0.1194</td>
<td>-0.3218</td>
<td>0.903</td>
</tr>
</tbody>
</table>

The screen test displays the values of the first three components of the principal component analysis. Climate condition variables are indicated by the following: rainfall, average temperature, max. temperature, THI and relative humidity. Temperature: average temperature. Max. Temperature: maximum temperature. THI: temperature-humidity index.

### CONCLUSIONS

Some climate conditions, such as average temperature, maximum temperature and temperature-humidity index, can predict periods with lower risk of occurrence of antimicrobial residues in bulk tank milk, in contrast to relative humidity. In addition, the risk of macrocyclic lactone residues in bulk tank milk was higher in months with less rainfall.

### ACKNOWLEDGMENT

This study was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Ministério da Agricultura Pecuária e Abastecimento (MAPA), Pró-Reitoria de Pesquisa da Universidade Federal de Minas Gerais and Laboratório de Análise da Qualidade do Leite (LabUFMG) of the Escola de Veterinária from the Universidade Federal de Minas Gerais. We are also very grateful to Gabriel Rübensam, Doriane Sacheto, Louise Jank and Magda Targa Martin for their valuable assistance with the chemical analysis. Drs.
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