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Original Article

Different methods and times of milk conservation: physical-chemical composition and microbiological quality

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The objective of this study was to verify methods of preservation of raw milk samples for physical-chemical analysis and bacterial count patterns. In Experiment 1, the experimental design was a factorial arrangement with three preservatives × two temperatures × five storage times. At seven days of storage, the samples with no preservative had higher total bacterial counts (TBC). However, the fat content increased in the refrigerated samples. The levels of protein, lactose and defatted solids were influenced by the analysed variables and by the interaction between them. Milk pH was influenced by the type of preservative and the duration of storage. Experiment 2 involved an evaluation of the influence of initial TBC, temperature and storage days. The factorial arrangement involved two TBCs × two temperatures × five storage times. The TBC, pH and total solid content of milk were influenced by the analysed variables. There was a quadratic pattern for TBC over storage days. With regard to fat, there was an effect of the initial TBC and storage temperature. Protein and lactose increased, with subsequent stabilisation. Samples with high initial TBC presented higher total solids levels. There was an effect of the interaction between TBC and temperature, and between temperature and storage days. For TBC analysis, the use of azidiol as a preservative is dependent on the use of refrigeration during storage. For physical-chemical analysis, the use of bronopol is indicated.

INTRODUCTION

The production of dairy products is an increasingly competitive activity, requiring acontinual search for increased production and improvement in quality, aiming to meet national demand and to conquer international markets.

Currently, Instrução Normativa 62 (IN 62) is the technical regulation relating to the production, identification, quality, collection and transportation of milk (MAPA, 2011). This regulation specifies a minimum fat content of 3%,a minimum total protein of 2.9%,a defatted dry extract (DDE) of 8.4%,a maximum total bacterial count (TBC) of 100,000/mL and a maximum somatic cell count (SCC) of 400,000/mL. Similarly, it requires that milk samples be transported in hygienic thermal boxes, at a temperature and other conditions recommended by the laboratory that will carry out the analysis.However, due to the great territorial extension and large number of dairy farms in Brazil, it is practically impossible for milk samples to only be refrigerated (CASSOLI; MACHADO; COLDEBELLA, 2010). Therefore, it is necessary to add preservatives to the milk samples in order to maintain the quality of the material to be analysed.

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Among the available preservatives, bronopol is widely used and intended for the preservation of samples for the analysis of the physical and chemical composition of milk (GONZALO et al., 2004; SÁNCHEZ et al., 2005). In contrast, for the total bacterial count the main preservative forraw milk samples is azidiol. This allows the sample to be analysed up to seven days after collection if kept at 7 °C, without freezing and heating (CASSOLI; MACHADO; COLDEBELLA, 2010).

However, when chemical preservatives are used, it is recommended that the samples be refrigerated at temperatures below 4 °C (SÁNCHEZ et al., 2005). According to Sierra et al. (2006), the time between sample collection and analysis directly influences the results. Thus, they recommend a maximum time of four days for the analysis of milk composition without the use of preservatives. Cassoli; Machado; Coldebella (2010) did not observe changes in the fat, protein, lactose, total solids and SCC contents when analysing samples of raw milk up to seven days after collection, regardless of the storage temperature, provided that the material was conserved using bronopol.

The present study aimed to compare three methods of raw milk conservation (no added preservative, azidiol and bronopol) for the analysis of the total bacteria count (TBC) using the standard counting method, chemical composition (fat, protein, lactose, total solids and fat solids) and pH of milk. This research also aimed to study the influence of the storage period (number of days), temperature (cooled and uncooled) and the initial microbiological quality of raw milk samples.

MATERIAL AND METHODS

The experiment was conducted at the Milk and Derivatives Quality Laboratory at the Federal University of Santa Maria, Palmeira das Missões (UFSM-PM) campus, located in Palmeira das Missões, Rio Grande do Sul, Brazil.

Sample Collection

The milk samples were collected in two stages, comprising two experiments. In both experiments, collection took place directly from the expansion tank of two milk-producing units, located in the city of Palmeira das Missões.

Firstly, the milk was shaken for 10 minutes with the aid of an automatic stirrer attached to the refrigeration equipment,which had a temperature of 4 °C. Subsequently, a pre-established volume of milk was collected using a collecting cup and an analytical funnel to help fill the volumetric flask. After collection, the samples were transported to the Milk and Derivatives Quality Laboratory of the UFSM-PM, packed in isothermal boxes containing recyclable ice.

In Experiment 1, a 4,000mL sample of refrigerated raw milk was collected and divided into 100 sub-samples of 40 mL each, kept in sterile flasks. The sub-samples were treated as follows: 20 samples without the addition of a preservative; 40 samples with azidiol containing sodium azide and chloramphenicol (Tablet Azidiol, sodium azide and chloramphenicol, Laborclin, Brazil), and 40 samples with bronopol containing natamycin and bronopol (Tablet Brononata, bronopol and natamycin, Laborclin, Brazil)

After the addition of the preservatives, the vials were homogenised by repeated inversion until complete dissolution of the preservative in the milk.

The sub-samples were distributed between the treatments and identified according to the type of preservative (azidiol or bronopol) and storage temperature (ambient or refrigerated), as follows: T1 = azidol/ambient temperature; T2 = bronopol/ambient temperature; T3 = azidiol/refrigerated temperature; T4 = bronopol/refrigerated temperature and T5 = no preservative/refrigerated temperature. Samples were stored at ambient temperature (20.7 to 22.6 °C) or stored in a refrigerator at a temperature between 3.9 and 5 °C.

For Experiment 2, two 1,600mL milk samples were collected from two other farms, also located in Palmeira das Missões, but with different hygienic or sanitary conditions and with TBC values lower than 10,000 and higher than 425,000 colony-forming units.At this stage, the milk was divided into 80 sub-samples of 40 mL each, which were placed in sterile flasks together with a tablet containing sodium azide and chloramphenicol (approximate tablet weight between 41 and 50 mg). Samples were homogenised until complete dissolution of the preservative in the milk.

The sub-samples were distributed between the treatments and identified according to TBC level (low or high) and storage temperature, as follows: T6 = low initial TBC/room temperature; T7 = low initial TBC/refrigerated temperature; T8 = high initial TBC/room temperature and T9 = high initial TBC/refrigerated temperature. The room and refrigerated temperature ranges were the same as those in Experiment 1.

After incubation of the milk samples from both experiments at the two temperature ranges, the influence of storage time (0, 1, 3, 5 and 7 days) was also evaluated.

Analysis

All the milk samples were submitted to TBC and physical-chemical analysis, including the percentages of fat, protein, lactose, total solids, defatted solids and the milk's pH. TBC analysis was performed using the standard counting method foraerobic mesophilic microorganisms on plates (AOAC, 2002). For each sample, three successive decimal dilutions were used, employing 0.1% buffered peptone water as the diluent, following the incubation protocol described by Walters; Estridge; Reynolds (1998). After the incubation period, TBC was determined with a manual model colony counter and the results were transformed into a base ten logarithm (Log 10).

The physical-chemical analysis was performed with aMilk Tester (Foss Electric, Denmark), which used the infrared principle. Before performing the analysis, the samples were homogenised, and the equipment was sanitised for each treatment. The pH analysis was performed using a bench-top pH meter (Model mPA 210, MS Tecnopon, Piracicaba, São Paulo).

Statistical analysis

For Experiment 1, we used a 3 × 2 × 5 factorial statistical design(three preservation types, two incubation temperatures and five storage times). For Experiment 2, we used a 2 × 2 × 5 factorial arrangement (two TBC levels, two incubation temperatures and five storage times).

The results were analysed using SAS software (SAS, 2001), after verification of the normality of residues and the homogeneity of variances. For data analysis, the PROC MIXED procedure of SAS, followed by a Tukey test for the comparison of means, was used according to the model:

$$Y_{ijk} = \mu + T_i + C_j + D_k + (C_j \times D_k) + e(a)_{ijk} + (T_i \times C_j) + (T_i \times D_k) + (T_i \times C_j \times D_k) + e(b)_{ijk}$$

Where Yijk = the observed value; μ = average overall; T_i = fixed temperature effect i; C_j = fixed effect of preservative; D_k = fixed effect of the day of preservation; $(b)_{ijk}$= random error associated with each observation within the sub-plot; $T_i \times C_j$= fixed interaction effect between temperature and preservative; $T_i \times D_k$= fixed interaction effect between temperature and day; $C_j \times D_k$= interaction effect between preservative and day; $T_i \times C_j \times D_k$= fixed interaction effect between temperature, preservative and day; $(a)_{ijk}$= random error associated with each observation within the main plot; and $(b)_{ijk}$ = random error associated with each observation within the sub-plot.

The collection day effect was analysed as a time-repeated measure, where several error structures were investigated and the structure for each evaluated variable was chosen according to the Bayesian information criterion (BIC). Adjusted means were obtained using the LSMEANS command of the PROC MIXED procedure, followed by polynomial regression analysis. For all statistical analyses, significance was established when $P \leq 0.05$.

RESULTS AND DISCUSSION

The milk was collected directly from the cooling tanks of the properties, thus, it did not require direct contact with the animals and there was no need for the approval of the Ethics Committee.

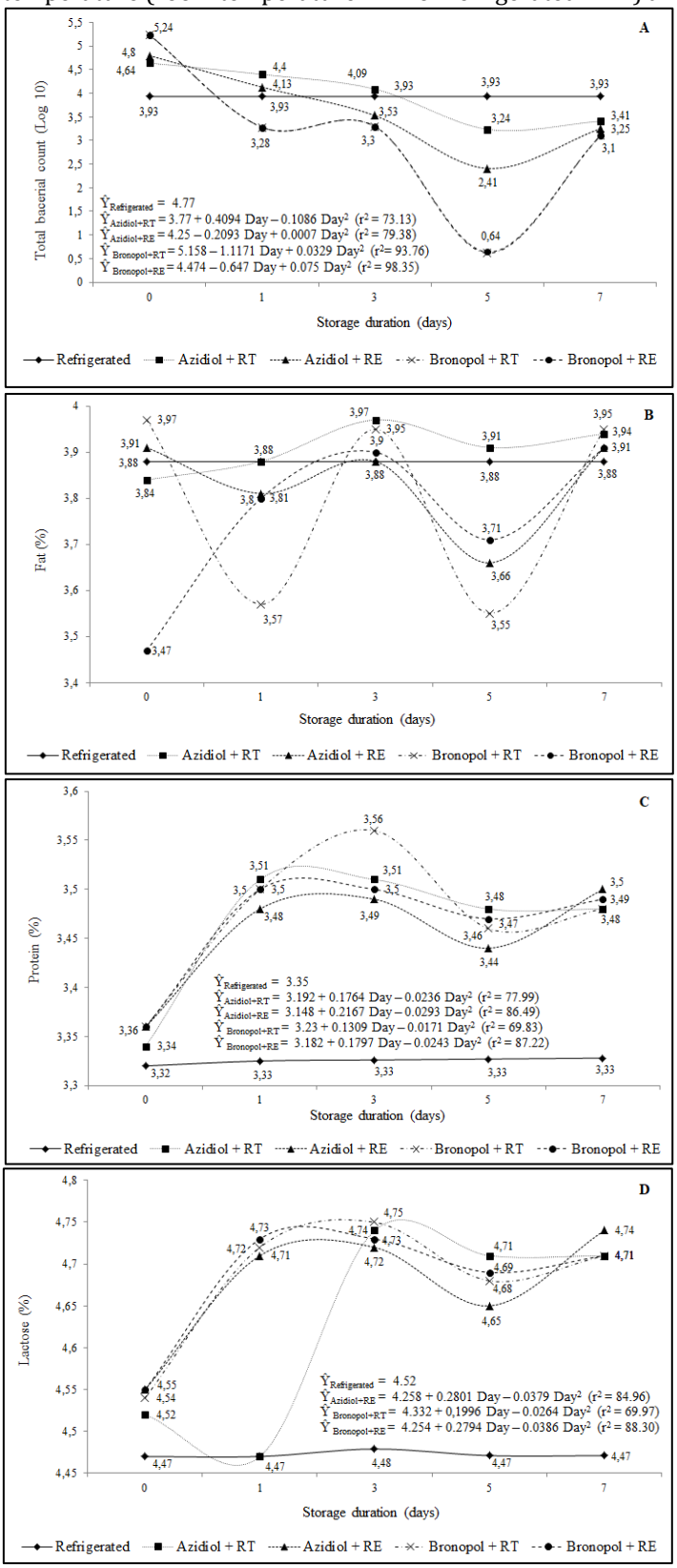
Milk total bacterial count (TBC) was influenced by the preservative, temperature and storage time ($P < 0.05$; Figure 1). During the seven days of storage, refrigerated and unpreserved milk samples showed higher TBC values, whereas samples with bronopol and without refrigeration showed a gradual reduction of TBC over time (Figure 1). Contradictory results were reported by Martins et al. (2009), who demonstrated a marked increase inmicrobial growth with the use of bronopol and a conservation temperature of 25 °C.

Ribas et al. (2006) evaluated the average age of samples, which showed that mean TBC values in samples on day 0 and day 2 of storage increased from1.36 (× 1000 CFU/mL) to 1.41 (× 1000 CFU/mL), but decreased from 1.37 (× 1000 CFU/mL) on day 3 to 1.27 (× 1000 CFU/mL) on day 7. The need for greater control of the refrigeration temperature (ideally between 1 °C and 7 °C) of the samples is evident, avoiding heating or freezing during transportation and storage. In this study, the average TBC values for the samples that contained no added preservative and were kept refrigerated did not differ over the seven days of storage ($P > 0.05$). However, as milk payment is made according to the quality of the product, an increase of 1.31 log TBC over the seven-day storage period (Figure 1) could cause financial harm to milk producers.

There was no effect of preservatives, temperature and days of storage on milk fat content (Figure 1), which presented an average value of 3.84%, which was above the 3% level recommended by IN 62 (MAPA, 2011).The milk protein content was lower when no preservative was used ($P < 0.05$), however, when using azidiol or bronopol, the means were larger and similar to each other. In addition, after two days of storage, protein levels improved in relation to day 0 and there was still an interaction between the type of preservative and storage days (Figure 3). According to Cassoli; Machado; Coldebella (2010), this behaviour may be associated with bacterial growth, because when the physical-

chemical constituents of milk are analysed by the infrared method, it is not possible to differentiate the true milk proteins (casein, albumin, lactoalbumin, lactoglobulin and immunoglobulins) from those formed by non-protein nitrogen, which are produced by some groups of bacteria.

Figure 1 – Total bacterial count (A), fat (B), protein (C) and lactose (D) contents according to the type of preservative (Azidiol or Bronopol), storage temperature (room temperature – RT or refrigerated – RE) and storage duration (days).



The milk lactose content was influenced by the type of preservative and storage day (Figure 1), and showed an interaction between preservative type and storage day ($P < 0.05$; Figure 3). At day 0, the milk lactose content was similar for all treatments ($P > 0.05$), witha mean content of 4.47%. From the first day of storage, the samples had an average 4.7% increase in lactose content, especially when stored with preservatives (Figure 1). By adding a preservative, an increase in the solids content may occur and the preservative itself may bind to water molecules, causing the measured lactose content to be higher. The evaporation of water, the main constituent of milk, probably also occurred throughout the storage period, resulting in a higher lactose content due to the concentration effect of the constituents. It should be noted that electronic equipment used for compositional analyses have acceptability limits for the repeatability standard deviation of 0.06%for protein, fat and lactose and 0.1% for total solid sand defatted solids (ALMEIDA et al., 2016). Ordóñez (2005) and Tronco (2013) found a reduction in lactose content with increasing storage time. This reduction was probably due to deterioration with increasing temperature, in which lactose is converted into acidic compounds, in addition to the deterioration caused by microorganisms that transform this constituent into lactic acid.

We observed no effect on the mean total solids content ($P > 0.05$; Figure 2), but there were correlations between storage days and type of preservative and between storage days and storage temperature ($P < 0.05$). Although the protein and lactose contents were modified throughout the seven days of storage, the fat content remained stable during this period (Figure 1); this may have diluted the effect of the other milk components, resulting in the absence of an effect of time on the total solids content of the milk. This result suggests that, after sample collection, the timing of the determination of chemical composition can alter the content of the above-mentioned components, as well as the total solids content.

The milk defatted solids content (DS) was influenced by the type of preservative and storage days, as well as a correlation between type of preservative and days of storage (Figure 2). From day 0 of storage, the DS content increased, maintaining the same pattern of behaviour that was reported for protein and lactose (Figure 1), since they are the main elements that make up this variable. This effect may be due to the fact that the preservative binds to water molecules, as mentioned previously.

Milk pH was influenced by the type of preservative and the storage days of the sample (Figure 2). For all samples, regardless of treatment, there was a slight reduction in pH values. When storage times were considered, the samples treated with azidiol and without preservative remained unchanged for 7 days.

The type of bacteria and the initial microbial load that are associated with the storage temperature are determinants for the possible proliferation of bacteria during the storage of raw milk (MENEZES et al., 2014). Thus, when samples containing azidiol were evaluated, with two initial levels of TBC at two storage temperatures, there was a reduction in TBC throughout the storage period. In contrast, fat content were influenced by the initial TBC level and storage temperature, where the highest fat content occurred when the initial TBC level was highest ($P < 0.05$; Figure 3). Further, when conserved at room temperature, there was a reduction in fat content on day 7 when the initial TBC was high and on day 5 when the initial TBC was low. This behaviour may be associated with the excessive viscosity of the azidiol-conserved samples at room temperature, which may have hindered milk homogenisation. These results corroborate those of Monardes et al. (1996), Gonzalo et al. (2004) and Sánchez et al. (2005). These authors recommended the addition of bronopol, independent of sample cooling, as the main strategy for preserving samples destined for physical and chemical composition, analysis, although for pH analysis, azidiol provides less oscillation of results over seven days of storage.

Figure 2 – Total solids (A), defatted solids (B) and milk pH (C) according to the types of preservatives (Azidiol or Bronopol), storage temperature (room temperature – RT or refrigerated – RE) and storage duration (days).

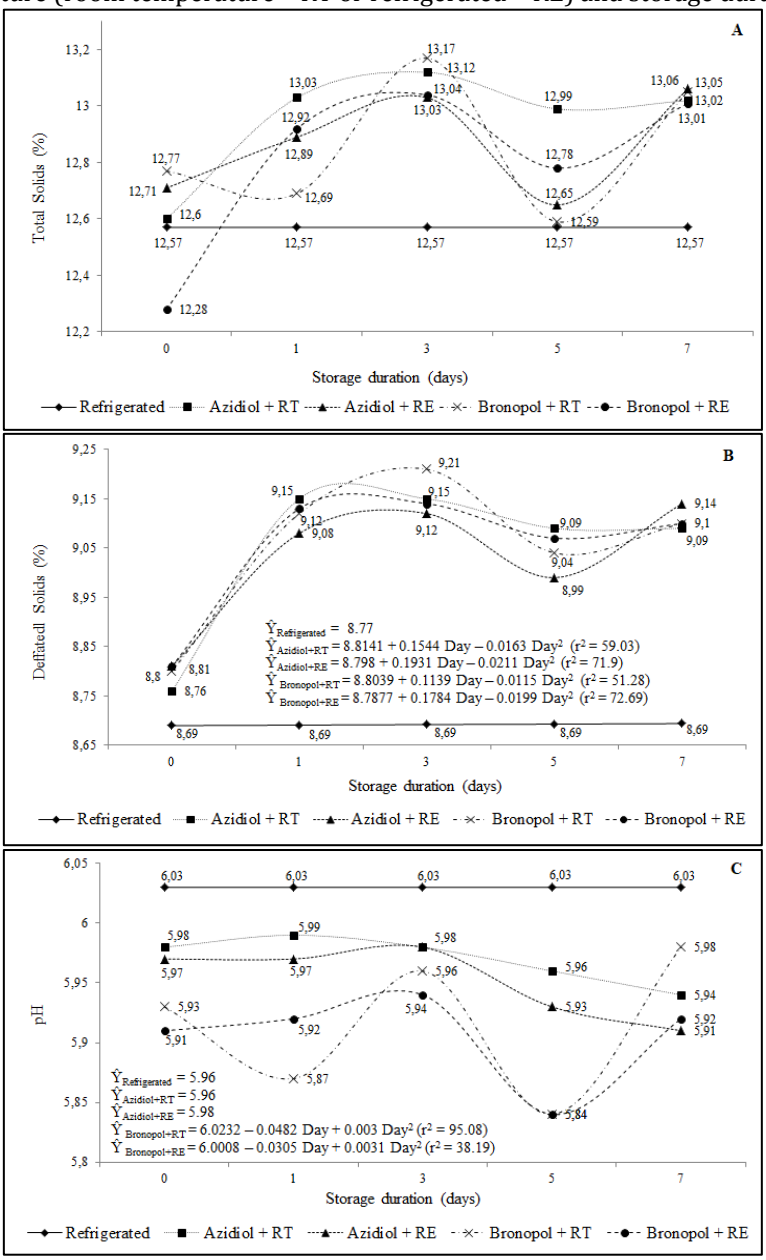
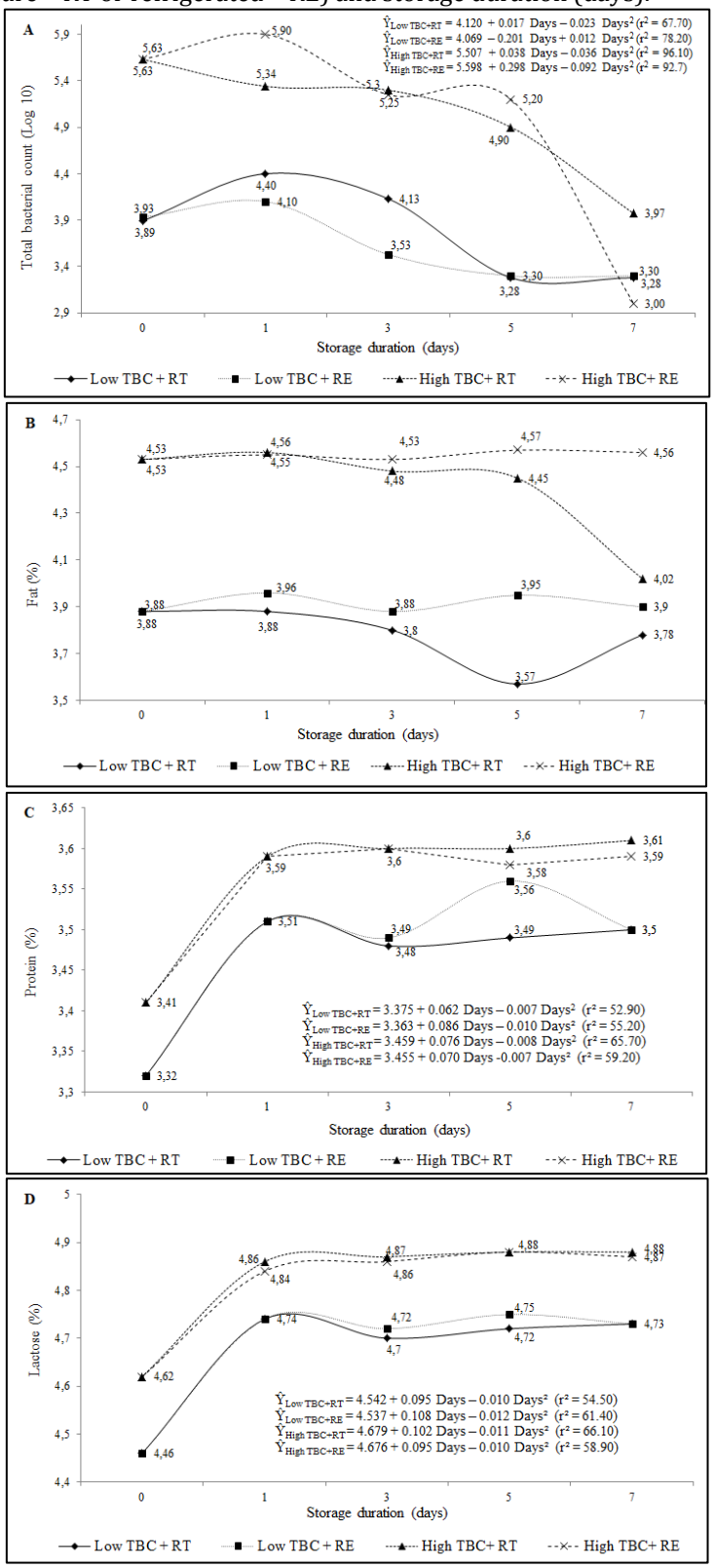


Figure 3 – Total bacterial count (A), fat (B), protein (C) and lactose (D) content of milk according to the initial TBC, storage temperatura (room temperature – RT or refrigerated – RE) and storage duration (days).



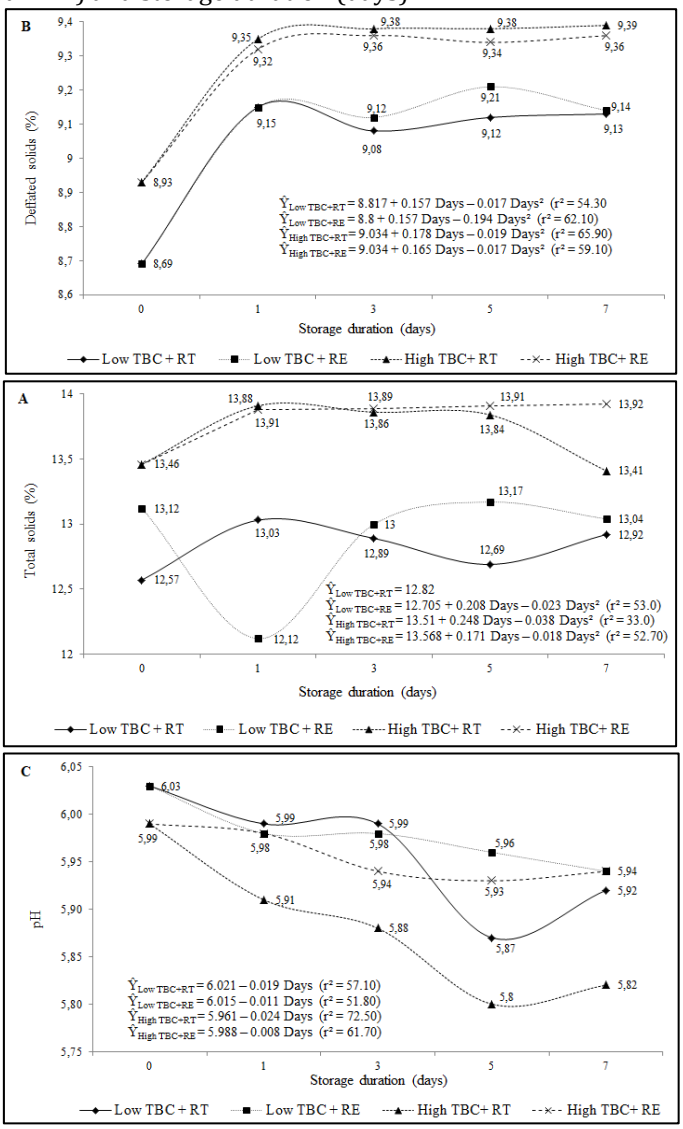
The initial TBC also influenced the protein content, where there was a quadratic effect for storage days, increasing until the first day, followed by stabilisation until the seventh day. In addition, milk with lower initial TBC had lower protein levels (Figure 3).

In the present study, the effect of TBC on milk composition parameters was less than that effect observed on the average fat and protein levels and in the volume of milk produced. However, Bueno et al. (2008) verified that protein levels increased with increased bacterial contamination. According to these authors, the degradation of the protein components requires greater metabolic activity of the microorganisms, and probably a relative concentration of protein, due to the degradation of lactose and fat components that reduce with an increase of the TBC.

The lactose content was influenced by the initial TBC and storage days, where lactose content increased until the first day and remained stable until the last day of evaluation. However, Ordóñez (2005) and Tronco (2013) both reported a decrease in lactose content with increasing milk storage time due to lactose deterioration into acidic compounds or a compensatory effect due to the reduction of fat content of the milk (Figure 3).Also, samples with low initial TBC had lower lactose contents compared to high TBC milk samples (Figure 3), which may be explained by the origin of the milk, coming from two farms using different feeds and cattle breeds.

Samples with high initial TBC showed higher levels of TS; the same was true for protein and lactose content ($P < 0.05$; Figure 4). In general, total solids values decreased over the evaluation period and were higher when refrigeration was used to preserve the samples ($P < 0.05$). In contrast, milk DS content was influenced by the storage period and the initial TBC due to the difference in the initial composition of the milk. Similarly, the DS content increased until day 1of storage, stabilising thereafter. This behaviour was in response to changes in milk protein and lactose content, since they are the main elements of the DS content.

Figure 4 – Total solids (A), defatted solids (B) and milk pH (C) according to initial TBC, storage temperature (room temperature – RT or refrigerated – RE) and storage duration (days).



Milk pH was influenced by the initial TBC, temperature and days of storage. In addition, there was an interaction between TBC and temperature and between temperature and storage days (Table 1). However, when comparing the mean values, there was only a difference between milk samples with high TBC that were kept at room temperature in comparison to the other treatments starting from the fifth day of storage (Figure 4).

Table 1 – Interactions between variables evaluated considering the type of preservative, initial TBC, storage temperatures and storage days.

Variables	Interactions			
	Preservative/temperature[1]			
	T x P	D x P	D x T	D x T x P
TBC (Log 10)	*	*	*	*
Fat (%)	ns	ns	*	ns
Protein (%)	ns	*	ns	ns
Lactose (%)	ns	*	ns	ns
Total Solids (%)	ns	*	*	ns
Defatted solids (%)	ns	*	ns	ns
pH	ns	ns	*	ns
Initial TBC/Temperature[2]				
	TBC x T	TBC x D	T x D	TBC x T x D
TBC (Log 10)	*	*	*	*
Fat (%)	ns	ns	ns	ns
Protein (%)	ns	ns	ns	ns
Lactose (%)	ns	ns	ns	ns
Total solids (%)	ns	ns	ns	ns
Defatted solids (%)	ns	ns	ns	ns
pH	*	ns	*	ns

According to Cassoli; Machado; Colbella (2010) it is necessary to maintain refrigeration during the transport of samples, since the use of preservatives does not promote an ideal bacteriostatic effect, especially when the initial TBC is high. It should be emphasised that milk carriers must receive specific instructions on the collection, handling and hygienic transportation of milk samples. The choice of milk collection and transport material, as well as the hygiene of these materials, are critical points for accurate results of the real quality of milk originating from rural properties (ELIZONDO et al., 2007). In a study conducted by Cunha et al. (2013), alack of homogenisation of milk stored in the expansion tanks of most transporters was demonstrated. This practice influenced the observed variations in SCC and bacterial counts between milk collection points, since rising fat can carry somatic and bacterial cells to the surface of stored milk.

CONCLUSIONS

For milk TBC analysis, the use of azidiol as a preservative, independent of refrigeration, is the most appropriate method for maintaining the milk's original microbiological characteristics over seven days of storage. With regard to the analysis of the physical-chemical composition of milk, specifically protein, lactose and defatted solids, the number of days of storage is an important factor that should be considered and standardised during farm milk collection; were commend the use of the preservative bronopol for this situation.

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