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Efficiency of chemical preservatives used in raw milk samples for bacterial counts by flow cytometry

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ABSTRACT - The objective of this study was to evaluate and compare two chemical preservatives in terms of their sample preservation capabilities, considering the individual bacterial count (IBC) and time and temperature variables. Samples were collected in expansion tanks in three commercial dairy farms located in the northwest of Rio Grande do Sul, characterized as G1: low IBC values, G2: average IBC values, and G3: high IBC values. The tanks were stored at three different temperatures (4, 10, and 25 °C) for 14 d. Samples supplemented with the preservative Azilat in G1 (lower IBC group) exhibited the best results at a temperature of 4 °C, whereas for G2 and G3, the results showed no statistically significant difference between temperatures 4 and 10 °C. The temperature 25 °C exhibited the worst results. For samples preserved with Azidiol, regardless of the studied group (G1, G2, and G3), the temperatures of 4 and 10 °C did not present a significant difference regarding the preservation of the samples, with the temperature of 25 °C exhibiting the worst results. Azilat was effective in keeping the samples conserved when they presented low IBC, being able to fluctuate with the increase in IBC and temperature variation. Azidiol was effective regardless of the initial IBC level.

Keywords: azidiol, azilat, IBC

1. Introduction

Bacterial counting is an important tool used by the Ministry of Agriculture, Livestock, and Supply to monitor the quality of milk produced on farms. Normative Instruction 76–2018, which sets the identity and quality characteristics of refrigerated raw milk, expresses the bacterial count in standard plate count, since the acceptable limit is a maximum value of 300,000 colony-forming units (cfu)/mL. This is equivalent to approximately 824,000 individual bacterial counts (IBC)/mL for quarterly geometric means (Brasil, 2018).

Microbiological contamination of raw milk can originate from a variety of sources, ranging from milking to storage. Hygienic storage conditions during sample collection and the period between milking and milk collection on the farm predispose milk to microbial proliferation (Sampaio et al., 2015; Skeie et al.,

2019). The preservation of raw milk samples to be representative from the moment of collection to analytical determination becomes a challenge in the milk production chain. In Brazil, especially under tropical and subtropical climate conditions, and with longer time periods between collection and analysis due to territorial distance, the process requires the addition of a bacteriostatic preservative that paralyzes the proliferation of the microbial population (Cassoli et al., 2010; Wentz et al., 2018).

Sodium azide and chloramphenicol are the active ingredients that constitute preservatives and have bacteriostatic properties, inhibiting protein synthesis, and thus prolonging the preservation time of refrigerated raw milk samples. In Brazil, azidiol (composed of sodium azide and chloramphenicol) is the only used bacteriostatic preservative currently allowed and supplied to be added to raw milk samples (Vancin et al., 2020). The use of a new chemical preservative for raw milk samples with the same composition may be another option for conservation. However, given the scarcity of information on the effectiveness of the new preservative launched on the market, the objective of this study was to evaluate and compare the two chemical preservatives in terms of their ability to preserve, considering the trinomial bacterial count index, time, and temperature.

2. Material and Methods

Milk was collected from three commercial dairy farms (G1, G2, and G3) located in northwest Rio Grande do Sul, Brazil. The selection of farms was determined from the IBC values/mL: G1, 27,466 (10,000 cfu/mL); G2, 76,464 (70,800 cfu/mL); and G3, 1,014,000 (369,750 cfu/mL).

The experimental design adopted was completely randomized. Twenty-five liters of refrigerated raw milk was collected from each farm, directly from the expansion refrigeration tank. Collection was performed in the morning, and the tanks contained milk produced at intervals of 24 h. Milk was collected after homogenization for a minimum period of 10 min, packed in isothermal boxes containing recyclable ice, and sent to the laboratory, where the milk was fractionated into 40 mL aliquots, totaling 683 samples. Thereafter, the sterile flasks were inverted three times every 5 min for 15 min for the dissolution of the preservatives in the whole milk mass. Samples were exposed to three forms of conservation: azilat preservative [9.58 mg (0.24 mg/mL) of sodium azide and 0.4 mg (0.010 mg/mL) of chloramphenicol], azidiol [4.79 mg (0.12 mg/mL) of sodium azide and 0.2 mg (0.005 mg/mL) of chloramphenicol], and no preservative (NP), and stored at three different storage temperatures (4, 10, and 25 °C) in controlled temperature environments (BOD incubators). Each group (G1, G2, and G3) was subjected to the same protocol with or without NP. Seven different storage times were evaluated: day 1 was considered as the day of sample collection, followed by days 3, 5, 7, 9, 11, and 14. A hundred and nine samples were analyzed on day 1; 97 samples on days 3, 5, 7, 9, and 11; and 89 samples on day 14. The IBC was analyzed using the Bentley BactoCount IBC® 150 Equipment (Bentley Instruments Inc., Chaska, USA) according to ISO 21187/IDF 196 standards (International Dairy Federation, 2004). Results were expressed as mean and standard deviation. Analysis of variance (ANOVA) was used to verify differences in IBC/mL levels. Tukey's post-hoc test was used to verify if there was a significant difference in the IBC levels/mL by the preservative factor, temperature, and time. For statistical analysis of data, the R software was used at a significance level of $P<0.05$.

3. Results

At 4 °C (Table 1), there were no statistically significant differences ($P<0.05$) for the studied groups. At 10 °C, days 1 ($P = 0.055$) and 3 ($P = 0.07$) differed significantly from the other analyzed days (5, 7, 9, and 12) with better IBC/mL averages. At 25 °C, there were no statistically significant differences in the results on days 1, 3, 5, 7, and 12 ($P<0.05$), showing the best means; however, significant differences were observed on days 9 ($P = 0.621$) and 14 ($P = 0.008$), demonstrating unsatisfactory IBC/mL results. Azilat in G1 (Table 2) obtained the best results at 4 °C (4.87), with an increase in IBC/mL at 10 °C (5.06) and 25 °C (6.39). For G2 and G3, there were no significant differences for temperatures 4 °C (5.75 and 5.87) and 10 °C (5.76 and 5.97), whereas 25 °C showed the worst average (6.27 and 6.43). As for azidiol

at 4 °C, G1, G2, and G3 did not differ significantly. The NP treatment showed the best averages at 25 °C (6.02), with an increase in IBC occurring at 4 °C (6.85) and 10 °C (7.05). The number of days in which the samples proved viable to be analyzed with the addition of azilat and azidiol was 14, and for the NP samples, only one day.

Table 1 - Analysis of variance of treatments according to temperatures (4, 10, and 25 °C) and storage times in days (1, 3, 5, 7, 9, 12, and 14)

T	Time (d)	SOS	dof	MS	F	Sig.	Noncent. Parameter	PO
				Preservative × IBC				
	1	0.82	4	0.20	26.05	0.00	104.20	1.00
	3	0.76	4	0.19	49.81	0.00	199.26	1.00
	5	0.69	4	0.17	7.86	0.00	31.46	0.99
4 °C	7	0.58	4	0.14	8.86	0.00	35.45	0.99
	9	0.79	4	0.19	10.82	0.00	43.31	0.99
	12	1.96	4	0.49	184.02	0.00	736.07	1.00
	14	1.40	3	0.46	18.10	0.00	54.31	1.00
	1	0.13	4	0.03	2.65	0.06	10.60	0.66
	3	0.61	4	0.15	4.40	0.00	17.62	0.89
	5	1.16	4	0.29	62.95	0.00	251.80	1.00
10 °C	7	1.36	4	0.34	30.09	0.00	120.35	1.00
	9	1.46	4	0.36	209.56	0.00	838.23	1.00
	12	2.02	4	0.50	79.91	0.00	319.64	1.00
	14	1.79	3	0.59	50.26	0.00	150.80	1.00
	1	1.87	4	0.46	22.44	0.00	89.78	1.00
	3	0.63	2	0.31	22.88	0.00	45.76	1.00
	5	2.32	2	1.16	22.52	0.00	45.04	1.00
25 °C	7	0.73	2	0.36	3.91	0.03	7.83	0.63
	9	0.15	2	0.07	0.49	0.62	0.97	0.12
	12	0.18	2	0.09	17.66	0.00	35.31	0.99
	14	0.17	2	0.08	6.48	0.00	12.95	0.85

T - temperature; SOS - sum of squares; dof - degree of freedom; MS - mean squared; PO - power observed.

Table 2 - Overall average of IBC/mL of the comparison between storage conditions, temperature, and conservation time of raw milk samples of the studied groups

Temperature	Group (IBC/mL)		
	G1	G2	G3
		Azilat	
4 °C	4.87A (14)	5.75A (14)	5.87A (14)
10 °C	5.06B (14)	5.76A (14)	5.97A (14)
25 °C	6.39C (14)	6.27B (14)	6.43B (14)
		Azidiol	
4 °C	4.70A (14)	5.68A (14)	5.97A (14)
10 °C	4.78A (14)	5.67A (14)	5.95A (14)
25 °C	6.34B (14)	6.67B (14)	6.35B (14)
		No preservative	
4 °C	6.85B (14)	7.04B (12)	7.36B (14)
10 °C	7.05C (14)	7.22C (12)	7.36B (14)
25 °C	6.02A (1)	5.37A (1)	6.46A (1)

( ) - number of days in which samples proved viable to be analyzed.
Means followed by the same letter do not differ in the column.
Tukey's test was performed to compare the preservatives at temperatures by groups.

At 4 °C (Table 3), there were no statistically significant differences between the preservatives azilat and azidiol during 14 d of analysis (P<0.05). The NP treatment yielded the worst results. At 10 °C on days 1, 7, and 14 of analysis, there were no differences between the preservatives (P<0.05), whereas 25 °C showed the worst results on all analyzed days (P>0.05), as it showed an excessive increase in IBC/mL means. At 25°C, there were no significant differences between azilat and azidiol until the five days of analysis (P>0.05), with days 7, 9, 12, and 14 showing the worst results.

Table 3 - Overall average in IBC/mL of the comparison of preservatives of the groups of samples studied at different temperatures and storage times

	1	3	5	7	9	12	14
				4 °C			
Azilat	5.47Aa	5.38Aa	5.47Aa	5.44Aa	5.45Aa	5.51Ba	5.75Aa
Azidiol	5.54Aa	5.44Aa	5.39Aa	5.36Aa	5.43Aa	5.41Aa	5.59Aa
NP	5.81Ba	6.52Bb	7.34Bc	7.35Bc	7.44Bc	7.49Cc	7.55Bc
				10 °C			
Azilat	5.51Aa	5.69Ba	5.72Ba	5.43Aa	5.52Ba	5.59Ba	5.75Aa
Azidiol	5.51Aa	5.37Aa	5.45Aa	5.36Aa	5.44Aa	5.51Aa	5.63Aa
NP	5.79Ba	7.09Cb	7.49Cc	7.56Bc	7.58Cc	7.51Cc	7.55Cc
				25 °C			
Azilat	5.43Aa	5.65Aa	6.21Bb	6.52Bb	6.22Bb	7.05Cc	7.01Cc
Azidiol	5.50Aa	5.63Aa	6.08ABab	6.66BCbc	6.42Bb	7.16Cc	7.13Cc
NP	5.95B	-	-	-	-	-	-

NP - no preservative.
Means followed by the same uppercase letter do not differ in the column; means followed by the same lowercase letter do not differ across the line.

4. Discussion

ANOVA was used to verify the influence of temperature on the integrity of the samples over time. At 4 °C, regardless of the studied group (G1, G2, or G3), the samples did not show significant differences over the 14 experimental days. At 10 °C, however, there were significant differences in the integrity of the samples between days 1 and 3 (P>0.05), making it necessary to investigate in which group and with which treatment this variation may have occurred (Table 2).

A comparison of treatment averages at different storage temperatures within the studied IBC groups is presented (Table 2). For the samples supplemented with the preservative azilat, in G1, the best storage temperature was 4 °C, whereas for G2 and G3, the results showed no significant difference between 4 and 10 °C. This can be explained by G2 and G3 starting from a higher IBC average than G1, which mitigates the differences between the groups when there is temperature oscillation, but does not attribute the best results to these groups. Thus, the influence of storage temperature on the efficiency of the preservative is evident; it maintains its antiseptic properties for a longer period the lower the temperature (Martins et al., 2009; Sešķēna and Jankevica, 2007). Milk obtained from different origins have diversified microbial populations that may become inactive based on the storage temperatures; thus, due to the IBC groups having different origins, the behavior of the groups according to the studied temperatures may be influenced (Sampaio et al., 2015).

For the samples preserved with azidiol, regardless of the IBC range studied (G1, G2, and G3), the temperatures 4 and 10 °C did not show a significant difference regarding the preservation of the samples, and the preservative was effective regardless of the initial IBC level. However, when stored at 25 °C, lower results were observed than for the other treatments. Therefore, it is important to store raw milk samples at low temperatures, even with the addition of chemical preservatives, as the use of the preservative regulates the metabolic activity of the bacterium, but this efficiency still depends on

the storage temperature (Vancin et al., 2020). Regardless of the group of samples evaluated, a storage temperature of 25 °C showed less efficiency in the conservation of the sample, as expected due to the temperature that favors the development of many microorganisms.

The better results were obtained for the NP treatment (Table 2) at 25 °C. However, this cannot be taken into account, as the samples stored at 25 °C could only be analyzed on the first day of collection (day 1) due to coagulation of samples. Thus, even when there is a bacterial count according to current regulations (<50,000 cfu/mL or ~ 145,000 IBC/mL), it is not possible to guarantee the conservation and fidelity of the results, especially in samples with no added preservatives (Vancin et al., 2020). For azidiol (Table 2), the results remained stable between 4 and 10 °C, proving its efficiency. The ability of azidiol to act as a bacteriostatic agent is related to the lowest temperature at which the samples were stored and was independent of the level of initial bacterial contamination. The results demonstrate the stability of azidiol at 4 °C during 14 d of analysis (Table 2).

At 4 °C (Table 3), samples with added preservatives azilat and azidiol did not show statistical differences between them for up to nine days of analysis and were significantly better than the NP samples. This demonstrates that at 4 °C, there is still a need for preservatives. The difference registered on day 12 occurred randomly, or it can be explained by the maximum guarantee limit stipulated by the manufacturers having been exceeded. Samples intended for analysis of total bacterial counts with the addition of azidiol, provided they are kept under refrigeration at 1–4 °C, can be analyzed for up to seven days after being collected (Martins et al., 2009). At 10 °C, on day 1 of analysis, there was no significant difference between the preservatives. However, from day 3 onwards, it was possible to observe that the samples started to demonstrate oscillatory behavior, as can be seen on days 3, 5, 9, and 12. On days 7 and 14, no difference was observed between the preservatives at the same temperatures. At 10 °C, azidiol was more effective. Azilat is safe to conserve samples only on the day 1 of analysis when kept at 10 °C, indicating that the increase in temperature may be causing an oscillation.

5. Conclusions

When raw milk samples are stored at 4 °C for 14 days, the preservatives do not differ among themselves and the mean IBC/mL remain constant. With the increase in temperature to 10 °C, from the third day on, there is a reduction in the efficiency of the preservative, as there is oscillation in the means of IBC/mL in the stored samples. The temperature of 25 °C is not indicated for the storage and transport of samples, even with the addition of preservatives. Azidiol showed the best conservation efficiency of the specimens.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

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