Journal of Dairy Research

cambridge.org/dar

Research Article

Cite this article: Martins FA, Marinho MT, Fischer V, Pereira JRA, Los PR and Martins AS (2024). Characterization of variables related to high stability of raw cow milk. *Journal of Dairy Research* **91**, 89–95. https://doi.org/10.1017/S0022029924000049

Received: 11 April 2023
Accepted: 11 December 2023
First published online: 19 February 2024

Keywords:
Ethanol stability; fat/protein ratio; ionized calcium; lactose; milk quality

Corresponding author:
Fernanda Antunes Martins;
Email: fernandaantunesmartins@outlook.com

Characterization of variables related to high stability of raw cow milk

Fernanda Antunes Martins[1], Marina Tolentino Marinho[2], Vivian Fischer[3], João Ricardo Alves Pereira[1], Paulo Ricardo Los[2] and Adriana de Souza Martins[1]

[1]Department of Animal Science, Ponta Grossa State University, Ponta Grossa, Paraná, Brazil; [2]Department of Food Engineering, Ponta Grossa State University, Ponta Grossa, Paraná, Brazil and [3]Department of Animal Science, Rio Grande do Sul Federal University, Porto Alegre, Rio Grande do Sul, Brazil

Abstract

This research paper analyzes the stability of raw cow milk in the alcohol test and seeks to understand to know the factors that influence milk stability and the occurrence of unstable non-acid milk. Milk samples were collected from the cooling tanks of rural farmers in the state of Paraná twice in summer and twice in winter. The farms were classified according to the production system: pasture with supplementation and feedlot. The following variables were analyzed: stability in the alcohol test, titratable acidity, ionized calcium concentration (iCa), chemical composition of milk, somatic cell count and standard plate count. The results showed that milk stability was greater in winter *vs.* summer, when the milk contained higher iCa, and in the feedlot *vs.* pasture system. The Pearson Correlation between variables (ethanol stability, milk composition, iCa, cooling tank temperature, milk volume, number of milking, number of cows milked, fat/protein ratio, distance and travel time) were analyzed. Stability was negatively correlated with iCa concentration and positively with lactose content. Logistic regression of the risk of unstable non-acid milk at 72% alcohol ($UNAM_{72}$) showed that only iCa and lactose were determinants, while evaluation of the same risk at 78% alcohol revealed iCa, titratable acidity, lactose and milk urea nitrogen as risk factors. Under the dairy farming conditions of Paraná state, the frequency of $UNAM_{72}$ was low (12.16%) and was higher in summer and in pasture systems with supplementation. In conclusion, in dairy herds bred with high technological level, with adequate nutritional and health management, the frequency of UNAM is low and is related to nutritional management abnd, perhaps, heat stress, factors that alter iCa and lactose levels.

The thermal stability of milk is extremely important for dairy industries since milk showing high stability can be subjected to high temperatures without undergoing coagulation (Loveday *et al.*, 2021). The use of unstable milk may reduce the yields of industrial processing since this type of milk can contain lower levels of lactose, protein and, in some situations, fat (Fischer *et al.*, 2011). Another problem caused by milk instability is precipitation during UHT processing, with the milk adhering to the equipment, which increases cleaning costs and milk discard (Rosa *et al.*, 2017) and reduces the quality of the end product. In Brazil, the thermal stability of milk is measured by the alcohol/alizarol test. The test is performed using a minimum alcohol concentration of 72 °GL (Brasil, 2018) before loading the milk into the tanker on the farm and later at the reception platform of the dairy plant. Studies have shown cases of instability in the absence of changes in the milk's titratable acidity, characterizing unstable non-acid milk (UNAM: Fischer et al. 2012). In an attempt to obtain milk with higher thermal stability, many dairy plants have increased the alcohol concentration in order to enhance the rigor of the test, thus increasing the percentage of rejected or undervalued milk due to problems of instability in the alcohol test (Zanela *et al.*, 2015).

Several factors affect milk stability, including nutrition (Stumpf *et al.*, 2013), feeding (Pinheiro *et al.*, 2022), genetics (Davis *et al.*, 2001), age or number of lactations, lactation stage (Omoarukhe *et al.*, 2010), environmental factors, mammary gland health including bacterial contamination (Zanela *et al.*, 2006), methodological factors (Zanela and Ribeiro, 2018), heat stress (Abreu *et al.*, 2020) and the concentration of ionized calcium (iCa) in milk (Akkerman *et al.*, 2019). These factors have already been identified and associated with cases of low milk stability on small farms, however, few studies have evaluated the effect of these factors on milk stability in herds with different levels of technical management. Thus, the aim of this study was to analyze the milk stability to different concentrations of ethanol of cows bred on different production systems. We hyptohesized that we could identify possible associated factors through correlation analysis, so as to promote strategies (on farm or during transport) that would encourage higher milk stability.

Materials and methods

The study was conducted at the Ponta Grossa State University (UEPG) between February and August 2021, in cooperation with the company Lactalis do Brasil. According to the classification of Köppen-Geiger (1948), the region has a temperate Cfb climate characterized by average temperatures lower than 22 °C in summer, frosts during winter and autumn with average temperatures lower than 14 °C, and average annual rainfall of 1,250 to 2000 mm.

A total of 814 milk samples were collected from the cooling tank of 319 farms located in 38 municipalities of the State of Paraná, Brazil. Samples were collected two times in summer (February and March) and two times in winter (July and August), totaling 425 and 389 samples, respectively. The dairy farms were classified according to the production system: pasture-based system with supplementation and feedlot system. Commercial concentrate and corn silage were supplied in both systems. The following data were recorded: number of milked cows, number of lactation cows, milk volume per month, cooling tank temperature, distance from the farm to the dairy plant and transport time from the farm to the dairy plant. Details of the dairy farms are given in the online Supplementary File.

Laboratory analyses

The milk samples were stored in 200-ml plastic flasks and transported in thermal boxes with recyclable ice to the laboratory of the Mesoregional Center of Excellence in Milk Technology for the laboratory analyses. The samples were kept uncovered for the volatilization of carbon dioxide and under refrigeration at 4 °C in order to maintain the ideal milk cooling temperature, according to Normative Instruction (NI) #77 (BRASIL, 2018). Milk composition, somatic cell count (SCC), and standard plate count (SPC) were analyzed by the Paraná Association of Holstein Cattle Breeders. The SCC was determined by flow cytometry (Bentley Instruments®) according to International Organization for Standardization (ISO) 13366-2 (2006). The concentrations of fat, protein, lactose, total solids, and milk urea nitrogen (MUN) were determined by Fourier transform infrared spectroscopy according to ISO 9622 (2013b). The SPC was analyzed by flow cytometry (Bentley Instruments®) according to ISO 4833-1 (2013a).

Milk stability in the alcohol test was analyzed as described by Tronco (2010) using concentrations of 72, 76, 78, 80, 82 and 86 °GL. The results were expressed as the lowest alcohol concentration that caused coagulation (Zanela *et al.*, 2015), i.e. unstable non acid milk (UNAM). Titratable acidity (g lactic acid/100 ml) was determined by titration with Dornic solution (0.11 N sodium hydroxide) according to Tronco (2010). A potentiometer (Orion Star A214, Thermo Scientific Orion®) was used to evaluate iCa in the milk samples. The potentiometer has a calcium-selective ion electrode that directly measures the ion in mg/l. A detailed description of the materials and methods is provided in the online Supplementary File.

Statistical analysis

The variables were first analyzed descriptively using the UNIVARIATE and FREQ procedures of SAS® (Statistical Analysis System, version 9.3) for calculation of the mean, standard deviation, and maximum and minimum values of each variable. Pearson's correlation coefficient between variables was obtained using the PROC CORR procedure of SAS® and was classified as follows: weak (0–0.29), moderate (0.30–0.59), strong (0.60–0.89), and very strong (0.90–1.00) (Callegari-Jacques, 2008). In analysis of variance (ANOVA), season of the year (winter or summer) and production system (feedlot or pasture with supplementation) were evaluated separately using the GLM procedure of SAS® and Fisher's LS means option for determining means. A P value < 0.05 was considered significant. Additionally, logistic regression analysis was performed using the Logistic procedure of SAS® (backward option) to assess the probability of $UNAM_{72}$ occurrence (UNAM at an alcohol concentration of 72%) and $UNAM_{78}$ (UNAM at an alcohol concentration of 78%) considering the following variables: iCa, titratable acidity, protein, lactose, total solids, non-fat solids, SCC, SPC, MUN, cooling tank temperature, milk volume, number of cow milkings, distance traveled and transport time from dairy farm to milk plant processing. The variables that influenced the occurrence of UNAM were retained in the model. The following logistic regression model was used:

$$\mathrm{logit}(P) = \beta_0 + \beta_1 x_{i1} \qquad ,$$

where *(P)* is the probability of occurrence of UNAM, × is the independent variable, and β is the regression coefficient. To determine which variables affect milk stability, the dependent variable of the model, multiple linear regression analysis was performed using the REG procedure of the SAS® statistical package (stepwise selection of variables).

Results

Table 1 shows the percentage of observations regarding the physicochemical composition of milk. The results of the analyses were divided into intervals according to the requirements of the current legislation (NI #77; BRASIL, 2018). Fat, protein and total solid levels were lower than the minimum limits established by NI #77 in less than 4% of the observations. The SCC was less than 500 000 cells/ml in approximately 56% of the observations, which is compatible with the recommendations of the current legislation. For SPC, the percentage of observations that complied with the legislation was high (approximately 82%), with counts below 100 000 CFU/ml.

Most observations (89.28%) showed titratable acidity between 0.14 and 0.18 g/100 ml, indicating that the milk samples had a normal pH. There was a higher percentage of alkaline milk samples (7.28%) compared to acid milk samples (3.44%). High milk stability was observed in the alcohol test, with 87.8 and 65.4% of the samples showing ethanol stability at concentrations > 72 or ≥ 80 °GL, respectively. Regarding iCa, concentrations were < 80 mg/l in 14.55% of the observations and ≥ 100 mg/l in 56.96%. The descriptive statistics of the physicochemical parameters of milk are given in Supplementary Table S1.

The linear correlations between variables are presented in Table 2. Ethanol stability showed a weak negative correlation ($P < 0.01$) with iCa concentration in milk ($r = -0.26$) and SPC ($r = -0.11$), while a positive correlation was observed with MUN ($r = 0.11$), titratable acidity ($r = 0.23$), and lactose ($r = 0.35$). There was a moderate negative correlation ($P < 0.01$) of SCC and SPC with lactose (−0.50 and −0.18, respectively). No association was observed between stability and distance traveled or transport time, but distance was positively correlated with iCa ($r = 0.17$).

Table 1. Percentage of observations (producers × number of days evaluated) and recommended levels of fat, protein, lactose, total solids, dry defatted extract (DDE), somatic cell count (SCC), standard plate count (SPC), titratable acidity, ethanol stability, and ionized calcium (iCa) concentration in milk samples of the cooling tanks

Variable	Interval of variable (%)		
Fat (g/100 g)	< 3.0 1.87	–	≥ 3.0[a] 98.13
Protein (g/100 g)	< 2.9 2.91	–	≥ 2.9[a] 97.09
Lactose (g/100 g)	< 4.3 17.0	–	≥ 4.3[a] 82.9
Total solids (g/100 g)	< 11.4 3.84	–	≥ 11.4[a] 96.16
DDE (g/100 g)	< 8.4 20.79	–	≥ 8.4[a] 79.21
SCC (cells/ml × 10^3)	< 200 14.83	200–500[a] 40.98	> 500 43.0
SPC (CFU/ml × 10^3)	< 100 81.7	100–300[a] 11.36	> 300 6.63
Titratable acidity (g lactic acid/100 ml)	< 0.14 7.28	0.14–0.18[a] 89.28	> 0.18 3.44
Ethanol stability (°GL)	< 72 12.19	74–78 22.41	≥ 80 65.4
iCa (mg/l)	< 80 14.55	80–100 28.48	≥ 100 56.96

[a]Reference range according to Normative Instruction #77 (BRASIL, 2018).

Milk stability was greater ($P < 0.001$) in winter compared to summer (83.56 *vs.* 79.16 °GL) (Table 3). On the other hand, iCa concentrations were lower in winter compared to summer (92.78 *vs.* 126.07 mg/l). The milk samples analyzed in winter had higher values of titratable acidity, fat, protein, lactose, total solids, dry defatted extract (DDE) and fat/protein ratio compared to summer samples. Likewise, the milk volume produced on the farms was higher in winter compared to the summer season. Regarding the production system, feedlot-raised cows produced more stable milk (81.97 °GL) than cows raised on pasture with supplementation (80.75 °GL: $P < 0.05$). Lactose and DDE content, as well as the milk volume produced, were higher in the feedlot system ($P < 0.01$), while SCC and SPC were lower compared to milk of cows raised on pasture with supplementation.

Ionized Ca and lactose remained in the logistic regression model of UNAM$_{72}$. The regression equation was as follows: $\text{UNAM}_{72} = 22.782 + 0.014 \times \text{iCa} - 6.089 \times \text{lactose}$. Thus, each additional unit of iCa increases the probability of occurrence of UNAM$_{72}$ by 1.014 times and each additional unit of lactose decreases the probability of occurrence by 0.002 times ($P < 0.05$). Considering UNAM$_{78}$, iCa, titratable acidity, lactose and MUN remained in the model. The regression equation was as follows: $\text{UNAM}_{78} = 16.773 + 0.01 \times \text{iCa} - 0.155 \times \text{titratable acidity} - 3.439 \times \text{lactose} - 0.092 \times \text{MUN}$. Thus, each unit increase in iCa increases the probability of occurrence of UNAM$_{78}$ by 1.01 times and each unit increase in titratable acidity, lactose or MUN decreases the probability of occurrence of UNAM$_{78}$ by 0.856, 0.032 and 0.912 times, respectively ($P < 0.05$).

Multiple linear regression analysis provided the following predictive model of milk stability: $\text{stability} = 10.856 - 0.03 \times \text{iCa} + 0.327 \times \text{titratable acidity} + 9.475 \times \text{lactose} + 0.870 \times \text{SCC} - 0.294 \times \text{SPC} + 0.106 \times \text{MUN} + 0.003 \times \text{transport time}$, which explained 26.6% of the total variation (total $r^2 = 0.27$). Lactose (partial $r^2 = 0.13$) and iCa (partial $r^2 = 0.06$) were the variables that explained most of the variability in milk stability.

Discussion

We aimed to evaluate the stability of raw cow milk in the alcohol test and to characterize the factors that influence stability in a region of very intensive dairy farming. The main contribution of this study was to highlight the high quality and stability of the milk produced in this region, which is the largest dairy basin in Brazil. The dairy farms are characterized by a predominance of animals of European origin specialized for milk production that have a high breeding value. The animals are housed in high-tech facilities that ensure animal welfare and permit constant monitoring by technicians, resulting in high productivity and high-quality milk.

One of the most important quality parameters for dairy industries is the thermal stability of milk because of the use of a high proportion of fluid milk resulting from UHT processing. According to Shew (1981), milk is stable at an alcohol concentration of 76 °GL or higher. Approximately 76% of the samples showed stability at a concentration higher than 76 °GL, indicating that the nutrition, management, environmental, and welfare conditions of the herds were satisfactory (Table 1). Nevertheless, we observed a negative effect of iCa levels on milk stability, in agreement with the results of Akkerman *et al.* (2021) and Marques *et al.* (2011). Higher iCa concentrations have usually been associated with higher titratable acidity or lower milk pH (Marques *et al.*, 2011) and with ruminal (Werncke et al. 2016) and metabolic acidosis (Marques *et al.*, 2011; Martins *et al.*, 2015). However, milk stability was positively correlated with titratable acidity, probably as a result of protein and phosphate concentrations (although not determined) since the bacterial count was low (81.7% of the samples had SPC < 100 000 CFU/ml). This would also explain the positive correlation of acidity with lactose concentration.

The moderate positive correlation between milk stability and lactose may be due in part to the low to moderate SCC and low SPC of the milk samples, in addition to the fact that the nutritional requirements of the animals were adequately met. A positive association between lactose and milk stability has been reported by Stumpf *et al.* (2013), who demonstrated that food restriction increases the permeability of tight junctions in the mammary gland, which induces the release of lactose into the blood, thus reducing this component in milk and increasing sodium levels. This increased permeability results in ethanol instability due to salt imbalance. Martins *et al.* (2019), who evaluated the effect of subclinical mastitis on the ethanol stability of milk, found lactose to be the only milk composition variable that was positively correlated with stability ($r = 0.18$).

Transport logistics can also interfere with the quality of the milk that arrives at the dairy plant. An increase in the distance between the farm and receiving plant and in the transport time can increase the shaking of milk and bacterial activity, events that reduce milk stability (Warminska *et al.*, 2006). Since the transport truck is isothermal, i.e., it only maintains the temperature of the milk, road quality and the distance traveled are determinant factors in maintaining the milk's characteristics. This fact is recognized by some cooperatives that add a bonus to the price paid per liter to the producer. Within this context, a negative

Table 2. Linear correlation coefficients between ethanol stability (STAB), ionized calcium (iCa), titratable acidity (TA), fat (F), protein (P), lactose (L), total solids (TS), somatic cell count (SCC), standard plate count (SPC), milk urea nitrogen (MUN), cooling tank temperature (T_{CT}), fat/protein ratio (F/P), distance from farm to dairy plant (Dist) and transport time (TT) from dairy farm to milk plant processing

	STAB	iCa	TA	F	P	L	TS	SCC	SPC	MUN	T_{CT}	F/P	Dist	TT
STAB	100	−0.26**	0.23**	0.00	0.11*	0.35**	0.15**	−0.04	−0.11*	0.11*	0.05	−0.08*	0.04	0.05
iCa		100	−0.07*	−0.06	−0.09*	−0.12*	−0.11*	0.02	0.05	0.02	−0.05	−0.00	0.17**	0.08
TA			100	0.22**	0.31**	0.28**	0.31**	−0.14*	0.04	0.19**	−0.00	−0.08*	−0.16*	−0.03
F				100	0.59**	0.03	0.89**	−0.03	−0.01	0.19**	−0.10*	−0.01	−0.09*	0.01
P					100	0.11*	0.75**	−0.00	−0.01	0.20**	−0.02	−0.03	−0.09*	−0.04
L						100	0.36**	−0.50**	−0.18**	0.10*	0.04	−0.04	0.03	−0.06
TS							100	−0.20**	−0.06	0.21**	−0.04	0.49**	−0.07	−0.02
SCC								100	0.19**	0.02	0.01	−0.03	−0.02	0.04
SPC									100	−0.05	−0.02	−0.01	−0.02	0.02
MUN										100	0.00	0.09*	−0.08	0.07
T_{CT}											100	−0.11*	−0.15*	−0.09*
F/P												100	–	–
Dist													100	0.47**
TT														100

** Variables with significant correlations < 0.01.
* Variables with significant correlations < 0.05.
Red: weak correlation; blue: moderate correlation; green: strong correlation.

Table 3. Mean values of ethanol stability (STAB), ionized calcium (iCa), titratable acidity (TA), fat, protein, lactose, total solids (TS), Dry defatted extract (DDE), somatic cell count (SCC), standard plate count (SPC), milk urea nitrogen (MUN), cooling tank temperature (T_{CT}), fat/protein ratio (F/P), cooling tank temperature (T_{CT}), milk volume, number of lactation cows (NLC), and number of daily milkings (NDM) according to season (winter or summer) and production system (feedlot or pasture with supplementation)

	Season			Production system		
Variable	Winter	Summer	*P*-value	Feedlot	Pasture[a]	*P*-value
STAB (°GL)	83.56	79.16	<0.01	81.97	80.75	<0.05
iCa (mg/l)	92.78	126.07	<0.01	107.06	111.78	0.2467
TA (g lactic acid/100 ml)	0.168	0.1503	<0.01	0.1598	0.1586	0.4666
Fat (g/100 g)	3.83	3.68	<0.01	3.73	3.77	0.3142
Protein (g/100 g)	3.28	3.21	<0.01	3.25	3.23	0.3294
Fat/protein ratio	1.17	1.15	<0.05	1.15	1.17	<0.05
Lactose (g/100 g)	4.50	4.43	<0.01	4.51	4.42	<0.01
Total solids (g/100 g)	12.56	12.25	<0.01	12.44	12.37	0.2584
Dry defatted extract (g/100 g)	8.73	8.59	<0.01	8.71	8.61	<0.01
SCC (Log_{10})	12.81	12.90	0.0961	12.76	12.96	<0.05
SPC (Log_{10})	10.31	10.16	0.1487	10.08	10.39	<0.05
MUN (mg/dl)	12.88	12.13	<0.05	12.96	12.06	0.069
T_{CT} (°C)	3.36	3.34	0.642	3.38	3.31	0.156
Milk volume (l/month)	39 587	33 956	<0.05	60 119	13 424	<0.01
NLC	70	66	0.1945	112	25	<0.01
NDM	2.03	2.04	0.8006	2.11	1.96	<0.01

[a] Pasture supplemented with corn silage and commercial concentrate.

correlation of distance and transport time with milk instability, titratable acidity and SPC was expected (Table 2). However, such correlation was not observed, probably indicating that the distance traveled (maximum of 255 km), the transport time (maximum of 12 h), and the milk temperature on the farm (maximum of 4 °C) were sufficient to maintain the quality of transported milk in terms of stability and SPC. Road quality was found to be adequate, a fact that may have reduced the shaking and vibration of milk during transport.

The lower ethanol stability of milk in summer compared to winter was related to the higher iCa content observed in summer (Table 3). According to Philippe *et al.* (2003), an increase in milk iCa content causes micellar disruption and increases hydrophobicity, events that increase the aggregation of micelles and, consequently, instability. Furthermore, free iCa can induce a decrease in the electrostatic repulsion of casein submicelles, rendering them more susceptible to destabilization and coagulation when exposed to the dehydrating action of alcohol (Marques *et al.*, 2011).

The titratable acidity of milk observed in both summer and winter met the standard recommended by the current legislation, however, the higher acidity observed in winter can be explained by the milk's composition. According to Velloso (1998), natural acidity is mainly caused by caseins (5 to 6 °Dornic) and phosphates (5 °Dornic). Consequently, the higher the content of these components, as observed for protein in winter, the higher the natural acidity of the milk. A high percentage of the present samples had a SPC < 100 000 CFU/ml. Thus, the titratable acidity values were not due to the fermentation of lactose by mesophilic bacteria but rather to the composition of the milk, particularly the high levels of protein and phosphates, although the latter were not evaluated.

The lower milk stability of pasture-raised cows compared to feedlot cows may be due to the greater difficulty of the former in meeting nutritional requirements because of the variations in forage amount and composition, in addition to greater exposure of the animals to the environment. A parallel can be drawn with studies that compared the supply of corn silage and sugar cane and found greater milk stability when corn silage was provided (Andrade *et al.*, 2016; Pinheiro *et al.*, 2022). The authors attributed this finding to the higher concentration of fermentable sugars from sugarcane, which may have caused mild ruminal and metabolic acidosis, increasing iCa concentration (Pinheiro *et al.*, 2022).

The analysis of titratable acidity according to production system showed that acidity remained within the levels recommended by the legislation in both systems. This finding indicates that the pasture and feedlot systems were efficient in terms of hygiene and maintenance of milk temperature, which are key factors to ensure normal milk acidity levels. On the other hand, the lower lactose and DDE levels and the higher SCC and SPC of the herds in the pasture system compared to the feedlot system may indicate problems in balancing diets, lack of supplementation during periods of forage shortage and greater exposure of animals to mastitis-causing microorganisms (paddocks, mud during rainy periods), with a consequent increase in SCC.

The incidence of UNAM at 72 °GL (12.16%) found in the herds from the State of Paraná was lower than those reported by other authors. Marx *et al.* (2011) analyzed 69 raw milk samples collected in the western region of Paraná in summer 2010–2011 and found 33% of cases. Fagnani *et al.* (2016) identified 42.8% of UNAM cases in a study conducted in northern Paraná that analyzed 322 milk samples.

Regarding the logistic regression results, iCa was the variable associated with the highest risk of instability, while lactose was associated with the lowest risk of instability. Loveday *et al.* (2021) found iCa to be the main predictor of the heat coagulation time of milk. According to the authors, electrostatic stability is weaker at lower pH due to lower ζ potential closer to the isoelectric point. Consequently, milk becomes more susceptible to iCa-induced salt bridging and coagulation of casein micelles occurs.

The occurrence of milk instability showed a positive and weak correlation with MUN. According to Huppertz (2016), when milk is subjected to high temperatures, urea slowly decomposes into ammonia and carbon dioxide and the former can stabilize milk against heat-induced acidification. Therefore, if on the one hand the increase in MUN levels is not desirable in terms of milk characteristics (proportion of caseins and whey proteins), on the other hand, this variable can contribute to improving milk stability.

In conclusion, despite the high stability of milk in the alcohol test, we showed that factors related to nutritional and environmental management can increase iCa levels, reducing stability. The most important variables that increase the risk of $UNAM_{72}$ and $UNAM_{78}$ were higher iCa concentrations and lower lactose concentrations.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029924000049

Acknowledgements. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil (Finance Code 001). The authors thank Lactalis do Brasil for providing the data of dairy farmers.

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