ABSTRACT

We aimed to evaluate the incidence of unstable non-acid milk (UNAM) in cows fed either sugarcane or corn silage. Second, we aimed to evaluate the effect of daily variation (d 1 to 4) and alcohol grades (72, 78, and 80%) on UNAM incidence. The experiment was conducted as a split-plot crossover design, with 2 periods and 2 roughage types (sugarcane or corn silage). Thirteen multiparous Holstein cows with an average of 281 ± 29 d in milk were randomly distributed into 2 diets. Individual blood (analysis of total proteins, albumin, urea, calcium, phosphorus, magnesium, iron, chloride, glucose, and lactate) and milk samples (analysis of protein, fat, lactose and total solids, somatic cell count, and characterization of the protein profile) were collected during the last 4 d of each period. For UNAM identification, the alcohol test was conducted in milk samples at 4°C; specifically, if the sample presented the formation of clots, this would be noted as positive for UNAM. In addition, the Dornic acidity analysis was performed in the same samples to evaluate the true milk acidity. The use of sugarcane and higher degrees of alcohol were associated with increased UNAM. We observed no daily variation in UNAM. Nevertheless, we found no roughage type effect on the variables most commonly associated with UNAM, such as changes in salts in the casein micelle and, consequently, the zeta potential and the κ-casein (CN) fraction. The Pearson correlation analysis showed that the zeta potential and the concentrations of αS2-CN, blood ionic calcium, lactate, and glucose increased as the incidence of UNAM increased, showing a positive correlation among these variables. In contrast, the concentrations of lactose, phosphorus, and potassium decreased as UNAM increased, presenting a negative correlation. This study brought important discoveries to unveil why cows manifest UNAM. For instance, higher alcohol grades and cows fed with sugarcane had increased the incidence of UNAM. Additionally, animals with a higher incidence of UNAM (sugarcane-fed cows) were related to increased ionic calcium and glucose and changes in milk protein profile, with lower levels of BSA, β-CN, and α-lactalbumin and greater αS1-CN content, all of which were correlated with UNAM. Nonetheless, this trial also provides evidence for the need for further studies to better understand the physiological mechanisms that directly affect the stability of milk protein. Key words: unstable milk, heat tolerance, farm alcohol test

INTRODUCTION

Bovine milk is a colloidal system synthesized in the mammary glands of female bovines through precursors that come from nutrition and metabolism. The physicochemical properties of this system are modified through intermolecular interactions of the continuous and dispersed phases, where the dispersed phases are formed by fat globules, casein micelles, and whey proteins, and the continuous phase is composed of water, lactose, soluble salts, and vitamins (Stebnitz and Sommer, 1936). It is already known that the main factors responsible for changes in milk production and composition are breed, lactation stage, udder health, age, heat stress, nutrient balance, metabolic disorders, milking hygiene, and others (Barros, 2001; da Fonseca and dos Santos, 2001; Molina et al., 2001). However, milk composition, especially protein and fat, may be altered by diet extensively (up to 50%; Fredeen, 1996). Corn silage is the most used roughage for dairy animals in tropical countries; however, fresh chopped sugarcane is being suggested as a replacement for corn silage for medium- and low-production animals (≤ 20 L/d; Costa et al., 2005; Andrade et al., 2016). Nonetheless, sugarcane has also been linked to altering milk
physicochemical properties; more specifically, it was linked to an increased occurrence of unstable non-acid milk (UNAM; Andrade et al., 2016). Unstable non-acid milk is characterized when milk has a low stability in on-farm alcohol tests (72 to 80%), but normal levels of lactic acid (between 14 and 18°D; Zanela and Ribeiro, 2018).

There is a trend for increasing the on-farm milk quality measurements worldwide. As a result, many countries such as Japan (Yoshida, 1980), Italy (Pecorari et al., 1984), Iran (Sobhani et al., 1998), Cuba (Ponce, 1999), Uruguay (Barros et al., 1999), Argentina (Negri et al., 2001), Bolivia (Alderson, 2000), Chile (Barchiesi-Ferrari et al., 2007), and the Netherlands (Zanela and Ribeiro, 2018) adopt the alcohol/alizarin test as a standard test to measure the raw milk quality, using alcohol grades between 68 and 75°, in addition to SCC, SPC, or lactic acid concentration (Draaijer et al., 2009). Theoretically, a positive result in the alcohol test would indicate a high acidity, which results from lactose degradation to lactic acid by the microorganisms (Brito and Brito, 1998). However, studies revealed that many positive samples in alcohol tests had normal levels of acid lactic (Marques et al., 2007; Zanela et al., 2009), which was named UNAM (Zanela et al., 2006).

Unstable non-acid milk is a considerably new phenomenon, and only limited information is found in the literature, and the reasons it occurs are uncertain. In the past, this was also named “the Utrecht abnormality of milk,” but the explanation for that phenomenon was never reached (Yoshida, 1977). Although the literature is scarce on this subject, few studies claim that there is no relationship between UNAM and blood pH, bacterial count, and Dornic acidity (Fagnani et al., 2016), but it is suggested that UNAM can be related to the differences of attractive and repulsive forces between casein micelles of UNAM compared with those of regular milk (de Kruif, 1999).

The literature claims that UNAM is linked to heat stress (Abreu et al., 2020), diets composition such as level of minerals, protein, and energy (Marques et al., 2011; Oliveira et al., 2011; Gabbi et al., 2013), and feed restriction (Zanela et al., 2006; Gabbi et al., 2016). However, none of these studies could identify causality factors regarding UNAM, and the physicochemical alterations in milk to cause UNAM remains unknown.

Still, dairy consultants (personal communication: A. Trece, Trece Pecuaria & Desenvolvimento, Juiz de Fora-MG, Brazil; S. Sossai, Microvet, Vicoso-MG, Brazil; M. Castro, Cooperabaete, Abaete-MG, Brazil) have linked the incidence of UNAM with (1) feeding fresh chopped sugarcane in replacement to corn silage, even with diets balanced in nutrients to meet the requirement of the animals; (2) the ethanol degree in the milk stability test; and (3) high daily variation, although we could not find any report in the literature to explain those observations.

Furthermore, to ensure milk has better thermal stability and shelf life, the dairy industry generally instructs the carrier to use alcohols 78 and 80% for the on-farm alcohol tests (Fischer et al., 2012). However, these alcohol levels are dairy industry standards, and their limits are well above the legislation of countries that perform the alcohol test (72%; Molina et al., 2001; Oliveira et al., 2011; Brazil, 2018). The problem is that it is documented that increasing alcohol grade in the on-farm alcohol test increased the occurrence of UNAM, and the dairy industry may refuse to collect milk that has normal lactic acid levels (Silva et al., 2012).

Thus, investigating the reasons for UNAM incidence demand a more complex evaluation, and we aimed to evaluate the occurrence of UNAM under increasing alcohol grades in the test (72, 78, and 80%) and 2 roughage sources (corn silage and fresh sugarcane), as well as study its daily variation. First, we hypothesized that a diet with sugarcane causes a greater incidence of UNAM than corn silage. Second, we hypothesized that higher alcohol grades would result in a higher incidence of UNAM, with no significant daily variations.

MATERIALS AND METHODS

The experiment was conducted at the Teaching, Research, and Extension Unit in Dairy Cattle at the Federal University of Viçosa (Viçosa, Minas Gerais, Brazil; 20°450 S and 42°520 W) between October and December. The environment temperatures ranged from 8°C to 34.6°C, averaging 21.3°C throughout our study. The institutional ethics committee approved the experiment (protocol no. 28/2020).

Animals, Treatments, and Experimental Design

Before treatment assignments and the experimental period, all cows had ad libitum access (30 d) to an adaptation diet consisting of corn silage, corn-based concentrate, soybean meal, and minerals with a roughage:concentrate ratio of 60:40 (DM basis). At the end of the adaptation period, 13 multiparous Holstein cows with an average of 281 ± 29 d in lactation and an average production of 17.5 ± 1.3 L per day were randomly divided into 2 groups and housed collectively in 2 pens (42 m²) containing rubber mattresses as bedding, feeders, and free access to water. At first, we had 14 cows in the experiment, but 1 cow from the corn silage treatment (period 1) had mastitis and was removed from the study; thus, the experiment was carried out...
as an unbalanced crossover design, with 2 periods and 2 treatments (roughage type). Based on the authors' experience, late-lactation cows tend to have a higher incidence of UNAM than early-lactation cows. Therefore, late-lactation cows were used in this experiment. Nevertheless, late-lactation cows may not respond similarly to earlier-lactation cows due to differences in the permeability of the tight junctions of mammary epithelium cells, which may limit some physiological responses in the cows. Therefore, future studies should focus on understanding the UNAM incidence in early-lactation cows.

Animals were fed ad libitum twice daily (0600 and 1600 h), right after milking, allowing orts for up 5% of as-fed TMR. The treatments were defined by the type of roughage: sugarcane and corn silage. Experimental diets (Table 1) had a roughage:concentrate ratio of 50:50 (DM basis) and were formulated to meet CP, ME intake, and macro and trace minerals requirements of cows with an average BW 650 ± 33 kg and 17.5 L/d of milk production, according to NRC (2001). The experiment lasted 60 d, divided into 2 periods of 30 d each. In each period, the first 26 d were for the adaptation of the animals to experimental diets, and the last 4 d of each period were used to collect milk and blood samples.

**Milk Collection and Analysis**

We aimed to evaluate differences in UNAM and acidity between the days (1 to 4 d) that the milk samples were refrigerated (4°C). The individual milk collection (100 mL) was performed during morning milking using collecting cups, closed immediately, and refrigerated in a freezer (4°C). The milk sample was used to analyze UNAM and milk acidity (Dornic test) at a temperature of 4°C from d 1 (24 h after the milk collection) to 4.

**Alcohol Test, and Dornic and Milk Composition Analyses**

The evaluation of UNAM was performed using the alcohol test containing alizarol solution in its composition, which was carried out with different grades (72, 78, and 80%; Figure 1). Alizarol is a brown-colored solution, and its presence in the alcohol test can cause color changes according to the pH of the milk, improving visual perception for identifying acidic milk. In cases of extreme pH, this change is noticeable, in which the milk becomes purple. However, when the pH range is close to the normal range of milk (6.6 to 6.8), there is no change in color. The alizarol solution consists of an alcoholic solution with the addition of alizarin, a pH indicator (Zanela and Ribeiro, 2018). The alcohol test was performed with the addition of 2 mL of milk and 2 mL of the alizarol solution, with constant agitation for 60 s. The sample was reported as UNAM when clots of any size were observed, and titratable acidity observed was below or equal to 18°D. Then, the number of positive and negative samples (yes/no) for UNAM were recorded and compared with the total of samples analyzed at the end of the alcohol test to calculate their proportions (%). The acidity was evaluated according to the Dornic method (Tronco, 2003). Four drops of phenolphthalein were added to an Erlenmeyer flask containing 10 mL of milk. The samples were titrated with the standard Dornic solution (9:1), consisting of a sodium hydroxide solution of 0.1 N, where each 0.1 mL corresponds to 1°D, or 0.1 g of lactic acid/L (Almeida, 2007).

The milk composition analyses were performed at the end of each period. Individual samples were collected in bottles containing bronopol and stored under refrigeration until transport to the Clínica do Leite laboratory (Piracicaba, Brazil). Milk was analyzed for protein, fat, lactose, and TS contents using a MilkoScan FT 120

| Item          | Unit    | Sugarcane | Corn silage |
|---------------|---------|-----------|-------------|
| Sugarcane     | % DM    | 50.00     | —           |
| Corn silage   | 50.00   |           |             |
| Ground corn   | 31.52   | 24.75     |             |
| Soybean meal  | 14.12   | 23.19     |             |
| Limestone     | 0.69    | 1.14      |             |
| Dicalcium phosphate | 0.91 | 0.31      |             |
| Salt          | 0.62    | 0.61      |             |
| Urea          | 1.68    | —         |             |
| Ammonium sulfate | 0.45    | —         | 0.31        |
| Cobalt sulfate | mg/kg of DM | —   | 0.31        |
| Copper sulfate | 15.87 | 7.95      |             |
| Potassium iodide | 1.19 | 1.17      |             |
| Sodium selenite | 0.78 | 0.76      |             |
| Vitamin blend | 0.38    | 0.40      |             |
| Diet          | % as fed | 43.90     | 50.18       |
| DM            | % DM    | 16.96     | 17.03       |
| CP            | 12.56   | 11.83     |             |
| RDP           | 4.39    | 5.21      |             |
| NDF           | 33.49   | 27.60     |             |
| Starch        | 25.41   | 30.76     |             |
| Ether extract | 2.35    | 3.71      |             |
| Calcium       | 0.64    | 0.66      |             |
| Phosphorus    | 0.37    | 0.38      |             |
| Magnesium     | 0.19    | 0.27      |             |
| Sodium        | 0.28    | 0.29      |             |
| Potassium     | 0.84    | 1.00      |             |
| Sulfur        | 0.21    | 0.15      |             |
| NE2           | 1.62    | 1.67      |             |
| Cobalt        | 0.22    | 0.15      |             |
| Copper        | 10.17   | 10.53     |             |
| Iodine        | 0.71    | 0.73      |             |
| Manganese     | 35.52   | 24.82     |             |
| Selenite      | 0.39    | 0.41      |             |
| Zinc          | 35.65   | 25.93     |             |
| Iron          | 15.12   | 104.46    |             |
The milk yield values were corrected for 3.5% fat, according to (Sklan et al., 1992). The SCC was performed using Fossomatic FC (Foss Electric).

**Electrophoretic Profile**

Milk (30 mL) samples were lyophilized for protein electrophoretic profiles. First, the samples were thawed at 8°C and skimmed by centrifugation at 2,100 × g for 30 min at 32°C. After lyophilization, the electrophoretic profile analysis was carried out according to (Egito et al., 2006). The equipment for electrophoresis analysis consisted of glass plates, where the gels were conditioned, and a vertical acrylic bowl Bio-Rad Mini Trans-Blot Cell model. The glass plates containing the gels were submerged, in a running buffer solution containing 25 mmol/L of Tris-HCl, 192 mmol/L of EDTA, and 0.1% SDS inside the vertical acrylic bowl, during the entire process of loading the proteins onto the gel. The SDS-PAGE had gels with a concentration of 4.9% in 125 mmol/L of Tris-HCl buffer solution, pH 6.8, and separation gels with 15.4% of polyacrylamide in 380 mmol/L of Tris-HCl buffer solution, pH 8.8 with 0.1% SDS. The lyophilized milk samples (2 mg of dry milk) were dissolved in 1 mL of Tris-HCl buffer, pH 6.8, with 0.1% of SDS, 5% β-mercaptoethanol, and placed in Eppendorf tubes. Soon after, the solutions were heated at 100°C for 3 min in a water bath, and 10 μL were placed on the gels (Egito et al., 2006). Next, the protein was fixed on a gel with 12% trichloroacetic acid for 30 min, stained with 0.1% Coomassie blue R250, and dissolved in a mixture of 50% ethanol and 2% trichloroacetic acid for 120 min. The discoloration was performed overnight with a solution of 30% ethanol and 7.5% acetic acid (Egito et al., 2006).

The gels were scanned, processed, and analyzed by ImageJ Software (version 6.0 program). We quantified the optical density through the molecular weight of each protein band per line in the gel, and the pixel amount of each fraction of them generated by the software was used. Then, the values in pixels of each protein were relativized with the total amount of protein (%) for better visualization of the results, according to Barros (2001). Protein fractions of milk samples identified and quantified were lactoferrin (80 kDa); BSA (66.2 kDa); casein fractions: αS1-CN (25 kDa), αS2-CN (24 kDa), β-CN (23 kDa), κ-CN (19 kDa); and soluble proteins: β-LG (18.3 kDa) and α-LA (14.2 kDa).

**Casein Micelle Purification**

Milk samples were skimmed by centrifugation at 1,500 × g for 30 min at 4°C, and then samples were stored...
with 0.05\% (wt/vol) sodium azide to avoid microbial growth. Purification of the casein micelle occurred by centrifugation at 25,000 \( \times g \) for 30 min at 20°C. The supernatant was discarded, and the pellet remained. Following the procedure, we added a Tris buffer solution (10 mM, pH 7.4) containing 10 mM CaCl\(_2\) to the pellet and subjected it to centrifugation. This process was repeated 5 times to eliminate whey proteins (Sahu et al., 2008).

**Mineral Analysis in the Casein Micelle**

The mineral solution was performed according to techniques described by Detmann et al. (2012), where the casein micelle solution was analyzed for macro minerals (Ca, P, Mg, K, and Na). We added strontium to purify the sample from any residue in the casein solution. Calcium and Mg readings were performed through atomic absorption spectrometry (GBC Avanta Sigma, GBC Scientific Equipment; method 968.08; AOAC International, 2000), whereas K concentrations were determined using flame emission spectrometry (Corning 400; method 985.35; AOAC International, 2000). The P contents were performed by reducing the P-molybdate complex with ascorbic acid, and the readings were performed in a calorimeter spectrophotometer (method 965.17; AOAC International, 2000).

**Zeta Potential and Micelle Size**

These measurements were obtained with the Zetasizer Nano ZS90 (Malvern Instruments and PANalytical). We used a laser wavelength of 663 nm with a 90° dispersion angle. The bucket used was of the “disposable folded capillary cell” type and refractive index of 1.330. All data were obtained using the average of 3 readings per sample and parameter. The result of a reading of the equipment is equivalent on average to 30 readings per parameter.

**Blood Collection and Analysis**

Two blood samples were collected through the middle coccygeal vein of the cows using vacuum tubes (BD Vacutainer SST II Advance) immediately after milking in the first 2 d of the collection period. One of the samples was collected in tubes with a clot activator and gel for serum separation to determine total proteins, albumin, urea, calcium, phosphorus, magnesium, iron, ionic calcium, and chloride concentration. The sample collected in the second tube, with EDTA and sodium fluoride (BD Vacutainer Fluoreto/EDTA), was used to measure glucose and lactate in the plasma. Then, samples were centrifuged at 1,000 \( \times g \) for 10 min at 25°C and stored in Eppendorf tubes at −20°C until further analysis. These constituents were assayed in duplicate using commercial colorimetric kits of the BioClin brand (BioClin Quibasa), according to the manufacturer’s recommendations. All the analyzes mentioned above, except chloride, were performed by an automatic biochemical analyzer (Mindray BS-200E). The plasma chloride level was measured using a commercially available kit (BioClin Quibasa).

**Statistical Analysis**

The first step of our statistical analysis was to identify variations in UNAM and milk acidity across days of evaluation, alcohol degree (only for UNAM responses), and treatment (forage used). We used a split-split-plot crossover design, considering day as a repeated measure, and including day, alcohol grade (only for UNAM), treatment, and all 2-way and 3-way interactions. Group, lactation number, and period were added to the model as random effects. The animal nested within period was identified as the subject in the model to account for variations in measurements taken in the same animal within each period.

As day and its interactions were not significant for UNAM analysis \((P > 0.689, \text{Figure 2})\), milk and blood samples from 2 d were pooled (analyses were performed separately and averaged) for all other response variables described above. We used a crossover design with treatment as fixed effect and group, lactation number, and period as random effects.

For all significant responses, Student’s \( t \)-test was used to identify differences across least squared means. All analyses were performed using PROC GLIMMIX of SAS University Edition (SAS Institute Inc.), considering statistical differences when \( P < 0.05 \) and trends when \( 0.05 < P < 0.10 \). The Kenward Roger procedure was used to correct the degrees of freedom for all our analyses. To attempt to identify unseen factors affecting UNAM in late-lactating cows, we ran a Pearson correlation analysis, including our response variables and the alcohol grade. For this specific analysis, the forage type was not considered in the model.

Last, a regression analysis using the logistic function of PROC GLIMMIX was performed to evaluate the risk of increasing UNAM relating to the type of forage and alcohol concentration.

The regression model included UNAM as response variable (binary), and alcohol levels \([(A_i) \ “i,”]\), forage type \([(F_j) \ “j = 1 \ “sugarcane; j = 2 \ “corn silage”]\), and their interaction as independent variables. Forage type was included as a classificatory effect and alcohol levels as a quantitative effect in the model. Day, period, and animal were included as random effects. The nonsig-
significant variables were removed from the model ($P > 0.05$).

**RESULTS**

The production data and composition of the milk samples are shown in Table 2. Roughage did not influence milk yield, with an average production of 17.5 L/d for animals ($P = 0.782$). The group fed with sugarcane had increased percentages of fat (+$11.34\%$, $P = 0.039$) and protein (+$2.56\%$, $P = 0.027$) when compared with cows fed with corn silage. In contrast, cows fed corn silage had a greater ($P = 0.001$) MUN (17.70 mg/dL) when compared with cows fed sugarcane (13.95 mg/dL).

Unstable non-acid milk was affected by roughage type and alcohol grade ($P < 0.046$), but unaffected by days ($P = 0.689$) (Figure 2). Furthermore, the greater the alcohol level, the greater the incidence of UNAM (Figure 2), and samples from cows fed with sugarcane showed greater UNAM than cows fed with corn silage. The group fed with sugarcane had an increase in the frequency of UNAM of 16.58\% with 72\% alcohol, an increase of 31.8\% with 78\% alcohol, and an increase of 13.16\% with 80\% alcohol, compared with corn silage.

The UNAM is characterized when the milk samples have normal lactic acid levels (degrees Dornic, Figure 3) but clots when the alcohol test is performed. In our study, all samples had normal Dornic levels (°D < 16.0, Figure 3), which indicates that all samples that clotted

![Table 2. Production and composition of milk samples from cows fed corn silage or sugarcane](image)

**Figure 2.** Frequency of unstable non-acid milk of cows fed with corn silage or sugarcane, conditioned to 3 alcohol degree tests (72, 78, or 80\%), and 4 d of collection. Error bars indicate SEM; different uppercase letters indicate a significant difference among treatments within groups (roughage or alcohol).
were indeed UNAM (Figure 2). As expected, the roughage type did not affect the titratable acidity of the milk ($P = 0.108$).

The zeta potential, the size of the casein micelles, and the mineral composition are reported in Table 3. The samples from the sugarcane group showed an average casein micelle size of 281.68 nm ($P = 0.906$), with a zeta potential of $-9.38$ mV ($P = 0.754$), which were no different from the corn silage group. The corn silage group had an average size of 277.16 nm ($P = 0.906$) and a zeta potential of $-9.61$ mV ($P = 0.754$).

The protein profile of milk samples (Table 4) from the sugarcane group showed 21.93% of $\alpha S_1$-CN ($P = 0.001$), which is 16.52% greater than that observed for the corn silage group ($P = 0.001$). Animals fed sugarcane had a BSA 17.21% lower than those fed with corn silage ($P = 0.003$). All other milk proteins did not differ between roughage types ($P > 0.103$). The analysis of blood plasma and serum (Table 5) showed a greater concentration of ionic Ca (2.54% increase, $P = 0.012$) and glucose (3.95% increase, $P = 0.005$) in sugarcane-fed cows. In contrast, magnesium and urea were reduced by 8.13% ($P = 0.012$) and 14.26% ($P = 0.004$), respectively, in sugarcane-fed cows. All other variables were unaffected by roughage type ($P > 0.421$).

To attempt to understand what may have caused this variation in UNAM, we performed a correlation analysis between UNAM and all response variables (Figure 4). The zeta potential was positively correlated, whereas chloride was negatively correlated with UNAM in alcohol at 72% ($P < 0.05$). For UNAM samples treated with 78% alcohol, we had a positive correlation with zeta potential and a negative correlation with lactose and $\alpha$-albumin ($P < 0.05$). Last, phosphorus,

![Figure 3. Acidity in Dornic degrees of milk of cows fed with corn silage or sugarcane, conditioned to 3 alcohol degree tests (72, 78, or 80%), and 4 d of collection. Error bars indicate SEM; different uppercase letters indicate a significant difference among treatments within groups (roughage or alcohol).](image)

Table 3. Zeta potential, size of the casein micelle, and concentration of the main minerals in the casein micelles of the milk of cows fed with corn silage or sugarcane

| Item              | Roughage | SEM    | $P$-value |
|-------------------|----------|--------|-----------|
| Size, nm          | Sugarcane| Corn silage | SEM        | $P$-value |
| Zeta potential, mV| $-9.38$  | $-9.61$| 0.495     | 0.358     |
| Calcium, mg/mL    | 0.064    | 0.062  | 0.007     | 0.579     |
| Phosphorus, mg/mL | 0.025    | 0.025  | 0.054     | 0.978     |
| Potassium, mg/mL  | 0.032    | 0.032  | 0.011     | 0.956     |
| Magnesium, mg/mL  | 0.0027   | 0.0027 | 0.507     | 0.688     |
potassium, and magnesium were negatively correlated, whereas glucose was positively correlated with UNAM in alcohol at 80% concentration.

The logistic regression analysis showed that the incidence of UNAM was not affected by the interaction, whereas the effect of alcohol levels and forage type did affect UNAM incidence ($P < 0.001$). Therefore, for every 1 unit increase in the alcohol level, the probability of UNAM increases by 0.3839 on average. Furthermore, sugarcane was 91.95% more likely to cause UNAM than corn silage.

The regression final regression model (fixed components) was UNAM = $-30.2610 \pm 3.8627 + 0.3839 \pm 0.0493 \times$ alcohol + $0.9195 \pm 2.729 \times$ sugarcane + $0 \times$ corn silage. Thus, the predicted average UNAM incidence per forage type and alcohol level were 14.5% UNAM at 70% ethanol, 62.8% UNAM at 72% ethanol, and 78.4% UNAM at 80% ethanol.

**DISCUSSION**

Studies about UNAM have been increasing in number in the last decade; however, advances in understanding the metabolic processes of animals that present this condition are still limited (Negri et al., 2004; Singh, 2004; Philippe et al., 2005a; Gaucheron, 2005). In addition, many factors act on the animal and can lead to a condition of UNAM, such as genetic factors (Botaro et al., 2009a), food restriction (Stumpf et al., 2013), degree of casein mineralization (Lin et al., 2016), and others. In our case, we seek to understand the effects of changing the roughage on the physicochemical aspects of milk, and the blood metabolic profile of animals with UNAM.

Animals fed with sugarcane were 91.95% more likely to incur UNAM in cows compared with the corn silage diet. Furthermore, the UNAM tends to appear as the concentration of alcohol increases, mainly in alcohol at 80% (Figure 2). For every 1 unit that increases in the level of alcohol, the probability of UNAM increases by 38.39% on average. Although recent studies diverge on the use of the alcohol test to verify the thermal stability of milk, other researchers (Molina et al., 2001; Chaves et al., 2004; Fagnani et al., 2016) have concluded that using a 75° ethanol solution is the most suitable to estimate the thermostability of milk. In this study, as expected, an increase in the incidence of UNAM was observed when the alcohol grades increased. This is due to greater dehydrating action on milk casein as the alcohol concentration increases, leading to a greater occurrence of UNAM (Zanela and Ribeiro, 2018).

**Table 4.** Composition of the protein fraction of milk from cows fed corn silage or sugarcane

| Item, %         | Sugarcane | Corn silage | SEM   | $P$-value |
|----------------|-----------|-------------|-------|-----------|
| Lactoferrin    | 1.43      | 1.49        | 0.137 | 0.264     |
| BSA            | 1.01      | 1.22        | 0.094 | 0.001     |
| $\alpha_S1-CN$ | 21.93     | 18.82       | 1.134 | 0.001     |
| $\alpha_S2-CN$ | 14.48     | 14.64       | 0.466 | 0.001     |
| $\beta-CN$     | 19.02     | 20.10       | 0.944 | 0.041     |
| $\kappa-CN$    | 16.36     | 16.10       | 1.203 | 0.041     |
| $\beta-LG$     | 11.24     | 11.71       | 0.603 | 0.363     |
| $\alpha-LA$    | 14.68     | 16.04       | 0.895 | 0.005     |

**Table 5.** Blood metabolites of cows fed with corn silage or sugarcane

| Item                      | Sugarcane | Corn silage | SEM   | $P$-value |
|---------------------------|-----------|-------------|-------|-----------|
| Ionic calcium, mg/dL      | 4.85      | 4.73        | 0.062 | 0.012     |
| Lactate, mg/dL            | 5.93      | 6.50        | 1.541 | 0.675     |
| Glucose, mg/dL            | 59.18     | 56.96       | 1.537 | 0.005     |
| Chloride, mEq/L           | 108.54    | 108.46      | 3.159 | 0.953     |
| Calcium, mg/dL            | 8.56      | 8.55        | 0.086 | 0.925     |
| Phosphorus, mg/dL         | 5.61      | 5.65        | 0.229 | 0.793     |
| Magnesium, mg/dL          | 1.92      | 2.09        | 0.055 | 0.012     |
| Iron, $\mu$g/dL           | 142.07    | 137.32      | 8.941 | 0.595     |
| Albumin, g/dL             | 2.97      | 2.92        | 0.094 | 0.421     |
| Total protein, g/dL       | 7.18      | 7.20        | 0.186 | 0.878     |
| Urea, mg/dL               | 33.91     | 39.55       | 2.199 | 0.004     |
Other studies using sugarcane as a source of roughage also found a higher incidence of UNAM (Ponce and Hernández, 2001; Andrade et al., 2016). According to Andrade et al. (2016), the change in roughage with less digestibility causes a reduction in the casein content and increases the concentration of ions in the milk, especially calcium. Although calcium levels were balanced in the animals’ diet and their concentrations were similar in the milk in both groups, we believe that the increased blood ionic calcium in sugarcane-fed animals may have been reflected in higher concentrations of ionic calcium in the milk, as reported in the study by Marques et al. (2011), in which cows with metabolic acidosis had increased blood and milk ionic calcium. Thus, we believe that the higher concentrations of fermentable sugars from the digestion of sugarcane lowered the ruminal pH of the animals and perhaps the blood, increasing the dissociation of ionic calcium from the blood and, consequently, from the milk (Martins et al., 2015). This possible change in the ionic status may have compromised the permeability of the mammary gland epithelium (Neville and Peaker, 1981), causing an imbalance of milk proteins and, even momentarily, increasing the repulsion between them and their instability. However, even if there is a significant difference in ionic calcium between the groups of animals fed with sugarcane or corn silage, this value is small and may not be biologically determinant for the higher incidence of UNAM.

Forage quality may increase the incidence of UNAM in cows as well. Marques et al. (2007) evaluated 9,892 samples for the occurrence of UNAM in the southern region of the state of Rio Grande do Sul in Brazil and found a maximum incidence of UNAM in April 2002, with 77.88%, and the lowest in September 2003, with 31.01%. The authors associated the higher incidence of UNAM with the reduction in the development of native summer forages and the initial stage of winter forages, thus relating the quality of the roughage offered and the stability of alcohol-proof milk.

In our study, milk samples were influenced by day, showing greater acidity on d 1 and 2 (Figure 3). According to Andrade (2013), one of the factors that most affects milk acidity over the days is inefficient refrigeration. This quantitative analysis measures the concentration of lactic acid, which, in turn, estimates the microbiological quality of milk (Brito et al., 1998). However, the SCC data did not differ between treatments and, therefore, the hypothesis that the milk was poorly refrigerated must be ruled out. Furthermore, the greater concentration of calcium ions and an imbalance of protein, lactose, minerals, density, and casein:protein ratio of milk can lead to increased acidity. Nevertheless, these variables were not measured over the days; thus, future studies should address that issue. Last, as these results are not linked with our hypotheses, they will not be further discussed.

The animals fed with corn silage had a lower incidence of UNAM. Our first hypothesis is that this result may be related to urea metabolism (Sutoh et al., 1996) because a greater concentration of urea nitrogen was observed in these samples, which presented 3.5% more MUN than that of sugarcane-fed cows. The urea metabolism is modified by sucrose intake, and this modification occurs by decreasing the concentration of rumen ammonia in high sugar diets (Sutoh et al., 1996), such as sugarcane-based diets. This implies a lower flow of plasma urea in sugarcane diets, reflected in lower MUN

**Figure 4.** Correlation between unstable non-acid milk and several response variables. Correlations followed by * indicate significance at \( P < 0.05 \).
of glucose (3.7% higher, \( P < 0.005 \)) when compared to the normal control (Sutoh et al., 1996). Singh and Creamer (1992) and Negri (2002) observed a negative correlation between UNAM and MUN, as we did in our study. According to these authors, non-protein nitrogen precludes milk acidification and its conversion into cyanide that reacts with milk protein. Then, the negative charge of the micelles and their electrostatic repulsion is increased, improving the thermal stability (Sweetser and Muir, 1981). However, our study showed that the type of roughage did not influence the zeta potential of casein micelles (Table 3). In any case, further studies are warranted to confirm this theory as our results could not do it. It is important to emphasize that the UNAM occurrence is a multifactorial process (Fagnani et al., 2016), making it challenging to point to only one responsible factor.

Sugarcane diets also had greater blood concentration of glucose (3.7% higher, \( P < 0.005 \)) when compared with corn silage diets, which is also likely linked to a greater sugar intake by sugarcane-fed cows. Diets rich in sugar increase the raw energy in the rumen and availability of propionate, thus increasing glucose flow to the bloodstream (Sutoh et al., 1996). A greater glucose was correlated with a higher incidence of UNAM (for all animals); however, we did not find a clear physiological link between UNAM and higher blood glucose.

A negative correlation was observed between lactose and UNAM (all cows included in this analysis). According to Fagnani et al. (2016), the lower lactose can lead to a more significant displacement of minerals from the blood to the milk. This causes changes in the minerals’ distribution between the continuous and colloidal phases, reflecting an increased ionic strength of the colloidal phase (i.e., solvation layer of proteins), a reduction in pH, and an increased concentration of blood ionic calcium (as observed in this study), generating precipitation of the protein due to their destabilization. Although some of this discussion is only speculation, we also found a strong positive correlation between blood ionic calcium and UNAM (Table 5), which might help to explain the higher UNAM in sugarcane-fed cows.

It is known that dairy cows require a higher mineral intake to meet the requirement for maintenance, fetal growth, and production. From the blood profile analysis, we expected to identify metabolic changes in the animals presenting UNAM that justified the casein instability. In the present study, animals fed with sugarcane showed a 2.4% increase in serum ionic calcium compared with those fed with corn silage, without showing an increase in the total calcium content. Mellau et al. (2004) studied the responses of anion supplements and rapidly fermentable carbohydrates in relation to calcium homeostasis. Cows previously fed with a rapidly fermentable carbohydrate diet recovered more quickly from the induced hypocalcemia, similar to animals submitted to an anionic diet. As mentioned before, this increase in ionic calcium levels is likely related to the sugar content in sugarcane-based diets because they stimulate calcium metabolism in a similar way to that of anionic salts (Mellau et al., 2004). According to Martins et al. (2015), a reduction in the cation-anionic balance of the diet causes an increase in the contents of casein and ionic calcium in milk. Additionally, the increase in the concentration of cations in milk increases the casein content in the casein micelles (Table 4), with a consequent reduction in their degree of hydration (Philippe et al., 2005b). These changes usually generate disturbances in the zeta potential and hydrophobicity, whereas the size of the micelles remains without significant changes. In the present study, we found a positive correlation between the concentration of blood serum ionic calcium and the incidence of UNAM. Still, based on our responses, we believe that the increase in blood serum ionic calcium may have caused an increase in the milk ionic calcium, causing a greater instability in milk protein (Barros et al., 1999; Oliveira and Timm, 2007; Marques et al., 2011).

The normal serum magnesium levels range from 2.0 to 3.0 mg/dL (González and Scheffer, 2002), and these concentrations are directly linked to the magnesium content present in the diet (González and Scheffer, 2002). The animals submitted to the sugarcane diet had a serum magnesium level of 1.92 mg/dL, which is lower than the reference literature. According to Goff (2018), levels below 2 mg/dL should indicate subclinical hypomagnesemia. As the diet offered to animals ensured adequate magnesium supplementation, the problem may be in its absorption rate, which is, in turn, affected by the magnesium solubility. One of the factors that interfere with magnesium solubility is the ruminal pH. When rumen pH is above 6.5, magnesium has its solubility reduced. As the sugarcane diet had 33.5% NDF and the corn silage diet had 27.6%, sugarcane-fed cows likely had higher rumen pH (although we could not measure it), reducing Mg solubility. Another relevant factor is that both diets were formulated to have similar dietary levels of Ca (0.66% DM) and Mg (0.28% DM); however, the sugarcane used in this study had a lower percentage of Mg than predicted (table value), and the diets were 30% short in Mg. Rumen fluid Ca\(^{2+}\) may compete with Mg\(^{2+}\) for entry through the electrical potential difference-dependent cation channel of the rumen epithelium apical membrane (Leonhard-Marek et al., 2005), reducing the Mg absorption through the rumen wall when too much Ca is fed. As the Ca/Mg relationship increased from 2.44 in corn silage diets to 3.37 in sugarcane diets, this increased competition is likely affecting Mg absorption negatively, leading to
lower Mg blood levels. Unfortunately, we do not have rumen pH and Mg solubility and absorption data, so further experiments should confirm this speculation.

Our results indicated that sugarcane diets could somehow induce milk protein instability. Thus, we expected that sugarcane diets could also induce changes in the casein micelle, such as an increase in diameter (Dalgleish and Corredig, 2012; de Kruijf et al., 2012) and colloidal calcium levels (Chavez et al., 2004), and reduced zeta potential (Singh, 2004; Lin et al., 2006). However, we found no significant differences in any of these variables. Furthermore, although we observed significant correlations between UNAM and the zeta potential, phosphorus, and potassium, roughage type did not influence those variables (Table 3), suggesting that unknown variables still play an essential role in UNAM incidence.

The presence of ethanol in liquids such as milk may change many properties of the system, especially those related to the κ-CN solubility, to maintain the stability of casein micelle in milk. Several factors, such as a high concentration of lactic acid, reduced zeta potential, high SPC, concentrations of κ-CN, contribute to the instability of the casein micelles. Thus, if milk has any of these factors, the presence of ethanol would be sufficient to promote the precipitation of the casein micelles (Horne, 1992). It is frequently suggested that the precipitation of UNAM occurs due to changes in the surface of the casein micelle, such as, for instance, the imbalance of ions at the micellar interface (Oliveira and Timm, 2007). However, in the present study, no significant differences were observed in the zeta potential (Table 3) of milk samples from cows fed with sugarcane or corn silage, which suggests that the effect of instability may have additional causes. This result should be carefully analyzed because the zeta potential measurement differentiates the electric potential in the electrical double layer of colloidal particles. However, it cannot yield information on the composition of ions in the electrical double layer and the bulk.

The addition of ethanol in liquids, such as milk, promotes changes in the 3-dimensional structure of the water molecules around the solutes (Javadian et al., 2008). Furthermore, it is well known that ions also affect the 3-dimensional water structure (Hribar et al., 2002). Therefore, many different and “invisible” factors can contribute to the alcohol instability of milk. For example, we observed (1) that when comparing the milk composition of our 2 diets (Table 2), milk from cows fed with corn silage showed a higher MUN, but was still within the normal range for lactating cows (Botaro et al., 2009b). Hence, we speculate that the effect of ethanol on the interface of the casein micelles was lower in corn silage-fed cows; that is, less incidence of UNAM due to the ethanol partition for the interface of the casein micelles and the solvation layer of the urea molecules present in the medium (House and House, 2017). In contrast, in milk samples from cows fed with sugarcane, the lower concentration of urea in the solution causes ethanol to partition toward the interface of the casein micelles, exerting its effect on precipitation over the κ-CN. Nevertheless, we could not find any study in the literature to corroborate this hypothesis, and future studies on this topic are warranted.

In our study, we also observed (2) no difference in the proportions of κ-CN between treatments, which agrees with Botaro et al. (2007) and Lopes (2008). However, the proportions of the electrophoretic profile of milk proteins were affected by roughage type, and sugarcane-fed cows showed the highest instability in the alcohol test. We observed an increase of 16.52% in the α_{s1}-CN content, and reductions of 17.21, 5.37, and 8.48% of BSA, β-CN, and α-LA (Table 4), respectively. The increase in α-LA corn silage-based diets, whose group had the higher protein stability in the face of alcohol testing. The α-LA has a high affinity for Ca^{2+} ions, and this Ca^{2+} sequestration of the solution promotes better thermal stability to the protein (Stuart et al., 1986). Nevertheless, we did not measure ionic Ca in milk, and future studies should confirm this speculation. Last, we observed (3) a lower β-CN in sugarcane-fed cows, which may be associated with the action of the enzyme plasmin present in milk. When β-CN is cleaved by plasmin, γ-CN is generated, blocking potassium channels (Silanikove et al., 2009). Because potassium and sodium are monovalent ions that act on ionic strength, any change in their concentrations can generate protein instability.

Based on the discussion above, our results lead us to believe that the alcohol instability is a consequence of changes in the water structuration promoted by the difference in ion distribution between casein micelle electrical double layer and system bulk. Therefore, any change in ion content may favor the UNAM occurrence.

**Conclusions**

This study demonstrated that sugarcane-fed cows and the higher degree of ethanol alcohol led to a higher incidence of UNAM with no significant daily variations. The higher degree of ethanol alcohol led to greater dehydrating action on the milk casein micelle, increasing its instability. Furthermore, sugarcane-fed cows were associated with higher levels of ionic calcium and plasma glucose, and reduced levels of magnesium and urea. In addition, sugarcane-fed animals also had an altered milk proteins profile, all of which were correlated with the incidence of UNAM. Nevertheless, we found no roughage type effect on some of the variables.
most associated with UNAM, such as changes in salts in the casein micelle, zeta potential, κ-CN, β-LG, and lactose, showing us clearly that other factors such as the 3-dimensional water structure plays an essential role on the milk stability to alcohol.

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