UNIVERSIDADE FEDERAL DE VIÇOSA

LETHIANE GARCIA ROCHA

UNVEILING UNSTABLE NON-ACID INCIDENCE IN HOLSTEIN COWS FED WITH CORN SILAGE OR SUGARCANE

VIÇOSA - MINAS GERAIS
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Dissertation submitted to the Universidade Federal de Viçosa as partial fulfillment of the requirements of the Animal Science Graduate Program for the degree of *Magister Scientiae*.

Adviser: Marcos Inácio Marcondes

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Assent:

Lethiane Garcia Rocha
Author

Marcos Inácio Marcondes
Adviser

DEDICATION

I dedicate this work to my advisor Marcos, who from the beginning believed that I was able to do it.

To my parents Cleunice and José, who without them, none of this would be possible, and if necessary, I would do everything the same again.

To my brothers Thiago and Larissa, your support was decisive for this work to materialize.

To the memory of João Victor, my little brother.

To my life partner, Rafael, who was by my side at all times, good and bad, and who brought me calm when nothing made sense.

For the oncologist who has been treating my mother since 2019, Sabrina. She is the angel of our family, thanks to her my mother can be here right now.

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BIOGRAPHY

LETHIANE GARCIA ROCHA, daughter of Cleunice Leia Garcia Rocha and José Carlos Rocha, was born on October 12, 1990, in the city of Rio Grande, Rio Grande do Sul, Brazil.

She started her undergraduate degree in Animal Science at the Federal University of Pelotas in 2013, and stayed there for two years. In 2015 she joined the same course at the Federal University of Viçosa, and became a bachelor of science in animal science in January 2018.

In March 2018 she started her master's degree in the postgraduate program in animal science at the Federal University of Viçosa, where she concentrated his studies in the area in nutrition and dairy cattle production, under the guidance of Professor Marcos Inácio Marcondes.

Today, March 2, 2021, she submits her dissertation to obtain a master's degree in animal science.

ABSTRACT

ROCHA, Lethiane Garcia, M.Sc., Universidade Federal de Viçosa, March, 2021. **Unveiling unstable non-acid incidence in holstein cows fed with corn silage or sugarcane**. Adviser: Marcos inácio Marcondes.

We aimed to evaluate the incidence of unstable non-acid milk (UNAM) in cows fed either sugarcane or corn silage. Secondly, we aimed to evaluate the effect of temperature, daily variation and alcohol grades (72º, 78º and 80º) on UNAM incidence. The experiment was conducted as split-plot cross-over design with two periods and two roughage types (sugarcane or corn silage). Thirteen multiparous Holstein cows with an average of 281 days in milk were randomly distributed into two 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, profile protein electrophoretic) were collected during the last 4 days of each period. Alcohol test and dornic acidity were conducted in milk samples at 36ºC and 4ºC. Analyzes of zeta potential, micelle size and macrominerals in the casein micelle (calcium, phosphorus, magnesium and potassium) were also determined in refrigerated milk samples. The use of sugarcane, the degree of alcohol and the temperature of the milk at the time of the analysis affected the incidence of UNAM. The zeta potential, αS2-casein, blood ionic calcium, lactate and glucose were positively correlated with UNAM while lactose, phosphorus and potassium were negatively correlated with UNAM. Sugarcane-fed cows had increased levels of ionic calcium and glucose in the plasma, and reduced the levels of magnesium and urea, also significantly altered the protein profile of milk with lower levels of bovine serum albumin (BSA), β-casein and α-lactalbumin and greater αS1-casein content, all of which were correlated with the incidence of UNAM. Nevertheless, we found no roughage type effect on the variables mostly associated with UNAM, which are: changes in salts in the casein micelle, and consequently the zeta potential, and the k-casein fraction. This study brought important discoveries to unveil why cows manifest UNAM, however it also evidenced the need of further studies to better understand the physiological mechanisms that directly affect the stability of milk protein.

Keywords: Electrophoretic profile. Casein micelle. Zeta potential

RESUMO

ROCHA, Lethiane Garcia, M.Sc., Universidade Federal de Viçosa, março de 2021. **Estudo investigativo sobre a incidência de leite instável não-ácido em vacas Holandesas alimentadas com silagem de milho ou cana-de-açúcar.** Orientador: Marcos Inácio Marcondes.

Nosso objetivo foi avaliar a incidência de leite instável não ácido (LINA) em vacas alimentadas com cana-de-açúcar ou silagem de milho. Em segundo lugar, objetivamos avaliar o efeito da temperatura, variação diária e graus de álcool (72º, 78º e 80º) na incidência da LINA. O experimento foi conduzido em delineamento cruzado em parcelas subdivididas com dois períodos e dois tipos de forragem (cana-de-açúcar ou silagem de milho). Treze vacas holandesas multíparas com média de 281 dias de leite foram distribuídas aleatoriamente em duas dietas. Amostras individuais de sangue (análise de proteínas totais, albumina, uréia, cálcio, fósforo, magnésio, ferro, cloreto, glicose e lactato) e de leite (análise de proteína, gordura, lactose e sólidos totais, contagem de células somáticas, perfil de proteína eletroforética) foram coletadas nos últimos 4 dias de cada período. O teste do álcool e acidez dornic foram realizados em amostras de leite a 36ºC e a 4ºC. As análises de potencial zeta, tamanho da micela e macrominerais na micela de caseína (cálcio, fósforo, magnésio e potássio) também foram determinados em amostras de leite refrigerado a 4ºC. O uso da cana-de-açúcar, grau de álcool e temperatura do leite no momento da análise afetaram a incidência da LINA. O potencial zeta, αS2-caseína, cálcio iônico, lactato e glicose foram positivamente correlacionados com LINA, enquanto lactose, fósforo e potássio foram negativamente correlacionados com LINA. As vacas alimentadas com cana-de-açúcar tiveram níveis elevados de cálcio iônico e glicose no plasma, e reduziram os níveis de magnésio e uréia, também alteraram significativamente o perfil protéico do leite com níveis mais baixos de albumina sérica bovina (BSA), β-caseína e α-lactalbumina e maior conteúdo de αS1-caseína, todos os quais foram correlacionados com a incidência de LINA. No entanto, não encontramos efeito do tipo de forragem nas variáveis mais associadas ao LINA, que são: alterações de sais na micela de caseína, e consequentemente o potencial zeta, e a fração de k-caseína. Este estudo trouxe importantes descobertas para desvendar porque as vacas manifestam LINA, porém também evidenciou a necessidade de mais estudos para melhor compreender os mecanismos fisiológicos que afetam diretamente a estabilidade da proteína do leite.

Palavras-chave: Perfil eletroforético. Micela de caseína. Potencial zeta.

SUMMARY

1. Introduction

Bovine milk is a colloidal system synthesized in the mammary gland of bovine females through precursors that comes 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 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; Fonseca and Santos, 2001; Molina et al., 2001). However, milk composition, especially protein and fat, may be extensively (up to 50%) altered by diet (Fredeen, 1996).

Corn silage is the most used roughage for dairy animals in tropical countries; however, fresh chopped sugarcane is being suggested as replacement for corn silage for medium and low production animals (≤ 20 L/d, Costa et al., 2005; Andrade et al., 2016). Nonetheless, the use of sugarcane has been also being linked to alter milk physicochemical properties, more specifically it was linked to an increasing occurrence of unstable non-acid milk (**UNAM**) (Andrade et al, 2016). The UNAM is characterized when milk has a low stability in on-farm alcohol test (72º to 80º) but normal levels of lactic acid (between 14 and 18ºD).

There is a trend for increasing the on-farm milk quality measurements over the world. Many countries adopt the alcohol/alizarin test as a standard test to measure the raw milk quality, using alcohol grades between 68-75º, besides somatic cell count (**SCC**), standard plate count (**SPC**) and lactic acid concentration (Jurjen Draaiyer et al., 2009). Theoretically, a positive result in alcohol test would indicate a high acidity, which is a result of lactose degradation to lactic acid by the microorganisms (Brito and Brito, 1998). However, studies revelated that many positive samples in alcohol test had normal levels of acid lactic. (Marques et al., 2007; Zanela et al., 2009), which was named “unstable non-acid milk” (**UNAM**) (Zanela et al., 2006).

The UNAM is a considerably new phenomena and only limited information is found in the literature, and the reasons it occurs remains uncertain to this point. Although the literature is scarce on this subject, the 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 normal milk.(De Kruif and Holt, 1999).

The literature claims that UNAM is linked to heat stress (Zanela et al., 2006), dietary mineral levels (especially Ca and P) (Marques et al., 2011), feed restriction (Fischer et al., 2012) and dietary roughage (Oliveira et al., 2011). However, none of these studies could identify factors of causality regarding UNAM and the physicochemical alterations in milk to cause UNAM remains unknown. Nevertheless, dairy consultants (personal communication) have also linked a great incidence of UNAM with 1) feeding fresh chopped sugarcane in replacement to corn silage; 2) the temperature of milk at the moment of the test; and 3) high daily variation, although we couldn't find any report in the literature to explain those observations.

Furthermore, the dairy industry, to ensure greater milk thermal stability and shelf life, generally instructs the carrier to use alcohol 78º and 80º for on-farm alcohol tests (Fischer et al., 2012), but literature recommendations varies from 72 to 80º (Molina et al., 2001; Oliveira et al., 2011; Brasil, 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 a milk that has normal lactic acid levels (Silva et al., 2012).

Thus, investigating reasons of 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%), different temperatures (4ºC or 36ºC), and two roughage sources (corn silage and fresh sugarcane), as well as study its daily variation. We hypothesize that diet with sugarcane causes a greater incidence of UNAM than corn silage. Secondly, we hypothesized that cold milk would have a lower incidence of UNAM, with no significant daily variations.

2. Material 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 with an average of 21,3°C throughout our study. The institutional ethics committee approved the experiment (protocol no. 28/2020).

Animal, treatments and experimental design

Before treatment assignments and the experimental period, all cows had ad libitum access (30 days) to an adaptation diet consisted 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, thirteen multiparous Holstein cows with an average of 281 days in lactation and an average production of 17.5 liters per day were randomly divided into two groups and housed collectively in two pens (42 m^2) containing rubber mattresses as bedding, feeders and free access to water. The experiment was carried out as an unbalanced crossover design, with two periods and two treatments (roughage type).

Animals were fed ad libitum twice daily (06:00 and 16:00 h), right after milking, allowing orts for up 5% of as-fed TMR. The treatments were defined by type of roughage: sugarcane and corn silage. Experimental diets (Table 1) had roughage:concentrate ratio of 50:50 (DM basis) and were formulated to meet crude protein (CP), metabolizable energy intake and macro- and trace minerals requirements of cows with an average BW 650 kg and 17.5 L/d of milk production, according to NRC (2001).

The experiment lasted sixty days, divided into two periods of thirty days each. In each period, the first twenty-six days were for the adaptation of the animals to experimental diets and the last four days 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 refrigerated (4ºC) and non-refrigerated milk (36ºC). The individual milk collection (100 mL) was performed during the morning milking using collecting cups attached to the parlor. Each sample was equally distributed in two disposable polypropylene containers. The first sample was classified as freshly milked milk and it was used for analyses of Unstable non-acid milk (**UNAM**) and Dornic analyzes at temperature of 36ºC and refrigerated at 4ºC, and the second sample was immediately stored in a freezer at a temperature of -20ºC for later analysis.

Alcohol Test, Dornic and Milk Composition analysis

The evaluation of UNAM was performed using the alcohol test, that was carried out

with different grades (72º, 78º e 80º) of alizarol solution (Figure 1). The alizarol solution consists of an alcoholic solution with the addition of alizarin, a pH indicator. 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 seconds. The sample was evaluated as UNAM when there was the appearance of clot of any size, and titratable acidity below or equal to 18ºD. After carrying out the test, milk samples refrigerated to repeat UNAM analysis after 24 h, when the samples were at 4 °C.

The acidity was evaluated according the Dornic method (Tronco, 2003). Four drops of phenolphthalein were added in an Erlenmeyer containing 10 mL of milk. The samples were titrated with the standard Dornic Solution (9:1), which consists of a solution of sodium hydroxide N/9, where each 0.1 mL corresponds to 1ºD or 0.1 g of lactic acid/L. After carrying out the test, milk samples refrigerated to repeat acidity analysis after 24 h, when the samples were at 4 ° C.

The milk composition analyzes were performed at the end of each period, with individual samples being collected in bottles containing Bronopol® and stored under refrigeration until the moment of transport to the Clínica do Leite's Lab. Milk was for analyzed for protein, fat, lactose, and total solids contents, using a MilkoScan® FT 120 analyzer (Foss Electric, Hillerod, Denmark). The milk yield values were corrected for 3.5 % fat according to Sklan et al. (1992). The somatic cell count was performed using FossomaticTM FC (Foss Electric, Hillerod, Denmark).

Electrophoretic profile

Milk (30mL) samples were lyophilized for protein electrophoretic profiles. First, the samples were thawed at 8 ºC and skimmed by centrifugation at 2,100 g for 30 minutes at 32ºC. After lyophilization, the electrophoretic profile analysis was carried out according Egito et al. (2006). This analysis used acrylic bowl for vertical electrophoresis Bio Rad Mini Trans-Blot® Cell model. SDS-page had gels with 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) were dissolved in 1 mL of Tris-HCl buffer, pH 6.8, with 0.1% of SDS, 5% β-mercaptoethanol and placed on eppendorfs. Soon after, the solutions were heated at 100ºC for three minutes at water bath and 10 µL were placed on the gels (Egito et al., 2006). Then, the protein was fixed on a gel with 12% trichloroacetic acid

(TCA) during 30 minutes and then stained with 0.1% Coomassie blue R250, dissolved in a mixture of 50% ethanol and 2 % TCA, for 120 minutes. 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 6.0 program. We quantified the optical density, and the pixel amount of each protein fraction was used. Protein fractions of milk samples identified and quantified were lactoferrin; bovine serum albumin (BSA); casein fractions (CN): αS1- CN and αS2-CN, β-CN, κ-CN; and soluble proteins: β-lactoglobulin and α-lactoalbumin.

Micelles Casein Purification

Milk samples were skimmed by centrifugation at 1500g for 30 minutes at 4 ºC, than samples were stored with 0.05% (w/v) sodium azide to avoid the microbial growth. Purification of the casein micelle occurred by centrifugation at 25000g for 30 minutes at 20ºC. The supernatant was discarded remaining the pellet. Following the procedure, we added a Tris buffer solution (10 mM, pH 7.4) containing 10 mM $CaCl_2$ to the pellet and subjected to centrifugation. This process was repeated five times in order 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 macrominerals (calcium, phosphorus, magnesium, potassium and sodium). In the casein solution, we added strontium to purify the sample from any residue. Calcium and Mg readings were performed through atomic absorption spectrometry (GBC Avanta Sigma, GBC Scientific Equipment, Hampshire, IL; method 968.08; AOAC International, 2000) while K concentrations were determined using flame emission spectrometry (Corning 400, Corning, NY; method 985.35; AOAC International, 2000). The P contents were performed by reduction of 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 equipment (Malvern, UK). We used a laser wavelength of 663 nm with a 90º dispersion angle. The bucket used was of the "disposable folded capillary cell" type and refractive index of 1330. All data were obtained through the average of three readings per sample and per 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 immediately after milking in the first two days of the collection period. One of the samples was collected in tubes with clot activator and gel for serum separation (BD Vacutainer® SST® II Advance®) for determine the total proteins, albumin, urea, calcium, phosphorus, magnesium, iron and chloride concentration. The sample collected in the second tube, with EDTA and sodium fluoride (BD Vacutainer® Fluoreto/EDTA, São Paulo, Brazil), was used to measure glucose and lactate in plasma. Then, samples were centrifuged at 1000 × g for 10 min and were stored in Eppendorf tubes at −20°C until to further analysis. These constituents were assayed in duplicate using colorimetric commercial kits of the BioClin® brand (Bioclin, Quibasa; Belo Horizonte, Brazil) according to the manufacturer's recommendations. All the aforementioned analyzes, except chloride, were performed by an automatic biochemical analyzer (Mindray BS-200E, China). The plasma chloride level was measured using a commercially available kit (Bioclin® Quibasa, Belo Horizonte, Brazil).

Statistics

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), milk temperature at the time of the test, and treatment (roughage used). We used a split-split-plot crossover design considering day as a repeated measure, including day, alcohol grade (only for UNAM), milk temperature, treatment and all two-way, three-way and four-way interactions. Group, order of lactation and period were added to the model as random effects. Animal within period was identified as 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 ($P > 0.689$, Figure 2), milk and blood samples from two days of were pooled (analyses were performed separately and averaged) for all other response variables described above. We used crossover design with treatment as fixed effect and group, order of lactation and period as random effects.

For all significant responses, Student's t-test was used to identify difference across least squared means. All analyses were performed using PROC GLIMMIX of SAS University Edition, considering statistical differences when $P < 0.05$ and trends when $0.05 < P < 0.10$.

3. Results

The UNAM was affected by roughage type, alcohol grade and milk temperature ($P < 0.046$), but unaffected by days ($P = 0.689$) (Figure 2). We also observed a roughage × alcohol interaction and sugarcane had greater incidence of UNAM when milk was tested using alcohol 72% or 78%, but roughages were not different when milk was tested using alcohol 80%. Additionally, the greater the alcohol level, the greater the incidence of UNAM (Figure 2), and overall sugarcane fed cows had greater UNAM than corn silage fed cows. The group fed with sugarcane had an increase in the frequency of UNAM of 16.58% with 72% alcohol, and an increase of 31.8% with alcohol 78% when compared with corn silage. Lastly, cooling milk samples reduced UNAM incidence in 7.21 % ($P = 0.018$).

The UNAM is characterized when the milk samples have normal levels of lactic acid (degrees Dornic, Figure 3) but clogs when the alcohol test is done. In our study, all samples had normal Dornic level (< 16.0, Figure 3), which indicates that all samples that clogged were indeed UNAM (Figure 2). Nevertheless, we observed a milk temperature × day interaction ($P = 0.011$) for Dornic degrees, were there was no day effect in warm milk, but Dornic degree increased in day 4 (ºD = 14.54) when compared to days 1 and 2, likely a refrigeration problem. As expected, the roughage type did not affect Dornic degrees ($P = 0.108$).

To try to understand what may have caused this variation in UNAM, we first performed a correlation analysis between UNAM and all response variables. The milk lactose had a high negative correlation with Cold 78% (−0.556), Warm 72% (−0.506) and Warm 78% (−0.532) milk samples. A positive correlation was found between the zeta

potential of the casein micelles and UNAM in Cold 72% (0.610), Cold 78% (0.472), Warm 72% (0.397) and Warm 78% (0.435) milk samples. In the casein micelles, negative correlations of phosphorus were found in Cold 80% (− 0.433), potassium in Cold 80% (− 0.507) and Warm 80% (−0.534), magnesium in cold 80% (-0.407) milk samples. Regarding the milk protein fractions, the αS1-casein showed a positive correlation with Warm 78% (0.501) and Warm 80% (0.440) milk samples. The β-casein showed a negative correlation in warm samples at 80%. The lactalbumin showed a negative correlation in cold samples at 78% (-0,499). The blood composition showed positive correlations between UNAM and ionic calcium in Warm 78% (0.406), lactate in Cold 72% (0.427), as well as glucose in Cold 80% (0.490) milk samples. It also showed a negative correlation for chloride levels (-0.396).

The production data and composition of the milk samples are shown in Table 2. Roughage did not influence milk yield, with a production of 17.39 L/d for animals fed with sugarcane and a production of 17.61 L/d for animals fed with corn silage (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. On the other hand, cows fed corn silage had a greater (P = 0.001) milk urea nitrogen (17.70 mg/dL) when compared with cows fed sugarcane (13.95 mg/dL).

The zeta potential and the size of the casein micelles, as well as 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 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).

There was no significant difference between the types of roughage for the mineral composition of the casein micelle (Table 3). The values found for the two treatments were similar, where sugarcane showed an increase of 1.63 % in micelle size, 2.40 % in zeta potential, 0.19 % in calcium content and 0.12 % in phosphor. The data on potassium and magnesium from sugarcane showed a decrease of 2.40 % and 1.11 %, respectively.

The protein profile of milk samples (Table 4) from the sugarcane group showed 21.93 % of αS1-casein (P = 0.001), which is 16.52% greater than that observed for 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. On the other hand, 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).

4. Discussion

Non-acid unstable milk and alcoholic grades

The UNAM has been studied a lot 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; Gaucheron, 2005). There are many factors that act on the animal and may lead to an UNAM's condition, such as genetic factors (Botaro et al., 2009), 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 in the blood metabolic profile of animals with UNAM.

Animals fed with sugarcane has greatly increased the incidence of UNAM when compared to the corn silage diet, but this difference tends to disappear with the use of 80% alcohol in the test (Figure 2). In this case, using 80% alcohol, we will not only increase the incidence of UNAM, but we may also find false positives for UNAM. According to Molina et al. (2001) the use of 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 found according to the increase in alcoholic grades in the tests.

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 Oliveira and Timm (2007) the change in roughage with less digestibility causes a reduction in the casein content and an increase in the concentration of ions, especially calcium. We found similar results in terms of ionic calcium content, but no difference in casein content. 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 (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 roughages and the initial stage of development of winter roughages, thus relating the

quality of the roughage offered and the stability of alcohol-proof milk.

Regarding the temperature, for the alcohol test, our samples at 36ºC presented a frequency of 7.21% more than UNAM than the refrigerated samples at 4ºC (Figure 2). The higher incidence of UNAM in post-milking samples (warm samples) is likely due to a higher concentration of dissolved carbon dioxide. The reaction of CO_2 in solution forms carbonic acid, thus making them momentarily more acidic due to the release of H^+ ions (Loss and Hotchkiss, 2003). According to Paula et al. (2012) carbon dioxide acts reversibly at pH and permanent in colloidal calcium phosphate bridges, reducing pH increases the solubility of colloidal calcium phosphate and weakens calcium bonds with caseins (Dalgleish and Law, 1989). This higher concentration of CO_2 dissolved in the hot sample increases the dissociation of ionic calcium and reduces the repulsion between negatively charged micelles, causing greater protein instability (Lin et al., 1972; Barros et al., 1999; Lin et al., 2009).

Dornic acidity

In our study, refrigerated samples were influenced by day, showing greater acidity in days 3 and 4 (Figure 3). According to Andrade (2013), inefficient refrigeration causes an increase in acidity. This quantitative analysis measures the concentration of lactic acid, which in turn estimates the microbiological quality of milk (Brito and Brito, 1998). The increase in the lactic acid content is an indication that the standard plate count (SPC) is high, and the high concentration of lactic acid generated by microorganisms causes a reduction in pH (Andrade, 2013). Thus, manipulation of the cooling tank or possible electrical failures of the milk storage equipment in our study may have caused inadequate cooling of the milk, increasing its acidity. Nevertheless, as these results are not linked with our hypotheses, they will not be further discussed.

Milk production and composition

Milk production and composition data showed that animals fed with sugarcane had greater contents of fat, protein, and total solids. However, MUN levels were reduced in the milk of animals fed with sugarcane. The composition of milk is of paramount importance to the industry, as it dictates the yield of dairy products, especially the contents of the fat and protein components. Currently, to guarantee the levels of these constituents, the IN-76

(Brasil, 2018), the current Brazilian legislation, requires that refrigerated raw milk must have minimum levels of 3.0% and 2.9% of fat and protein, respectively. According to Walstra et al. (2006) the average levels of fat and protein in the milk of Holstein cows are 3.6% and 3.3%, respectively. In this study, the average values were 4.32% fat and 3.59% protein for cows fed sugar cane while for animals fed corn silage it was 3.88% fat and 3.5% of protein. These levels are higher than the results found in the literature for the Holstein breed. In addition, the greater contents of fat and protein in milk samples of cows fed with sugarcane contrasts with the results of Costa et al., (2005), Magalhães et al., (2000;2004). These studies analyzed the effects of total and partial replacement of corn silage by sugarcane and the impacts caused on milk composition. The authors concluded that there was a reduction in the levels of protein and fat when cows are fresh chopped sugarcane. Magalhães et al. (2004) used a roughage: concentrate ratio of 40:60, aiming at a moderate concentrate intake and consequent reduction in pH. Although the roughage:concentrate (50:50) ratio of our study had been similar between treatments, this was different from Magalhães et al. (2004). We believe that the increased supply of roughage may have increased the levels of fat and protein in the milk of cows fed sugarcane. Firstly, this is likely associated with a greater acetate:propionate ratio, since sugarcane has a greater NDF content (Preston, 1982; Magalhães et al., 2004; Rodrigues et al., 2005). Secondly, the greater sugar content in sugarcane-based diets will favor the increase in the population of protozoa of the genus Dasytricha, improving ruminal stability and digestibilities of dry matter and organic matter and energy (Sutoh et al. 1996; Valvasori et al. (2001), favoring NDF digestibility and microbial protein synthesis, with positive consequences in milk fat and protein. Further, milk total solids of animals fed with sugarcane had showed an increase of 0.5% in relation to those fed with corn silage. This value reflects the greater fat and protein content present in the milk samples of cows fed with sugarcane. According to Fonseca and Santos (2000), a greater value of total solids causes greater acidity in the milk, which may cause instability in the casein micelles. In our study, type of roughage did not influence acidity (measured by the Dornic degrees) but did influence milk stability to alcohol (UNAM), evidencing that milk total solids don't play a big role in casein micelles stability.

The animals fed with corn silage had a lower incidence of UNAM. Our first hypothesis is that this result may be related to a urea metabolism (Sutoh et al., 1996), since a greater concentration of urea nitrogen was observed in these samples, which presented 3.5% more MUN than 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 in a lower flow of plasma urea in sugarcane diets, which will reflect in lower MUN (Sutoh et al., 1996). Although we found no correlation between UNAM and MUN, Singh & Creamer (1992) and Negri et al. (2002) observed a negative correlation between UNAM and MUN. According to these authors, non-protein nitrogen precludes the milk acidification and its conversion into cyanide that reacts with milk protein. Then, the negative charge of the micelles and their electrostatic repulsion are increased, improving the thermal stability (Sweetsur and Muir, 1981). In any case, further studies are warranted to confirm this theory as our results could not do it.

Sugarcane diets had also a greater blood glucose concentration (4% 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 blood stream (Sutoh et al., 1996). This greater glucose concentration was negatively correlated with UNAM but had not impact on milk yield or milk lactose (Table 2). Following the same trend as glucose, a negative correlation was found between lactose and UNAM. According to Fagnani et al. (2016), the lower lactose content can lead to a greater displacement of minerals from the blood to the milk. This causes changes between the continuous and colloidal phases destabilizing their conformations. This increases the ionic strength of the colloidal solution, a reduction in pH and an increase in the concentration of blood ionic calcium (as observed in this study), generating a precipitation of the protein due to their destabilization. In agreement with these results, we also found a strong positive correlation between ionic calcium and UNAM (Table 5), which might explain the higher UNAM in sugarcane-fed cows.

Blood profile

It is known that dairy cows require a higher mineral intake to meet the requirement for maintenance, fetal growth and production. From the analysis of the blood profile 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 in relation to 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 anionic diet. As mentioned before, this increase in the levels of ionic calcium is likely related to the content of sugar 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., 2005). These changes usually generate disturbances in the zeta potential and hydrophobicity, while the size of the micelles remains without significant changes. In the present study, we found a positive correlation between the concentration of serum ionic calcium and the incidence of UNAM. We did not find any study that correlated the content of serum ionic calcium with the content of ionic calcium present in milk, but we believe, based on our responses, that the increase in serum 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 2.0-3.0 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 sugarcane treatment had a serum magnesium level of 1.92 mg/dL, which is then lower than the reference literature. According to Goff (2018), levels below 2 mg/dL should be considered indicative of 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.

Another relevant factor is source of roughage. Both diets were formulated to have the 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 and the diets was 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 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.

Casein micelles

Our results indicated that sugarcane diets can 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 Kruif et al., 2012) and colloidal calcium levels (Chavez et al., 2004) and reduced zeta potential (Singh, 2004; Lin et al., 2006). But there were no significant difference in any of these variables. 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 unidentified variables are still playing an important role in UNAM incidence.

The addition of ethanol in liquids, such as milk, promotes changes in the three-dimensional structure of the water molecules around the solutes (Javadian et al., 2008). Ethanol has amphiphilic characteristics because its structure is composed of a hydrophobic portion (hydrocarbon chain) and a hydrophilic portion (polar hydroxyl head group) (MacDonald, MacLennan, & Marangoni, 2020). Therefore, when added to milk, it preferably links to proteins interfaces, more especially the casein micelles, where the k-casein fractions (k-cas) are found, which, like ethanol, also have amphiphilic properties. The penetration of ethanol into the hairy layer of the casein micelles, composed of k-cas, leads to an increase in the surface area and, therefore, to a decrease in the charge density of the micellar surface. The presence of ethanol also decreases the dielectric constant of the solvent, causing a reduction in the electrostatic and steric repulsions that existed between the casein micelles. Several factors, such as a high concentration of lactic acid, reduced zeta potential, high SPC, concentrations of k-cas, contribute to the instability of the casein micelles. Thus, if milk has any of these factors, the presence of ethanol will be sufficient to promote the precipitation of the casein micelles. This destabilization phenomenon is known as the collapse of the hairy layer (exposed C-terminal segment of κ-casein) 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. When comparing the milk composition of our two diets (Table 2), we noticed that milk from cows fed with corn silage showed a higher MUN content, but still within the normal range for lactating cows (Botaro et al., 2009). Nevertheless, 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 for the solvation layer of the urea molecules present in the medium (House & House, 2017). On the other side, in milk samples from cows fed with sugarcane, the lower concentration of urea in solution causes ethanol to partition towards the interface of the casein micelles, exerting its effect on precipitation over the k-cas. Nevertheless, we could not find any study in the literature to corroborate this hypothesis and future studies in this topic are warranted.

Milk protein profile

Some studies show that colloidal stability is associated with a higher content of k-casein and the steric stability generated by its hair layer (Creamer et al., 1998; Tuinier and Kruif, 2002). In our study, we observed no difference in the proportions of k-casein between treatments, which agrees with the results of 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 greatest instability in the alcohol test. We observed an increase in 16.52% in the αS1-casein content, and reductions of 17.21%, 5.37% and 8.48% of BSA, β-casein and α-lactalbumin (Table 4), respectively, in the milk protein of those animals. According to Ordoñez et al. (2005) the content of α-lactalbumin is related to the content of lactose, since it is part of the enzymatic system of its synthesis, however we did not observed changes in milk lactose between roughage types (Table 2). On the other hand, we found an increase in α-lactalbumin corn silage based diets, whose group had the greater protein stability in the face of alcohol testing. The α-lactoalbumin has high affinity for Ca^{2+} ions, and this Ca^{2+} sequestration of the solution promotes greater thermal stability to the protein (Stuart et al., 1986). The BSA is a whey protein associated with the permeability of the cellular junctions of the mammary gland, and its increase is usually linked to inflammations or infections of the mammary gland (Litwinczuk et al., 2011), corn silage-fed cows, which showed greater concentration of BSA, showed normal levels of SCC. Thus, we suspect that other factor than infections may also play a role in

BSA synthesis.

We observed a lower β-casein in sugarcane-fed cows, which may be associated with the action of the enzyme plasmin present in milk. When β-casein is cleaved by plasmin, γ - casein is generated, which blocks potassium channels (Silanikove et al., 2009). Since potassium and sodium are monovalent ions that act on ionic strength, any change in their concentrations can generate protein instability. The αS1-casein was also increased in sugarcane-fed cows which may also be related to the higher incidence of UNAM in those animals. However, as much as this result is similar to those obtained by Barbosa et al. (2012), there is still not enough information to support a biological connection between αS1-casein and UNAM.

5. Conclusion

In summary, higher alcohol grades, non-refrigerated milk, and cows fed with sugarcane had increased incidence of UNAM. The zeta potential, αS2-casein, ionic calcium, lactate and glucose were positively correlated with UNAM while lactose, phosphorus and potassium were negatively correlated with UNAM. Sugarcane-fed cows had increased levels of ionic calcium and glucose in the plasma, and reduced the levels of magnesium and urea, also significantly altered the protein profile of milk with lower levels of BSA, β-casein and α-lactalbumin and greater αS1-casein content, all of which were correlated with the incidence of UNAM. Nevertheless, we found no roughage type effect on the variables mostly associated with UNAM, which are changes in salts in the casein micelle, zeta potential, k-casein, β-lactoglobulin and lactose. This study brought important discoveries to unveil why cows manifest UNAM, however it also evidenced the need of further studies to better understand the physiological mechanisms that directly affect the stability of milk protein.

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7. Tables and figures results

ALIMENTOS	Unit	Sugarcane	Corn Silage
		Ingredients	
Sugarcane		50.00	-
Corn silage		-	50.00
Ground corn		31.52	24.75
Soybean meal		14.12	23.19
Limestone	% DM	0.69	1.14
Dicalcium phosphate		0.91	0.31
Salt		0.62	0.61
Urea		1.68	-
Ammonium sulfate		0.45	-
Cobalt sulfate		-	0.31
Copper sulfate		15.87	7.95
Potassium iodide	mg/kg DM	1.19	1.17
Sodium selenite		0.78	0.76
Vitamin blend		0.38	0.40
		diet	
Dry matter	% as fed	43.90	50.18
Crude Protein		16.96	17.03
Rumen degradable protein		12.56	11.83
Rumen undegradable protein		4.39	5.21
Neutral Detergent Fiber		33.49	27.60
Starch		25.41	30.76
Ether Extract	% DM	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
Net Energy for Lactation	Mcal/kgDM	1.62	1.67
Cobalt		0.22	0.15
Cupper		10.17	10.53
Iodine		0.71	0.73
Manganese		35.52	24.82
Selenite	mg/kgDM	0.39	0.41
Zinc		35.65	25.93
Iron		15.12	104.46

Table 1 – Proportions of feeds in the concentrate, and the diet and concentrate or cows fed with corn silage or sugarcane.

Table 2 – Production and composition of milk samples from cows fed corn silage or sugarcane.

Item	Roughage		SEM	P value
	Sugarcane	Corn silage		
Milk yield, kg/d	17.39	17.61	1.515	0.782
Fat, %	4.32	3.88	0.269	0.039
Protein, %	3.61	3.52	0.090	0.022
Lactose, %	4.41	4.44	0.079	0.523
Total solids, %	13.35	12.84	0.297	0.025
Non-fat solids, %	9.04	8.98	0.268	0.326
SCC, log (x 1000)	2.43	2.33	0.186	0.092
MUN, mg/dL	13.95	17.70	1.095	0.001

SCC=somatic cell count; MUN=milk urea nitrogen

Table 3 – Zeta potential and 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
	Sugarcane	Corn silage		
Size, nm	281.68	277.16	66.526	0.906
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

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

Item	Roughage		SEM	P value
	Sugarcane	Corn silage		
Lactoferrin, %	1.43	1.49	0.137	0.264
Bovine serum albumin, %	1.01	1.22	0.094	0.001
αS1-casein, %	21.93	18.82	1.134	0.001
αS2-casein, %	14.48	14.64	0.466	0.697
β-casein, %	19.02	20.10	0.944	0.041
κ-casein, %	16.36	16.10	1.203	0.621
β-lactoglobulin, %	11.24	11.71	0.603	0.363
α-lactalbumin, %	14.68	16.04	0.895	0.005

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

Item	Roughage		SEM	P value
	Sugarcane	Corn silage		
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	0.958
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, mcg/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	0.876
Urea, mg/dL	33.91	39.55	2.199	0.004

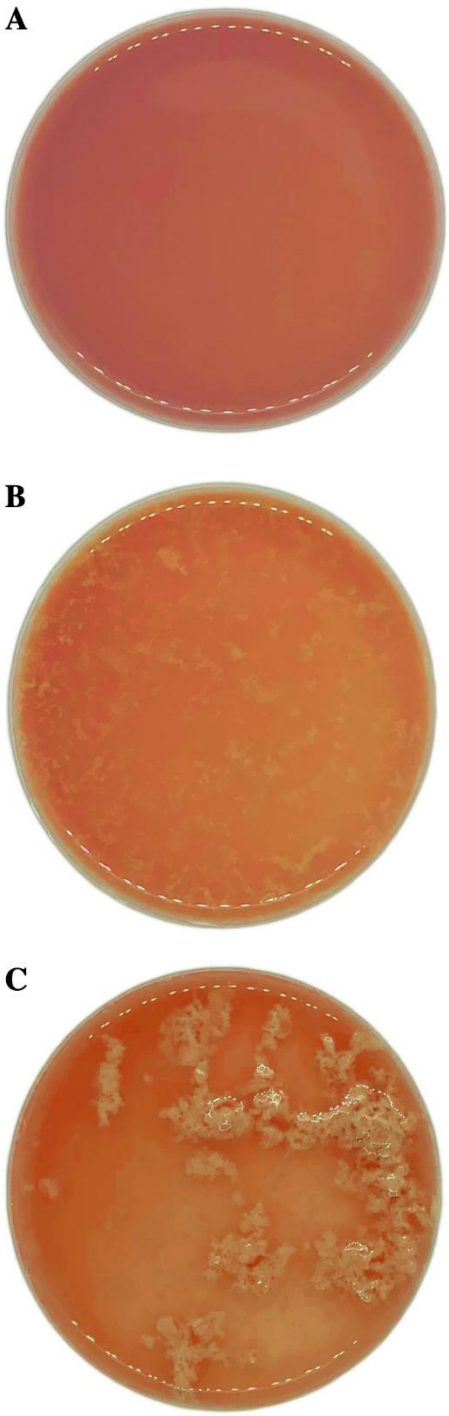

Figure 1 – A - Solution with 2 mL of normal milk and 2 mL of alizarine/alcohol 78º; B – Solution with 2 mL of unstable non-acid milk and 2 mL of alizarine/alcohol 72º; C – Solution with 2 mL of unstable non-acid milk and 2 mL of alizarine/alcohol 78º.

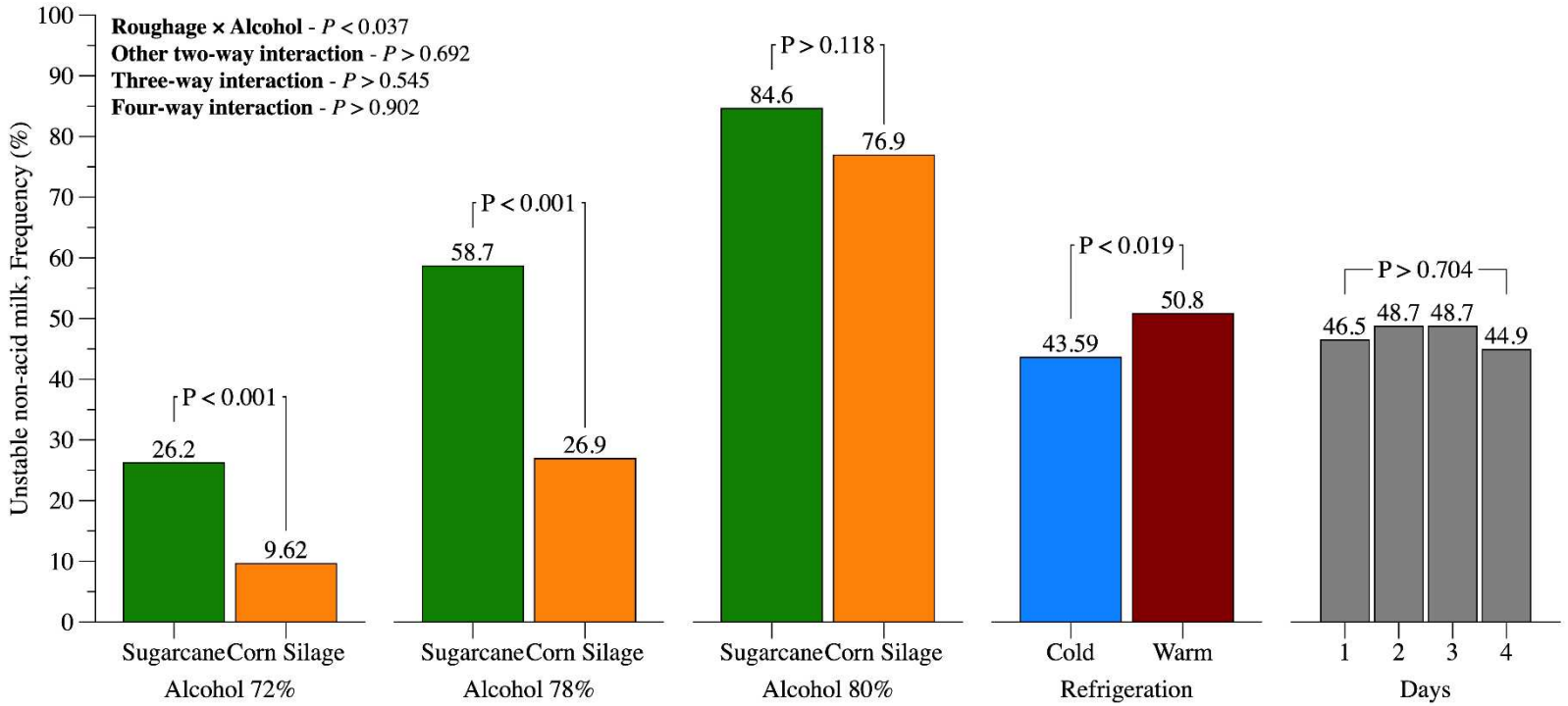

Figure 2 – Frequency of unstable non-acid milk of cows fed with corn silage or sugarcane, conditioned to three alcohol degrees (72, 78 or 80%), two temperatures (36º C after milking or after refrigeration at 4º C) and four days of collection.

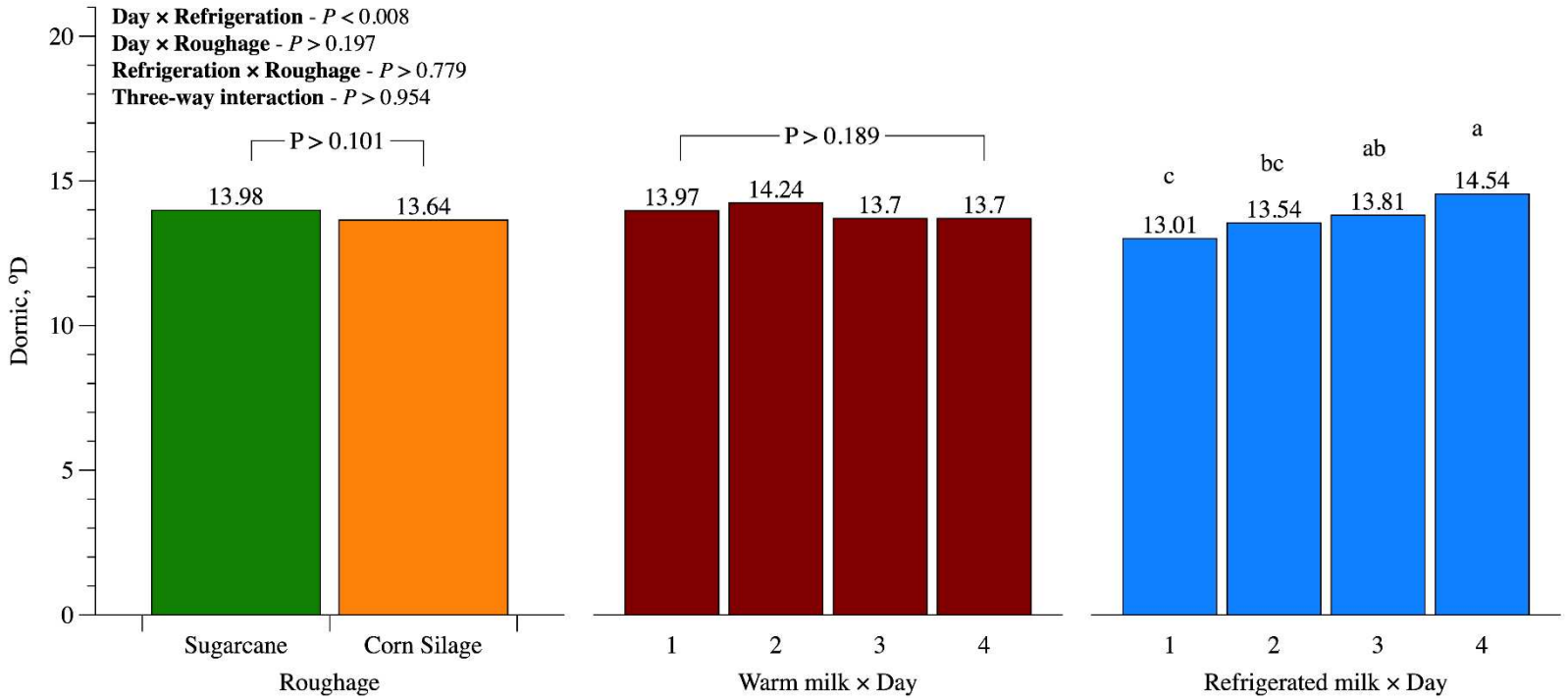

Figure 3 – Acidity in Dornic degrees of milk of cows fed with corn silage or sugarcane, conditioned to three alcohol degrees (72, 78 or 80%), two temperatures (36º C after milking or after refrigeration at 4º C) and four days of collection.

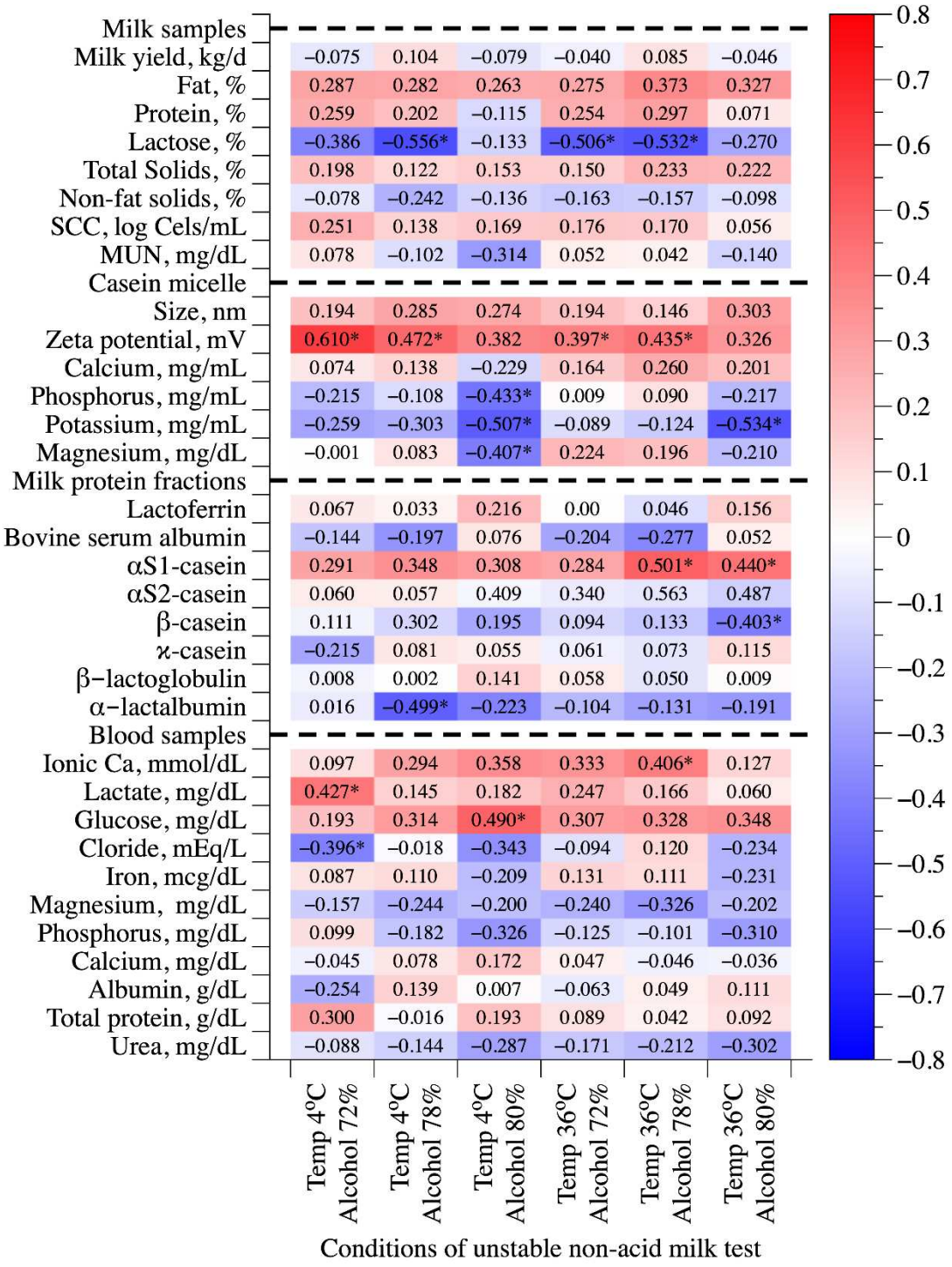

*Figure 4 - Correlation between unstable non-acid milk and several response variables. Correlations followed by * indicate significant at $P < 0.05$.*