Effect of dietary cation-anion difference on performance of lactating dairy cows and stability of milk proteins

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ABSTRACT

Casein micelle stability is negatively correlated with milk concentrations of ionic calcium, which may change according to the metabolic and nutritional status of dairy cows. The present study aimed to evaluate the effect of dietary cation-anion difference (DCAD) on concentrations of casein subunits, whey proteins, ionic calcium, and milk heat and ethanol stability. Sixteen Holstein cows were distributed in 4 contemporary 4 × 4 Latin square designs, which consisted of 4 periods of 21 d and 4 treatments according to DCAD: 290, 192, 98, and −71 mEq/kg of dry matter (DM). The milk concentrations of ionic calcium and κ -casein were reduced as DCAD increased, whereas the milk urea nitrogen and β -lactoglobulin concentrations were increased. As a result of these alterations, the milk ethanol stability and milk stability during heating at 140°C were increased linearly with increasing DCAD [$Y = 74.87$ (standard error = 0.87) + 0.01174 (standard error = 0.0025) × DCAD (mEq/kg of DM) and $Y = 3.95$ (standard error = 1.02) + 0.01234 (standard error = 0.0032) × DCAD (mEq/kg of DM), respectively]. In addition, 3.5% fat-corrected milk and fat, lactose, and total milk solids contents were linearly increased by 13.52, 8.78, 2.5, and 2.6%, respectively, according to DCAD increases from −71 to 290 mEq/kg of DM, whereas crude protein and casein content were linearly reduced by 4.83 and 4.49%, respectively. In conclusion, control of metabolic changes in lactating dairy cows to maintain blood acid-base equilibrium plays an important role in keeping milk stable to ethanol and during heat treatments.

Key words: casein, dietary cation-anion difference, ionic calcium, milk ethanol stability

INTRODUCTION

Stability of milk proteins is an important issue in the dairy industry, especially for the manufacture of milk products that require intense heat treatments, such as UHT and powdered milk. Also, high raw milk stability is needed to avoid losses to dairy farmers due to price penalty or rejection of milk, to prevent coagulation of milk during heat treatment (Fischer et al., 2012), and also to reduce the age-related gelation of heat-treated milks during storage (García-Risco et al., 1999). Thus, the milk ethanol stability (MES) test is still used worldwide in receiving platforms of dairy plants to predict the stability of milk proteins (Fischer et al., 2012).

The casein micelle in milk is formed by casein subunits (α , β , and κ) interconnected by micellar calcium phosphate (Walstra, 1999). The calcium, besides the colloidal phase, is also present in milk in a free form (ionic calcium), which is negatively correlated with casein micelle stability (Barros et al., 1999). The increase in milk concentrations of ionic calcium (iCa) may reduce the negative charges of the casein micelles and the strength of electrostatic repulsion between them, which reduces casein resistance to form clots during contact with ethanol or during milk heat treatments (Barros et al., 1999). The α - and β -CN are unstable when in contact with milk iCa. However, κ -CN, which is located in the outer layer of the micelle, is hydrophilic and stable to iCa reaction and therefore protects the micelle hydrophobic core (composed of α - and β -CN) from water contact and iCa ionization (Creamer et al., 1998; Walstra, 1999). Thus, the structural arrangement of α -, β -, and κ -CN in the micelle, the milk concentrations of divalent cations, and the electrostatic repulsion strength between micelles may determine the heat stability (Jeurnink and De Kruif, 1995; Barros et al., 1999; Fischer et al., 2012), the formation of β -LG/ κ -CN complexes on heating, and, subsequently, formation of irreversible gel in UHT milk during storage (McMahon, 1995; García-Risco et al., 1999).

High incidences of unstable milk showing normal acidity values have been reported worldwide, especially in countries using UHT as the main form of fluid milk

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processing (Chavez et al., 2004; Oliveira et al., 2013). Moreover, most studies reported that the nutritional and metabolic conditions of dairy cows were the main factors that changed the milk stability (Zanela et al., 2006; Marques et al., 2011; Barbosa et al., 2012). Barbosa et al. (2012) reported that cows fed with nutrient-deficient diets produced milk with lower concentrations of κ -CN and, consequently, lower ethanol stability when compared with milk from cows fed balanced diets. Alternatively, Marques et al. (2011) reported that the metabolic acidosis of dairy cows, through the action of parathyroid and 1,25-dihydroxycholecalciferol hormones, may increase milk concentrations of iCa and reduce the MES. Besides the iCa and κ -CN concentrations, other dairy components were also associated with lower milk stability, such as the formation of β -LG protein complexes with κ -CN in the micelle, MUN, temperature, pH, and concentrations of citrate and phosphate in milk (Jeurnink and De Kruif, 1995; Singh, 2004; Lewis, 2011).

Milk constituents associated with milk stability can be changed directly by the nutrition and metabolic status of dairy cows (Marques et al., 2011; Barbosa et al., 2012; Fischer et al., 2012). However, few studies on the individual, nutritional, or metabolic factors that may alter milk protein stability exist, as both deficient and excess nutrients (through metabolic acidosis resulting from ruminal acidosis) can reduce milk stability by different means (Marques et al., 2011; Barbosa et al., 2012; Fischer et al., 2012). The use of anionic diets can be applied to study the effect of metabolic acidosis in lactating dairy cows on milk composition and stability without changing the protein and energy content of the diet (Marques et al., 2011). However, the effect of the intensity of metabolic changes of dairy cows by a gradual decrease in DCAD on heat and ethanol stability of milk has not been determined. In addition, Chavez et al. (2004) and Fischer et al. (2012) reported that the MES had limited capability to estimate milk stability during heat treatments, which limits the usefulness of results from studies based only on ethanol stability to define corrective measures of unstable, nonacid milk. Therefore, the present study aimed to evaluate the effect of DCAD of dairy cows on concentrations of milk casein subunits, whey proteins, iCa, and milk ethanol and heat stability.

MATERIALS AND METHODS

Experimental Design and Animals

At the beginning of the trial, 16 Holstein cows averaging (mean $\pm$ SD) 22 ± 4.5 kg of milk/d, 158 ± 52 DIM, and 618 ± 57 kg of BW were distributed in 4 contem-

porary 4×4 Latin squares, consisting of 4 treatments and 4 periods of 21 d divided into 14 d of adaptation and 7 d of sample collection. The experimental treatments consisted of 4 DCAD levels, calculated according to the Na, K, Cl, and S concentrations of the dietetic ingredients (Table 1) as 290, 192, 98, and -71 mEq/kg of DM. The DCAD levels were calculated based on Tucker et al. (1991) using the formula: mEq/kg of DM = $[(\% \text{ Na in DM}/0.023) + (\% \text{ K in DM}/0.039)] - [(\% \text{ Cl in DM}/0.0355) + (\% \text{ S in DM}/0.016)]$. The diets were formulated to be isoenergetic (1.58 Mcal/kg of DM) and to have equal amounts of protein (160 g/kg of DM), according to NRC (2001) recommendations. Throughout the experiment, the cows were housed in individual stalls in a freestall system, fed a TMR once a day, and milked twice daily.

Feed Analysis and DMI

For determination of DMI, the amounts of feed offered and orts were weighed for each cow from d 14 to 21. The cows were fed according to DMI on the previous day, to keep daily orts of 5 to 10% of the amount offered. Samples of the feed supplied and orts were collected during the last 4 d of each experimental period, and were stored at -20°C , to compose a sample by period for each animal based on the dry weight for chemical analysis. The DM, ash, CP, and ether extract (EE) content in samples of feed and orts were determined according to AOAC International (1995). Acid detergent insoluble CP (ADICP), neutral detergent insoluble CP (NDICP), ADL, NDF, and ADF contents were obtained according to Van Soest et al. (1991). The NDF analysis was performed according to adaptations described by Mertens (2002) using α -amylase without sodium sulfite. The samples were ground, dry digested, and solubilized before mineral analysis. The Ca, P, Na, and K content were determined by flame emission spectrophotometer (Thermo Fisher Scientific, 2008), and chloride content was determined according to the Mohr method (AOAC International, 1995). The S concentration was obtained by colorimetry (molybdenum blue) according to methodology described by Malavolta et al. (1997). The NFC content was calculated according to Hall (2000) and the TDN and NE_L were calculated according to NRC (2001). The DMI and ingested amounts of CP, NDF, EE, NFC, TDN, Ca, P, Na, K, Cl, and S were calculated based on the chemical composition of feed and orts and according to NRC (2001).

Milk Sampling and Analysis

Milk yield was measured daily and the value was used to calculate the 3.5% FCM according to Sklan et

Table 1. Ingredient proportion and chemical composition of diets of dairy cows according to DCAD

Item	DCAD, mEq/kg of DM			
	−71	98	192	290
Ingredient, g/kg of DM				
Maize silage	600	600	600	600
Ground corn	205.63	212.38	214.32	209.72
46% CP soybean meal	153.45	146.47	145.78	150.23
Mineral mixture ¹	15.34	15.34	15.34	15.34
NaCl	5.11	7.67	7.93	—
Sodium bicarbonate	—	2.25	3.58	11.66
Ammonium sulfate	14.83	5.68	—	—
Urea	2.56	7.16	9.97	9.97
Dicalcium phosphate	2.56	2.56	2.56	2.56
Magnesium oxide	0.56	0.56	0.56	0.56
Chemical composition, g/kg of DM (unless otherwise noted)				
DM, g/kg of natural matter	609	609	608.9	608.9
OM	918.60	918.70	919.90	919.60
CP	163.17	161.13	161.13	163.07
Ether extract	27.70	27.90	27.98	27.80
TDN ²	665.17	670.56	674.33	677.00
NE _L ² Mcal/kg of DM	1.51	1.52	1.53	1.54
NDF	351.41	350.90	350.90	351.41
NDFap ³	321.65	321.87	321.91	321.97
ADF	190.70	190.10	190.10	190.50
Neutral detergent insoluble CP	29.76	29.02	28.98	29.44
Acid detergent insoluble CP	11.77	11.74	11.76	11.77
NFC ⁴	401.59	409.73	414.18	417.40
ADL	21.30	21.20	21.20	21.30
Ash	6.54	6.51	6.39	5.84
Ca	6.14	6.14	6.14	6.14
P	3.15	3.13	3.13	3.07
Na	3.38	4.96	5.42	4.65
K	10.23	10.08	10.08	10.13
Cl	3.89	5.12	5.52	0.87
S	5.93	3.71	2.35	2.35

¹Mineral mixture composition per kilogram: 242 g of Ca [minimum (min)], 30 mg of Co (min), 1,008 mg of Cu (min), 80 g of S (min), 390 mg of Fe (max), 39 g of P (min), 60 mg of I (min), 20 g of Mg (min), 2,998 mg of Mn (min), 1,100 mg of monensin sodium (min), 30 mg of Se (min), 4,032 mg of Zn (min), 400,000 IU of vitamin A (min), 40,000 IU of vitamin D₃ (min), and 1,450 IU of vitamin E (min).

²Estimate by NRC (2001).

³NDFap = neutral detergent fiber corrected to ash and protein.

⁴Calculated according to Hall (2000).

al. (1994). Milk samples were collected from d 15 to 16 of each period, chilled, and preserved with 2-bromo-2-nitropropane-1,3-diol (0.05%, wt/vol) for determination of fat, lactose, TS, casein, and CP by infrared absorption (Bentley Instruments, 1995) and milk freezing point by Fourier transform infrared spectroscopy (Foss Analytical, 2008). Milk SNF was obtained by the difference between fat and TS content. The MUN content was determined by an enzymatic and colorimetric methodology (Bentley Instruments, 1998).

In each experimental period, milk samples were collected for immediate determination of iCa concentration using a potentiometer with selective electrode and pH by potentiometry (Barros et al., 1999). Individual milk samples from all cows were collected and frozen at −20°C for analysis of Ca, P, Na, and K by flame

emission spectrophotometer (Thermo Fisher Scientific, 2008), and the chloride (Cl) content was determined according to the Mohr method (AOAC International, 1995).

Composite milk samples from 2 daily milkings were collected and stored for 12 h at 5°C before the determination of MES at the following concentrations (vol/vol): 68, 70, 72, 74, 76, 78, and 80% ethanol (Zanela et al., 2006). Results were expressed by the lowest concentration of alcohol in which coagulation occurred. In addition, milk heat stability was assessed by determination of coagulation time as follows: glass capillaries (7.00 cm long, 0.15 cm external diameter, and 0.1 cm internal diameter) were individually filled with milk samples, heat sealed, and submitted to immersion in glycerin to heating at 140°C. The time taken for the milk samples

to clot was recorded from the capillary introduction in the glycerin bath at 140°C until the appearance of the first visible signs of coagulation (Negri et al., 2003).

Representative milk samples from 2 daily milkings were collected and frozen (−20°C) until analysis of the milk caseins subunits (α , β , and κ) and whey proteins (α -LA and β -LG) concentration. Each sample was evaluated in duplicate by HPLC according to the methodology proposed by Bobe et al. (1998). Separation and identification of the proteins were performed at 220 nm in a HPLC system (Shimadzu, Kyoto, Japan) equipped with UV detector and Jupiter C18 column (4 μ m, 4.6 $\times$ 150 mm; Phenomenex, Torrance, CA). Chromatographic run was carried out at room temperature using mobile phases solvent A (acetonitrile:water:trifluoroacetic acid, 100:900:1) and solvent B (acetonitrile:water:trifluoroacetic acid; 900:100:1). The gradient program started with 25% solvent B, and the proportion of the solvent was gradually increased after the injection of the sample [34 (4), 48 (11), 50 (13), and 10% (17 min)] and returned to the initial conditions after 2 min. Flow rate was adjusted at 1.0 mL/min (Oliveira et al., 2013). Retention times for α -S-, β -, and κ -CN, α -LA, and β -LG, respectively, were 12.3, 12.9, 9.1, 14.4, and 14.1 min.

Quantification of casein fractions (α -S₁, α -S₂, β , and κ) and whey proteins was performed by measuring peak areas of the samples and then plotting them against the calibration curves of each casein fraction. Purified α -S-, β -, and κ -CN, α -La, and β -LG standards (Sigma, St. Louis, MO) were diluted in distilled water, and aliquots were frozen at −20°C. Individual protein standards were prepared in the same way described for milk samples, at the following concentrations: α -S-CN: 0.5, 1.0, 2.0, and 4.0 mg/mL; β -CN: 0.375, 0.75, 1.50, and 3.0 mg/mL; κ -CN: 0.187, 0.375, 0.75, and 1.50 mg/mL; α -LA: 0.125, 0.250, 0.375, and 0.5 mg/mL; β -LG: 0.25, 0.5, 0.75 and 1.0 mg/mL. The α -S₁: α -S₂-CN ratio was assumed to be 4:1 (wt/wt; Bobe et al., 1998).

Statistical Analysis

The data were analyzed using SAS (version 9.2, SAS Institute Inc., Cary, NC) after testing for residual normality and homogeneity of variance. The data were analyzed using the MIXED procedure of SAS according to the following model:

$$Y_{ijkl} = \mu + T_i + S_j + C_{k(j)} + P_l + e_{ijkl},$$

where Y_{ijkl} = dependent variable; μ = overall mean; T_i = fixed effect of treatment i (3 df); S_j = fixed effect of Latin square j [1 to 4 (3 df)]; $C_{k(j)}$ = random effect of cow k within each Latin square [$k = 1$ to 16 (12 df)]; P_l

= fixed effect of period l [1 to 4 (3 df)]; e_{ijkl} = random error associated to each observation.

The orthogonal polynomials coefficients were obtained using the IML procedure of SAS because the treatments were not equally spaced. The treatment effect was decomposed into 3 orthogonal polynomial contrasts (linear, quadratic, and deviation). Intercepts and slopes were calculated using the estimate option of the MIXED procedure of SAS. In addition, Pearson correlation was used to evaluate the association between heat and ethanol milk stability. For all statistical analyses, significance was declared at $P \leq 0.05$ and trends at $P \leq 0.10$.

RESULTS

In the present study, the DMI increased in a quadratic ($P < 0.0001$) form with increasing DCAD. According to the equation [$Y = 22.63$ (SE = 0.94) + 0.0185 (SE = 0.0032) $\times$ DCAD (mEq/kg of DM) − 0.00008 (SE = 0.000013) $\times$ DCAD² (mEq/kg of DM²)], the DMI peak was obtained with DCAD of 115.62 mEq/kg of DM, where the DMI estimate was 23.70 kg/d. Similar to DMI, ingestion of CP, NDF, EE, Ca, P, Na, and K were also increased in a quadratic form as DCAD increased. The maximum nutrient intake (CP, NDF, EE, Ca, P, Na, and K) was observed when DCAD was between 100 and 150 mEq/kg of DM, where the maximum consumption of CP and NDF was obtained with DCAD of 125 and 118.33 mEq/kg of DM, respectively (Table 2).

Changes of DMI and metabolic status of dairy cows may alter milk yield and composition. In the current study, milk yield (MY) and FCM were increased in the quadratic ($P = 0.020$) and linear ($P < 0.0001$) form, respectively, according to the increase of DCAD. The point of maximum milk yield (22.35 kg/d) was obtained with DCAD of 171.67 mEq/kg of DM [$Y = 21.47$ (SE = 1.22) + 0.0103 (SE = 0.0038) $\times$ DCAD (mEq/kg of DM) − 0.00003 (SE = 0.000012) $\times$ DCAD² (mEq/kg of DM²)]. By increasing the DCAD from −71 to 290 mEq/kg of DM, the FCM increased 2.9 kg/d [$Y = 22.23$ (SE = 1.19) + 0.008798 (SE = 0.0016) $\times$ DCAD (mEq/kg of DM)]. Conversely, productive efficiency (MY/DMI) was reduced in a quadratic ($P = 0.004$) form with increasing DCAD. Milk concentrations of fat, lactose, TS, and MUN increased linearly with increasing DCAD, whereas milk content of SNF increased in a quadratic form. According to the increase of DCAD from −71 to 290 mEq/kg of DM, the milk fat content was 8.71% higher [$Y = 3.82$ (SE = 0.15) + 0.0008 (SE = 0, 0003) $\times$ DCAD (mEq/kg of DM)]. In contrast, the milk content of CP and casein decreased linearly ($P = 0.004$ and $P = 0.039$, respectively) as DCAD increased.

Table 2. Effect of DCAD of dairy cows on dry matter and nutrients intake

Intake, kg/d (unless otherwise noted)	DCAD, mEq/kg of DM					P-value ¹		
	–71	98	192	290	SEM	L	Q	D
DM, % of BW	3.41	3.82	3.85	3.45	0.062	0.215	<0.0001	0.255
NDF, % of BW	1.21	1.36	1.37	1.22	0.024	0.300	<0.0001	0.239
DM	20.96	23.44	23.53	21.17	0.337	0.227	<0.0001	0.326
OM	19.38	21.69	21.91	19.62	0.359	0.181	<0.0001	0.228
CP	3.16	3.49	3.50	3.19	0.054	0.332	<0.0001	0.421
Ether extract	0.60	0.67	0.68	0.63	0.010	0.015	<0.0001	0.572
NDF	7.62	8.55	8.61	7.64	0.154	0.304	<0.0001	0.237
Ca	0.117	0.130	0.131	0.118	0.002	0.333	<0.0001	0.407
P	0.094	0.105	0.105	0.094	0.002	0.459	<0.0001	0.338
Na	0.062	0.098	0.107	0.084	0.002	<0.0001	<0.0001	0.002
K	0.214	0.235	0.247	0.216	0.004	0.123	<0.0001	0.011
Cl	0.074	0.110	0.110	0.018	0.005	<0.0001	<0.0001	<0.0001
S	0.112	0.080	0.052	0.046	0.003	<0.0001	0.003	<0.0001

¹L = probability of linear effect, Q = probability of quadratic effect, D = deviation.

As a result of the effects of DCAD on milk yield and composition, the daily production of milk components was also modified by the DCAD levels studied. An increase in milk fat production was observed in a linear ($P = 0.0003$) form according to increase of DCAD, whereas CP, casein, lactose, SNF, and TS production increased in a quadratic form (Table 3). In response to the increased milk production, the point of maximum daily milk TS production was achieved when the DCAD was 170.21 mEq/kg of DM. However, the point of maximum production of CP and casein were reached when the DCAD was 118.57 and 130 mEq/kg of DM, respectively.

In the present study, no effect of DCAD was noted on concentrations of α - (s_1 and s_2) and β -CN and α -LA.

However, milk content of κ -CN decreased linearly ($P = 0.031$) with increasing DCAD, whereas milk content of β -LG increased in a linear ($P = 0.002$) form. Therefore, when the DCAD increased from –71 to 290 mEq/kg of DM, the milk κ -CN concentration reduced 7.69%, whereas the β -LG level increased 17.14%. However, a tendency ($P = 0.089$) was noted for a linear reduction in the proportion of κ -CN in total CP as DCAD increased. The concentration of β -LG linearly ($P = 0.006$) increased in 0.79 g/100 g of CP according to an increase in the DCAD from –71 to 290 mEq/kg of DM (Table 4).

The mineral composition of milk was affected by DCAD, as the milk concentrations of iCa and chloride increased in a quadratic ($P = 0.009$) and linear ($P =$

Table 3. Effect of DCAD of dairy cows on milk yield and composition

Item	DCAD, mEq/kg of DM					P-value ¹		
	–71	98	192	290	SEM	L	Q	D
Milk yield, kg/d	20.71	22.17	22.66	22.05	0.599	0.004	0.020	0.520
FCM, kg/d	21.42	23.32	24.49	24.32	0.611	<0.0001	0.127	0.367
Fat, kg/d	0.76	0.84	0.88	0.87	0.022	0.001	0.095	0.434
CP, kg/d	0.66	0.69	0.72	0.66	0.012	0.509	0.017	0.213
Casein, kg/d	0.49	0.51	0.53	0.48	0.009	0.612	0.008	0.120
Lactose, kg/d	0.89	0.97	0.99	0.97	0.027	0.001	0.001	0.514
SNF, kg/d	1.73	1.88	1.91	1.82	0.042	0.011	0.002	0.455
TS, kg/d	2.51	2.72	2.80	2.69	0.061	0.002	0.008	0.396
Fat, g/100 g	3.76	3.86	4.00	4.09	0.071	0.001	0.661	0.644
CP, g/100 g	3.31	3.25	3.26	3.15	0.052	0.004	0.352	0.219
Casein, g/100 g	2.45	2.39	2.42	2.34	0.046	0.039	0.586	0.177
Casein, % CP	73.95	73.73	74.14	73.30	0.370	0.242	0.296	0.121
MUN, mg/dL	17.71	19.07	18.90	21.43	0.522	0.002	0.264	0.241
Lactose, g/100 g	4.32	4.39	4.42	4.43	0.047	<0.0001	0.340	0.902
SNF, g/100 g	8.55	8.58	8.64	8.53	0.063	0.909	0.182	0.191
TS, g/100 g	12.32	12.44	12.64	12.64	0.120	0.005	0.857	0.313
BW, kg	617	617.5	620.44	615.94	57.540	0.977	0.646	0.581
Productive efficiency (milk yield/DMI)	1.04	0.98	0.99	1.03	0.034	0.675	0.004	0.911

¹L = probability of linear effect, Q = probability of quadratic effect, D = deviation.

Table 4. Effect of DCAD of dairy cows on caseins subunits and whey proteins

Protein	DCAD, mEq/kg of DM					<i>P</i> -value ¹		
	−71	98	192	290	SEM	L	Q	D
α _{S1} -CN, mg/mL of milk	6.38	6.36	6.52	6.17	0.120	0.404	0.165	0.118
α _{S2} -CN, mg/mL of milk	1.58	1.58	1.62	1.53	0.030	0.455	0.167	0.113
β-CN, mg/mL of milk	7.34	7.29	7.38	7.10	0.195	0.423	0.482	0.449
κ-CN, mg/mL of milk	5.07	4.96	4.95	4.68	0.154	0.031	0.361	0.478
β-LG, mg/mL of milk	1.40	1.53	1.55	1.64	0.077	0.002	0.951	0.580
α-LA, mg/mL of milk	0.84	0.76	0.74	0.77	0.053	0.115	0.172	0.815
α _{S1} -CN, g/100 g of CP	19.58	19.96	20.35	19.90	0.341	0.274	0.281	0.419
α _{S2} -CN, g/100 g of CP	4.90	4.99	5.04	4.97	0.080	0.376	0.392	0.716
β-CN, g/100 g of CP	22.63	22.88	23.38	23.06	0.607	0.325	0.660	0.517
κ-CN, g/100 g of CP	15.19	14.39	14.47	14.20	0.300	0.089	0.568	0.642
β-LG, g/100 g of CP	4.39	4.57	4.61	5.18	0.277	0.006	0.164	0.402
α-LA, g/100 g of CP	2.55	2.40	2.27	2.46	0.164	0.360	0.210	0.410

¹L = probability of linear effect, Q = probability of quadratic effect, D = deviation.

0.014) form, respectively, by reducing the DCAD. The lowest milk concentrations of iCa were obtained when DCAD was 169 mEq/kg of DM. In addition, the reduction of DCAD tended ($P = 0.070$) to linearly increase the K concentration and, in a quadratic form ($P = 0.096$), the Na concentration in the milk. However, the daily production (kg/d per cow) of P, Na, K, and Cl was not affected by DCAD (Table 5).

In addition to changing the performance of lactating dairy cows, the DCAD may alter the resistance of milk proteins to form clots during the ethanol reaction or during heat treatment. In the present study, the milk ethanol and heat stability linearly ($P < 0.0001$ and $P = 0.0005$, respectively) decreased according to the DCAD reduction (Table 6). By reducing the DCAD, from 290 to −71 mEq/kg of DM, the resistance of milk to form clots during heating at 140°C was reduced by 4.77 min. Similarly, by reducing the DCAD, the ethanol concentration required in the alcohol solution was reduced by 4.6% to cause milk precipitation (Figure 1). The milk freezing point and pH of milk were not

altered by DCAD. In addition, we observed medium ($r = 0.45$) significant ($P = 0.01$) correlation between heat and ethanol stability.

DISCUSSION

DMI and Milk Yield

In the present study, the DM and nutrients (CP, NDF, EE, Ca, P, Na, and K) intake increased in a quadratic form with increasing DCAD ($P < 0.0001$). Similar results were reported by Hu and Murphy (2004), who conducted a meta-analysis and found that increasing DCAD levels quadratically elevated the DMI of dairy cows (maximum intake observed when DCAD was 395.52 mEq/kg of DM). However, Hu and Murphy (2004) did not include S in the DCAD calculation and used the following formula: $\text{DCAD} = \text{Na} + \text{K} - \text{Cl}$. This difference of the calculation methodology of DCAD may affect the optimal DCAD recommended for lactating dairy cows when compared with other studies

Table 5. Effect of DCAD of dairy cows on mineral milk content and daily secretion

Minerals, g/100 g (unless otherwise noted)	DCAD, mEq/kg of DM					<i>P</i> -value ¹		
	−71	98	192	290	SEM	L	Q	D
Milk content								
iCa, mg/L	92.34	80.10	78.61	82.17	1.984	0.002	0.009	0.914
K	0.101	0.101	0.095	0.092	0.003	0.070	0.544	0.478
Na	0.074	0.070	0.069	0.074	0.002	0.757	0.096	0.599
Cl	0.130	0.116	0.118	0.117	0.003	0.014	0.101	0.319
P	0.074	0.076	0.077	0.073	0.001	0.977	0.137	0.456
Daily secretion								
K	0.020	0.024	0.022	0.020	0.001	0.942	0.080	0.452
Na	0.015	0.015	0.015	0.016	0.000	0.327	0.770	0.646
Cl	0.026	0.026	0.027	0.025	0.001	0.770	0.326	0.231
P	0.017	0.016	0.017	0.016	0.001	0.337	0.517	0.084

¹L = probability of linear effect, Q = probability of quadratic effect, D = deviation.

Table 6. Effect of DCAD of dairy cows on milk ethanol and heat stability

Item	DCAD, mEq/kg of DM				SEM	<i>P</i> -value ¹		
	−71	98	192	290		L	Q	D
Milk ethanol stability, ² %	73.42	76.61	76.40	78.02	0.560	<0.0001	0.468	0.251
Coagulation time, ² min	2.42	5.69	6.12	7.19	0.550	0.001	0.403	0.628
pH	6.73	6.75	6.70	6.80	0.021	0.130	0.080	0.060
Cryoscopy, °H	−0.535	−0.537	−0.539	−0.533	0.002	0.880	0.066	0.385

¹L = probability of linear effect, Q = probability of quadratic effect, D = deviation.

²Milk ethanol stability = minimum concentration of ethanol in the alcohol solution (vol/vol) necessary for precipitation; coagulation time = necessary time for milk precipitation at 140°C. Correlation between milk ethanol stability and coagulation time: $r = 0.45$ ($P < 0.01$).

that included S in the calculation. Delaquis and Block (1995) studied cows in the middle stage of lactation and reported increased DMI from 15.6 to 17.0 kg/d when the DCAD increased from 140.2 to 372.7 mEq/kg of DM. The lower DMI observed by reducing the DCAD, particularly in diets with a negative DCAD, may be associated with the addition of anionic salts, such as ammonium sulfate used in our study. Anionic salts (usually sodium chloride and sulfur sources) have low acceptance by cows and can therefore reduce the DMI (West et al., 1991). The DMI may also be reduced by decreasing the DCAD in response to metabolic acidosis, by reducing serum concentrations of bicarbonate (Sharif et al., 2010). Changes in rumen microbial activity due to the reduction of DCAD were also associated with the DMI decrease. To reduce the DCAD, the replacement of sodium bicarbonate by anionic salts may reduce ruminal pH, and therefore decrease the optimum conditions for the activity of cellulolytic bacteria and reduce the rate of NDF digestion, the rate of passage through total gastrointestinal tract, and, consequently, the DMI (Sharif et al., 2010).

Changes of DMI, ruminal fermentation, and blood ionic balance are factors that can directly affect the flow of nutrients to the mammary gland and thus the MY (Hu and Murphy, 2004; Sharif et al., 2010). In our study, the MY increased in a quadratic form as DCAD increased. Similarly, Hu and Murphy (2004) also reported that the increase of DCAD increased the MY in a quadratic form and that the DCAD of 490 mEq/kg of DM was the optimum level for FCM (4% of fat). Similar results were described by West et al. (1991) and Delaquis and Block (1995), who reported a positive association between MY and DCAD. Alternately, Roche et al. (2005) studied the effects of high DCAD of early lactation dairy cows grazing pasture and reported no effect of increasing DCAD (from 230 to 880 mEq/kg of DM) on DMI and MY. In the present study, MY increased in response to the DCAD increase (from −71 to 290 mEq/kg of DM) probably due to the higher DM and nutrient intake. In response to the higher consumption, the availability of nutrients to ruminal fermenta-

tion and intestinal absorption may be increased, which may result in a higher flow of nutrients to the mammary gland to be used in milk synthesis. In addition, with the increasing DCAD, available acids in the bloodstream can be neutralized by increasing the concentration of current HCO_3^- , which facilitates the entry of glucose into cells due to high insulin activity. With the higher uptake of glucose into cells, the lactose synthesis in the mammary gland may be increased, which may contribute to the rise of MY by osmotic pressure exerted by lactose in the milk synthesis (Block, 1994; Hu and Murphy, 2004; Sharif et al., 2010).

Milk Composition and Stability

Fat content is an important component for dairy processing yield and may vary according to the diet, especially when changes occur in rumen microbial activity. In our study, the fat content of milk increased linearly with increasing DCAD. Similar results were reported by Delaquis and Block (1995), Roche et al. (2005), and Apper-Bossard et al. (2010), who reported higher fat content in milk when cows were fed increasing DCAD. This increased fat content of milk may be a response to the positive effect of DCAD on rumen pH after feeding (Apper-Bossard et al., 2010), which may result in a more stable rumen microbial activity, especially of cellulolytic bacteria that present great growth at around neutral pH (Allen, 1997). Thus, with proper conditions for ruminal microbial activity, the biohydrogenation process of unsaturated FA can be higher than when the ruminal pH is lowered by decreasing the formation of intermediate FA (*trans*), which are negatively associated with the synthesis of fat in the mammary gland (Bauman and Griinari, 2003). Additionally, Roche et al. (2005) reported that increasing DCAD might increase DMI as well as short-chain FA synthesis in the rumen, providing more substrate for *de novo* FA production, which may contribute to increase the milk fat content.

Lactose is an important osmoregulator of MY, and the milk content of lactose is less influenced by nutritional factors when compared with other milk compo-

Figure 1. Effect of DCAD (mEq/kg of DM) on concentrations of κ -CN [$Y = 5.02$ (SE = 0.30) $- 0.00098$ (SE = 0.0004) $\times$ DCAD (mEq/kg of DM)], β -LG [$Y = 1.46$ (SE = 0.15) $+ 0.00065$ (SE = 0.0002) $\times$ DCAD (mEq/kg of DM)], ionic calcium [iCa; $Y = 85.78$ (SE = 4.98) $- 0.08$ (SE = 0.01) $\times$ DCAD (mEq/kg of DM) $+ 0.000237$ (SE = 0.00008) $\times$ DCAD² (mEq/kg of DM)²], MUN [$Y = 18.22$ (SE = 1.36) $+ 0.0094$ (SE = 0.0026) $\times$ DCAD (mEq/kg of DM)], milk ethanol stability [MES; level of ethanol in alcohol solution necessary to cause milk precipitation; $Y = 74.87$ (SE = 0.87) $+ 0.01174$ (SE = 0.0025) $\times$ DCAD (mEq/kg of DM)], and coagulation time [CT; necessary time to coagulate the milk during the heating at 140°C; $Y = 3.95$ (SE = 1.02) $+ 0.01234$ (SE = 0.0032) $\times$ DCAD (mEq/kg of DM)] in milk.

nents, such as fat and protein. In our study, the milk content of lactose increased linearly in response to the DCAD increase. Similar results were observed by Hu et al. (2007), who reported that the concentration of lactose in milk increased linearly according to the increase of DCAD. The increased lactose content of the milk resulting from increased DCAD may be associated with increased DMI and greater availability of glucose

in the mammary gland, as by reducing the DCAD the availability of acid in the blood can be increased due to high consumption of anions, which could hamper insulin activity and the entry of glucose into cells (Block, 1994).

As a result of the increase of milk fat and lactose content, the TS content also increased linearly according to the increase of DCAD. However, the milk content

of SNF was not changed by diet. This may be a result of the negative effect of increased DCAD on the protein content, offset by an increase of the lactose milk content, which resulted in the lack of DCAD effect on milk SNF content. The reduction in CP content of milk in response to the increased DCAD probably occurred by linear reduction of milk content of casein. These results differ from those reported by Hu et al. (2007), who observed an increase in protein content of milk when evaluating DCAD of 30, 220, and 470 mEq/kg of DM. Otherwise, Hu and Murphy (2004) and Roche et al. (2005) found no effect of DCAD on the CP content of milk.

Milk mineral composition is relatively constant, but can vary according to the metabolic status of dairy cows. In our study, the reduction of DCAD only tended to alter the milk concentration of Na and K, and did not change the P content. However, in the present study, chloride concentration in the milk was increased linearly as DCAD reduced. The increase in milk concentration of chloride may have occurred in response to metabolic acidosis resulting from the DCAD reduction, as the excretion of anions by body fluids, such as milk and urine, may be increased to compensate acidosis (Block, 1994). The iCa concentration in milk was high with the DCAD reduction. This result probably occurred through mechanisms controlled by hormones such as the parathyroid, calcitonin, and 1,25-dihydroxycholecalciferol, which may alter the intestinal absorption, bone reabsorption, and renal calcium excretion to increase the concentration of this mineral in the blood in an attempt to compensate for the metabolic acidosis (Block, 1994; Marques et al., 2011). As a result of this increase of iCa in blood, the concentration of iCa in milk may also be high with the metabolic acidosis (Marques et al., 2011). Using a different experimental design, Roche et al. (2005) reported no effects of high DCAD (230 to 880 mEq/kg of DM) on the mineral composition of milk.

Calcium may be present in the colloidal phase (bound to casein micelles) or in the soluble phase of milk, and the ionic balance between these 2 phases and with other milk minerals is important to maintain casein stability. In the present study, in response to increasing iCa concentration of milk, the milk ethanol and heat stability were linearly reduced. A similar result was reported by Marques et al. (2011), who found decreased ethanol stability with increasing iCa concentration in milk. According to Barros et al. (1999), when milk concentrations of iCa increased a reduction was noted in the negative charges of casein micelles and in the electrostatic repulsion forces between them. Thus, increased iCa activity in milk may reduce the electrostatic and steric repulsion between casein micelles

and probably increases their Van der Waals attraction, which may raise the micelle interactions and reduce the heat stability of milk (Jeurnink and De Kruif, 1995).

In addition to altering the ionic balance between the subunits of casein, the milk concentration of iCa may enhance the interactions between the κ -CN, which is hydrophilic and stays in the outer layer of the micelle, and β -LG, which has reactivity with κ -CN and can be associated with milk stability. In our study, the milk content of κ -CN increased linearly with DCAD reduction. A similar result was described by Marques et al. (2011), who observed a trend ($P = 0.09$) for increased κ -CN content of milk from cows fed an anionic diet compared with milk from cows fed a cationic diet. In a different form, Roche et al. (2005) no found effects of high DCAD on the concentrations of casein subunits and whey proteins. In our study, the reduction of DCAD led to a linear decrease in the concentration of β -LG in milk. The β -LG shows reactivity with κ -CN in the micelle, with the intensity of interactions between these proteins depending on the milk concentration of free calcium (Singh and Fox, 1987). Jeurnink and De Kruif (1995) reported that β -LG might react in 3 ways with others proteins: forming aggregates with other β -LG molecules, forming a complex with κ -CN at the surface of the casein micelle, and forming a complex with the κ -CN in the serum phase of milk. The milk concentration of free calcium is a factor that determines the intensity and the site of formation of protein complexes, and according to Jeurnink and De Kruif (1995) with increasing the calcium ion activity a β -LG/ κ -CN complex is formed at the surface of the casein micelle. Thus, in our study, in response to metabolic acidosis resulting from the DCAD reduction, the increase in iCa concentration in the milk may have enhanced the association of β -LG with κ -CN in the micelle, which can facilitate the formation of protein complex between these 2 proteins (Jeurnink and De Kruif, 1995; Holt et al., 2013).

Besides the iCa concentrations and interactions between milk proteins, other factors, such as the MUN concentration, can contribute to change the milk stability. In our study, the MUN concentration reduced according to the DCAD reduction, which may have contributed to reducing milk stability. The MUN can inhibit the formation of acids and act on the thiol groups of proteins. In addition, urea may be converted to cyanide, which unlike calcium, reacts with milk protein and increases the negative charges of the micelles, which increases the repulsive forces among them and, consequently, milk stability (Sweetsur and Muir, 1981).

The metabolic acidosis in lactating dairy cows can occur as a result of DCAD reduction or from ruminal acidosis or high metabolic rate of dairy cows (Barros

et al., 1999; Marques et al., 2011; Fischer et al., 2012). Thus, diets with high levels of NFC or low eNDF may also contribute to the occurrence of low milk stability, as ruminal acidosis may result in metabolic acidosis (Ponce and Hernández, 2005; Fischer et al., 2012). Moreover, as described by Barbosa et al. (2012), the deficiency of dietary nutrients can also reduce milk stability. Stumpf et al. (2013) reported that feed restriction increases permeability of mammary gland cell tight junctions and reduces the MES, probably due a higher passage of ions to milk. Therefore, to prevent the occurrence of low milk stability, the high nutrient demand of lactating dairy cows must be met by the use of concentrated feeds; however, measures such as adequate supply of NDF or eNDF and the use of buffering or alkaline in the diet of dairy cows must be considered to avoid overproduction of VFA in the rumen fluid that can contribute to acidification and changes in the acid or base blood balance. Furthermore, changes in the forage or concentrated ingredients or mineral supplements without prior adaptation should also be avoided to prevent digestive problems (Fischer et al., 2012), changes in DCAD, and, consequently, problems of low milk stability.

In Brazil, the main exported dairy products are UHT milk, powdered milk, and condensed milk (Brazil, 2014); thus, milk instability causes high financial losses to the dairy agroindustrial chain (Fischer et al., 2012). Marques et al. (2007) reported that during a 17-mo evaluation period, 58% of the samples were unstable to the reaction with ethanol (76% vol/vol ethanol in the alcohol solution), but without acquired acidity (titratable acidity average of 15.81°D). The low stability of milk causes losses to the dairy farmers, who suffer a penalty in milk payment; for the dairy industry, which cannot use this low-stability milk for the production of dairy products, such as UHT and powdered milk; and for the consumer, as milk with low stability may have a reduced shelf life because the β -LG/ κ -CN complex can be formed during high temperature treatment of milk and can lead to irreversible gel formation during storage (i.e., micelle aggregation to form a continuous 3-dimensional network; McMahon, 1995; García-Risco et al., 1999; Holt et al., 2013). McMahon (1995) observed by electron micrographs that casein micelles from aged UHT milk sometimes reveal protuberances, spikes, or tendrils comprising complexes of β -LG and κ -CN. According to McMahon (1995), during UHT milk storage at room temperature the casein micelle size increases, and some factors were associated with gelation, such as proteolysis, physiochemical changes, and aggregation of β -LG/ κ -CN complexes that form and subsequently dissociate from the casein micelle in UHT milk. Thus, the

metabolic acidosis in lactating dairy cows may enhance the gel formation during milk storage, as the increased milk concentration of iCa may potentiate the β -LG and κ -CN interaction (Hambling et al., 1992; Jeurink and De Kruif, 1995).

In our study, a moderate correlation was observed between milk stability to ethanol and stability at 140°C ($r = 0.45$; $P = 0.01$). Similar to our results, Molina et al. (2001) and Chavez et al. (2004) reported low or medium correlation between milk stability to ethanol and other heat treatment measurements, such as clotting on the boiling test and coagulation time. In many countries that use UHT as the main form of fluid milk processing, some dairy industries have been using higher ethanol concentrations (from 72 to 82%) in the alcohol test aiming to select milk with high stability. However, the use of MES as a screening test for acceptance raw milk for heat processing may not provide satisfactory results, as MES had limited capacity to estimate milk heat stability. Thus, future studies are need to propose accurate and precise methods to estimate milk heat stability, which can improve the efficiency of dairy agroindustrial chain, as low-stability milk without acquired acidity by microbial contamination could be adequately identified and subjected to less intense heat processing, such as pasteurization (Fischer et al., 2012).

CONCLUSIONS

Concentrations of iCa and κ -CN in milk increase according to the DCAD reduction, whereas MUN and β -LG concentrations decrease. Because of these changes, milk stability to ethanol and during heating at 140°C is decreased linearly with DCAD reduction due to changes in the milk ionic balance and the interactions among the milk proteins in micelle. Thus, the results of the present study suggest that the control of metabolic changes in lactating dairy cows to maintain the blood acid-base status in equilibrium play an important role to maintain milk stable to ethanol and during heat treatments.

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