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Feeding Protected Lysine and Methionine Modifies Milk Protein Profile in Grazing Dairy Cows

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Abstract

The experiment was designed to determine the effect of protected lysine (Lys) and methionine (Met) supply on milk protein profile in grazing dairy cows specifically in the caseins (CNs) and α-lactalbumin fractions. Twelve multiparous mid lactation Holstein cows producing 24 (±4.76) kg of milk were assigned to one of two treatments (six cows per treatment) during an experimental period of 21 days. In the control (C) group, cows grazed a *Pennisetum clandestinum* pasture and were supplemented with a commercial concentrate according to milk production. In the Met-Lys treatment, cows received the same ration supplemented with protected Lys and Met. Milk yield and composition and milk protein profile were measured at the start and the end (21st day) of the experimental period. The Tricine-SDS-PAGE and the Gel-Quant Express Analysis (Invitrogen) software were used to determine milk protein composition. Statistical analysis was performed using the SAS's PROC MIXED procedure through a mixed model that included the animal as a random effect and the treatments as a fixed effect adjusted by covariables. Milk production averaged 23.7 (±2.0) kg cow^{-1} day^{-1} without differences between treatments ($P < 0.96$). Yield of fat corrected milk (4% FCM) tended ($P < 0.10$) to increase in the Met-Lys treatment (26.0 kg cow^{-1} day^{-1}) compared to C (24.2 kg cow^{-1} day^{-1}). Milk protein content (g/kg) did not differ (C = 30.4; Met-Lys = 31.1) and lactose content tended ($P < 0.08$) to be higher in the Met-Lys (47.4) group compared to C (45.9). Milk protein content (g/kg) of αS1-CN resulted higher ($P < 0.046$) in Met-Lys (10.58) compared to C (9.44). Concentration of β-CN also increased ($P < 0.05$) after protected aminoacid supply (C = 9.58; Met-Lys = 10.35). It can be concluded that milk protein composition was improved by protected Lys-Met supply without altering other compositional parameters of milk composition. Milk nutritional quality and its potential yield for cheese-making were positively enhanced.

Keywords

Grazing Dairy Cows, Protected Amino Acids, Milk Proteins

1. Introduction

Milk is an important food source both, in its natural form and also in a wide variety of processed dairy products. Approximately 80% of milk proteins are made up of caseins (CNs) being some of the most valuable components of dairy products because of their nutritional value and their important role and influence in the quality of cheese production [1]. Milk proteins and related peptides are classified into four different groups: CNs made up of αS1-CN, αS2-CN, β-CN, and κ-CN; serum proteins which include α-lactalbumin (α-LA), β-lactoglobulin (β-Lg), bovine serum albumin (BSA); immunoglobulins (Igs) and other minor milk serum proteins, such as proteases and proteins of the fat globule membranes [2]. CNs are a product of the expression of four genes which encode four polypeptide chains αS1-CN, αS2-CN in a 4:1 ratio, β-CN and κ-CN in a 4:1 ratio. CNs undergo posttranscriptional modifications, which allow them to interact with calcium phosphate ($Ca_3(PO_4)_2$ and thus form micelles. The phosphoserine cluster of αS1-CN, αS2-CN and β-CN stabilizes phosphate nanoclusters of calcium, crossing each other and thus increasing the size of the micelles [3] [4] [5]. Phosphorylation levels of αS-CN, according to some authors, could stabilize the internal structure of the micelle, which would positively affect the properties of nutritional and industrial milk [3] [6]. The following variations in CN types have been found in the *Bos* genus [7]: 9 of αS1-CN (A, B, C, D, E, F, G, H, I), 4 of αS2-CN (A, B, C, D), 12 of β-CN (A1, A2, A3, B, C, D, E, F, G, H1, H2, I), 14 of κ-CNs (A, A1, B, B2, C, D, E, F1, F2, G1, G2, H, I, J), 3 of α-LA 100 (A, B, C), and 11 of β-Lg (A, B, C, D, E, F, G, H, I, J, W) [8].

The protein synthesis in the mammary gland depends on the availability of specific amino acids (AAs) [9] being Met and Lys considered to be the most important ones [10] [11] [12]. These two AAs are the most limiting in dairy production grazing systems in which concentrates based on soy cake and corn grain are fed [13]. This deficiency can be verified by comparing the amino-acid profile of these feedstuffs with that of microbial protein and bovine milk [14].

The production and manufacture of CNs occur in the rough endoplasmic reticulum and their phosphorylation takes place in the Golgi apparatus being this event essential for the binding of organic and inorganic calcium and other metal ions. Phosphorylation is required for the building and integrity of micelles, and it has a potential influence on functional properties such as rennet-induced coagulation [15]. Although the four CNs (αS1-CN, αS2-CN, β-CN, and κ-CN) are phosphoproteins, αS1-CN and αS2-CN showed higher levels of phosphorylation and their phosphorylation profiles are more heterogeneous than those of β-CN and κ-CN. The amount of αS1-CN is 35% of the total CN in bovine milk,

and its phosphorylation level is one of the factors affecting the industrial properties of milk. Therefore, there is a growing interest to know if any dietary factors may contribute to the variation in the CNs profile of milk [16]. The study was designed to determine changes in the composition of several milk proteins such as CNs and *α*-LA when lactating dairy cows were supplemented with protected Met and Lys.

2. Materials and Methods

2.1. Animals and Treatments

The experiment was conducted at the "Betania" farm, located in Santa Rosa de Osos (Antioquia, Colombia) at 2500 meters above sea level with an average temperature of 14˚C and a relative humidity of 79%. The average annual rainfall is 2500 mm. Twelve lactating Holstein cows (547 ± 56.1 kg body weight) in their second to fourth lactation with 124.8 (±14.0) days in milk and a body condition score of 3.02 (±0.17) were used. At the start of the experiment cows were producing 24 (±4.76) kg milk with 28.9 (±1.7) g/100g protein and 34.5 (±4.2) g/100g fat. Cows grazeda pasture composed by kikuyugrass (*Pennisetum clandestinum*) in a rotational grazing system in the same paddock throughout the experiment with fresh water available all the time. Herbage biomass (kg DM ha^{-1}) was estimated by cutting samples of forage at the ground level with manual scissors in an area delimited by a metal frame of 0.125 m^2 in a total cutting area of 1 m^2 in each sampling. The total sample (8 subsamples of 0.125 m^2) was dried (65˚C for 48 hours) to determine the dry matter (DM) content. The area of the daily strip was established according to pasture allowance and adjusted to offer 30 kg DM cow^{-1} day^{-1} using electric fences. The cows were milked twice daily at 4:00 a.m. and 2:00 p.m.

The animals were randomly divided into two groups of six cows balanced by lactation number (C = 2.83 ± 0.98 and Met-Lys = 2.67 ± 0.82, respectively), body condition score (BCS, 3.00 ± 0.16 and 3.08 ± 0.20) and days in milk (131 ± 13.42 and 120 ± 13.86). In the C treatment, cows grazed the kikuyu pasture and were individually supplemented with a commercial concentrate during each milking time according to milk production. In the Met-Lys treatment, cows received the same ration with the addition of protected Lys and Met in the concentrate. The chemical composition of the pasture and the concentrate (**Table 1**) was determined according to methods described by AOAC [17]. Non-structural carbohydrates (NSC) and net energy of lactation (NE_L) were calculated using the following equations: NSC = 100 − (%NDF + % CP + % EE + % Ash), NE_L = 0.0245*TDN (%) − 0.12. Met and Lys concentration in the forage and concentrate were reported previously [18].

The Amino-Cow software [19] was used to estimate cows requirements of Met and Lys. Data showed a Met and Lys deficiency of 29.7% and 20.5% respectively in the total diet. In consequence, protected-Met (Mepron®, Evonik, Degussa AG, Germany) and protected-Lys (AjiProTM-L Ajinomoto, Tokyo, Japan)

Table 1. Chemical composition of the kikuyu pasture and concentrate.

	Kikuyu	Concentrate	RP-Met	RP-Lys
DM, g/kg	109	902	982	974
CP, g/kg DM	182	171	439	563
TDN, g/kg DM	604	760	-	-
EE, g/kg DM	31.2	39	10	427
NDF, g/kg DM	564	218	30	-
ADF, g/kg DM	314	165	-	-
ADL, g/kg DM	63.5	38.3		
ADICP, g/kg DM	18	11	-	-
NSC, g/kg DM	114	472		
NE_L, Mcal/kg DM	1.36	1.77	1.941	3.262
Ash, g/kg DM	109	100	1.501	
Calcium, g/kg DM	3.4	-	-	-
Phosphorus,	3.1	-	-	-
Soy lecithin, g/kg DM	-	-	-	10.02
Met, g/kg DM	3.1	5.8	453	
Lys, g/kg DM	9.5	10.9	-	377

RPMet: Rumen protected methionine (Mepron), LysP: Rumen protected lysine (AjiPro-L); DM: dry matter; CP: Crude protein, TDN = total digestible nutrients; EE: Ether extract, NDF: Neutral detergent fiber, ADF: Acid detergent fiber, ADL: Acid detergent lignin, ADICP: Acid detergent insoluble crude protein, NSC: Non-structural carbohydrates = 100 − (%NDF + % CP + % EE + % Ash), NEL: Net energy of lactation = 0.0245 *TDN (%) − 0.12, Met: Methionine, Lys: Lysine.

were added to the concentrate in sufficient quantities (7.03 ± 2.93 g of P-Met and 23.9 ± 3.82 g of P-Lys) to be released in the small intestine. The cows were offered the experimental diets from days 1st to 21th of the trial.

2.2. Pasture and Concentrate Intake

Pasture and concentrate intakes were estimated using chromium oxide (Cr_2O_3) as an external marker and ADL as an internal marker [20] [21]. Chromium oxide was dosed (10 g/day) twice daily after the morning and afternoon milkings in 5 g of shredded paper during 9 days which corresponds to day 12 after the start of the experimental period.: The first 6 days were used to attain the equilibrium between intake and excretion of the marker. On the 7th day, fecal samples (250 g each) were taken manually from rectum immediately after morning and afternoon milkings during three consecutive days. Fecal samples were stored at −20˚C, dried at 60˚C in a forced-draught oven and were ground to pass a 1-mm mesh sieve and stored in plastic containers for subsequent chemical analysis. Fecal samples were analyzed for chromium oxide using an atomic absorption spectrophotometer, according to the methodology described by Souza *et al.* [22]. Concentrate DM intake was measured by weighing the quantities offered and refused. Fecal production and forage DMI were estimated using the following formulae as proposed by [23].

$$\mathrm{FP}\left(\left(\mathrm{g\ DM/cow}\right)/\mathrm{d}\right)=\frac{\left(\text{Chromium administered, g/d}\right)}{\text{Chrome concentration in feces, g/g DM}}$$

where FP = Fecal production, g of DM/day.

$$\mathrm{DMIf}\left(\mathrm{kg/cow/d}\right)=\frac{\left(\mathrm{ADL\ feces}*\mathrm{FP}\right)-\left(\mathrm{ADLc}*\mathrm{DMIc}\right)}{\mathrm{ADLf}}$$

DMIf = Dry matter intake of the forage, kg/cow/day, FP = Total fecal production, kg DM/day, ADL feces = Acid detergent lignin found in the animal's feces, %, ADLc = Acid detergent lignin of the concentrated food, %, DMIc = Dry matter intake of the concentrated food, kg/cow/day, ADLf = Acid detergent lignin of the forage. The fecal chromium recovery rate was assumed to be 80%. Total intake was computed as pasture plus concentrate intake.

2.3. Sampling Measurements and Laboratory Procedures

The sampling protocols were accepted by the Ethics Committee of the University of Antioquia (Procedural number: 71, June 17, 2011). Milk production was daily and individually measured and milk samples were collected, composited according to the corresponding volume measured at each milking time and analyzed by infrared spectrophotometry (MilkoScanTM; FOSS Electric, Hillerod, Denmark) according to ISO/IDF standard method. Yield of 4% FCM was computed using the following formula: 4% FCM = (0.4 × milk production) + (15 × fat production). On days 1st and 21st milk samples (15 mL) were also assayed for CNs and α-LAcontent. The relative semiquantification of protein concentrations was performed using a calibration curve with BSA of 66-kDa molecular weight (BSA Standards ELISA Quantitation Set, Cat. No. 23209, Thermo Fisher Scientific Inc., Rockford IL USA). Dilutions of BSA were made at concentrations of 1.000, 2.500, 1.250, 625, 312.5, 156.25 μg/ml, corroborated in the ELISA kit, at 562 nm (microplates reader ELx800NB). The different BSA concentrations were deposited in Tricine-SDS-PAGE gels (see Figure 1). After migration over a 6 h,

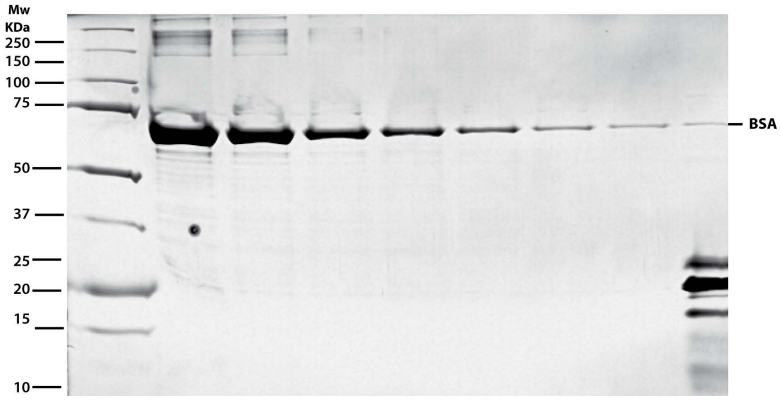

Figure 1. Electrophoresis pattern of bovine serum albumin (BSA) performed with Tricine-SDS-PAGE gels to obtain the standard curve. Line 1. Bio-Rad molecular-weight marker (Precision Plus protein Dual Color Standards). Lines 2 - 8: Serial dilutions of bovine serum albumin (BSA, 97% purity) with a molecular weight of 66 kDa.

photos of the gels were taken with the DNR's Gel capture mini software (Invitrogen), and the areas and densities of the bands obtained with the GelQuant Express Analysis software (Invitrogen) were analyzed. A standard curve of the densities of the bands and their concentration was plotted, which makes it possible to establish the regression equation used to estimate the concentrations of the samples of interest.

2.4. Individual Determination of Milk Proteins (αS1, αS2, β, κ, α-LA)

Pre-Fractionation of the Sample

The milk fat was removed and 2 mL of each sample was centrifuged for 15 min at 2500 relative centrifugal force. The soluble fraction was extracted and 10 µL were diluted in 240 µL of distilled water (1:25). Separation of lactoproteins: One-dimensional electrophoresis was performed using Mini-Protean III Cell Electrophoresis (Bio-Rad Laboratories, Richmond, CA) and Tricine-SDS-PAGE gels [24]. The separation gels according to [25], were 10% T, 3% C, and the concentration gel of 4% T and 3% C that separates proteins in molecular-weight ranges of 5 - 30 kDa.

The conditions for the protein migration program in electrophoresis were 40 min × 30 V (constant) and then 6 - 7 h at 40 mA (constant current). Gels were stained at room temperature with Coomassie Brilliant Blue R-250 under constant stirring conditions for 20 min [26]. They were destained for 16 h while being agitated. DNR Bio-imaging Systems (MiniBIS Pro) was used for the photos. The semiquantification of the different proteins was carried out on the basis of the color intensity and individual areas of the bands of each lactoprotein in each of the samples, using the GelQuant Express Analysis software (Invitrogen) (See **Figure 2**).

3. Statistical Analysis

Data were analyzed using a mixed model considering the animals as a random effect and the treatments as a fixed effect. Milk production at the start of the trial, days in milk and body condition score were used as covariates. Differences were considered as significant with $P < 0.05$ and trends with $P < 0.1$, using the SAS's LSMEANS procedure [28].

4. Results

4.1. Chemical Composition of the Diet and Intake

According to pre-planned design, intakes of protected Met and Lys resulted higher in the Met-Lys treatment (**Table 2**) due to the increased supply of rumen protected AA.

4.2. Milk Production and Milk Protein Composition

Supplementation with rumen-protected Met and Lys modified milk protein profile

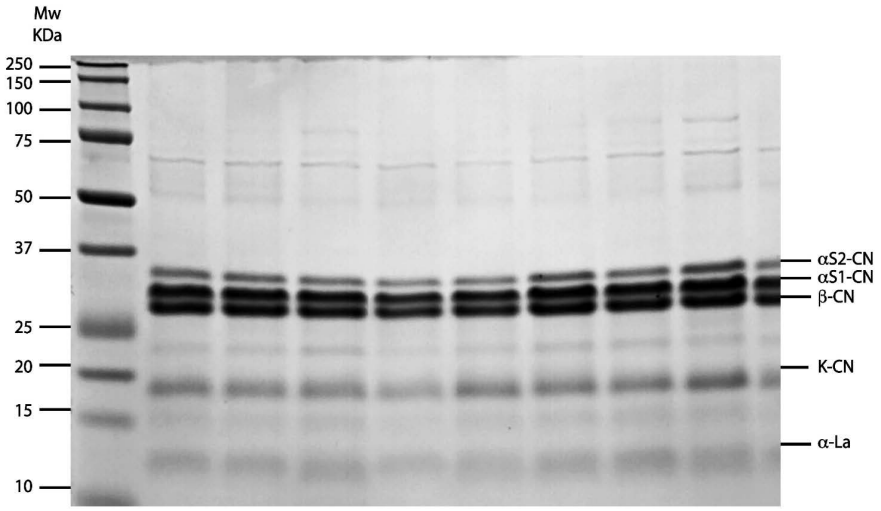

Figure 2. Electrophoresis patterns of bovine caseins (CN) and *α*-LA obtained from Tricine-SDS-PAGE gels. Line 1. Bio-Rad molecular-weight marker (Precision Plus Protein Dual Color Standards). From top to bottom, with molecular weights of 250, 150, 100, 75, 50, 37, 25, 20, 15, 10 KDa. Line 2 - 10. Bovine milk samples assessed, corresponding to caseins *α*S2-CN (25,266 KDa), *α*S1-CN (23,615 KDa), *β*-CN (23,983 KDa), *κ*-CN (19,037 KDa) according to [27]. Line 2, 4, 6, 8, 10 corresponding to control cow samples and line 3, 5, 7, 9 to experimental cow samples.

Table 2. Chemical composition of the experimental diets and dry matter intake (DMI) in lactating dairy cows supplemented with protected methionine (Met) and lysine (Lys).

Variable	Control	Met-Lys	EE	*P*<
Chemical composition of the diet				
Crude Protein, % total DMI	17.8[b]	18.4[a]	0.07	<0.001
NDF, % total DMI	42.7	42.3	1.80	0.66
EE, % total DMI	3.43[b]	3.83[a]	0.07	0.004
TDN, % total DMI	66.5	67.3	1.86	0.39
NE_L, Mcal/kg total DMI	1.49	1.50	0.02	0.60
DMI, kg cow^{-1} day^{-1}				
Pasture	11.5	11.9	2.67	0.58
Concentrate	7.58	7.56	0.57	0.97
Total	19.1	19.6	1.30	0.69
Protein Intake and Amino Acid Supply				
Protein, g DM cow^{-1} day^{-1}	3476	3525	233	0.84
Met supply, g DM cow^{-1} day^{-1}	79.7[b]	165[a]	7.47	0.006
Lys supply, g DM cow^{-1} day^{-1}	193[b]	264[a]	15.6	0.006

[a,b]: In the same row means with different letters differs($P \leq 0.05$). TDN: Total digestible nutrients, EE: Ether extract, NDF: Neutral detergent fiber, NE_L: Net energy for lactation. Each chemical fraction for the Control treatment was calculated as: (Kg of chemical fraction provided by each of the sources-forage, concentrate/Total DMI) * 100 and for MetLys group as: (Kg of chemical fraction provided by each of the sources – forage, RPMet, RPLys/Kg of Total DMI) * 100, Total Crude Protein Intake = (forage DMI in Kg * % forage CP * 1000) + (concentrate DMI * % concentrate CP * 1000). InMet-Lys group quantities provided from rumen protected Met and Lys sources were added. Met DMI or Lys DMI (g/d): Intake of Met or Lys = Intake of Dry Matter from feed in Kg * % Amino acid in the feed (as % Dry Matter) * 1000.

(Table 3) increasing the production of α-CN and β-CN ($P < 0.05$). There were no significant effects on yields of milk and 4% FCM, concentration of protein and κ-CN, αS2-CN, or αLa.

5. Discussion

Methionine and Lys are considered as the most limiting AAs for milk yield and protein production in pasture-based diets for lactating dairy cows but the addition of these protected AAs in the concentrate increased Met and Lys intake (Table 2). The potential improvement in the AA balance in turn increased milk protein concentration. In addition, Lys and Met are recognized as the limiting AAs for milk production directing the mRNA expression of the JaK-STAT and mTOR pathways and regulating both, the production of protein and the expression of AA transporters at the epithelial cells level of the mammary gland [29] [30]. In addition, it was reported that Lys and Met increase contents of some milk proteins [31] and the presence of Met and leucine in αS1-CN and leucine in β-CN respectively has been documented [32].

These findings from the literature are consistent with results observed in the present experiment and partially explain the observed increase inαS1-CN and β-CN levels as well as the increase in milk production observed in cows supplemented with rumen-protected Lys and Met. The αS1-CN is a highly phosphorylated protein, and phosphorylation increases its chaperone activity thereby increasing its ability to stabilize and increase the growth of micelles [33] [34]. It is well-known that the stability and size of micelles improve the industrial qualities of milk, especially its cheese yield. It was reported that the high concentration of the αS1-CN isoform (αS1-CN-8P) in bovine milk has a great benefit in the production of raw cheese curd because this protein is hydrolyzed more efficiently by the chymosin during maturity [35] [36].

Table 3. Milk yield and milk protein concentrations in lactating dairy cows supplemented with protected methionine (Met) and lysine (Lys).

Variables	Control	Met-Lys	EE	*P*
Milk yield, kg cow^{-1} day^{-1}	23.7	23.8	2.05	0.96
Yield of 4% FCM, cow^{-1} day^{-1}	24.2	26.0	3.42	0.10
Milk lactose content, g/kg	45.9	47.4	0.69	0.08
Milk protein content, g/kg	30.4	31.1	0.692	0.159
αS1-CN, g/kg	9.44^{b}	10.58^{a}	0.593	0.046
αS2-CN, g/kg	2.55	2.94	0.582	0.125
β-CN, g/kg	9.58^{b}	10.35^{a}	0.592	0.050
κ-CN, g/kg	3.25	3.49	0.572	0.663
αLa, g/kg	1.13	1.20	0.702	0.159

^{a,b}: In the same row means with different letters differs ($p < 0.05$). 4% FCM: Fat corrected milk (0.4 × milk production) + (15 × production of fat), αS1-CN: αS1 Casein, αS2-CN: αS2 casein, β-CN: β-casein, κ-CN: κ casein, α-LA: lactalbumin, EE: Standard error.

Recent studies have demonstrated the important role of some AAs in the diet due to their effects on the transcription and translation of genes in the mammary gland [37]. When Met and Lys were added (*in vitro*) to bovine mammary gland cells at a 3:1 ratio, an effect on the expression of related genes was observed with the transcription and translation of milk proteins, such as CSN1S1, CSN1S2, CSN2, CSN3, LALBA, JAK2, STAT5, ELF5, mTOR, and EIF4EBP1 which subsequently led to an increase in the CN synthesis [30]. It is also very important to point out that the cheese yield (grams of dry cheese/100g of milk protein) is improved when milk was selected from cows with high concentrations of αS1-CN, β-CN, and κ-CN [32].

The αS1-CN, the most prevalent form of CNs in bovine milk, has antioxidant properties with elimination of free radicals. In turn, this protein contains casecid in which exhibits *in vitro* activity against *Staphylococcus*, *Sarcina*, *Bacillus subtilis*, *Diplococcus pneumoniae*, and *Streptococcus pyogenes* [33]. The β-CN and its fragments have been implicated in numerous biological functions, such as the f(63-680) and f(8191-103), which has been reported as an activator of macrophage phagocytosis and peroxidase release. Apart from stimulating the proliferation of lymphocytes and macrophages, it promotes the synthesis of antibodies and the regulation of cytokines [38] [39]. The β-CN is also a source of peptides such as casomorphins that exhibit opioid activity, bind to receptors in the intestinal lumen and act as exogenous modulators of motility, permeability, and release of intestinal hormones. Because of these reasons, there is currently a great interest for the possible nutraceutical role of β-CN in cases of diarrhea [40].

The 4:1 ratio between αS1-CN and αS2-CN and between β-CN and κ-CN reported by [2] was not observed in this study, possibly because of the sensitivity of the analytical technique used here. Among many other studies related to ours, significant differences in α-CN and β-CN concentrations were reported without determining if the change was in αS1-CN or αS2-CN [41]. On the other hand, the effect of rumen-protected Met supply on grazing systems in New Zealand was reported to decrease the β-CN production alone [42].

It is concluded that feeding rumen-protected Met and Lys to grazing dairy cows supplemented with concentrates based on soy cake and corn grain positively modified milk protein profiles by increasing the levels of αS1-CN and β-CN without affecting the total milk protein content but increasing the yield of 4% FCM. These results justify the search for additional information up to the achievement of the best diets so that cows are able to express all their qualitative and quantitative potential for milk production.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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