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Influence of lipid supplementation on milk components and fatty acid profile

Henrique Valentim Nunes Machado[1*], José Carlos Pereira[2], Vitor Pereira Bettero[3], Fernando de Paula Leonel[1], Raphael Pavesi Araújo[4], Leonardo Marmo Moreira[1], Rafael Bastos Teixeira[5], Joanis Tilemahos Zervoudakis[6]

[1] Universidade Federal de São João del-Rei, Departamento de Zootecnia, São João del-Rei, MG, Brazil.
[2] Universidade Federal de Viçosa, Departamento de Zootecnia, Viçosa, MG, Brazil.
[3] Vaccinar Nutrição e Saúde Animal, Belo Horizonte, MG, Brazil.
[4] Instituto Federal de Educação, Ciência e Tecnologia do Tocantins, Colinas do Tocantins, TO, Brazil.
[5] Instituto Federal de Minas Gerais, Bambuí, MG, Brazil.
[6] Universidade Federal de Mato Grosso, Cuiabá, MT, Brazil.

ABSTRACT - The objective of this study was to evaluate the effects of different lipid sources in diets for lactating cows on milk yield and composition, conjugated linoleic acid (CLA) content, and fatty acid profile in the milk fat. Five primiparous Holstein cows were distributed in a 5 × 5 Latin square design. Treatments were: control (no lipid addition) and four other diets containing different lipids sources – ground raw soybean, cottonseed, soybean oil, and calcium salts of soybean fatty acids (CSSFA). The greater milk yield (kg/day) and milk lactose (g/kg) and solids non-fat (g/kg) contents were obtained with the animals fed diets with CSSFA. Regarding the fatty acid profile in the milk fat, the diets with CSSFA and ground raw soybeans produced the greatest concentrations of polyunsaturated fatty acids and $C_{18:2}$. Supplementation with CSSFA provided a greater production (g/day) of CLA, resulting in almost twice the values shown by the other treatments. The use of different lipid sources does not affect the milk total solids (protein, fat, and lactose) and CSSFA has a positive influence on the fatty acid profile of the milk fat and amount of CLA produced. Additionally, milk yield is not affected by this supplement.

Key Words: biohydrogenation, dairy production, fatty acids, supplementation

Introduction

There is a new utilitarian perspective to consider food in addition to its nutritive function. There is an increasing identification of the components of many foods with properties that are beneficial to humans. This fact shows a new way for research involving food science and animal science, among other correlated areas, such as the sector of functional foods (Roberfroid, 2002).

Thus, it is possible to detect functional aspects in products of animal origin and, consequently, provide expectations in terms of adding value to them, by making them differentiated and more attractive products. In this context, we can mention that milk composition includes a significant amount of conjugated linoleic acid (CLA). The CLA are trans fatty acids that have been the focus of studies demonstrating their beneficial action on the health of human beings, e.g., anticarcinogenic effect, increase in immune response, and assistance in the human development, among others (Wang and Jones, 2004).

Lipid supplementation to lactating cows shows a relevant potential to increase the milk CLA content, in addition to improving its fat profile (Glasser et al., 2008). In contrast, some lipid supplements may reduce the digestibility of other chemical fractions of the food, depending on the quality and quantity added to the diet (Palmquist et al., 2005).

There are many ways to supplement diet of cows with lipids. However, it is necessary to choose feedstuffs and their availability to the farmers. Besides, the price of the feedstuff is an important aspect to be considered.

Therefore, we chose ground raw soybeans, cottonseed, soybean oil, and calcium salts of soybean fatty acids as lipids sources, aiming to study their effects on fatty acid profile and conjugated linoleic acid content in milk.

Material and Methods

Five primiparous Holstein cows averaging 500±50 kg body weight (BW), with 100±20 days of lactation, and

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***Corresponding author:** henriquemachado@ufsj.edu.br
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milk yield of 25±4 kg/day were distributed in a 5 × 5 Latin square design. The animals were housed in individual stalls and feed was supplied twice daily, at 7:00 and 17:00 h. The animals received a total mixed ration.

The experiment lasted 105 days and each period consisted of 21 days, with the first 14 days used for adaptation and the other seven for data collection. The treatments were control (no lipid addition) and four other diets containing different lipid sources – ground raw soybean, cottonseed, soybean oil, and calcium salts of soybean fatty acids (CSSFA).

The five diets were adjusted to be isoproteic. It is important to notice that the four diets supplemented with lipid contained 5% of crude fat (Table 1).

The procedures described by AOAC (2000) were used to determine the dry matter (DM, method 967.03; AOAC, 1990), crude protein (CP, method 984.13; AOAC, 1990), and crude fat (CF, method 2003.06; Thiex et al., 2003). The neutral detergent fiber (NDF) was assayed with a heat-stable amylase and expressed inclusive of residual ash (Van Soest et al., 1991). The concentration of non-fibrous carbohydrates (NFC) was calculated as OM − (NDF + CP + CF), in which OM = organic matter.

Table 1 - Balance, chemical composition, and some fatty acid contents in the experimental diets

Ingredient (g/kg)	Experimental diet or treatment				
	Control	SB	CS	SO	CSSFA
Corn silage	550.0	550.0	550.0	550.0	550.0
Grain corn	247.0	240.0	210.0	250.0	250.0
Ground raw soybeans	0.0	120.0	0.0	0.0	0.0
Soybean meal	120.0	60.0	110.0	150.0	150.0
Calcium salts	0.0	0.0	0.0	0.0	25.0
Cottonseed	0.0	0.0	100.0	0.0	0.0
Soybean oil	0.0	0.0	0.0	20.0	0.0
Urea/ammonium sulfate (9:1)	13.0	10.0	10.0	10.0	10.0
Wheat bran	50.0	0.0	0.0	0.0	0.0
Na bicarbonate/Mg oxide (2:1)	4.0	4.0	4.0	4.0	4.0
Vitamins A-D-E mix	1.0	1.0	1.0	1.0	1.0
Mineral mix[1]	15.0	15.0	15.0	15.0	10.0
Chemical composition of the experimental diets (g/kg of dry matter)					
Dry matter	587	593	592	589	588
Organic matter	936	936	936	934	934
Crude protein	159	160	158	157	157
Crude fat	33	54	50	51	53
Non-fibrous carbohydrates	382	361	348	376	373
Neutral detergent fiber	380	376	395	365	365
Some fatty acid contents in the experimental diets (g/100 g of crude fat)					
$C_{14:0}$	0.81	0.74	0.67	0.72	0.55
$C_{16:0}$	16.44	14.46	18.62	13.89	12.93
$C_{18:0}$	3.13	4.49	2.86	3.44	2.89
$C_{18:1\ cis}$	13.03	19.72	13.47	16.18	16.55
$C_{18:2}$	31.70	37.27	40.29	37.65	35.59
$C_{18:3}$	7.19	6.47	4.47	6.34	5.70
Others	3.13	2.04	3.66	3.89	4.00

SB - ground raw soybeans; CS - cottonseed; SO - soybean oil; CSSFA - calcium salts of soybean fatty acids (Megalac-E®).
[1] Composition: 425 g/kg dicalcium phosphate; 250 g/kg limestone; 210 g/kg common salt; 75 g/kg potassium chloride; 25 g/kg ammonium sulfate; 12.5 g/kg zinc sulfate; 2.50 g/kg copper sulfate; 0.15 g/kg cobalt sulfate; 0.05 g/kg sodium selenite.

Cows were milked twice daily and milk yield was automatically recorded at each milking activity. Milk samples were taken on the third collection day of each experimental period at 6:00 and 16:00 h milking activities. Thus, a sample of approximately 300 mL was prepared, which was proportional to the yield in each milking, for the analyses of milk composition.

A 3.5% fat-corrected milk (FCM) yield was estimated according to Sklan et al. (1992), using the following equation: FCM = (0.432 + 0.1625 × % milk fat) × milk yield in kg/day.

On the fourth day of each collection period, a 2% aliquot of the milk production was collected and frozen for the analysis of the fatty acid profile, following the methodology described by Feng et al. (2004). Aliquots of 30 mL were centrifuged at 17,800 × *g* for 20 min at 4 ºC (Centrifuge Himac CR21, Hitachi Ltd., Katsuda, Japan), forming a supernatant milk cream ("fat cake") that was removed and frozen. Approximately 1 g of the fat cake was transferred to 1.5 mL Eppendorf® microtubes and centrifuged at 17,500 × *g* for 20 min at room temperature (Force 14 centrifuge – Denver Instrument Company, Denver, CO, USA). After centrifugation, the lipid fraction remained in the upper part of the tube, where it was collected with micropipettes and conditioned in 1-mL Eppendorf® tubes, which were frozen at –10 ºC until the preparation of the methyl esters.

The methyl esters were prepared using the method elaborated by Hartman and Lago (1986). Samples of 40 µL of the fat were transferred to test tubes with screw caps. Lipids were hydrolyzed with the addition of 2.5 mL of the NaOH solution at 0.5 N in methanol under 70 ºC for 15 min to obtain the methyl esters.

After chilling, 2 mL of NaOH 20% solution and 2 mL of hexane (HPLC grade) were added and then the tube was agitated by vortex so that approximately 1 mL of the upper phase containing the methyl esters was collected. Subsequently, another 1 mL of hexane (HPLC grade) was added to the tube, from which, again, approximately 1 mL of the upper phase was extracted. The methyl esters were stocked in amber-colored glass bottles and frozen at −18 ºC for later analyses.

Analyses of fatty acid methyl esters (FAME) in hexane from the milk fat were conducted in a gas chromatograph (GCMS-QP 5000 – Gas Chromatography Mass Spectrometer – Shimadzu, S.A., Kyoto, Japan) and the components of the methyl esters were separated in a Carbowax column (30 m × 0.25 mm).

The chromatography followed these conditions: injector temperature, 220 ºC; interface temperature, 240 ºC; gas flow in the column, 1.3 mL/min; column pressure, 67.7 kpa; linear velocity, 40.6 cm/s; outlet orifice, 3; injected volume, 1 µL; and carrier gas, helium (inert gas).

The identification of fatty acid peaks was performed by comparison with the retention times of fatty acids in a standard mixture of FAME (Supelco™ 37FAME Mix). The identification of specific CLA peaks was achieved by difference, comparing the retention times of the methyl esters from the mixture of conjugated fatty acids *cis*-9, *trans*-11 and *trans*-10, *cis*-12 of a pure commercial product (05632 – SIGMA).

The statistical model was:

$$Y_{ijk} = \mu + \gamma_i + a_j + \beta_k + e_{ijk},$$

in which µ is the intercept, γ_i corresponds to the effect of the *i*-th lipid supplement (i = 1 to 5); a_j is the effect of the *j*-th animal (j = 1 to 5); β_k is the effect of the *k*-th experimental period (k = 1 to 5); and e_{ijk} is the random error assumed *iid* N(0, σ^2). Treatments (γ_i) were considered as fixed effect; animals (a_j), experimental period (β_k), and the error term (e_{ijk}) are random effects. The data were subjected to analysis of variance and test of means, using the PROC MIXED procedure of SAS (Statistical Analysis System, version 9.0) with the command for repeated measures over time, applying Tukey's test for comparison of means and adopting the 5% level of confidence.

Results

There was no significant interaction between period and treatment in any of the studied variables. Milk fat, protein, lactose, total solids, and solids non-fat contents in grams per kilo did not show differences (P>0.05) in relation to the treatments (Table 2).

The average milk and lactose yield of animals receiving CSSFA were only greater than the productions of the cows fed the control and soybean oil diets. The animals that received the diet with ground raw soybeans and diet with cottonseed meal showed an intermediate performance, not differing (P>0.05) from the rest.

Regarding the production of solids non-fat, only the diet containing CSSFA and the control presented difference (P<0.05), being the higher value corresponding to the CSSFA-containing diet. All the other treatments showed an intermediate performance, not differing from the rest. The daily 3.5% of yield and the milk contents did not show differences (P>0.05) among the five diets (Table 2).

The polyunsaturated fatty acid (PUFA) was the only one to present difference (P<0.05) among the treatments. The highest PUFA content was obtained with the milk from animals fed CSSFA and ground raw soybeans in relation to diets containing cottonseed and the control treatment. The diet containing soybean oil, however, was superior only to that containing cottonseed and the latter resulted in the milk with the lowest PUFA levels (Table 3). None of the other fatty acids was affected (P>0.05) by lipid supplementation.

When the fatty acids were analyzed individually, no effect of diet was observed for most of them. However, the fat in the milk produced from animals consuming cottonseed showed a higher (P<0.05) stearic acid ($C_{18:0}$) content than the control treatment, whereas the other

Table 2 - Average values for daily yield and milk composition

Variable	Experimental diet or treatment					SEM	P-value
	Control	SB	CS	SO	CSSFA		
	Composition (g/kg)						
Fat	37.8	31.9	37.7	37.4	33.1	0.22	0.187
Protein	31.9	33.0	32.0	32.6	30.1	0.13	0.087
Lactose	44.4	47.5	44.9	44.5	45.3	0.09	0.127
Total solids	125.2	123.6	125.6	125.7	119.5	0.36	0.520
Solids non-fat	87.4	91.8	88.0	88.4	86.4	0.21	0.098
	Yield (kg/day)						
Milk	18.5b	19.9ab	19.1ab	18.7b	21.9a	2.46	0.024
3.5% FCM	19.3	18.2	19.8	19.5	21.2	2.42	0.423
Fat	0.7	0.6	0.7	0.7	0.7	0.09	0.489
Protein	0.6	0.6	0.6	0.6	0.7	0.06	0.264
Lactose	0.8b	0.9ab	0.9ab	0.8b	1.0a	0.11	0.008
Total solids	2.3	2.4	2.4	2.4	3.6	0.27	0.272
Solids non-fat	1.6b	1.8ab	1.7ab	1.7ab	1.9a	0.19	0.038

SB - ground raw soybeans; CS - cottonseed; SO - soybean oil; CSSFA - calcium salts of soybean fatty acids (Megalac-E®); SEM - standard error of the mean; FCM - fat-corrected milk.
Means followed by the same letter in the row do not differ by Tukey's test at 5% significance.

Table 3 - Average values for milk fatty acid profile (g/kg fat) separated by chain length and number of double bonds

Variable	Experimental diet or treatment					SEM	P-value
	Control	SB	CS	SO	CSSFA		
SCFA	63.5	54.1	58.7	59.3	56.3	0.43	0.561
MCFA	545.5	490.4	513.7	505.5	490.5	2.51	0.439
LCFA	356.0	420.2	399.2	398.0	418.4	2.58	0.405
OCFA	31.7	31.0	29.1	29.3	26.1	0.15	0.191
SFA	715.4	677.6	718.0	692.6	659.6	2.20	0.299
UFA	279.4	315.3	277.4	299.0	333.3	2.15	0.298
MUFA	253.1	281.3	256.2	268.3	299.2	2.08	0.515
PUFA	26.1bc	34.6a	21.1c	30.9ab	33.9a	0.22	0.000

SB - ground raw soybeans; CS - cottonseed; SO - soybean oil; CSSFA - calcium salts of soybean fatty acids (Megalac-E®); SEM - standard error of the mean; SCFA - short-chain fatty acids; MCFA - medium-chain fatty acids; LCFA - long-chain fatty acid; OCFA - odd-chain fatty acids; SFA - saturated fatty acid; UFA - unsaturated fatty acids; MUFA - monounsaturated fatty acids; PUFA - polyunsaturated fatty acids.
Means followed by the same letter in the row do not differ by Tukey's test at 5% significance.

treatments showed intermediate values. The highest linoleic acid ($C_{18:2}$) content was detected in the milk from cows fed ground raw soybeans as compared with the control and the diet with cottonseed (Table 4).

The cows fed CSSFA produced higher CLA content in the milk fat than cows fed ground raw soybeans or cottonseed, but was not higher than cows in control or soybean oil treatments, which did not differ from the others. This same trend was observed for the amount of CLA per liter of milk (Table 4).

Discussion

The diet supplemented with CSSFA was efficient in increasing the milk yield in relation to the control and to supplementation with soybean oil. This is probably due to the lower availability (protection) of the unsaturated fatty acid contained in the CSSFA and to the ruminal processes of interaction with the microorganisms, including biohydrogenation. Thus, the smaller contact of the rumen microbes with the unsaturated fatty acid prevents toxic effects caused by these acids and, therefore, they do not affect the fiber digestibility and the microbial protein synthesis. Besides, the supplement also increases the energy density of the diet, which allows for increments in the milk yield.

Results of research studies are inconclusive in linking elevated milk yield to lipid supplementation, much less to some specific type of supplement. In this aspect, positive results for milk yield with fat-supplemented diets were obtained by Costa et al. (2007) and absence of responses by Eifert et al. (2006), Huang et al. (2008), and Ganjkhanlou et al. (2009).

The results obtained for 3.5% of FCM yield did not follow the same trend observed for milk yield. The greater production obtained with the treatment with CSSFA, when adjusted for 3.5% fat, became similar to the other treatments. This suggests that a smaller fat content in the milk produced by animals fed this supplement reduced the yield, resulting in this equality, although the milk fat concentrations did not show significant differences.

There is a great concern over the reduction of the milk total solid components, especially regarding the concentration of fat (Harvatine and Allen, 2005; Abu-Ghazaleh et al. (2003). In spite of this, in the present work, the lipid supplementation did not affect the milk fat. It probably happened because the lipid inclusion in the diet was not enough to promote the ruminal production of the *trans*-10, *cis*-12 CLA, and *trans*-10 18:1 to cause the syndrome of low milk fat content. These intermediate fatty acids have the greatest evidence of depressing the milk fat (Bauman and Griinari, 2003; Harvatine et al. 2009; Harvatine and Bauman, 2011).

Some researchers, among them Eifert et al. (2006) and Huang et al. (2008), observed a decrease in the fat content of milk produced by cows fed soybean oil. On the other hand,

Table 4 - Average values for milk fatty acid contents (g/kg fat)

Fatty acid profile	Experimental diet or treatment					SEM	P-value
	Control	SB	CS	SO	CSSFA		
$C_{6:0}$	26.8	24.3	27.0	26.7	27.7	0.13	0.369
$C_{8:0}$	15.0	10.7	12.1	12.0	9.3	0.21	0.430
$C_{10:0}$	36.6	29.4	27.9	33.2	27.8	0.25	0.122
$C_{12:0}$	44.1	35.5	32.6	39.7	3.5	0.27	0.076
$C_{13:0}$	1.1	0.9	0.8	0.9	0.8	0.00	0.080
$C_{14:0}$	127.7	111.9	109.4	121.4	115.0	0.51	0.065
$C_{15:0}$	12.5	12.7	10.6	11.6	9.2	0.09	0.100
$C_{16:0}$	310.4	288.5	321.1	287.2	290.6	1.71	0.418
$C_{16:1}$	13.1	11.5	11.3	11.5	11.6	0.12	0.603
$C_{17:0}$	8.2	7.7	8.1	7.4	8.5	0.05	0.579
$C_{17:1}$	1.7	1.1	1.9	1.4	1.6	0.03	0.310
$C_{18:0}$	101.6b	126.4ab	139.3a	123.1ab	108.4ab	0.85	0.030
$C_{18:1}$	222.9	255.8	232.1	241.0	272.2	2.02	0.479
$C_{18:2}$	21.5bc	29.2a	17.8c	25.1abc	29.1ab	0.21	0.003
CLA[1]	3.3ab	2.5b	2.0b	3.3ab	6.1a	0.06	0.008
Others	56.7	54.3	48.0	57.8	52.8	0.23	0.061
CLA (g/day)	2.30b	1.51b	1.41b	2.34b	4.47a	0.39	0.003
CLA (g/L)	0.13ab	0.08b	0.08b	0.12ab	0.19a	0.02	0.015

SB - ground raw soybeans; CS - cottonseed; SO - soybean oil; CSSFA - calcium salts of soybean fatty acids (Megalac-E®); SEM - standard error of the mean; CLA - conjugated linoleic acid.
Means followed by the same letter in the row do not differ by Tukey's test at 5% significance.
[1] Isomers *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA.

the results obtained in the present experiment corroborate Avila et al. (2000), Santos et al. (2001), and Ganjkhanlou et al. (2009), who did not find any effects of lipid supplements on the fat and protein contents of milk.

The lipid supplementation has a small effect on milk protein percentage (Linn, 1983; Palmquist and Jenkins, 1980) and when it happens, it is probably due to the inability of microorganisms to utilize lipids as a source of energy, impairing the microbial protein synthesis or by deficiency of glucose, as mentioned by Garnsworthy (2002). However, we did not find significant changes in this component, probably because the lipid inclusion was not enough to promote this process. Likewise, the lactose is the constant constituent in the milk, despite nutritional plan (Davies et al., 1983; Jenness, 1985). This can explain the lack of variation in the solids non-fat content of the milk.

The production of fat, protein, and total solids in kg/day did not follow the values obtained for milk yield and remained equal for all treatments. Hence, a dilution effect could be suggested, since there was a higher milk yield for the treatment with CSSFA, characterizing a lower ratio between the milk components and milk yield, in kg/day. Yet, the concentrations as a percentage of these same components did not present differences (Table 2).

The lipid supplementation had no effect on short-chain fatty acids (SCFA), medium-chain fatty acids (MCFA), and long-chain fatty acids (LCFA) in the milk fat. The metabolic origin of these fatty acids can be useful to explain this. Fatty acids from C_4 to C_{14} are originated from *de novo* synthesis within the cells of the mammary parenchyma, C_{16} arises from both diet and *de novo* synthesis, whereas LCFA ($>C_{16}$) derive from the absorption of circulating lipids (from the diet or from the mobilization of the body reserve) (Chilliard et al., 2000). The fatty acids containing six to 16 carbons could suffer reductions due to the inhibition of the *de novo* synthesis in the mammary gland by several *trans* 18 isomers, as reported by Shingfield and Griinari (2007). However, we did not observe this effect in present work, probably due to the lower amount of lipid supplements in the diet. Glasser et al. (2008) found a reduction in the value of these fatty acids, but they worked with animals that received higher levels of lipids in the diet than the animals in the present study (484 to 868 g/day versus approximately 380 g/day).

The CSSFA, soybean oil, and ground raw soybeans are feedstuffs rich in PUFA and are probably more resistant to the rumen biohydrogenation process, consequently resulting in greater PUFA escapes to the intestine to be incorporated in the milk fat. This is probably the cause of the highest PUFA values in milk fat of the cows that received these ingredients in the diets.

Regarding the fatty acid profile, the control diet resulted in the lowest $C_{18:0}$ content and the likely reason for this is the higher amount of LCFA contained in the lipid supplements utilized, which, depending on the extent of their biohydrogenation, produce stearic acid (Palmquist and Jenkins, 1980).

The fat of the milk from cows fed cottonseed had the highest and lowest values for $C_{18:0}$ and $C_{18:2}$, respectively. This happened because $C_{18:2}$ present in feeds are highly bio-hydrogenated in the rumen and the concentrations of these elements in the milk fat are not very affected when animals are supplemented with lipids, unless these lipids are protected (Glasser et al. 2008), like CSSFA in the present work (Table 4). A higher concentration of stearic acid in milk fat from cows supplemented with fat sources was also found by Huang et al. (2008) and by Weiss and Pinos-Rodríguez (2009).

The CSSFA supplement provided higher ($P<0.05$) CLA in milk than ground raw soybean and cottonseed (Table 4). Although not statistically significant, the CLA in milk fat of the cows which fed CSSFA was almost twice as much as the concentrations observed for cows fed the control and soybean oil diets. Regarding the ground raw soybeans and cottonseed supplements, the difference was significant, with CSSFA being responsible for an increase greater than 140% in the CLA content.

The physiological sources of CLA are biohydrogenation, in which CLA is intermediate, and the desaturation of vaccenic acid provoked by the activity of the Δ^9-desaturase enzyme in the mammary gland. Thus, both CLA and *trans*-11 18:1, which escape the biohydrogenation process, are responsible for the deposition of CLA in the fat of cow milk (Palmquist, 2007; Jenkins at al., 2008). Consequently, protected lipid supplements or those that present traits favorable to greater ruminal passages of vaccenic acid and CLA to the intestine can be effective in increasing the milk CLA content (Harvatine and Allen, 2006), as is the case with CSSFA.

Several studies indicated that there was not a single lipid supplement responsible for supporting the increase in milk CLA (Huang et al., 2008; Boken et al., 2005; Harvatine and Allen, 2006; Mosley at al., 2006). However, there was one characteristic in common: these supplements should be rich in PUFA.

The CLA concentrations in the milk fat were also analyzed in g/L of milk (Table 4), which showed the same trend as the contents of this fatty acid in the milk

fat. Another result is the daily production of CLA in g/day, wherein both the concentrations of *cis*-9, *trans*-11 CLA in the fat and the milk yield obtained for the treatment with CSSFA were responsible for the greater quantity of CLA produced with this treatment.

Conclusions

The "protected" lipid (calcium salts of soybean fatty acids) shows a positive influence on the profile of fatty acids contained in milk fat, providing greater quantities (g/day) of polyunsaturated fatty acids, especially in terms of conjugated linoleic acid. Ground raw soybeans promote an increase in the levels of polyunsaturated fatty acids in milk, but do not change the profile of conjugated linoleic acid. Cottonseed negatively modifies the milk fatty acid profile, increasing the content of saturated fatty acids. Soybean oil is not related to changes in the profile of milk fatty acids. Milk yield and milk composition are not affected by these supplements.

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