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Ruminal biohydrogenation and abomasal flow of fatty acids in lactating cows: Oilseed provides ruminal protection for fatty acids

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ABSTRACT

Fat sources, besides the energy-rich content, have featured beneficial effects on dairy cow production, reproduction and health. This work aimed to study the biohydrogenation process and fatty acid abomasal flow in lactating dairy cows fed different fat sources. Eight rumen and abomasum cannulated cows (188 ± 27.3 days in milk, 18.9 ± 3.24 kg of milk yield, and 572 ± 59.6 kg of body weight) were used in a 4 × 4 Latin square design. Control (CON) diet without fat source, soybean oil (SO), raw soybean grain (SG) and calcium salts of unsaturated fatty acids (CS) were evaluated. Fat sources decreased dry matter (DM), crude protein and neutral detergent fiber (NDF) intake and increased ether extract (EE) intake and ruminal pH ($P < 0.05$). Acetate to propionate ratio was lower in animals fed diets with fat ($P < 0.05$). Diets had no effect on microbial protein synthesis, and energy and nitrogen balances. NDF digestibility and DM passage rate were lower in animals fed diets with fat sources ($P < 0.05$), while protected sources (SG and CS) tended to increase ruminal fiber digestibility ($P = 0.092$) in relation to SO diet. Intake and abomasal flow of FA were higher ($P < 0.05$) for animals supplemented with fat sources than those fed CON. Protected sources (SG and CS) promoted greater abomasal flow of linoleic acid (C18:2) and lower biohydrogenation rate compared to the SO diet. Fat sources increased unsaturated milk fatty acids and serum cholesterol concentration while protected sources (SG and CS) increased milk C18:2 *cis* concentration ($P < 0.05$). Fat sources improved ruminal fermentation without compromise nutrients digestion and increasing fatty acids abomasal flow and milk concentration. Raw soybean grain had higher ruminal biohydrogenation protection than calcium salts.

Abbreviations: aADF, acid detergent fiber; aNDF, neutral detergent fiber; A:P, acetate to propionate ratio; CLA, conjugated linoleic acid; CON, control; CP, crude protein; CS, calcium salts of fatty acids; DC, digestibility coefficient; DM, dry matter; DMI, dry matter intake; EE, ether extract; FA, fatty acids; FCM, fat corrected milk; Kd, digestibility rate; Kp, passage rate; iNDF, indigestible neutral detergent fiber; N, nitrogen; NEL, net energy for lactation; NH_3-N, ammonia nitrogen; PUFA, polyunsaturated fatty acid; SEM, standard error of the mean; SCFA, short-chain fatty acids; SG, raw soybean grain; SO, soybean oil; tdNDF, truly digestible NDF.

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1. Introduction

Dairy farming productivity worldwide heavily relies on intensive practices that include providing animals with ideal ratios of available grains, roughage, and commercial feeds. Fat content and composition represent key aspects of dairy cow feeds. In this sense, fat sources have been extensively studied, not only as a means of maintaining energy balance with low heat increment for dairy cows, but also as metabolic modulators and animal health promoters (Onetti and Grummer, 2004; Jenkins et al., 2008). Altering the fatty acid (FA) supply to dairy cows affects immune function (Silvestre et al., 2011), nutrient flow (Harvatine and Allen, 2006), animal performance and, ultimately, the composition and quality of dairy products (Mansbridge and Blake, 1997).

Ruminants absorb lipids mostly in the duodenum. The quality and degree of fat absorption by duodenum depends on the dietary FA composition and ruminal metabolism (Harvatine and Allen, 2006). In turn, this process varies with the amount of polyunsaturated fatty acids (PUFA) reaching the rumen. High PUFA contents affect ruminal microorganisms reducing fiber digestibility (Eastridge and Firkins, 1991). As a defense mechanism, these microorganisms bio-hydrogenate PUFA in a process that depends on pH (VanNevel and Demeyer, 1996) and on the amount and profile of fat (Beam et al., 2000).

Producers should attempt to optimize ruminal metabolic processes, fatty acids absorption in the duodenum, and productivity following breed guidelines and providing animals with an appropriate amount and quality of dietary FA. Few studies focused on the extent in which dietary FA are protected and reach duodenum for absorption. However, it is well established that the degree of FA protection will heavily depends on the source. For example, oilseeds (Chilliard et al., 2000) and calcium salts of FA (Jenkins and Bridges, 2007) provide greater protection to FA against rumen biohydrogenation than vegetable oils.

Given the importance of absorbed FA to animal health and performance, specifically regarding dairy cows and being linoleic acid the major unsaturated FA in conventional fat sources, we evaluated the effects of different fat sources, rich in linoleic acid on nutrients intake and digestibility, ruminal kinetics, biohydrogenation, and abomasal flow of FA. We hypothesized that ruminal protection (mechanic or chemical) inhibits ruminal biohydrogenation, increases unsaturated FA abomasal flow and milk secretion, without affecting performance and ruminal kinetics and fermentation of lactating dairy cows.

2. Material and methods

The Ethics Committee of the School of Veterinary Medicine and Animal Science of the University of Sao Paulo approved the experimental procedures approval number 1603/2009.

2.1. Animals, experimental design and diets

The study used eight Holstein cows cannulated in rumen and abomasum, which averaged 188 ± 27.3 days in milk, 572 ± 59.6 kg of body weight, and 18.9 ± 3.24 kg/d of milk yield at the beginning of the trial. Animals were housed in individual pens, with 17.5 m^2 of area, sand beds and forced ventilation and were mechanically milked twice daily. The animals were allocated into two Latin squares to receive one of four experimental diets (Table 1) formulated according to the NRC (2001) recommendations, as follows: CON: control diet with no fat source; SO: soybean oil, with 30 g/kg of soybean oil; SG: soybean grain diet, with 160 g/kg of whole raw soybean grain; and CS: calcium salts, with 30 g/kg of calcium salts of fatty acids (Megalac E®, Church & Dwight Company and distributed by Química Geral do Nordeste, Nova Ponte, Brazil). Each experimental period lasted for 21 days, consisting of 14 days for adaptation to diets and seven days for sampling.

2.2. Data and sample collection

2.2.1. Nutrients intake and apparent digestibility

Cows were fed a total mixed ration twice daily, at 06:00 and 13:00, according to the amount of orts from the previous day to maintain a percentage of refusal between 50 and 100 g/kg of the feed offered. Samples of feed and refusals were collected throughout the sampling period and frozen until chemical analyses. Fecal samples (500 g) were collected every nine hours on days 15, 16, and 17 of the experimental period, forming a composite sample.

Feed, refusal, and fecal samples were analyzed for dry matter (method 930.15; AOAC, 2000), crude protein ($N \times 6.25$; method 984.13; AOAC, 2000), ether extract (method 920.39; AOAC, 2000), acid-detergent fiber and lignin (ADF-ADF and Lignin (sa), method 973.18; AOAC, 2000), ash (method 942.05; AOAC, 2000), neutral-detergent fiber and neutral-detergent fiber corrected for ash and protein (aNDF-NDF), and using α-amylase without addition of sodium sulfite (VanSoest et al., 1991).

Feed samples were evaluated for neutral-detergent insoluble crude protein and acid-detergent insoluble crude protein, according to the methods described in the NRC (2001) for assessing net energy of lactation (NEL_{3x}).

Fecal excretion was estimated using chromic oxide as marker. From day 9–17 of each experimental period, 15 g/d of chromium oxide were inserted in the rumen through the cannula. Phosphoric acid was used in the fecal sample digestion

Table 1
Experimental diets composition and fatty acid profile.

Item	CON[a]	SO[b]	SG[c]	CS[d]
Ingredients, g/kg				
Corn silage	650	650	650	650
Ground corn	210	180	139	180
Soybean meal	107	106	20.0	106
Soybean oil	–	30.2	–	–
Raw soybean grain	–	–	160	–
Calcium salts of fatty acids	–	–	–	30.2
Urea	8.0	9.5	4.0	8.0
Ammonium sulfate	2.0	2.0	1.0	2.0
Magnesium oxide	1.6	1.6	1.6	1.6
Dicalcium phosphate	6.3	6.3	6.2	6.3
Limestone	7.4	7.4	8.9	–
Minerals[e]	2.1	2.1	2.1	2.1
Nutrient composition				
Dry matter, g/kg	575	577	582	557
Neutral detergent fiber, g/kg	364	360	346	360
Crude protein, g/kg	141	141	139	141
Ash, g/kg	80.3	87.4	82.9	87.4
Ether extract, g/kg	27.3	53.4	56.6	53.4
$NE_{L3\times}$[f] MJ/kg	7.87	8.37	8.49	8.28
Fatty acid, g/kg of FA				
C14:0	3.1	3.3	2.7	3.1
C16:0	113	116	124	113
C18:0	32.3	32.1	33.6	32.1
cis C18:1	228	224	219	225
C18:2	504	503	507	501
C18:3	55.8	54.2	56.9	56.3
Others	11.7	5.4	3.4	4.1
Total unsaturated fatty acids	799	787	786	786

[a] Control (CON).
[b] Soybean oil (SO).
[c] Soybean grain (SG).
[d] Calcium salts of fatty acids (CS) (Megalac-E®).
[e] Each kg contains: 125 mg of Co, 5625 mg of Cu, 9 mg of S, 312 mg of I, 5000 mg of Fe, 18,125 mg of Mn, 144 mg of Se, 23,750 mg of Zn, 2000 IU of Vitamin A, 500 IU of Vitamin D, 12,500 IU of Vitamin E.
[f] Estimated according to NRC (2001): NE_{L3x} (MJ/kg MS) = [0.245 * TDN_{1x} (g/kg) − 0.12] * 4.184, were total digestible nutrients(TDN) were estimated as: TDN_{1X} (g/kg) = tdNFC(g/kg) + tdCP(g/kg) + (tdFA(g/kg) * 2.25) + tdNDF(g/kg); where tdNFC, tdCP, tdFA and tdNDF were the truly digestible fraction of non fiber carbohydrate, crude protein, fatty acids and neutral detergent fiber, respectively.

(Williams et al., 1962) and readings were made in a spectrophotometer (Biochrom Asys Expert, Holliston, MA, United States). Fecal excretion and apparent digestibility coefficient (DC) were estimated according the following equations:

$$\text{Fecal excretion}\left(\frac{\text{kg}}{\text{day}}\right) = \frac{\text{Cromiumoxidedoseddaily}\left(\frac{\text{g}}{\text{day}}\right)}{\text{Cromium oxide fecal concentration}\left(\frac{\text{g}}{\text{kg}}\right)}$$

$$\text{DC}\left(\frac{\text{g}}{\text{g}}\right) = \frac{\text{Intake(kg)} - \text{Fecal excretion(kg)}}{\text{Intake (kg)}}$$

2.2.2. Ruminal fermentation

For ruminal fermentation evaluation, liquid was collected on day 17 of each experimental period at zero, 2, 4, 6, 8, 10, and 12 h after the morning feeding. The rumen pH value was recorded immediately after collection with a digital pH meter (MB-10, Marte Científica, Santa Rita do Sapucaí, Brazil). The concentrations of short chain fatty acids (SCFA) in the rumen liquid were measured as described by Shen et al. (2004), and the NH_3-N content was analyzed using phenol-hypochlorite method (Broderick and Kang, 1980).

2.2.3. Ruminal kinetics

Ruminal (1000 g) and abomasal (1000 g) samples were collected every nine hours on days 15, 16, and 17 of each experimental period, creating a composite sample representative of a 24-h cycle. Ruminal samples were separated with an 1 mm pore cheesecloth into liquid and solid content and after ground were reconstituted at original DM ratio (Faichney, 1975).

Rumen contents were manually evacuated through ruminal cannula 12:30 (4.5 h after feeding) on the 20th day of each period and 5:30 (2.5 h before feeding) on day 21 of each period (Harvatine and Allen, 2006). Feed, refused feed, ruminal, and abomasal samples were packed in bags of non-woven tissue (100 g/m^2) and incubated for 288 h in the rumen of two

Holstein cows (Casali et al., 2008). Residues were analyzed for NDF (VanSoest et al., 1991) to assess the indigestible neutral detergent fiber (iNDF), which was used as internal marker.

Rumen turnover rate was calculated as iNDF intake and iNDF rumen content ratio. According to a two-compartment model for cellulose digestion (Waldo et al., 1972), ruminal passage rate (k_p) of iNDF was calculated dividing the size of the rumen compartment by iNDF intake. Ruminal digestion rate (k_d) of NDF was calculated as the difference between NDF clearance and passage rate (Allen and Mertens, 1988). The NDF ruminal apparent digestibility was assessed as: $k_d/(k_d + k_p)$. Truly digestible NDF (tdNDF) were calculated from the difference between NDF and iNDF (NRC, 2001), tdNDF ruminal digestion were estimated similarly to NDF.

Ruminal fluid turnover was estimated using a 4000 molecular weight Polyethylene glycol (PEG) as marker. Before the morning feeding, on day 18 of each experimental period, 100 g of PEG (Synth, Diadema, Brazil) were diluted in 400 mL of water and placed in the rumen. Samples were performed 0, 1, 2, 4, 8 and 24 h after infusion. PEG concentration were analyzed according to the Hyden (1955) methodology. Liquid passage rate were obtained by linear natural logarithmic regression in function of time.

2.2.4. FA flow and biohydrogenation rate

Feed and refusals, ruminal and abomasal content samples were ground using a Walley mill 5 mm sieve, liquid N at −192 °C, to prevent oxidation of FA (Harvatine and Allen, 2006). Lipid extraction was performed according to the method proposed by Folch et al. (1957) and methylation performed according to Kramer et al. (1997). Fatty acids were quantified by gas chromatography (GC Shimatzu 2010, São Paulo, Brazil) using SP-2560 capillary column (Supelco, Bellefonte, PA).

The biohydrogenation rates were calculated by the Jenkins and Bridges (2007) model, using the following equation:

$$BRPUFA = (PUFA_{af} - \mathrm{PUFA_i})/\mathrm{PUFA_i}$$

where *BRPUFA*: biohydrogenation rate of polyunsaturated fatty acid; $PUFA_{af}$: polyunsaturated fatty acid abomasal flow; and $PUFA_i$: polyunsaturated fatty acid intake.

2.2.5. Milk yield and composition

Cows were mechanically milked twice a day, at 06:30 and at 15:30. Milk yield was recorded at each milking on day 14th to 21th, and samples were collected at 16th to 21th day and analyzed for crude protein, fat and lactose, using ultrasonic milk analyzer MCC (Milcotronic Company, Nova Zagora, 8900, Bulgaria). Milk yield was corrected for 35 g/kg of fat (FCM), according Sklan et al. (1992).

Milk samples used for evaluating fatty acids profile were centrifuged at 17.800*g* for 30 min at 4 °C and next for 19.300 × g for 20 min at 4 °C, according to Feng et al. (2004). The separated fat (0.300–0.400 g) was methylated and the methyl esters were formed according to (Kramer et al., 1997). Two internal C18:0 and C19:0 FA standards were used for correcting losses during the process of methylation.

The fatty acids were quantified by chromatography gas (Shimadzu GC 2010 with automatic injection) using capillary column SP-2560 (100 m × 0.25 mm i.d. with 0.02 mm of film thickness, Supelco, Bellefonte, PA, USA). The initial temperature was 70 °C for four minutes (13 °C/minute) until it reached 175 °C, maintaining for 27 min. Afterward, a new increase of 4 °C/minute was initiated until 215 °C, maintaining for 31 min. Hydrogen (H_2) was used as carrier gas with flux of 40 cm/s. During the process of identification four standards were used: standard C4-C24 FA (Supelco® TM 37), vaccenic acid C18:1 *trans*-11 (V038-1G, Sigma®), CLA C18:2 *trans*-10, *cis*-12 (UC-61 M 100 MG), and CLA C18:2 *cis*-9, *trans*-11 (UC-60 M 100 MG), (NU-CHEK-PREP USA®) for identification of fatty acids that are formed during the biohydrogenation.

2.2.6. Nitrogen balance and microbial protein synthesis

The daily urinary volume was estimated from the concentration of creatinine (g/L) in spot samples obtained on the 16th day of each experimental period, four hours after the morning feeding. The creatinine concentrations were analyzed with a biochemical colorimetric kit (kinetic creatinine: cat. no. K-067, Bioclin, Belo Horizonte, Brazil) in a semi-automatic spectrophotometer (SBA 200, CELM, São Caetano do Sul, Brazil), and a daily creatinine excretion rate of 0.024 g/kg of body weight was assumed (Chizzotti et al., 2008). The total excretion of uric acid (uric acid stable liquid: cat. no. K-052, Bioclin, Belo Horizonte, Brazil; determined in a semi-automatic spectrophotometer SBA 200, CELM, São Caetano do Sul, Brazil) and allantoin in urine and milk (Fujihara and Yamaguchi, 1978) were considered as the total excretion of purine derivatives. Microbial protein synthesis was estimated from these concentrations in accordance with the Chen and Gomes (1992) method. Estimation of energy and protein balance was performed according to NRC (2001) equations.

2.2.7. Serum glucose and cholesterol

Blood samples were collected on the 19th day of each experimental period by puncture of a coccygeal vein or artery, before the morning feeding. The samples were centrifuged at 800*g* for 10 min, and the serum was collected and stored at −20 °C. The analyses were performed with commercially available colorimetric kits (glucose: cat. no. K-082; total cholesterol: cat. no. K-083; Bioclin®, Belo Horizonte, Brazil). The readings were determined with a semi-automatic spectrophotometer (SBA 200, CELM, São Caetano do Sul, Brazil).

Table 2
Nutrient intake and total apparent digestibility of cows fed different fat sources.

Item	Diet[a]				SEM[b]	P[c]		
	CON	SO	SG	CS		C1	C2	C3
Intake								
Dry matter, kg/d	21.9	19.5	20.0	19.5	0.80	0.032	0.822	0.662
NDF[d], kg/d	7.91	6.95	7.31	6.88	0.31	0.044	0.739	0.394
Crude protein, kg/d	2.76	2.45	2.43	2.54	0.09	0.019	0.780	0.441
Ether extract, kg/d	0.61	1.03	1.00	1.09	0.05	0.001	0.713	0.166
$NE_{L3\times}$ [e], MJ/d	172	164	170	162	5.98	0.343	0.629	0.193
Apparent digestibility								
Dry matter	0.692	0.651	0.641	0.661	0.015	0.071	0.740	0.520
Crude protein	0.798	0.776	0.759	0.771	0.015	0.111	0.543	0.443
Ether extract	0.922	0.950	0.938	0.945	0.005	0.017	0.294	0.641
NDF[d]	0.568	0.526	0.538	0.529	0.009	0.052	0.504	0.108

[a] Control (CON); soybean oil (SO); soybean grain (SG); calcium salts of fatty acids (CS) (Megalac-E®).
[b] Standard error of mean.
[c] Probability: C1 = CON vs fat sources (SO. SG. and CS); C2 = SO vs SG and CS; C3 = SG vs CS.
[d] NDF: Neutral detergent fiber.
[e] Estimated according to NRC (2001): NE_{L3x} (MJ/kg MS) = [0.245 * TDN_{1x} (g/kg) − 0.12] * 4.184, were total digestible nutrients(TDN) were estimated as: TDN_{1X} (g/kg) = tdNFC(g/kg) + tdCP(g/kg) + (tdFA(g/kg) * 2.25) + tdNDF(g/kg); where tdNFC, tdCP, tdFA and tdNDF were the truly digestible fraction of non fiber carbohydrate, crude protein, fatty acids and neutral detergent fiber, respectively.

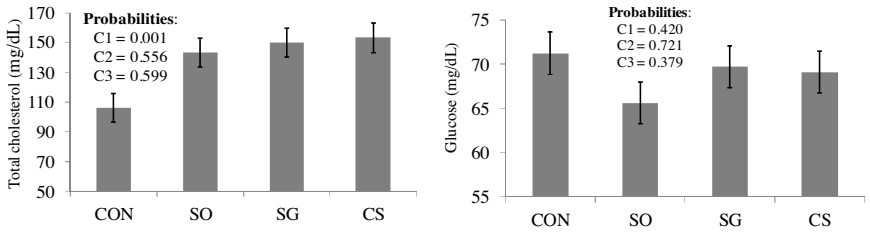

Fig. 1. Serum total cholesterol and glucose concentrations in cows fed different fat sources.
Mean ± standard error of the mean. Control (CON); soybean oil (SO); soybean grain (SG); calcium salts of fatty acids (CS) (Megalac-E®). Probability: C1 = CON vs fat sources (SO, SG, and CS); C2 = SO vs protected sources (SG and CS); C3 = SG vs CS.

2.3. Statistical analyses

Data were analyzed using PROC MIXED (Statistical Analysis System for Windows 9.0, SAS Institute Inc., Cary, USA), according to the following model:

$$Y_{ijkl} = m + D_i + P_j + S_k + a_l(s_k) + e_{ijkl}$$

where: Y_{ijklm} was the observed value in the animal l, from k square, in the j^{th} period, which received the i^{th} experimental diet; μ = overall mean; D_i was the fixed effect of diet; P_j was the fixed effect of experimental period; S_k was the fixed effect of the Latin square; $a_l(s_k)$ was the random effect of animal within Latin square; e_{ijkl} was the random residual error.

Diets effect were studied in three orthogonal contrasts: C1: fat source effect (CON vs SO + SG + CS); C2: protected fat source effect (SO vs SG + CS); C3: protection type effect (SG vs CS). Degrees of freedom correction was made according to Kenward and Roger (1997). Means shown were adjusted by LSMEANS function.

The ruminal fermentation variables (pH, NH_3-N, acetate, propionate, butyrate, and total SCFA) were analyzed as repeated measures in PROC MIXED of SAS 9.0, considering in the statistical model the effects of animal, period, and experimental diet, beyond the effects of time with their interactions with the other effects mentioned above. Akakike criterion was used for choosing covariance structure. Differences were considered significant at the 0.05 level. Trends were considered at $P < 0.10$.

3. Results

3.1. Feed intake and digestibility

Animals fed with fat source diets had lower DM intake in relation to those fed control diet ($P < 0.05$; Table 2). Similarly, animals fed fat sources had lower CP and NDF intake and higher EE intake than those fed CON ($P < 0.05$). Then, the observed decrease in DM and nutrient intake was directly related to the higher energy density of fat source diets (Table 1).

The experimental diets had no effect on net energy intake, DM and CP apparent digestibility and serum glucose concentration (Fig. 1). Although, fat sources increased ether extract digestibility and serum cholesterol concentration ($P < 0.05$) and tended to decrease NDF ($P = 0.052$) and dry matter ($P = 0.071$) apparent digestibility.

Table 3
Ruminal fermentation of dairy cows fed different fat sources.

Item	Diet[a]				SEM[b]	*P*[c]				
	CON	SO	SG	CS		Time	D*T I	C1	C2	C3
pH	6.01	6.03	5.97	6.28	0.07	<0.001	0.281	0.037	0.023	<0.001
NH_3-N, g/L	0.214	0.222	0.215	0.230	0.005	0.001	0.930	0.451	0.988	0.246
Total SCFA[d],mMol/L	183	187	183	177	2.28	<0.001	0.756	0.726	0.085	0.199
Acetate, mMol/L	121	121	119	117	1.47	<0.001	0.792	0.448	0.315	0.564
Propionate, mMol/L	35.4	40.0	37.3	34.4	0.57	<0.001	0.954	0.053	<0.001	0.012
Butyrate, mMol/L	18.4	17.1	17.1	16.7	0.28	0.001	0.458	0.005	0.661	0.466
A:P[e]	3.45	3.08	3.25	3.45	0.02	<0.001	0.999	<0.001	<0.001	0.002

[a] Control (CON); soybean oil (SO); soybean grain (SG); calcium salts of fatty acids (CS) (Megalac-E®).
[b] Standard error of the mean.
[c] Probability: D*T I.: diet*time interaction; C1 = CON vs fat sources (SO. SG. and CS); C2 = SO vs SG and CS; C3 = SG vs CS.
[d] SCFA: short-chain fatty acids.
[e] A:P: Acetate to propionate concentrations ratio.

Table 4
Ruminal kinetics in cows of cows fed different fat sources.

Item	Diet[a]				SEM[b]	*P*[c]		
	CON	SO	SG	CS		C1	C2	C3
Ruminal content, kg	68.8	67.7	70.0	66.5	3.29	0.746	0.823	0.224
Digestion rate, h^{-1}								
Dry matter	0.033	0.031	0.034	0.035	0.002	0.908	0.367	0.653
NDF[d]	0.015	0.012	0.015	0.017	0.001	0.991	0.044	0.347
Passage rate, h^{-1}								
Dry matter	0.036	0.032	0.031	0.030	0.002	0.054	0.613	0.712
NDF [d]	0.022	0.021	0.021	0.019	0.001	0.402	0.465	0.220
Ruminal removal rate, h^{-1}								
Dry matter	0.069	0.063	0.064	0.064	0.002	0.075	0.497	0.782
NDF [d]	0.037	0.034	0.037	0.036	0.001	0.384	0.272	0.649
iNDF[e]	0.025	0.026	0.026	0.023	0.001	0.848	0.633	0.235
tdNDF[6]	0.049	0.044	0.048	0.052	0.003	0.877	0.335	0.604
Intake, kg/d								
NDF [d]	7.91	6.95	7.43	7.16	0.27	0.016	0.253	0.444
tdNDF[6]	5.34	4.41	5.00	4.91	0.30	0.121	0.157	0.839
Ruminal digestibility								
NDF[d], kg/d	3.30	2.63	3.15	3.57	0.32	0.653	0.092	0.396
NDF[d], kg/kg	0.394	0.357	0.399	0.463	0.034	0.768	0.052	0.212
tdNDF[6], kg/kg	0.570	0.553	0.549	0.550	0.032	0.076	0.969	0.987

[a] Control (CON); soybean oil (SO); soybean grain (SG); calcium salts of fatty acids (CS) (Megalac-E®).
[b] Standard error of the mean.
[c] Probability: C1 = CON vs fat sources (SO. SG. and CS); C2 = SO vs SG and CS; C3 = SG vs CS.
[d] NDF: neutral detergent fiber.
[e] iNDF: indigestible neutral detergent fiber; tdNDF: truly digestible neutral detergent fiber.

3.2. Ruminal fermentation

There was an expected physiological time effect on the ruminal variables evaluated regardless of animal diet. When analyzed independently, fat sources addition increased ruminal pH ($P < 0.05$; Table 3), decreased rumen concentration of butyrate and acetate to propionate (A:P) ratio ($P < 0.05$), and tended to increase ruminal propionate concentration ($P = 0.053$).

Fat protected sources (SG and CS) increased pH and A:P ratio ($P < 0.05$), and decreased ruminal propionate ($P < 0.05$) when compared to unprotected source (SO). Furthermore, between protected sources, SG increased ruminal propionate concentration and decreased A:P ratio and pH, in relation to the CS diet ($P < 0.05$). Altogether, our results show that dietary fat sources, especially unprotected ones, affect ruminal fermentation. However, parameters were all within the normal range for animals at this lactation stage.

3.3. Ruminal kinetics

Fat source diets had no effects on DM digestion and NDF passage rates. However, fat addition tended to decrease DM passage ($P = 0.052$; Table 4) and ruminal removal rates ($P = 0.075$). Animals fed protected sources had higher ruminal NDF digestion rate than those fed SO diet ($P < 0.05$).

Table 5
Fatty acid (FA) intake, abomasal flow, and ruminal biohydrogenation rate in cows fed different fat sources.

Item	Diet[a]				SEM[b]	*P*[c]		
	CON	SO	SG	CS		C1	C2	C3
Intake, g/d								
Total FA	488	857	797	860	60.6	<0.001	0.583	0.304
16:0	55.3	99.1	98.7	97.6	7.09	<0.001	0.878	0.873
18:0	15.8	27.5	26.8	27.6	1.97	<0.001	0.849	0.678
cis 18:1	111	192	174	193	13.5	<0.001	0.456	0.165
18:2	246	431	404	431	30.5	<0.001	0.600	0.377
18:3	27.2	46.5	45.4	48.4	3.38	<0.001	0.884	0.374
Abomasal flow, g/d								
Total FA	611	984	984	903	118	<0.001	0.639	0.420
16:0	103	142	172	160	24.2	0.011	0.280	0.637
18:0	438	727	684	610	82.5	<0.001	0.210	0.309
trans 18:1	13.8	44.8	11.3	33.6	5.86	0.014	0.002	0.006
cis 18:1	19.0	22.9	58.9	38.8	16.2	0.207	0.148	0.325
18:2	9.0	10.5	18.1	12.8	2.22	0.045	0.049	0.064
18:3	0.19	0.22	0.28	0.33	0.15	0.522	0.559	0.781
Biohydrogenation rate								
18:1	0.706	0.659	0.604	0.619	0.096	0.405	0.635	0.894
18:2	0.962	0.975	0.955	0.968	0.006	0.530	0.039	0.091
18:3	0.992	0.996	0.994	0.991	0.003	0.649	0.382	0.506

[a] Control (CON); soybean oil (SO); soybean grain (SG); calcium salts of fatty acids (CS) (Megalac-E®).
[b] Standard error of the mean.
[c] Probability: C1 = CON vs fat sources (SO. SG. and CS); C2 = SO vs SG and CS; C3 = SG vs CS.

Table 6
Milk production and composition of cows fed different fat sources.

Item	Diet[a]				SEM[b]	P[c]		
	CON	SO	SG	CS		C1	C2	C3
Production, kg/d								
Milk	15.5	15	14.9	15.5	0.67	0.570	0.538	0.473
FCM[d]	16.8	15.5	17.2	16.0	0.60	0.419	0.13	0.153
Fat	0.62	0.55	0.66	0.56	0.02	0.514	0.698	0.008
Protein	0.48	0.45	0.46	0.47	0.02	0.348	0.91	0.279
Lactose	0.72	0.68	0.69	0.71	0.03	0.297	0.799	0.261
Milk composition, g/kg								
Fat	40.8	38.2	46.4	37.9	0.18	0.987	0.174	0.017
Protein	31.0	29.9	31.0	30.7	0.04	0.610	0.294	0.734
Lactose	46.5	45.0	46.5	46.0	0.06	0.635	0.337	0.755

[a] Control (CON); soybean oil (SO); soybean grain (SG); calcium salts of fatty acids (CS) (Megalac-E®).
[b] Standard error of the mean.
[c] Probability: C1 = CON vs fat sources (SO, SG, and CS); C2 = SO vs SG and CS; C3 = SG vs CS.
[d] FCM: Fat corrected milk, estimated according Sklan et al. (1992).

Fat protected sources tended to decrease ruminal NDF digestibility in relation to SO diet (P = 0.092). This effect was replicated when we evaluated NDF digestibility coefficient (P = 0.052). In addition, lipid sources tended to decrease ruminal tdNDF digestibility coefficient (P = 0.076) and had no effect on liquid ruminal turnover.

3.4. Abomasal flow and ruminal biohydrogenation of fatty acids

Dietary fat sources potentiated the intake of all evaluated fatty acids (P < 0.05; Table 5). Similarly, animals fed fat source diets had increased abomasal flows of C18:2, *trans* C18:1, C18:0, C16:0, and total fatty acids. Protected lipid sources resulted in higher C18:2 abomasal flow and lower *trans* C18:1 flow in relation to SO (P < 0.05). Between protected fat sources, SG had lower *trans* C18:1 abomasal flow, and tended to had higher C18:2 abomasal flow (P = 0.064) than CS.

Dietary treatments had no effect on the biohydrogenation rate of C18:1 and C18:3. Fat protected sources decreased C18:2 biohydrogenation rate compared to the SO diet. Soybean grain tended to decrease C18:2 biohydrogenation in relation to CS (P = 0.091).

3.5. Milk yield, composition and fatty acids profile

Fatty acids addition had no effect on milk yield and composition (P > 0.05; Table 6). However, animals fed with CS showed lower milk fat yield and concentration when compared with those fed SG (P < 0.05).

Table 7
Milk fatty acid (FA) profile of dairy cows fed different fat sources.

Item	Diet[a]				SEM[b]	*P*[c]		
	CON	SO	SG	CS		C1	C2	C3
FA, g/kg total FA								
C4:0	11.0	7.0	7.1	12.1	1.84	0.203	0.170	0.027
C6:0	13.4	7.2	9.1	10.4	1.82	0.023	0.210	0.575
C8:0	10.0	6.1	7.7	6.5	1.07	0.005	0.373	0.365
C10:0	25.6	19.3	21.1	15.3	1.91	0.003	0.619	0.033
C11:0	2.4	1.5	1.6	0.8	0.25	<0.001	0.415	0.041
C12:0	33.9	25.7	26.5	20.7	2.22	<0.001	0.420	0.055
C14:0	121	98	100	89	4.50	<0.001	0.563	0.122
C14:1	5.4	4.5	4.5	4.2	0.53	<0.001	0.736	0.523
C15:0	3.3	2.4	1.9	1.6	0.40	<0.001	0.077	0.440
C16:0	351	306	307	301	11.1	<0.001	0.892	0.717
C16:1 *cis*	14.5	12.5	10.8	11.0	1.36	0.014	0.206	0.849
C17:0	2.1	1.9	2.1	1.9	0.36	0.616	0.795	0.458
C18:0	116	158	176	160	12.3	<0.001	0.360	0.205
C18:1 *trans 11*	12.2	21.2	6.0	21.1	3.64	0.281	0.057	0.002
C18:1*cis* 9	277	317	302	322	17.1	0.024	0.762	0.293
C18:2 *n*-6. *cis*	20.6	17.7	29.4	23.2	2.76	0.148	<0.001	0.015
C18:3	1.0	1.2	1.5	1.1	0.25	0.297	0.541	0.213
C20:0	1.1	1.4	1.5	1.2	0.12	0.023	0.414	0.026
cis-9.*trans*-11 CLA	5.0	8.8	1.7	7.5	1.94	0.598	0.046	0.020
<C16	228	173	181	162	11.4	<0.001	0.903	0.256
C16	351	306	307	301	11.1	0.001	0.892	0.717
>C16	450	541	532	552	25.1	0.003	0.982	0.548
Unsaturated C18	310	356	338	369	20.3	0.022	0.890	0.164
Saturated C18	116	158	176	160	12.3	<0.001	0.360	0.205
Saturated	693	636	663	622	17.4	0.004	0.715	0.068
Unsaturated	336	384	356	393	22.0	0.035	0.628	0.117
U:S FA ratio[d]	2.15	1.73	1.88	1.60	0.15	0.006	0.917	0.109

[a] Control (CON); soybean oil (SO); soybean grain (SG); calcium salts of fatty acids (CS) (Megalac-E®).
[b] Standard error of the mean.
[c] Probability: C1 = CON vs fat sources (SO. SG. and CS); C2 = SO vs SG and CS; C3 = SG vs CS.
[d] Unsaturated to saturated fatty acids ratio.

Animals fed with fat source diets had lower milk concentration of short chain fatty acids, including C6:0, C8:0, C10:0, C11:0, C12:0, C14:0, C14:1, C15:0, C16:0 and C16:1 FA in relation to animals fed CON diet ($P < 0.05$; Table 7). Soybean grain increased C10:0, C11:0 e C12:0 milk concentration in relation to SC ($P < 0.05$). In addition, cows fed diets with lipid sources had higher milk concentration of C18:0, C20:0, *cis*9 C18:1, and total saturated and unsaturated C18 FA than those fed CON ($P < 0.05$).

Dietary fat addition increased milk unsaturated FA concentration ($P < 0.05$). Animals fed with protected fat source diets had higher milk C18:2 *n*-6, and lower *cis* 9 *trans 11* CLA concentrations compared those fed SO ($P < 0.05$). Soybean grain decreased C18:2 *n*-6 and increased *trans11* C18:1 and *cis*9 *trans11* CLA milk concentrations than CS ($P < 0.05$).

3.6. Energy and nitrogen balances and microbial protein synthesis

Diets had no effect on nitrogen balance, nitrogen utilization efficiency, microbial protein synthesis, and microbial growth efficiency ($P > 0.05$; Table 8).

4. Discussion

The absorption of FAs in the duodenum of dairy cows affects animal health, performance, and ultimately product quality. We evaluated how different fat sources affect ruminal kinetics and FA flow, and found that oilseed results in lower ruminal biohydrogenation rate and increased abomasal flow of unsaturated FAs, when compared to vegetable oil and unsaturated FA calcium salts.

In the current study, fat-supplemented diets decreased dry matter intake (DMI) in relation to the control diet. Several authors have reported this effect of dietary fat addition (Palmquist and Jenkins, 1980; Casper et al., 1990; Schauff et al., 1992). The mechanisms underlying fat-induced reduction in DMI have yet to be fully established but may result from the acceptability of high fat content diets, release of gut hormones, and fat oxidation in the liver (Allen, 2000). Alternatively, gut motility associated with increased cholecystokinin (CCK) and slow gastric emptying may also contribute to the reduction in DMI (Liddle et al., 1985). We speculate based on our results that this effect may also reflect the distinct energy density of fat-containing diets (Table 1). Because we formulated diets to have similar CP and NDF levels, fat supplementation also reduced the intake of these nutrients.

Table 8
Nitrogen and energy balances, and microbial protein synthesis in cows fed different fat sources.

Item	Diet[a]				SEM[b]	*P*[c]		
	CON	SO	SG	CS		C1	C2	C3
N intake, g/d	442	393	390	407	15.4	0.019	0.601	0.532
N balance, g/d	206	159	168	198	14.8	0.146	0.653	0.136
N efficiency[d]	0.167	0.173	0.191	0.181	0.007	0.236	0.303	0.601
NE_L[e]/DEI[f]	0.282	0.295	0.284	0.293	0.012	0.847	0.785	0.813
Energy balance, MJ/d	48.5	36.3	44.6	35.8	7.11	0.458	0.678	0.563
CPmic[g], g/d	1032	1128	1019	1020	24.0	0.961	0.245	0.765
Microbial efficiency[h], g/kg	64.5	76.7	67.9	68.4	1.79	0.507	0.174	0.897

[a] Control (CON); soybean oil (SO); soybean grain (SG); calcium salts of fatty acids (CS) (Megalac-E®).
[b] Standard error of the mean.
[c] Probability: C1 = CON vs fat sources (SO. SG. and CS); C2 = SO vs SG and CS; C3 = SG vs CS.
[d] N efficiency: N milk to N intake ratio.
[e] NEL: Net energy for lactation.
[f] DEI: Digestible energy intake.
[g] CPmic: Microbial crude protein.
[h] Microbial efficiency: g de microbial crude protein to kg of TDN intake.

The three fat-supplemented diets decreased DMI without altering total weight of rumen content. Thus, dietary fat decreased DM passage rate and ruminal removal rate. Digestion and passage rates oppose each other in that a slower passage rate correlates with higher digestion (Allen and Mertens, 1988). Therefore, we would expect that the decrease in passage rate caused by dietary fat should result in improved digestion. However, we observed that supplemental fat tended to decrease the tdNDF ruminal digestion (relative value). This difference resulted from the inhibitory effects of the free fat in the SO diet on rumen fermentation that pulled down the average NDF digestibility of fat-containing diets as a group. The inhibitory effects of free fat on ruminal NDF digestibility has been previously reported (AbuGhazaleh et al., 2004).

An increase in rumen propionate concentration and decrease in A:P ratio was observed in animals fed fat-rich diets. The effect on rumen SCFA concentration may result of cellulolytic bacteria growth inhibition (Yang et al., 2009) promoting amylolytic bacteria growth (Patra and Yu, 2013) which favor propionate production.

Animals fed fat-supplemented diets had increased ruminal pH when compared to control. This difference mostly results from a pH increase induced by the CS diet, which raised the average for the combined fat-supplemented diets. This effect of FA calcium salt diets has not been described previously and the mechanisms underlying it remain unknown. Rumen pH of animals fed CS was 0.31 greater than that of SG-fed animals. Lower ruminal pH can change the microbial population, altering usual ruminal biohydrogenation routes by changing the formation of *cis*9 *trans*11 C18:2 and *trans*11 C18:1 by *trans*10 *cis*12 C18:2 and *trans*10 C18:1 formation, key intermediates of linoleic acid biohydrogenation (Griinari et al., 1998; Bauman and Griinari, 2003; Shingfield et al., 2010). In the present study, even with lower rumen pH in SG-fed animals, biohydrogenation rates were lower in comparison with CS-fed animals.

Fat-supplemented diets increased abomasal flow of all evaluated FAs, except for *cis*-C18:1 and C18:3. Diets containing low concentrations of these unsaturated FAs are also more subjected to the biohydrogenation process (Harvatine and Allen, 2006). Protected FA sources decreased C18:2 biohydrogenation, with a consequent increase in its abomasal flow. Protected fat sources decreased *trans* C18:1 flow, a known intermediate product of C18:2 biohydrogenation (Mosley et al., 2002). When compared to the CS diet, the SG diet decreased biohydrogenation and increased the flow of C18:2 while reduces *trans* C18:1 abomasal flow (Table 5). This result shows that an oilseed diet (SG) provides greater FA protection than calcium salts. The digestion of commercially available FA calcium salts should begin in the low pH environment of the abomasum. However, the 5.6 pKa of soybean calcium salts lies below the lower limits of rumen pH (Sukhija and Palmquist, 1990). Thus, the desired protective effect against ruminal biohydrogenation does not occur at the advertised rate.

Milk short-chain fatty acids are mainly synthesized in the epithelial cells of the mammary gland, from acetate and β-hidroxibutirate, which are originated in the rumen. In this study, milk short chain FA profile results suggests inhibition of short chain FA synthesis of milk fat in cows fed with fat sources which can be explained by reduced ruminal acetate to propionate ratio, and, the fatty acids supply for the mammary gland (Bauman and Griinari, 2003).

An assessment of the inclusion and fat digestion processes in ruminants shows that most fatty acids are changed by ruminal metabolism, so biohydrogenation is generally not complete, resulting in a wide variety of fatty acids (Byers and Schehing, 1993). So when an incomplete biohydrogenation of PUFA occurs, there is an increase in duodenal flow of fatty acids C18:1 *trans* and conjugated linoleic acids CLA *cis*-9,-11and *trans*-11and CLA *trans*-10, *cis*-12, the latter of which presents an attested inhibiting effect over milk fat synthesis (Bauman and Griinari, 2001). As observed in this study, fat sources diets changed milk fatty acids profile and animals fed SG diets had higher concentration of FA C18:2 on milk and in the abomasum, and lower concentration of intermediaries the biohydrogenation (C18:1 *trans;* CLA *cis*-9,-11 *trans*-11 and CLA *trans*-10, *cis*-12) than animals fed CS diet.

The protection of whole raw soybean grain FA against ruminal bio-hydrogenation, improves abomasal C18:2 concentration and decreases isomer formation, which are associated with milk fat depression. This result demonstrates the power of protection and the slow liberation of soybean grain lipids.

5. Conclusion

The natural physical barriers of the oilseed more efficiently protected FAs against ruminal biohydrogenation than the chemical barrier provided by calcium salts. This finding may guide the development of new rumen inert products. Alternatively, future studies should evaluate the FA content and flow of other oilseeds that might have greater technical and economic viability than commercial products.

Conflict of interests

The authors declare that are no conflicts of interest to the current manuscript.

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