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Different profiles of fatty acids in Ca soaps on dissociation and modification by biohydrogenation *in vitro*

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ABSTRACT - This study evaluated the effects of rumen pH on *in vitro* Ca dissociation of different sources of Ca soaps of fatty acids (CSFA). Treatments were distributed in a 6×4 factorial design and consisted of six CSFA sources and four rumen fluid pH. The CSFA sources were: two sources of palm oil (PO1 and PO2), soybean oil (SO), palm + soybean oil (PSO), palm + cottonseed oil (PCO), and palm + cottonseed + soybean oil (PCSO). All CSFA samples were added to a pool of rumen fluid and adjusted to four different pH (5.5, 6.0, 6.5, and 7.0), and then, incubated at 37 °C for 1 h. This procedure was replicated over three consecutive days. Effect of CSFA source × rumen pH was detected for *in vitro* relative Ca dissociation and change (*Δ*) in concentrations of oleic, linoleic, and linolenic acids. Calcium dissociation did not differ among CSFA sources in pH 7.0 or 6.5, but was greater for SO vs. PO2, PSO, PCO, and PCSO in pH 6.0. Relative Ca dissociation in pH 5.5 was lower for PO1 and PSO vs. SO, but greater for PO1 and PSO vs. PCO and PCSO. The *Δ* of oleic acid was greater for PO2 vs. PCO in pH 6.5 and PO1 and PCSO vs. PO2 and SO in pH 7.0. The *Δ* of linoleic acid was greatest for SO across all pH evaluated and did not differ between PO1 and PO2, but both had a reduced *Δ* of linoleic acid than other CSFA sources in pH 5.5 and 7.0. The *Δ* of linolenic acid concentrations did not differ between PO1 and PO2, but both had less *Δ* of linolenic acid concentrations than other CSFA sources across all pH. Besides, SO had greater *Δ* of linolenic acid compared with PSO, PCO, and PCSO in pH 5.5, 6.0, and 6.5. Combining palm + cottonseed oil and palm + cottonseed + soybean oil reduces Ca dissociation and maintains the original fatty acid profile of the CSFA source.

Keywords: cottonseed, palm, rumen, soybean

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1. Introduction

The use of Ca soaps of fatty acids (CSFA) is one alternative to mitigate the potential negative effects of lipid supplementation on rumen metabolism and function in ruminants (Palmquist and Jenkins, 1980; Relling and Reynolds, 2007; Jenkins and Harvatine, 2014; Freitas Jr. et al., 2018). The CSFA are expected to be stable at pH values above 6.0 and dissociate as pH levels gradually decrease (Jenkins and Palmquist 1984; Palmquist, 1984).

Sources of CSFA containing PUFA have greater dissociation percentages as rumen pH decreases compared with Ca sources of monounsaturated and saturated fatty acids (Sukhija and Palmquist, 1990). However, limited information is available regarding the effects of rumen pH on dissociation of multiple CSFA sources with different profiles of long-chain fatty acids. Traditionally, oilseeds (e.g., whole cottonseed and soybean) and palm oil are the most used sources of fat included into the diets

of cattle (Rabiee et al., 2012). Due to the yearly price fluctuation, different high oil-based commodities may be used as fat source to manufacture CSFA, potentially leading to fatty acid dissociation levels different from the expected. Therefore, the objective of the current study was to evaluate the effects of rumen pH on *in vitro* dissociation of CSFA obtained from different oil-based sources (cottonseed, palm, soybean, and mixtures of these oils).

2. Material and Methods

This study was conducted in Ona, Florida, USA (27°26' N and 82° 55' W) from January to April 2019. Animals were cared for in accordance with acceptable practices as outlined in the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010).

Treatments evaluated herein consisted of six CSFA sources: two sources of palm oil (PO1 and PO2), soybean oil (SO), palm + soybean oil (PSO), palm + cottonseed oil (PCO), and palm + cottonseed + soybean oil (PCSO) assigned to four rumen pH (5.5, 6.0, 6.5, and 7.0) in a 6×4 factorial design. All CSFA samples were obtained from Nutricorp Nutrição Animal (Araras, São Paulo, Brazil), whereas PO1 was obtained from Church & Dwight Co. Inc. (Megalac®, Princeton, NJ, USA). The fatty acid profile of each source used herein is described in Table 1. Manufacturing standards for quality and guaranteed levels were verified by each supplier according to their manufacturing procedures.

The procedures for the *in vitro* incubation used in this study were performed according to those previously reported by Sukhija and Palmquist (1990) with a few modifications (described below). A pool of ruminal fluid was generated using the ruminal content of three rumen-cannulated Angus

Table 1 - Composition of calcium soaps of fatty acids (CSFA) used in the *in vitro* incubations

Item[2]	Ca soaps of fatty acids[1]					
	PO1	PO2	SO	PSO	PCO	PCSO
Dry matter (g kg^{-1} DM)	963	965	955	968	972	969
Total fatty acids (g kg^{-1} DM)	883	888	832	820	792	820
Total calcium (g kg^{-1} DM)	97.3	92.2	88.5	96.7	98.9	96.4
Fatty acid profile (g 100 g^{-1} of fatty acid methyl esters)						
Myristic (14:0)	1.26	2.30	0.31	1.53	1.54	2.02
Palmitic (16:0)	45.6	42.6	22.6	29.6	28.6	34.0
Palmitoleic (16:1 *cis*-9)	0.19	0.21	0.14	0.23	0.50	0.25
Stearic (18:0)	4.50	4.28	5.62	4.66	4.87	4.31
Elaidic (18:1 *trans*-9)	0.25	0.20	5.58	0.48	1.31	0.40
Oleic (18:1 *cis*-9)	35.4	33.7	21.4	28.1	25.7	29.2
Vaccenic (18:1 *cis*-11)	0.85	0.85	1.95	1.22	1.30	1.00
Linoleic (18:2 *n*-6)	8.62	8.21	28.2	23.6	25.4	20.0
Linolenic (18:3 *n*-3)	0.29	0.28	2.04	1.98	2.12	1.32
Arachidic (20:0)	0.35	0.31	0.41	0.34	0.34	0.31
Gonodic (20:1 *n*-9)	0.12	0.12	0.22	0.16	0.20	0.14
Behenoic (22:0)	ND	0.05	0.64	0.32	0.38	0.20
Lignoceric (24:0)	0.05	0.12	0.39	0.21	0.19	0.12
Others (14:0 to 24:0)	0.20	0.27	0.38	0.34	0.26	0.20
Total SFA	51.8	49.8	30.1	36.7	36.0	41.0
Total MUFA	36.9	35.1	29.3	30.3	29.1	31.1
Total PUFA	8.96	8.57	30.4	25.7	27.7	21.4

SFA - saturated fatty acids; MUFA - monounsaturated fatty acids; PUFA - polyunsaturated fatty acids; ND - not detected.
[1] CSFA sources consisted of: palm oil [PO1 and PO2), soybean oil (SO), palm + soybean oil (PSO), palm + cottonseed oil (PCO), and palm + cottonseed + soybean oil (PCSO)].
[2] Composed sample taken during each day of incubation.

steers (approximately 500 kg body weight) before supplement feeding (07.00 h). The donor steers were housed in an open-sided barn and fed a diet composed of free-choice access to stargrass hay (*Cynodon nlemfuensis*) and 2 kg d^{-1} (as-fed basis) of a 50:50 soybean meal and cottonseed meal supplement offered once a day at 08.00 h. The steers had free access to fresh water and a mineral supplement [containing (dry matter basis) 168 g kg^{-1} of Ca; 40 mg kg^{-1} of P; 248 mg kg^{-1} of NaCl; 10 mg kg^{-1} of Mg; 5,000 mg kg^{-1} of Zn; 1,700 mg kg^{-1} of Cu; 60 mg kg^{-1} of Co; 350 mg kg^{-1} of I; 60 mg kg^{-1} of Se; 440,000 IU kg^{-1} of vitamin A; 33,000 IU kg^{-1} of vitamin D_3; 441 IU kg^{-1} of vitamin E].

The sampling of the pooled ruminal content and all *in vitro* incubations (all CSFA × pH combinations) were replicated three times on three consecutive days (n = 1 rumen fluid pool and 1 round of all incubations day^{-1}). Each day, the ruminal content was manually obtained from the ventral region of the rumen of each steer, filtered through four layers of cheesecloth into 3.8-L insulated bottles pre-heated at 39 °C by filling with hot water, and transferred to the laboratory within 10 min of sampling. The filtrate was then pooled among all steers and transferred to 50 mL polypropylene tubes, centrifuged at 6,000 × *g* for 15 min at 5 °C (Sorvall™ Legend™ X1R, Thermo Fisher Scientific, Waltham, MA, USA), and the supernatant was divided into four 500-mL beakers (200 mL rumen supernatant $beaker^{-1}$), so the pH could be adjusted to 5.5, 6.0, 6.5, or 7.0 (Accumet™ XL250, Fisher Scientific, Hampton, NH, USA) by adding 1 N H_3PO_4 or 1 N NaOH dropwise.

Samples of each CSFA source (approximately 1.5 g) were previously weighed (Ohaus E11140 Explorer Analytical Balance, Ohaus Corporation, Parsippany, NJ, USA) into Pyrex™ culture tubes (20 × 2 cm; n = 1 tube $CSFA^{-1}$ $rumen^{-1}$ pH^{-1} day^{-1}; total of 24 tubes day^{-1}). The rumen fluid supernatant adjusted to each respective pH was added (30 mL) into each tube using a 50-mL Pyrex™ glass graduated cylinder to obtain a 5% CSFA concentration and sealed with Teflon®-lined screw caps. Each tube was then vortexed (Fisherbrand™ Mini Vortex, Fisher Scientific, Hampton, NH, USA) for 1 min and incubated in a shaking water bath (GP 20, Precision Water Baths, Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C for 1 h. The content of Pyrex™ culture tubes were then transferred into 50-mL polypropylene tubes and centrifuged at 1,000 × *g* for 10 min at 5 °C (Sorvall™ Legend™ X1R, Thermo Fisher Scientific, Waltham, MA, USA). By using a Pasteur pipette, all clear subnatant solution was then transferred into 30-mL Falcon® tubes and stored at −21 °C for further analyses of soluble Ca and fatty acid composition.

Subsamples of each CSFA source before incubation (Table 1) and each CSFA source × rumen pH collected on each day of incubation were analysed for Ca and total fatty acids concentrations, as well as fatty acid profile, as described by Brandão et al. (2018). Fatty acid profile was determined according to AOAC (2005; method 996.06), preparation of fatty acid methyl esters was performed according to AOCS (2012; method Ce 2-66), n-3 FA analysis was performed according to AOCS (2012; method Ce 1d-91), and conjugated linoleic acid (CLA) analysis was performed according to AOCS (2012; method Ce 1h-05) using a Supelco SP2560 (100 m × 0.25 mm × 0.2 μm film) column. The gas chromatograph (Agilent 7890A with 7683B Autosampler; Agilent Technologies, Santa Clara, CA, USA) settings were as follows: oven temperature was set held for 5 min at 140 °C, increased at 4 °C min^{-1} to 200 °C (15 min), increased at 2 °C min^{-1} to 240 °C (20 min), and held for 15 min (total: 55 min). The inlet temperature was 250 °C, pressure was 35 psi, split ratio was 60:1, and injection volume was 2 μL. Individual FA are reported as percentage of total FA. Calcium was analyzed by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES; Method 985.01 A, B, and C; AOAC, 2005) after nitric-perchloric wet ash sample preparation with hydrochloric acid sample matrix [method 975.03 B(b); AOAC, 2005]. Ingredients were also analyzed for dry matter at 105 °C (method 930.15; AOAC, 1986). Relative Ca dissociation was calculated as the ratio of total amount of Ca present in each sample after incubation and the initial amount of Ca weighted in each culture tube before incubation. Fatty acid profile was expressed as g 100 g^{-1} of fatty acids and also as fatty acid variation (Δ) between the final and the initial concentration of each fatty acid isomer.

Data were analyzed using the GLIMMIX procedure of SAS (Statistical Analysis System, version 9.3) as a completely randomized design using a 6 × 4 factorial arrangement of treatments. Tube was considered the experimental unit. The model included the fixed effects of CSFA source, rumen pH, and CSFA source × rumen pH. *In vitro* batch and tube (batch × CSFA × rumen pH) were included as random effects.

The Satterthwaite approximation method was used to determine the denominator degrees of freedom for testing fixed effects. When interactions were not significant (P>0.05), main effects of CSFA sources and rumen pH were reported. When an interaction was considered significant (P≤0.05), the effects of CSFA source were evaluated within each rumen pH using the SLICEBY option of SAS.

The statistical model used was:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + B_k + T(B\alpha\beta)_{lijk} + \varepsilon_{ijkl},$$

in which Y_{ijkl} = observation of the effect of CSFA i per pH j in each tube l in each *in vitro* batch k; μ = overall mean; α = effect of CSFA; β = rumen pH; $\alpha\beta$ = interaction between CSFA × rumen pH; B = effect of *in vitro* batch; $T(B\alpha\beta)$ = tube(batch × CSFA × rumen pH); and ε_{ijkl} = random error associated with each observation.

Results were reported as least-squares means, whereas differences were declared significant at P≤0.05 using Tukey-Kramer test.

3. Results

A CSFA source × rumen pH interaction was detected (P≤0.04) for *in vitro* concentration of palmitic (16:0), oleic (18:1 *cis*-9), linoleic (18:2 *n*-6), rumenic (18:2 *cis*-9, *trans*-11), and linolenic (18:3 *n*-3) acids (Table 2). The *in vitro* concentration of palmitic acid decreased (P<0.01) for PO2 vs. PO1 in pH 6.0, but not (P≥0.16) in pH 5.5, 6.5, or 7.0. The concentration of palmitic acid decreased (P≤0.04) for SO vs. PO1 in pH 5.5, 6.0, and 6.5, but not in pH 7.0 (P = 0.07). Moreover, palmitic acid concentration decreased (P<0.001) for SO vs. PO2 only in pH 7.0. No differences were observed (P≥0.54) for palmitic acid concentration between PSO vs. SO and PO2 in pH 5.5, 6.0, and 6.5. In pH 7, palmitic acid concentrations did not differ (P = 1.00) between PSO and SO, but decreased (P<0.001) for PSO vs. PO2.

The concentrations of oleic acid did not differ (P≥0.08) between SO and PO2 in pH 6.5, but decreased (P≤0.001) in pH 5.5, 6.0, and 7.0 (Table 2). The concentrations of oleic acid also decreased (P≤0.03) for SO vs. PO1 across all pH levels. Moreover, concentrations of oleic acid did not differ (P≥0.07) in PCSO vs. PO1 in pH 6.5 and 7.0 and in PCSO vs. PO2 in pH 6.0 and 6.5. The concentrations of oleic acid decreased (P≤0.007) for PCSO vs. PO1 in pH 5.5 and 6.0 and for PCSO vs. PO2 in pH 5.5. Nonetheless, concentrations of oleic acid did not differ (P≥0.09) between PO1 and PO2 in all pH levels evaluated herein.

No differences were detected (P≥0.91) for the concentrations of linoleic acid between PO1 and PO2 in any of the pH evaluated herein (Table 2). Conversely, concentrations of linoleic acid decreased (P<0.001) for PO1 and PO2 compared with PCO and PSO across all pH levels. Moreover, SO had greater (P<0.001) concentrations of linoleic acid in pH 6.5 in PO1 and PO2, whereas PCO had greater (P≤0.05) concentrations of linoleic acid compared with all other CSFA sources in pH 5.5 and 6.0.

The concentrations of rumenic acid did not differ (P≥0.49) among PO1, PO2, and PCSO in pH 6.0 and 6.5, but these sources had greater (P<0.001) concentrations of that fatty acid than SO, PSO, and PCO in pH 6.0 (Table 2). The concentrations of rumenic acid decreased (P = 0.001) for PO2 vs. PO1 in pH 7.0. Moreover, PCSO had greater (P≤0.02) concentration of rumenic acid than SO, PSO, and PCO across all pH levels evaluated herein.

The PSO and PCO sources had greater (P≤0.01) concentrations of linolenic acid compared with all other CSFA sources across all pH levels, whereas no differences were detected (P≥0.06) between these two sources in pH 6.0, 6.5, and 7.0 (Table 2). Regardless of pH, the concentrations of linolenic acid did not differ (P≥0.88) between PO1 and PO2, but both had lower (P≤0.008) concentrations of linolenic acid compared with all other CSFA sources.

No effects of CSFA source, rumen pH, and CSFA × rumen pH were detected (P≥0.18) for concentrations of palmitoleic (16:1 *cis*-9), arachidic (20:0), and gonodic acids (20:1 *n*-9; Table 2). A CSFA source effect was detected (P≤0.05) for *in vitro* concentrations of myristic (14:0), stearic (18:0), and elaidic acids

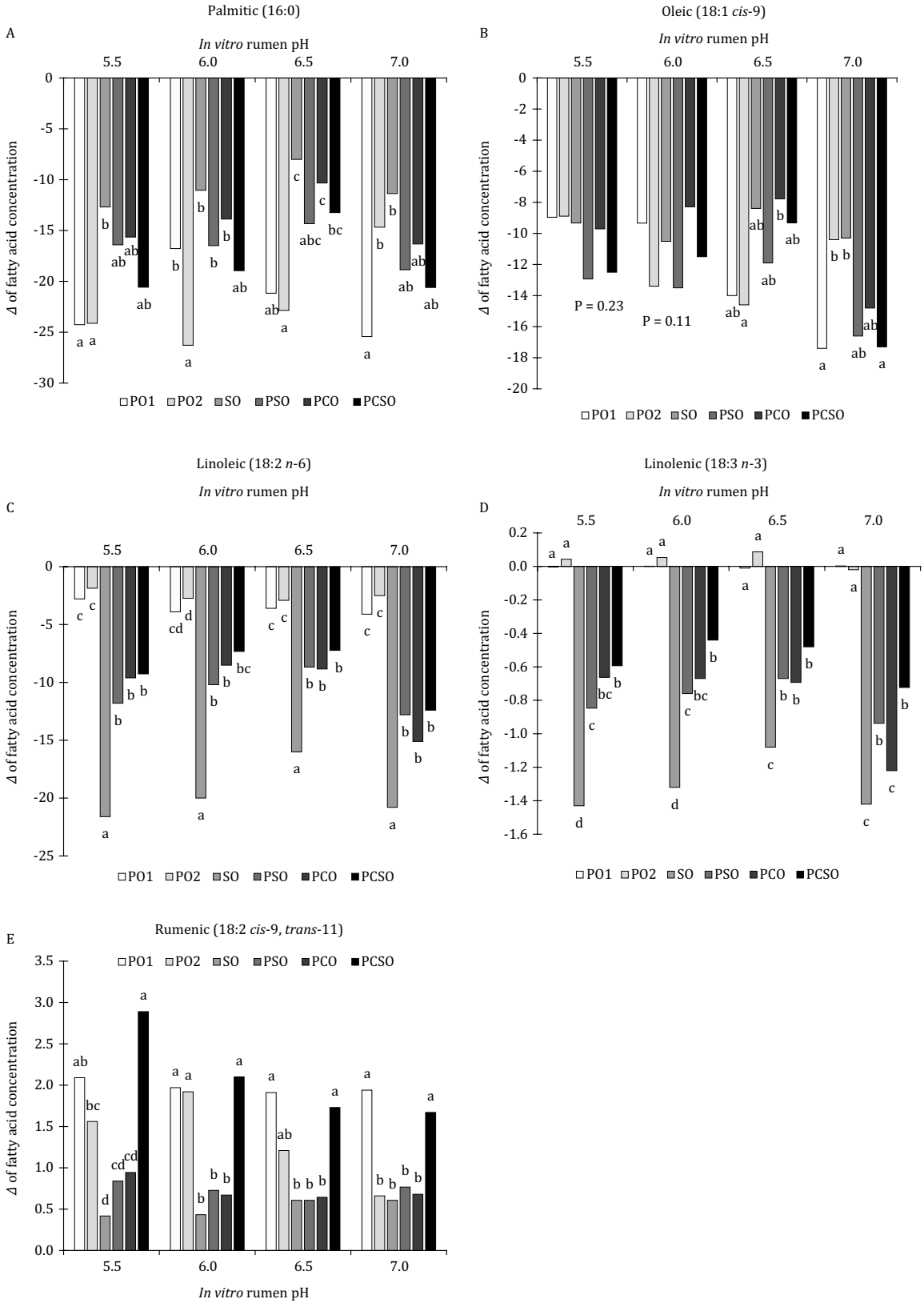
(18:1; Table 2). More specifically, myristic acid concentration was greater (P≤0.005) for PO2 and PCSO compared with all other CSFA sources, and also greater (P≤0.001) for PSO and PCO vs. SO. No differences in myristic acid concentration were observed (P≥0.32) between PO2 and PCSO and among PO1, PSO, and PCO. *In vitro* concentrations of stearic acid were reduced (P = 0.03) for PCO vs. PO1. The elaidic acid concentration was greater (P<0.001) for SO compared with all other CSFA sources, and greater (P≤0.04) for PCO vs. PO1, PO2, and PCSO (Table 2).

A CSFA source × rumen pH interaction was detected (P≤0.04) for *Δ* of concentrations of palmitic, oleic, linoleic, rumenic, and linolenic acids (Figure 1). The *Δ* of palmitic acid did not differ (P≥0.99) between PO1 and PO2 in pH 5.5 and 6.5 (Figure 1A), but decreased in pH 6.0 and increased in pH 7.0 for PO1 vs.

Table 2 - Effects of different sources of calcium soaps of fatty acids (CSFA) and rumen pH (5.5, 6.0, 6.5 and 7.0) on *in vitro* fatty acid profile

Item (g 100 g^{-1} of fatty acid methyl esters)	Ca soaps of fatty acids[1]						SEM	P-value		
	PO1	PO2	SO	PSO	PCO	PCSO		CSFA	Rumen pH	CSFA × rumen pH
Myristic (14:0)	1.29b	1.64a	0.973c	1.27b	1.29b	1.52a	0.044	<0.001	0.087	0.272
Palmitic (16:0)							2.43	<0.001	0.028	0.036
pH 5.5	21.4a	18.5ab	9.94b	13.1ab	13.0ab	13.4ab				
pH 6.0	28.8a	16.4b	11.6b	13.1b	14.8b	15.0b				
pH 6.5	24.4a	19.8ab	14.6b	15.2ab	18.3ab	20.7ab				
pH 7.0	20.2ab	28.0a	11.3bc	10.7c	12.3bc	13.7bc				
Palmitoleic (16:1 *cis*-9)	1.05	1.09	1.23	1.01	1.26	1.04	0.087	0.212	0.441	0.470
Stearic (18:0)	6.72a	6.12ab	6.55ab	6.43ab	6.07b	6.40ab	0.333	0.021	0.005	0.247
Elaidic (18:1 *trans*-9)	0.593c	0.626c	1.59a	0.666bc	0.933b	0.643c	0.069	<0.001	0.014	0.073
Oleic (18:1 *cis*-9)							1.12	<0.001	<0.001	0.007
pH 5.5	26.5a	24.8a	12.1b	15.2b	16.0b	16.8b				
pH 6.0	26.1a	20.3ab	10.9c	14.7bc	17.4b	17.7b				
pH 6.5	21.4a	19.3ab	13.0b	16.3ab	17.9ab	19.9a				
pH 7.0	18.0ab	23.3a	11.2c	11.6c	10.9c	11.9bc				
Linoleic (18:2 *n*-6)							0.595	<0.001	<0.001	<0.001
pH 5.5	5.84c	6.35c	6.53c	11.8b	15.8a	10.8b				
pH 6.0	4.72c	5.48c	8.20c	13.4b	16.9a	12.7b				
pH 6.5	5.02b	5.30b	12.2a	14.9a	16.6a	12.8a				
pH 7.0	4.52b	5.71b	7.41ab	10.9a	10.4a	7.61ab				
Rumenic (18:2 *cis*-9, *trans*-11)							0.187	<0.001	<0.001	0.031
pH 5.5	2.09ab	1.56bc	0.417d	0.840cd	0.943cd	2.89a				
pH 6.0	1.97a	1.92a	0.433b	0.727b	0.670b	2.10a				
pH 6.5	1.91a	1.21ab	0.607b	0.607b	0.643b	1.73a				
pH 7.0	1.94a	0.660b	0.607b	0.767b	0.680b	1.67a				
Linolenic (18:3 *n*-3)							0.032	<0.001	<0.001	<0.001
pH 5.5	0.287d	0.323d	0.610c	1.13b	1.460a	0.727c				
pH 6.0	0.290c	0.333c	0.720b	1.22a	1.450a	0.880b				
pH 6.5	0.280c	0.367c	0.963b	1.310a	1.430a	0.840b				
pH 7.0	0.293c	0.260c	0.617b	1.040a	0.897a	0.597b				
Arachidic (20:0)	0.678	0.628	0.609	0.636	0.667	0.624	0.048	0.911	0.754	0.178
Gonodic (20:1 *n*-9)	0.107	0.155	0.136	0.168	0.209	0.090	0.031	0.070	0.007	0.552

[1] CSFA sources consisted of: palm oil [PO1 and PO2), soybean oil (SO), palm + soybean oil (PSO), palm + cottonseed oil (PCO), and palm + cottonseed + soybean oil (PCSO)].
a-d - Within rows, means with a different letter differ (P<0.05; Tukey's test).



Each bar is the mean of three replicates.
a-d - Within rumen pH, bars with a different letter differ (P≤0.05; Tukey-Kramer test).

Figure 1 - Effects of different sources of calcium soaps of fatty acids [CSFA; palm oil (PO1 and PO2), soybean oil (SO), palm + soybean oil (PSO), palm + cottonseed oil (PCO), and palm + cottonseed + soybean oil (PCSO)] and rumen pH (5.5, 6.0, 6.5 and 7.0) on *in vitro* variation of fatty acids composition (*Δ*; final – initial concentration [g 100 g^{-1}]).

PO2, respectively (P≤0.05). Further differences in Δ of palmitic acid were not detected (P≥0.19) among all other CSFA sources across all pH range (Figure 1A).

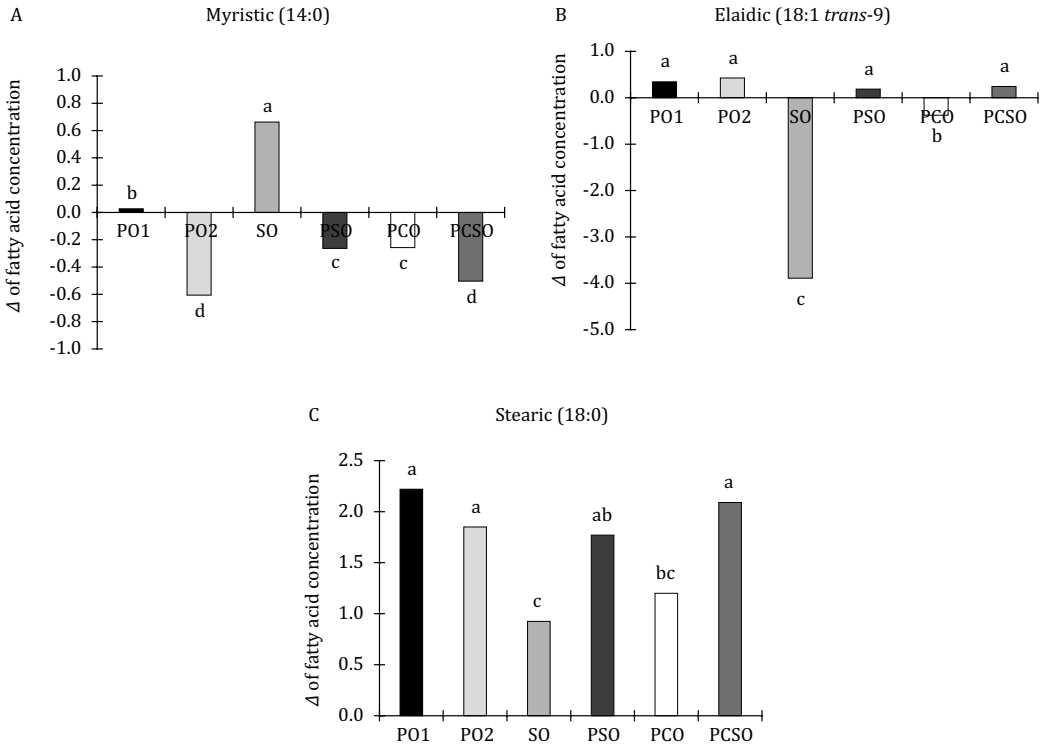
Effects of CSFA source were not detected (P≥0.11) for Δ of oleic acid in pH 5.5 and 6.0 (Figure 1B). However, the Δ of oleic acid was greater (P≤0.03) for PO2 vs. PCO in pH 6.5 (Figure 1B) and for PO1 and PCSO compared with PO2 and SO in pH 7.0 (Figure 1B).

The Δ of linoleic acid was greatest (P≤0.001) for SO across all pH evaluated (Figure 1C). The Δ of linoleic acid did not differ (P≥0.75) between PO1 and PO2, but both had a reduced Δ of linoleic acid than all other CSFA sources in pH 5.5 and 7.0 (P≤0.001; Figure 1C). Moreover, the Δ of linoleic acid did not differ (P≥0.06) between PCSO and PO1 in pH 6.0 (Figure 1C).

The Δ of linolenic acid concentrations did not differ (P≥0.82) between PO1 and PO2, but both had less (P≤0.001) Δ of linolenic acid concentrations compared with the other CSFA sources across all pH (Figure 1D). On the other hand, SO had greater Δ of linolenic acid compared with PSO, PCO, and PCSO in pH 5.5, 6.0, and 6.5 (P≤0.001; Figure 1D). In pH 7.0, PCO had greater (P<0.01) Δ of linolenic acid compared with PCSO and PSO (Figure 1D).

The Δ of rumenic acid did not differ (P≥0.09) between PO1 and PCSO (Figure 1E), but was greater (P≤0.04) for PO1 than SO, PSO, and PCO across all pH levels. In pH 7.0, PO1 resulted in greater (P = 0.001) Δ of rumenic acid than PO2 (Figure 1E). In contrast, Δ of rumenic acid did not differ (P≥0.48) among SO, PSO, and PCO in all pH levels (Figure 1E).

A CSFA source effect was detected (P≤0.001) for Δ of myristic, stearic, and elaidic acids (Figure 2). The SO had greater (P<0.001) Δ of myristic acid compared with PO1 (Figure 2A). Moreover, the Δ of myristic acid did not differ (P = 0.56) between PO2 and PCSO and both had greater (P≤0.002) Δ compared with PSO and PCO (Figure 2A). Moreover, Δ of elaidic acid was greater (P<0.001) for SO and PCO compared

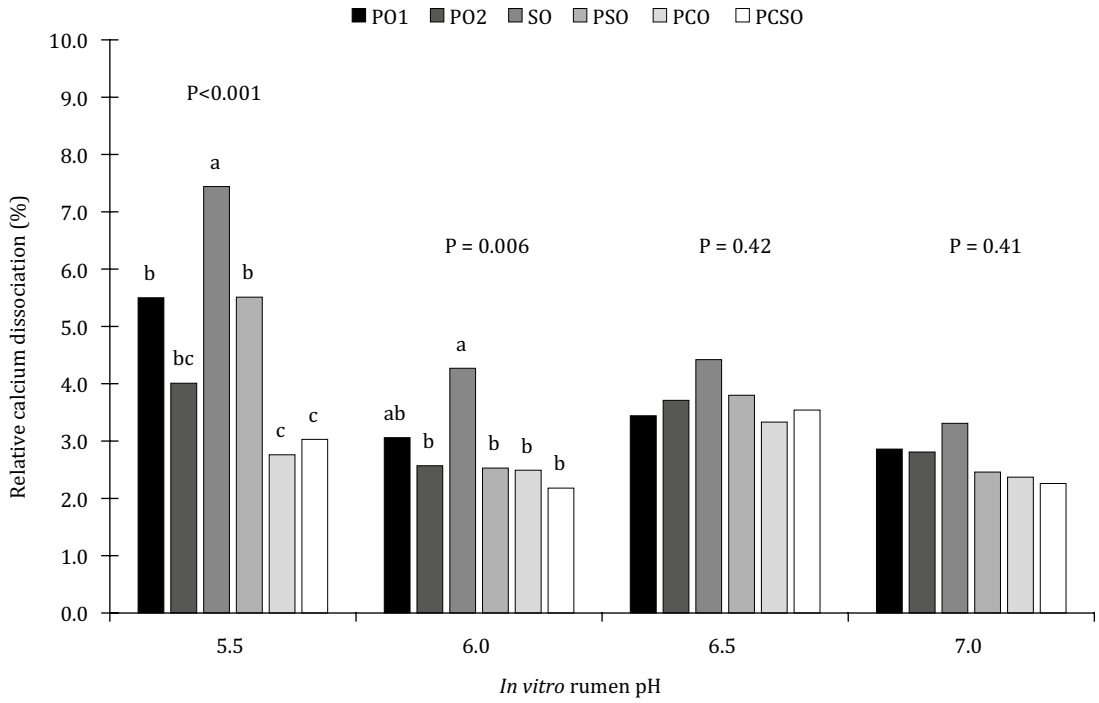


Each bar is the mean of three replicates.
a-c - Bars with a different letter differ (P≤0.05; Tukey-Kramer test).

Figure 2 - Effects of different sources of calcium soaps of fatty acids [CSFA; palm oil (PO1 and PO2), soybean oil (SO), palm + soybean oil (PSO), palm + cottonseed oil (PCO), and palm + cottonseed + soybean oil (PCSO)] on *in vitro* variation of fatty acids composition (Δ; final – initial concentration [g 100 g^{-1}]).

with PO1, PO2, PSO, and PCSO, and for SO vs. PCO (P<0.001; Figure 2B). The *Δ* of stearic acid was reduced (P≤0.002) for SO compared with PO1, PO2, PSO, and PCSO (Figure 2C) but did not differ (P = 0.77) between SO and PCO.

A CSFA source × rumen pH effect was detected (P<0.001) for relative Ca dissociation (Figure 3). Calcium dissociation did not differ (P≥0.41) among CSFA sources in pH 7.0 or 6.5. In contrast, Ca dissociation was greater (P≤0.04) for SO compared with PO2, PSO, PCO, and PCSO in pH 6.0, but not (P = 0.24) compared with PO1. No further differences were detected (P≥0.89) among PO1, PO2, PSO, PCO, and PCSO in pH 6.0. Relative Ca dissociation in pH 5.5 was greatest (P≤0.01) for SO compared with other CSFA sources (Figure 3). Relative Ca dissociation in pH 5.5 was low (P≤0.01) for PO1 and PSO compared with SO, but greater (P≤0.001) for PO1 and PSO compared with PCO and PCSO. Relative Ca dissociation did not differ (P≥0.09) between PO2 vs. PO1, PSO, PCO, and PCSO on pH 5.5.



Each bar is the mean of three replicates.
a-c - Within rumen pH, bars with a different letter differ (P≤0.05; SEM = 0.54; Tukey-Kramer test).

Figure 3 - *In vitro* dissociation of calcium soap of fatty acids [CSFA; palm oil (PO1 and PO2), soybean oil (SO), palm + soybean oil (PSO), palm + cottonseed oil (PCO), and palm + cottonseed + soybean oil (PCSO)] in rumen fluid at different pH (5.5; 6.0; 6.5 and 7.0).

4. Discussion

Normal rumen pH varies between 5.8 and 6.8 in cattle fed forage-based diets (NASEM, 2016), but may drop to 5.2 in feedlot cattle fed high-concentrate diets (Owens et al., 1998). Therefore, relatively small rates of CSFA dissociation would be expected using the pH range evaluated in the present study. The relative Ca dissociation ranged from 2.18 to 7.44 % across all rumen pH evaluated herein and was much lower than the values reported by Sukhija and Palmquist (1990). According to these authors, sodium acetate buffer complex free Ca forming soluble Ca acetate, overestimating the relative dissociation of CSFA, which is between 40 to 60% in pH 5.5 for tallow, soybean, and palm oil. In the current study, 1 N H_3PO_4 or 1 N NaOH was gradually added to reach the desired rumen pH, so Ca concentration and relative Ca dissociation obtained herein would be more precise than previously observed.

The relatively small dissociation of fatty acids in rumen pH 7.0 and 6.5 can be associated with the high stability of these sources in alkaline-based pH levels, but may also represent the Ca concentration naturally occurring in rumen fluid. As rumen pH decreased to 6.0 and 5.5, differences between CSFA were observed in relative Ca dissociation, suggesting that varying fatty acid composition may lead to differences on rumen protection and, consequently, degree of ruminal biohydrogenation of fatty acids. According to Sukhija and Palmquist (1990), unsaturated soaps were less satisfactory for maintaining normal rumen function because dissociation rate was greater than saturated sources. In agreement with these data, SO contained the greatest amount of unsaturated fatty acids and also greatest Ca dissociation in pH 5.5. The greater dissociation of SO was noticeable by the decrease in concentrations of elaidic (18:1 *trans*-9), linoleic (18:2 *n-6*), and linolenic acids (18:3 *n-3*). Nonetheless, as aforementioned, the degree of unsaturation was much lower than previously reported (Sukhija and Palmquist, 1990) and likely enabled a satisfactory delivery of fatty acids for subsequent gut absorption. Additionally, it is important to mention that CLA isomers (C18:2 *cis*-10, *trans*-12; C18:2 *trans*-10, *cis*-12; and C18:2 *trans*-9, *cis*-11) were not detected in any samples analyzed herein. Ruminal synthesis of CLA is associated with incomplete biohydrogenation of PUFA, often observed as ruminal concentrations of free linoleic acid increase, inhibiting the synthesis of stearic acid (Jenkins et al., 2008). Hence, the lack of detection of CLA isomers reinforces our results of relatively low rumen dissociation of CSFA tested in the current study.

Although Ca dissociation is an easy and quick methodology to access CSFA ruminal stability, it does not completely describe the magnitude of change in the amount of fatty acids reaching the gut. For instance, in this study, although SO had greater Ca dissociation compared with PO1 and PO2 in pH 5.5, the variation of palmitic and oleic acid concentrations (the main fatty acids in SO) were less or did not change compared with PO1 and PO2 in pH 5.5. Therefore, using the variation in fatty acids concentration may provide a useful and complementary information to compare different CSFA sources in addition to Ca dissociation.

Calcium salts of palm oil are frequently included in diets for dairy cows. Available information about Megalac (Church & Dwight Co. Inc., Princeton, NJ, USA) has been well documented in the literature (Rabiee et al., 2012). Hence, this product was included in our study as our standard treatment. However, overall differences in Ca dissociation and Δ of fatty acids concentration were not observed between PO1 and PO2.

Interactions among CSFA sources and rumen pH suggests that dietary factors, such as high starch or high soluble carbohydrate, monensin utilization, and low fiber level may impact the stability of products containing different fatty acids profile. According to Palmquist and Jenkins (2017), selection of fatty acids and Ca source could influence rates of insoluble Ca salt formation. In the present study, PCO and PCSO combined mainly palmitic, oleic, and linoleic acids had the least Ca dissociation at the lowest rumen pH and relatively small variation of fatty acid composition across all rumen pH levels evaluated herein, suggesting that these sources may be a potential alternative to be included in diets containing relatively high concentrations of starch.

5. Conclusions

Overall differences in Ca dissociation and Δ of fatty acids concentration were not observed between palm oil sources evaluated herein. Within the ruminal pH of 5.5 to 7.0, the combination of palm + cottonseed oil and palm + cottonseed + soybean oil reduces Ca dissociation and maintains the pre-incubation fatty acid profile of each source. The approach of using the variation in fatty acids concentration following *in vitro* incubation in different rumen pH provides a useful and complementary information to compare the effects of different sources of Ca soaps of fatty acids on final concentrations of fatty acids in rumen fluid.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: B.I. Cappellozza and P. Moriel. Data curation: L.F. Ferraretto. Formal analysis: L.F. Ferraretto. Funding acquisition: B.I. Cappellozza. Methodology: B.I. Cappellozza and L.F. Ferraretto. Supervision: P. Moriel. Writing-review & editing: L.F. Ferraretto, M. Vedovatto and P. Moriel.

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