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Dietary chitosan improves nitrogen use and feed conversion in diets for mid-lactation dairy cows

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ABSTRACT

Feed additives and fat sources have been used to meet high productive dairy cow energy requirements. This study aimed to evaluate dietary chitosan and soybean oil effects on mid-lactation dairy cow intake, digestibility, metabolism and productive performance. Twenty-four Holstein cows (134.7 ± 53.1 days in milk, 36.14 ± 5.32 kg/day of milk yield, and 581.2 ± 73.6 kg of body weight, Mean ± SD) were used in a replicated 4 × 4 Latin square design with 21-d periods, with 14 d of adaptation and 7 d for data collection. The treatment arrangement was a 2 × 2 factorial design with two levels of chitosan (0 and 4 g/kg of dietary dry matter – DM) and two levels of soybean oil (0 and 33 g/kg of dietary DM). Chitosan decreased intake only in diets without oil ($P < 0.05$). Regardless of fat addition, chitosan increased DM and CP digestibility ($P < 0.05$). Soybean oil and chitosan increased total serum cholesterol ($P < 0.05$). Chitosan diet had higher urea plasma concentration than control diet (CON) ($P < 0.05$). Over all, soybean oil increased propionate and decreased acetate ruminal molar proportion, and therefore decreased acetate:propionate ratio ($P < 0.05$). Chitosan decreased milk yield, nitrogen use and feed conversion efficiencies in oil-diets ($P < 0.05$). Soybean oil decreased short and medium milk fatty acids concentration ($P < 0.05$). Chitosan had no effect on long-chain milk fatty acids in diets with soybean oil ($P > 0.05$). However, in free oil-diets, chitosan increased milk polyunsaturated fatty acids concentration, nitrogen and energy efficiency. Chitosan addition in free-fat diets improved feed efficiency, increased milk unsaturated fatty acids concentration and association with soybean oil negatively affect animal performance.

1. Introduction

Energy requirements have been among the greatest challenges for ever-increasing lactation productivity dairy cows (Loften et al., 2014). One of the most studied strategies for this challenge is the dietary additives supplementation, to modulate ruminal digestion process (Van Nevel and Demeyer, 1988; Odongo et al., 2007; Silva et al., 2007). The most frequently used additives are primarily substances with antimicrobial activity, particularly ionophore, which has been successful in increasing the efficiency of protein and energy utilization (Van Nevel and Demeyer, 1988; Odongo et al., 2007; Silva et al., 2007). Antibiotic utilization in animal feeds, however, is facing reduced social acceptance because of the possible residues in animal products and the development of resistant strains of bacteria (Barton, 2000).

Goiri et al. (2009) proposed chitosan use as a modulator of ruminal fermentation and digestive processes. Chitosan is a nontoxic and biodegradable biopolymer that has been used on several applications in medicine and food preservation, primarily because of its antimicrobial activities. Chitosan is obtained by the deacetylation of chitin, the most abundant biopolymer in nature after cellulose, and an important component of the exoskeleton of insects and crustaceans (Kong et al., 2010). Goiri et al. (2010a) showed that chitosan inhibits *in vitro* biohydrogenation, and increases unsaturated fatty acids concentration in Rusitec® assay while in their other study Goiri et al. (2010b) found that rumen propionate increased, ammonium decreased and no effect on intake and total tract digestibility in sheep.

Another strategy to meet energy requirements of high productive ruminants is to use feeds with high energy density, such as those rich in lipids. Lipids are known to increase the energy density of diets and may affect ruminal fermentation, altering milk fatty acids profile (Jenkins, 1993). Both chitosan and fat sources, alter ruminal fermentation. We hypothesized that chitosan and lipids association may improve ruminal

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fermentation, productive performance, energy status and milk fatty acids profile. It is unknown if there are interaction effects between these two or if there are additive effects. This study evaluated the combined use of chitosan and soybean oil in dairy cow diets on ruminal fermentation, intake and digestibility, N balance, productive performance and milk fatty acids profile.

2. Material and methods

The Ethics Committee of the School of Veterinary Medicine and Animal Science of the University of São Paulo, under number 3057/2013, approved the experimental procedures.

2.1. Animals, experimental design and diets

Twenty-four Holstein (eight ruminal cannulated) with 134.7 ± 53.1 days in milk, 581.2 ± 73.6 kg of body weight and 36.14 ± 5.32 kg/d of milk yield (mean ± SD), were housed in individual pens, with 17.5 m^2 of area, on sand beds and forced ventilation. Animals were divided into six groups, according to ruminal cannula presence, days in milk and milk yield. Animals within groups were randomly allocated into a treatments sequence, in a Latin Square design. Experimental diets were formulated according NRC (2001) recommendations, as follows (Table 1): CON: control diet with no chitosan and no soybean oil; CHI: chitosan diet, with chitosan (4 g/kg of dry matter) and without soybean oil; SO: soybean oil diet, with no chitosan and with soybean oil (33 g/kg of dry matter); and CSO: chitosan and soybean oil, diet with chitosan and with soybean oil. The Chitosan was manufactured from Polymar® (Polymar Science and Nutrition, Fortaleza, Brazil) and dosage was determinated to provide almost 150 mg/kg body weight intake, according to Araújo et al. (2015) and Mingotti et al. (2016). Chitosan (average MW 500,000 g) had a pH of 8.81 and a deacetylation degree of 86.6%. Soybean oil level was added to the diet to provide 64 g/kg of ether extract in the diet dry matter (NRC, 2001). Corn silage, ground corn and soybean meal were the main ingredients of the diets. Chitosan and soybean oil were added to the concentrate and offered as total mixed ration.

Table 1
Ingredients and chemical composition of experimental diets containing chitosan and soybean oil.

Item	Experimental diets[a]			
	CON	CHI	SO	CSO
Ingredients (g/kg DM)				
Corn silage	500	500	500	500
Ground corn	282	279	242	240
Soybean meal	191	189	198	196
Soybean oil	–	–	33.0	32.7
Chitosan	–	4.0	–	4.0
Sodium bicarbonate	8.0	7.9	8.0	7.9
Limestone	7.0	6.9	7.0	6.9
Urea	3.2	3.2	3.2	3.2
Dicalcium phosphate	3.0	3.0	3.0	3.0
Salt	3.0	3.0	3.0	3.0
Mineral mixture[b]	2.0	2.0	2.0	2.0
Ammonium Sulphate	1.0	1.0	1.0	1.0
Chemical composition, g/kg DM				
Dry matter[c]	529	529	531	531
Non-fiber carbohydrates[d]	393	393	367	367
Neutral-detergent fiber (NDF)	349	348	344	343
Acid-detergent fiber	189	189	188	188
Crude Protein	172	173	171	172
Ash	60.8	60.7	60.4	60.3
Acid-detergent lignin	34.3	34.2	33.6	33.6
Ether Extract	31.1	30.9	64.1	63.7
Net energy $_{3x}$[e]	6.94	6.94	7.32	7.28

[a] CON: Control diet; CHI: Chitosan diet; SO: Soybean oil diet; and CSO: Chitosan and soybean oil diet.
[b] Each kg contains: 205 g of Ca; 60 g of P; 35 g of K; 70 g of Na; 20 g of S; 20 g of Mg; 2500 mg of Zn; 1600 mg of Mn; 700 mg of Cu; 700 mg of Fe; 40 mg of I; 19 mg of Se; 10 mg of Cr; 200,000 IU of Vitamin A, 50,000 IU of Vitamin D, and 1500 IU of Vitamin E.
[c] g/kg of natural matter.
[d] Non-fiber carbohydrate (g/kg) = 1000 – [(crude protein (g/kg) – crude protein from urea (g/kg) + urea (g/kg)) + NDF (g/kg) + ether extract (g/kg) + ash (g/kg)] in which values were expressed in percentage, from Hall (2000).
[e] Calculated according to NRC (2001): NE_{L3x} (MJ/kg DM) = [0.245* TDN_{1x} (g/kg) − 0.12] *4.184 MJ/Mcal, were TDN_{1X} (g/kg) = tdNFC + tdCP + (tdFA *2.25) + tdNDF.

2.2. Data and sample collection

Each experimental period lasted for 21 days, with 14 days for adaptation and seven days for sampling. Cows were fed twice daily, at 7:00 a.m. and 1:00 p.m., with the quantity determined according to the amount of refusal from the previous day to maintain a percentage of refusal between 5% and 10% of supplied diet. Samples of corn silage and refusals were daily collected throughout the sampling period, stored frozen, and pooled in a composite sample for subsequent chemical analyses. Concentrates were collected at the animal's food factory, once for each week of sampling.

On days 16–18 of each experimental period, fecal samples were taken from each cow, after a.m. and p.m. daily milking. Samples of feeds, refusals and feces were dried at 60 °C in a forced-air oven for 72 h and processed in a Wiley mill either through a 1 mm or a 2 mm sieve. Samples of feeds, refusals and feces, ground at 1-mm, were analyzed for dry matter (DM, method 930.15; AOAC, 2000), crude protein (CP, N × 6.25; Kjeldahl method −984.13; AOAC, 2000), ether extract (EE, method 920.39; AOAC, 2000), acid-detergent fiber and lignin (ADF and Lignin, method 973.18; AOAC, 2000), ash (method 942.05; AOAC, 2000) and neutral-detergent fiber (NDF) using α-amylase and without addition of sodium sulfite (Van Soest et al., 1991).

Indigestible acid-detergent fiber (iADF) was used as internal marker to estimate fecal excretion and apparent digestibility of nutrients. Samples of feeds, refusals and feces, ground at 2-mm, were placed in bags of nonwoven textile (100 g/m^2 of weight per area unit) and were incubated for 288 h in the rumen of two Holstein cows (Casali et al., 2008), previously adapted to a high-concentrate diet. After removal, the bags were washed in running tap water, dried at 60 °C in a forced-air oven and digested by an acid-detergent solution in an fiber analyzer (TE149®, Tecnal Equipamentos Científicos, Piracicaba, Brazil) to obtain the indigestible acid-detergent fiber (method 973.18; AOAC, 2000). Apparent digestibility coefficient were estimated through the intake and fecal excretion data.

Ruminal liquid was collected on day 20 of each experimental period at zero, two, four, six, eight, ten and 12 h after morning feeding from four rumen cannulated cows. 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 volatile fatty acids (VFA) in the rumen fluid were measured by gas chromatography as described by Shen et al. (2004), and the N-NH_3 content was analyzed using phenol-hypochlorite (Broderick and Kang, 1980).

Blood samples were collected on the 15th day of each experimental period, before the morning feeding, by coccygeal vein or artery puncture. Samples were centrifuged at 800 × *g* for 10 min, and the serum was collected and frozen. The analyses were performed with commercially available colorimetric kits (glucose: cat. no. K-082; total cholesterol: cat. no. K-083; high-density lipoprotein - HDL cholesterol: cat. no. K-015; urea: cat. no. K-056; aspartate aminotransferase – AST: cat. no. K-048; and gamma-glutamyl transpeptidase - GGT: cat. no. K-080; Bioclin®, Belo Horizonte, Brazil). Readings were determined with a semi-automatic spectrophotometer (SBA 200®, CELM, São Caetano do Sul, Brazil).

Daily urine volume was estimated from creatinine concentration (mg/L) in spot samples obtained on the 16th and 17th day of each experimental period, four hours after morning feeding. Creatinine concentrations were analyzed with a biochemical colorimetric kit

Table 2
Intake and digestibility of dry matter and nutrients in lactating dairy cows fed diets containing chitosan and soybean oil.

Item	Diets[1]				SEM[2]	*P*-value		
	CON	CHI	SO	CSO		Chitosan	Soy. oil[3]	CHI × SO[4]
Intake, kg/day								
Dry matter	22.5[a]	21.2[b]	20.0[c]	20.1[c]	0.40	0.020	< 0.001	0.009
NDF[5]	7.82[a]	7.32[b]	6.79[c]	6.83[c]	0.15	0.027	< 0.001	0.010
Crude protein	3.90[a]	3.69[b]	3.40[c]	3.45[c]	0.07	0.093	< 0.001	0.009
Ether extract	0.70	0.67	1.29	1.29	0.04	0.426	< 0.001	0.489
Total tract apparent digestibility, g/kg								
Dry matter	725	740	727	750	3.7	0.003	0.326	0.558
NDF[5]	601	599	584	603	5.3	0.341	0.479	0.253
Crude protein	742	767	754	782	4.2	< 0.001	0.055	0.881
Ether extract	851	841	891	898	7.2	0.889	< 0.001	0.443

[1] CON: Control diet; CHI: Chitosan diet; SO: Soybean oil diet; and CSO: Chitosan and soybean oil diet.
[2] SEM: Standard error of the mean.
[3] Soybean oil effect.
[4] Chitosan × Soybean oil interaction.
[5] NDF: Neutral-detergent fiber.
[a,b,c] Values within a row with different superscripts differ significantly at $P < 0.05$.

(kinetic creatinine: cat. no. K-067, Bioclin) in a semi-automatic spectrophotometer (SBA 200), and a daily creatinine excretion rate of 24.05 mg/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; determined in a semi-automatic spectrophotometer) and allantoin (Fujihara and Yamaguchi, 1978) in urine and milk were considered to be the total excretion of purine derivatives. Microbial protein synthesis was estimated from these concentrations considering molar excretion of allantoin (urine and milk) and uric acid (urine). We considered 0.116 of ratio between N purine and microbial N, and 0.83 of intestinal microbial purines digestibility (Chen and Gomes, 1992). According to González-Ronquillo et al. (2003), urinary recovery of absorbed purines was assumed to be 0.70 and endogenous contribution was estimated as 0.512 mmol/kg$^{0.75}$ BW. Energy intake was estimated according NRC (2001) equations. Nitrogen efficiency of use was assessed by N milk yield and N intake ratio (Arndt et al., 2015).

Cows were mechanically milked twice daily, at 6:00 a.m. and 4:00 p.m., and the milk yield was weighed daily for the last seven days of each experimental period. From 16th to 18th days of each period, milk samples of two daily milking, were collected for composition analyses. Fresh samples were analyzed for crude protein, fat and lactose, using ultrasonic milk analyzer MCC (Milcotronic Company, Nova Zagora, 8900, Bulgaria). Milk yields were corrected for 3.5% fat content, according to Sklan et al. (1994).

Milk fatty acid profiles were evaluated in the composite sample obtained on the 16th day of each experimental period. Fatty acid extraction was performed according to Feng et al. (2004) and methylated according to Kramer et al. (1997). Readings were performed in gas chromatograph (GC Shimatzu model GC-2010, Kyoto, Japan), using a capillary column (Sigma Aldrich Supelco SP-2560®, St. Louis, MO, USA) and hydrogen (H_2) as the carrier gas. Standards C4 to C24 fatty acids Sigma Aldrich Supelco TM37®, St. Louis, MO, USA; vaccenic acid (C18:1 trans-11) (Sigma Aldrich V038–1 G®, St. Louis, MO, USA), C18:2 trans-10, cis-12 (NU-Chek-Prep UC-61M 100 mg®, Elysian, MN, USA) and C18:2 cis-9, trans-11 (NU-Chek-Prep UC-60M 100 mg®, Elysian, MN, USA) were used. Internal standards of C19:0 was used to correct methylation losses.

2.3. Statistical analyses

Data were analyzed using PROC MIXED (Statistical Analysis System for Windows, version 9.0, SAS Institute Inc., Cary, NC, USA, 2003), according to the following statistical model:

$$Y_{ijklm} = \mu + C_i + SO_j + C^*SO_{ij} + P_k + S_l + a_m(s_l) + e_{ijklm}$$

where: Y_{ijklm} was the observed value in the animal m, from l square, in the kth period, which received the lth level of chitosan and the jth level of soybean oil; μ: overall mean; C_i was the fixed effect of chitosan; SO_j was the fixed effect of soybean oil; C^*SO_{ij} was the chitosan and soybean oil interaction effect; P_k was the fixed effect of experimental period; S_l was the fixed effect of the Latin square; $a_m(s_l)$ was the random effect of animal within square and e_{ijklm} was the random residual error. Degrees of freedom were corrected by Kenward and Roger (1997) methodology. Means were adjusted by LSMEANS option and when there was chitosan*soybean oil interaction effect, means were studied by the PDIFF means test.

The ruminal fermentation variables (pH, N-NH_3, acetate, propionate, butyrate and total VFA) were analyzed as repeated measures in PROC MIXED of SAS 9.0, considering in the statistical model the effects of animal, period, chitosan, and soybean oil and the effects of time with its interactions with the other effects above mentioned effects. Akaike methodology was used to choose the best covariance structure: TOEP was used for pH, total VFA concentration and propionate proportion; ARH(1) for N-NH3; AR(1) for acetate proportion and acetate to propionate ratio and CS for propionate proportion. Differences were considered significant at the 0.05 level.

3. Results

3.1. Intake and digestibility

A significant soybean oil × chitosan interaction effect was observed for DM, CP and NDF intakes ($P \leq 0.010$; Table 2). Chitosan supplementation to low fat diets decreased feed intake ($P \leq 0.05$), while chitosan had no effect on dry matter intake in fat supplemented diets ($P > 0.05$). Chitosan showed no effect on EE intake and digestibility coefficients ($P \geq 0.426$). Soybean oil increased EE intake and digestibility ($P < 0.001$) and chitosan increased DM and CP digestibility ($P \leq 0.003$).

3.2. Ruminal fermentation

There was an expected physiological time effect on evaluated ruminal variables ($P \leq 0.005$; Table 3 and Fig. 1). Chitosan had no effect on ruminal fermentation variables, regardless fat addition ($P \geq 0.111$). Soybean oil decreased ruminal acetate concentration and acetate to propionate ratio and increased ruminal propionate proportion ($P \leq 0.004$).

Table 3
Ruminal fermentation parameters of lactating dairy cows fed diets containing chitosan and soybean oil.

Item	Diets[a]				SEM[b]	P-value			
	CON	CHI	SO	CSO		Time	Chitosan	Soybean oil	Int. CHI × SO[c]
pH	6.34	6.46	6.43	6.48	0.048	0.001	0.353	0.580	0.632
N-NH_3, g/L	0.339	0.336	0.281	0.317	0.012	< 0.001	0.386	0.244	0.443
VFA[d], mMol/L	129	123	120	115	2.54	< 0.001	0.111	0.061	0.383
Molar proportion									
Acetate	0.660	0.661	0.619	0.641	0.004	< 0.001	0.136	0.004	0.235
Propionate	0.211	0.215	0.248	0.240	0.003	0.005	0.564	0.001	0.545
Butyrate	0.129	0.124	0.132	0.119	0.002	< 0.001	0.201	0.897	0.522
A:P[e]	3.17	3.13	2.56	2.74	0.053	< 0.001	0.395	0.001	0.365

[a] CON: Control diet; CHI: Chitosan diet; SO: Soybean oil diet; and CSO: Chitosan and soybean oil diet.
[b] SEM: Standard error of the mean.
[c] Soybean oil × chitosan interaction effect.
[d] VFA: total volatile fatty acids.
[e] A:P: Acetate to propionate ruminal molar ratio.

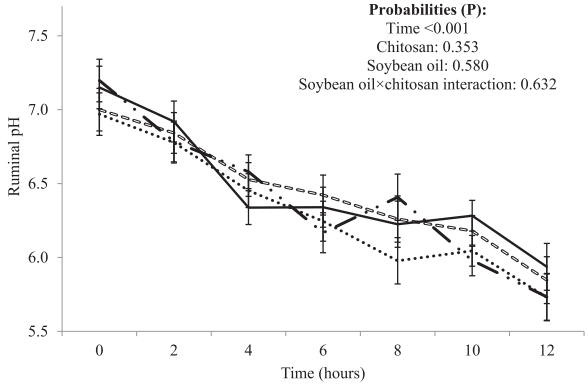

Fig. 1. Ruminal pH of dairy cows fed diets containing chitosan and soybean oil. Control diet (●●●●●●); Chitosan diet (⬭⬭⬭); Soybean oil diet (▬▬ ●); Chitosan and soybean oil diet (▬▬▬); hours of feeds (↑); Means (± SEM).

3.3. Blood metabolism

Experimental diets had no effect on glucose serum concentrations and GGT activity (P ≤ 0.191; Table 4). There was soybean oil × chitosan interaction effect on serum urea concentration (P < 0.001). Chitosan supplementation to low fat diets increased serum urea (P ≤ 0.05), while chitosan had no effect on serum urea on those animals fed with fat supplemented diets (P > 0.05). Regardless fat supplementation, chitosan increased serum total cholesterol (P = 0.012). Furthermore, dietary fat addition increased HDL-cholesterol, total cholesterol, and AST activity (P ≤ 0.010).

3.4. Milk yield and composition

There was a soybeans oil × chitosan interaction effect on milk, protein and lactose production and feed conversion efficiency (P ≤ 0.033; Table 5). Chitosan increased feed conversion efficiency (P ≤ 0.05) and had no effect on milk, protein and lactose production of animals fed diets without soybean oil (P > 0.05), while chitosan decreased milk yield and feed conversion efficiency of those animals fed fat supplemented diets (P ≤ 0.05). Soybean oil decreased fat-corrected milk and milk fat content, and increased lactose milk content (P ≤ 0.05). Chitosan in diets without oil had not affect on milk composition (P > 0.05).

3.5. Energy and N usage and microbial protein synthesis

A significant soybean oil × chitosan interaction effect was observed on net energy intake and efficiency of energy usage (P ≤ 0.014; Table 6). Animals fed diets without soybean oil showed decreased net energy intake and increased energy usage efficiency when chitosan was dietary added (P ≤ 0.05). However, chitosan decreased energy usage efficiency (P ≤ 0.05) and had no effect on net energy intake of those animals fed fat supplemented diets (P > 0.05). Regardless chitosan addition, soybean oil supplementation decreased milk net energy (P < 0.001).

There was a significant soybean oil × chitosan interaction effect on N intake, N milk secretion and N usage efficiency (P ≤ 0.047). In

Table 4
Serum parameters of lactating Holstein cows fed diets containing chitosan and soybean oil.

Item	Diets[1]				SEM[2]	P-value		
	CON	CHI	SO	CSO		Chitosan	Soybean oil	CHI × SO[3]
Glucose, g/L	0.674	0.676	0.689	0.647	0.015	0.497	0.797	0.441
Total Cholesterol, g/L	1.469	1.700	2.143	2.249	0.078	0.012	< 0.001	0.341
HDL Cholesterol[4], g/L	0.574	0.577	0.636	0.652	0.016	0.679	0.003	0.767
Urea, g/L	0.329[c]	0.418[a]	0.381[a,b]	0.359[b,c]	0.009	0.004	0.734	< 0.001
AST[5], IU/L	59.06	57.20	66.95	64.89	1.959	0.506	0.010	0.972
GGT[6], IU/L	35.90	37.70	37.31	38.69	1.003	0.191	0.320	0.863

[1] CON: Control diet; CHI: Chitosan diet; SO: Soybean oil diet; and CSO: Chitosan and soybean oil diet.
[2] SEM: Standard error of the mean.
[3] Soybean oil × chitosan interaction effect.
[4] HDL Cholesterol: high-density lipoprotein.
[5] AST: Aspartate aminotransferase.
[6] GGT: Gamma-glutamyl transferase.
[a,b,c] Values within a row with different superscripts differ significantly at $P < 0.05$.

Table 5
Milk yield and composition of lactating dairy cows fed diets containing chitosan and soybean oil.

Item	Diets[1]				SEM[2]	P-value		
	CON	CHI	SO	CSO		Chitosan	Soybean Oil	CHI × SO[3]
Production, kg/d								
Milk Yield	32.8[a]	33.6[a]	32.9[a]	31.3[b]	0.548	0.386	0.024	0.023
FCM[4]	34.5	34.7	30.9	30.4	0.636	0.866	< 0.001	0.606
Fat	1.25	1.24	1.03	1.04	0.027	0.936	< 0.001	0.789
Protein	1.00[a]	1.02[a]	1.01[a]	0.96[b]	0.018	0.228	0.099	0.030
Lactose	1.50[a]	1.53[a]	1.52[a]	1.43[b]	0.027	0.270	0.089	0.033
FCE[5], kg/kg	1.48[c]	1.62[a,b]	1.68[a]	1.59[b]	0.031	0.365	0.002	< 0.001
Milk concentration, g/kg								
Fat	38.0	37.2	31.7	33.5	0.773	0.607	< 0.001	0.198
Protein	30.5	30.4	30.9	30.7	0.154	0.316	0.092	0.872
Lactose	45.6	45.4	46.2	46.1	0.202	0.393	< 0.001	0.765

[1] CON: Control diet; CHI: Chitosan diet; SO: Soybean oil diet; and CSO: Chitosan and soybean oil diet.
[2] SEM: Standard error of the mean.
[3] Chitosan × Soybean oil interaction effect.
[4] FCM: 3.5% fat corrected milk.
[5] FCE: feed conversion efficiency (milk yield: dry matter intake ratio).
[a,b,c] Values within a row with different superscripts differ significantly at P < 0.05.

animals fed fat supplemented diets, chitosan addition had no effect on N intake (P > 0.05), decreased milk N secretion (P ≤ 0.05) and, therefore, decreased N usage efficiency (P ≤ 0.05). Although, chitosan decreased N intake (P ≤ 0.05), it had no effect on N milk secretion (P > 0.05) and increased N efficiency (P ≤ 0.05) in animals fed non-supplemented diets. Additionally, chitosan and soybean oil had no effect on microbial protein synthesis, allantoin: creatinine ratio, N urinary excretion and N balance (P > 0.05).

3.6. Milk fatty acids profile

Regardless dietary chitosan addition, soybean oil decreased medium to short chain and increased long chain fatty acids milk concentration (P < 0.001; Table 7). A significant soybean oil × chitosan interaction effect was observed on C16:0 FA, C18:2 *cis* − 9,12 FA, C18:3 *c* − 9,12,15 FA, saturated FA, polyunsaturated FA, n-3 FA and n-6 FA milk concentration (P < 0.038). Chitosan addition in fat supplemented diets had no effect on these milk fatty acids concentration (P > 0.05). However, chitosan increased C18:3 cis-9,12,15 FA, C18:2 cis-9,12 FA, poly-unsaturated fatty acids (PUFA), PUFA: saturated ratio, n-3 and n-6 FA concentration and decreased C16:0 FA, and saturated FA concentration when added to diets without soybean oil addition.

4. Discussion

Until the present study, there has been no study evaluating chitosan and fat source association in dairy cow diets. Mingoti et al. (2016), Araujo et al. (2015) and Goiri et al. (2010b) found no effect of chitosan on dry matter intake (DMI) in dairy cows, beef cattle and sheep, respectively. In the present study, however, chitosan decreased DMI in

Table 6
Nitrogen and energy usage and microbial protein synthesis of lactating dairy cows fed diets containing chitosan and soybean oil.

Item	Diets[1]				SEM[2]	P-value		
	CON	CHI	SO	CSO		Chitosan	Soybean Oil	CHI × SO[3]
Net energy, MJ/day								
Intake (NEI)[4]	159[a]	148[b]	148[b]	147[b]	2.81	0.008	0.001	0.014
Milk (NEL)[5]	96.6	96.6	87.9	86.2	1.75	0.638	< 0.001	0.583
Nitrogen, g/day								
Intake	623[a]	590[b]	545[c]	552[c]	11.6	0.093	< 0.001	0.009
Urinary	204	206	188	205	6.38	0.310	0.355	0.395
Fecal	162	137	133	122	3.98	< 0.001	< 0.001	0.183
Milk	158[a]	160[a]	159[a]	150[b]	2.82	0.236	0.102	0.047
Balance[6]	99.2	87.1	64.0	74.7	8.86	0.943	0.061	0.257
Microbial protein	284	299	303	303	10.9	0.644	0.488	0.636
Coefficients								
Energy efficiency[7]	0.336[b]	0.361[a]	0.342[b]	0.320[c]	0.0071	0.753	0.008	0.004
Nitrogen efficiency[8]	0.255[c]	0.277[b]	0.300[a]	0.277[b]	0.0054	0.936	< 0.001	< 0.001
Allantoin: Creatinine[9]	1.52	1.57	1.68	1.60	0.053	0.857	0.274	0.446

[1] CON: Control diet; CHI: Chitosan diet; SO: Soybean oil diet; and CSO: Chitosan and soybean oil diet.
[2] SEM: Standard error of the mean.
[3] Chitosan × Soybean oil interaction effect.
[4] NEI: Net energy intake.
[5] Net energy for lactation.
[6] Nitrogen balance (g/day): N intake - (urinary N + fecal N + milk N).
[7] Energy efficiency: net energy intake used for lactation (NEL) to digestible energy intake (DEI) ratio.
[8] Nitrogen efficiency: relation between milk nitrogen and nitrogen intake.
[9] Molar ratio.
[a,b,c] Values within a row with different superscripts differ significantly at *P* < 0.05.

Table 7
Milk fatty acids profile of dairy cows fed diets containing chitosan and soybean oil.

Item	Diets[1]				SEM[2]	P-value		
	CON	CHI	SO	CSO		Chitosan	Soybean oil	Int. Chi × SO[3]
Milk fatty acids concentration, g/kg								
C4:0	10.6	10.7	9.0	9.0	0.22	0.860	< 0.001	0.733
C6:0	12.8	12.8	8.5	8.3	0.32	0.734	< 0.001	0.741
C8:0	9.54	9.43	5.50	5.25	0.29	0.373	< 0.001	0.716
C10:0	24.5	23.9	13.4	12.7	0.81	0.323	< 0.001	0.947
C11:0	0.64	0.62	0.35	0.32	0.03	0.523	< 0.001	0.798
C12:0	31.2	30.0	17.7	16.9	0.99	0.227	< 0.001	0.798
C13:0	2.12	2.12	1.25	1.25	0.08	0.646	< 0.001	0.640
C14:0	104	99.4	71.9	70.7	2.22	0.104	< 0.001	0.351
C14:1 *cis*-9	10.6	10.0	7.0	6.9	0.34	0.317	< 0.001	0.541
C15:0	10.3	10.8	7.2	7.3	0.34	0.528	< 0.001	0.608
C16:0	311[a]	293[b]	235[c]	238[c]	4.48	0.039	< 0.001	0.003
C16:1 *cis*-9	16.8	15.8	12.5	12.7	0.46	0.464	< 0.001	0.320
C17:0	6.32	6.35	4.60	4.67	0.13	0.691	< 0.001	0.883
C18:0	95.6	99.9	141	143	3.43	0.371	< 0.001	0.715
C18:1 *trans*-9	2.65	2.82	6.54	6.09	0.25	0.527	< 0.001	0.159
C18:1 *trans*-11	9.84	12.4	28.9	27.5	1.49	0.717	< 0.001	0.243
C18:1 *cis*-9	259	268	317	320	4.88	0.176	< 0.001	0.549
C18:2 *cis*-9,12	28.4[c]	32.2[b]	38.0[a]	38.3[a]	0.80	0.012	0.319	0.031
C18:2 *c* − 9 *t*-11	3.92	4.74	8.02	7.54	0.34	0.726	< 0.001	0.181
C18:2 *t* − 10 *c*-12	0.02	0.01	0.27	0.28	0.02	0.927	< 0.001	0.867
C18:3 *c*-9,12,15	1.63[c]	2.05[b]	2.60[a]	2.58[a]	0.08	0.024	< 0.001	0.015
C20:0	1.05	1.09	1.43	1.46	0.03	0.300	< 0.001	0.978
C20:1 *cis*-11	0.40	0.40	0.54	0.53	0.01	0.710	< 0.001	0.845
C20:2 *cis*-11,14	0.06	0.09	0.05	0.09	0.02	0.279	0.809	0.809
C20:3 *c*-11,14,17	0.95[b]	1.10[a]	0.84[c]	0.78[c]	0.03	0.190	< 0.001	0.003
C20:4 *c*-5,8,11,14	1.75	1.92	1.25	1.27	0.04	0.047	< 0.001	0.107
C22:0	0.06	0.15	0.30	0.27	0.02	0.410	< 0.001	0.068
Others	44.1	45.5	52.3	52.8	0.83	0.492	0.859	0.738
SCFA[4]	23.4	23.5	17.5	17.3	0.51	0.944	< 0.001	0.703
MCFA[5]	68.0	64.7	38.2	36.5	2.10	0.122	< 0.001	0.622
LCFA[6]	864	866	888	892	2.42	0.268	< 0.001	0.555
SAT[7]	620[a]	600[b]	517[c]	519[c]	6.85	0.098	< 0.001	0.038
MUFA[8]	299	309	372	374	5.74	0.234	< 0.001	0.398
PUFA[9]	36.8[c]	42.1[b]	51.5[a]	50.9[a]	0.97	0.013	< 0.001	0.003
n-3	1.63[c]	2.05[b]	2.60[a]	2.58[a]	0.08	0.024	< 0.001	0.015
n-6	35.2[c]	40.1[b]	48.8[a]	48.4[a]	0.90	0.013	< 0.001	0.003
PUFA: SAT ratio	0.060[c]	0.071[b]	0.100[a]	0.099[a]	0.026	0.057	< 0.001	0.017

[1] CON: Control diet; CHI: Chitosan diet; SO: Soybean oil diet; and CSO: Chitosan and soybean oil diet.
[2] SEM: Standard error of the mean.
[3] Chitosan × Soybean oil interaction effect.
[4] Short chain fatty acids (C4:0 and C6:0).
[5] Medium chain fatty acids (C8:0 to C13:0 fatty acids).
[6] Long chain fatty acids (C14:0 and higher FA's).
[7] Saturated fatty acids.
[8] mono-unsaturated fatty acids.
[9] poly-unsaturated fatty acids.
[a,b,c] Values within a row with different superscripts differ significantly at $P < 0.05$.

diets without soybean oil. We expected a chitosan ruminal fermentation modulation effect (Goiri et al., 2010b), similarly to monensin, which could lead to decreased dry matter intake (Duffield et al., 2008). However, chitosan had no effect on ruminal fermentation and, only, increased DM and CP digestibility.

Chitosan increased DM and CP digestibility. Araujo et al. (2015), Paiva et al. (2017), and Mingoti et al. (2016) also found DM and CP digestibility increase using dietary chitosan. These studies and the present one used internal markers for daily fecal estimation. According to Benediktsdottir et al. (2014), in *in vitro* studies, chitosan can interact with intestinal components, increasing drugs epithelial permeability. These authors did not explain what the possible mechanisms were, and they were not sure whether chitosan acts on transcellular or paracellular processes or both mechanisms. We speculate that chitosan could, similarly, increase ruminant intestinal membranes permeability, increasing nutrients digestibility.

Soybean oil increased propionate concentration and consequently decreased acetate to propionate ratio. Fat effect on rumen fermentation can be attributed to direct action on ruminal microorganisms. Machmuller et al. (1998) found lipids action on protozoa, which are known to be inefficient in dietary energy use. Dohme et al. (2001) showed that lipids inhibited methane production not only by reducing the number of protozoa but also by direct action on other methanogenic populations. Whereby propionate and methane are competing substances for hydrogen reception, lipid addition can increase ruminal propionate concentration and decrease methane synthesis (Eugene et al. (2004)). Likewise, Wencelova et al. (2014) found that chitosan inhibits some protozoan populations, but with lower intensity than vegetables oils. In this study, however, chitosan had no effect on ruminal VFA profile and microbial protein synthesis, using purine derivatives as marker. Water solubility of chitosan is one of the most important characteristic that determines antimicrobial effect (Kong et al., 2010). Furthermore, lower solubility can be associated with small effects on ruminal fermentation and higher effects on intestinal

digestibility, as observed for Araujo et al. (2015) and Mingoti et al. (2016), using chitosan from Polymar. Fat supplementation can affect intestinal environmental and consequently change chitosan effect on digestion.

Chitosan increased serum urea of cows fed diets without soybean oil. Schelling (1984) suggested that monensin increased the flow of undegraded protein from the rumen to the small intestine because of the decrease in rumen ammonia concentrations likely occurred through the inhibition of deaminated bacteria. Chitosan did not change ruminal ammonia and we agree that serum ammonia increased in response to increased CP digestibility. Increased serum urea concentration indicate higher effect of chitosan on protein digestibility in those animals fed diets without soybean oil. Regardless chitosan addition, soybean oil increased total and HDL serum cholesterol, which can be associated with higher fatty acid intake of these animals. The AST and GGT results with the chitosan addition, regardless of supplementation with soybean oil, showed low hepatotoxicity of this additive, as was also reported by Araujo et al. (2015) and Mingoti et al. (2016).

Chitosan increased feed conversion efficiency in those animals fed diets without soybean oil. This effect seem associated with higher digestibility and lower intake. Paiva et al. (2017) observed milk yield increase and no effect on intake whith chitosan dietary use. However, in fat supplemented diets, chitosan addition decreased milk yield. Mingoti et al. (2016) observed no effect of chitosan on milk yield, using higher ether extract dietary level than Paiva et al. (2016) (42.3 *vs* 28.9 g/kg, respectively). One possibility to explain the reduction in feed conversion efficiency is chitosan chelating capacity with many metal ions (Kurita, 1998), which, associated with the changes in the absorptive mechanism of diets supplemented with soybean oil, like calcium salts of fatty acids formation, could determine changes in intermediary metabolism and reduce the efficiency of nutrient utilization.

Soybean oil supplementation, regardless chitosan inclusion, decreased milk fat yield and content. Shingfield et al. (2006) showed that unsaturated fatty acid addition in dairy cow diets decreased milk fat content, which was related to changes in rumen function (Jenkins, 1993). According to Von Soosten et al. (2012), milk fat synthesis might represent up to 50% of the energy requirement of a high-production dairy cow. Thus, in this study, soybean oil supplementation decreased fat corrected milk yield and energy used for lactation. Moreover, chitosan did not inhibit milk fat depression in diets containing soybean oil.

Soybean oil decreased milk short and medium chain fatty acid concentration and milk fat content. The inhibiting effect of unsaturated C18 on de-novo synthesis has been known in the past, but precise mechanisms involved in the studies were not yet well understood, even if some 18:2 isomers, such as C18:2 trans-10, cis-12, have been identified (Bauman and Griinari, 2001). Renno et al. (2013) also found milk fat depression and short-chain fatty acid concentration decrease in dairy cows fed with long-chain unsaturated fatty acids. In the present study, soybean oil increased milk C18:2 cis-9 trans-11, C18:2 trans-10 cis-12 and decreased short and medium chain fatty acid concentration and chitosan was not able to inhibit linoleic acid biohydrogenation in fat supplemented diets.

Yinghui et al. (2007), studying chitosan cytotoxicity, found specific chemical modifications of chitosan molecule associated with linoleic acid. Association changed molecular charge density and cationic functionalities, structure and conformational flexibility. This may be another reason for absence of effect of chitosan on milk fat profile of those animals fed with fat supplemented diets. Mingoti et al. (2016) also found no effect of chitosan on milk fatty acids profile using lower dietary concentration of ether extract from whole soybeans. In oil-free diets, chitosan increased linoleic and linolenic acid milk concentration. According to Goiri et al. (2010a), chitosan increases vaccenic acid and total conjugated linoleic acid (CLA) in the rumen and this can be associated with chitosan and free fatty acid association and inhibitory effect on the growth of microbial populations.

5. Conclusion

Chitosan improved animal performance and nutrient utilization efficiency, increasing long chain fatty acid concentration, showing a very promising feed additive in dairy cow diets without fat supplementation. The addition of chitosan in diets containing soybean oil compromised performance, showing negative interaction of chitosan with basal diet components.

Conflict of interests

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

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