Effects of chitosan and whole raw soybeans on ruminal fermentation and bacterial populations, and milk fatty acid profile in dairy cows

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ABSTRACT

The objective of this study was to evaluate whether providing chitosan (CHI) to cows fed diets supplemented with whole raw soybeans (WRS) would affect the nutrient intake and digestibility, ruminal fermentation and bacterial populations, microbial protein synthesis, N utilization, blood metabolites, and milk yield and composition of dairy cows. Twenty-four multiparous Holstein cows (141 ± 37.1 d in milk, 38.8 ± 6.42 kg/d of milk yield; mean $\pm$ SD) were enrolled to a 4×4 Latin square design experiment with 23-d periods. Cows were blocked within Latin squares according to milk yield, days in milk, body weight, and rumen cannula ($n = 8$). A 2×2 factorial treatment arrangement was randomly assigned to cows within blocks. Treatments were composed of diets with 2 inclusion rates of WRS (0 or 14% diet dry matter) and 2 doses of CHI (0 or 4 g/kg of dry matter, Polymar Ciência e Nutrição, Fortaleza, Brazil). In general, CHI+WRS negatively affected nutrient intake and digestibility of cows, decreasing milk yield and solids production. The CHI increased ruminal pH and decreased acetate to propionate ratio, and WRS reduced $\text{NH}_3\text{-N}$ concentration and acetate to propionate in the rumen. The CHI reduced the relative bacterial population of *Butyrivibrio* group, whereas WRS decreased the relative bacterial population of *Butyrivibrio* group, and *Fibrobacter succinogenes*, and increased the relative bacterial population of *Streptococcus bovis*. No interaction effects between CHI and WRS were observed on ruminal fermentation and bacterial populations. The CHI+WRS decreased N intake, microbial N synthesis, and N secreted in milk of cows. The WRS increased N excreted in feces and consequently decreased the N excreted in urine. The CHI had no effects on blood metabolites, but WRS decreased blood concentrations of

glucose and increased blood cholesterol concentration. The CHI and WRS improved efficiency of milk yield of cows in terms of fat-corrected milk, energy-corrected milk, and net energy of lactation. The CHI increased milk concentration [g/100 g of fatty acids (FA)] of 18:1 *trans*-11, 18:2 *cis*-9, *cis*-12, 18:3 *cis*-9, *cis*-12, *cis*-15, 18:1 *cis*-9, *trans*-11, total monounsaturated FA, and total polyunsaturated FA. The WRS increased total monounsaturated FA, polyunsaturated FA, and 18:0 to unsaturated FA ratio in milk of cows. Evidence indicates that supplementing diets with unsaturated fat sources along with CHI negatively affects nutrient intake and digestibility of cows, resulting in less milk production. Diet supplementation with CHI or WRS can improve feed efficiency and increases unsaturated FA concentration in milk of dairy cows.

Key words: chitin, fatty acid, oilseed, ruminal bacteria

INTRODUCTION

High-yielding dairy cows have relatively high energy requirement that often exceeds the nutrient intake capacity and consequently limits their milk production. In addition, the symbiotic relationship between ruminants and rumen microorganisms has energy and protein inefficiencies that results in methane eructation and excess of ammonia N production (Van Soest and Demeyer, 1988). Microbial fermentation can be modulated to favor fermentation pathways that minimize losses either by promoting dietary changes (Beauchemin et al., 2008) or providing antimicrobials (Van Soest and Demeyer, 1988). The use of ionophore antibiotics (e.g., monensin) in ruminant diets has shown to increase the rate of propionate production, decrease methane production, and reduce the ammonia accumulation in the rumen (reviewed by McGuffey et al., 2001). In addition, feeding ionophore antibiotics increased the total ruminating time during the day (Knowlton et al., 1996) and tended to decrease the meal interval time between meals in early lactating cows (Mullins et al., 2012).

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However, the ionophore utilization has been contested worldwide due to possible residues in animal products and the potential development of bacterial resistance, resulting in discontinued use in the European Union (European Commission, 2003). For these reasons, the search for alternative antimicrobials has attracted the attention of the scientific community.

Chitosan (**CHI**) is a biopolymer (*N*-acetyl-D-glycosamide) derived from the deacetylation of chitin—the second most abundant polysaccharide in nature and the major component of exoskeleton from crustaceans and insects (Senel and McClure, 2004). Chitosan has demonstrated antimicrobial activity against several bacteria, fungi, and yeasts (Kong et al., 2010) and is considered generally recognized as safe by the US Food and Drug Administration since 2012 (GRN#443; FDA, 2012). Chitosan interacts with the outer membrane proteins causing bacterial cell membrane disruption and cell death (Jeon et al., 2014). Although studies have evaluated CHI as an additive for silage preservation (Gandra et al., 2016a), or to prevent metritis (Daetz et al., 2016) and mastitis in dairy cattle (Lancôt et al., 2017), few studies have evaluated the effects of CHI on ruminal fermentation in in vivo trials, especially with lactating cows (Paiva et al., 2016; Del Valle et al., 2017). Goiri et al. (2010a), Araújo et al. (2015), and Paiva et al. (2016) demonstrated the potential of CHI in decreasing the propionate to acetate ratio in ruminal fluid of sheep, beef steers, and lactating cows, respectively. Besides the effects on VFA production, evidence suggests that CHI decreases biohydrogenation in the rumen (Goiri et al., 2010b; Del Valle et al., 2017). Recently, Del Valle et al. (2017) reported that CHI improved the feed efficiency and increased milk UFA concentration of cows fed a soybean-oil-free diet. However, when CHI was provided in diets with soybean oil, these authors reported a negative associative effect on performance of cows. The reasons for the latter associative effect are not clear because no differences in nutrient intake and digestibility, and ruminal VFA concentration were detected between cows fed the diet with soybean oil and the diet with soybean oil along with CHI.

Lipid supplementation is a strategy to increase energy density of high-yielding cow diets, but depending on the form of diet supplementation (rumen-protected or not and unsaturation degree), it can decrease specific bacteria populations (particularly those belonging to the *Butyrivibrio* group) due to cytotoxic effects of UFA (Maia et al., 2007, 2010). Dietary fatty acid (**FA**) supplementation using whole oilseeds instead of ground oilseeds or vegetable oils has shown small negative effects on ruminal fermentation (Chilliard et al., 2009; Weld

and Armentano, 2017). Whole raw soybeans (**WRS**) are considered a natural form of rumen-protected fat source that increases the abomasal flow of UFA (Barletta et al., 2016; Bettero et al., 2017). In addition, WRS have a lower price compared with their products (i.e., roasted soybeans) or other rumen-protected fat sources (i.e., calcium salts of FA).

We hypothesized that the negative interaction between CHI and UFA (soybean oil) supplementation on performance observed by Del Valle et al. (2017) would be eliminated by feeding CHI and WRS. We expected that CHI and WRS combination would improve ruminal fermentation, alter ruminal bacterial populations, and increase UFA concentration in milk of cows. The objective of this study was to evaluate the effects of CHI and WRS on nutrient intake and digestibility, ruminal fermentation and bacterial populations, microbial protein synthesis, N utilization, blood metabolites, and milk yield and composition of dairy cows.

MATERIALS AND METHODS

All procedures performed in the current study were approved (protocol no. 3059/2013) by the Ethics Committee in the Use of Animals from the School of Veterinary Medicine and Animal Sciences of University of São Paulo, São Paulo, Brazil.

Animals, Experimental Design, and Diets

Twenty-four multiparous Holstein cows (141 ± 37.1 DIM, 38.8 ± 6.42 kg/d of milk yield, and 615 ± 81.1 kg of BW at the start of experiment), 8 of which were rumen cannulated, were enrolled in this study. Cows were housed in a barn containing individual pens (17.5 m^2 of area) with concrete floor, bunks, sand beds, and free access to water. The experiment consisted of a 4×4 Latin square design with 23-d periods (last 8 d for sampling). Cows were blocked according to milk yield, DIM, BW, and rumen cannula. A 2×2 factorial treatment arrangement was randomly assigned to cows within blocks. Treatments were composed of diets with 2 inclusion rates of WRS (0 or 14% diet DM) and 2 doses of CHI (0 or 4 g/kg of DM, Polymar Ciência e Nutrição, Fortaleza, Brazil). Chitosan dose was based on a previous study with beef cattle of our research group (Araújo et al., 2015) and further supported by an experiment with lactating cows (Mingoti et al., 2016). According to the manufacturer, CHI had an apparent density of 0.32 g/mL, pH 7.9, viscosity of 50 cP at 20°C, and deacetylation degree of 86.3%. Chitosan was hand mixed into the concentrate before the morning feed. Cows were fed TMR (Table 1) twice daily at 0800

Table 1. Ingredient and chemical composition of experimental diets

Item	Diet ¹	
	CON	WRS
Ingredient, % of DM		
Corn silage	50.0	50.0
Ground corn	29.2	23.9
Soybean meal	18.0	9.50
Whole raw soybean		14.0
Urea	0.44	0.22
Limestone	0.15	0.15
Mineral and vitamin mix ²	2.00	2.00
Salt	0.20	0.20
Chemical, % DM, otherwise stated		
DM, % as fed	58.5	58.7
OM	93.3	93.5
CP	17.3	17.4
Insoluble protein in NDF	1.50	1.45
Ether extract	2.44	4.76
NFC	44.2	41.2
NDF	30.7	30.8
ADF	16.6	16.8
ADL	2.68	2.70
Ash	6.65	6.47
NE _L , Mcal/kg of DM	1.52	1.58
Fatty acids (FA), % of total FA		
14:0	0.40	0.39
16:0	15.8	15.3
18:0	3.14	3.22
<i>cis</i> -9 18:1	25.8	25.6
<i>cis</i> -9, <i>cis</i> -12 18:2	43.4	43.7
<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15 18:3	4.22	4.71
Σ MUFA	26.1	25.9
Σ PUFA	47.7	48.5
SFA-to-UFA ratio	0.28	0.28

¹Control (CON) and whole raw soybeans (WRS).

²Contained per kilogram of product: 100 g of Ca, 42 g of P, 18 g of S, 45 g of Mg, 20 g of K, 123 g of Na, 28 mg of I, 500 g of Cu, 14 g of Co, 1,050 mg of Fe, 1,400 mg of Mn, 18 mg of Se, 2,800 mg of Zn, 420 mg of F, 80 mg of biotin, 200,000 IU of vitamin A, 40,000 IU of vitamin D₃, and 1,200 IU of vitamin E.

and 1300 h in equal amounts, aiming refusals between 5 and 10% on an as-fed basis.

Feed Chemical Analyses

Feed ingredient and refusal samples were dried in a forced-air oven (60°C for 72 h) and ground in a 1-mm-screen Wiley mill (Arthur H. Thomas Co., Philadelphia, PA). Ground samples were analyzed for DM (method 930.15), CP (N × 6.25, Dumas method 990.03), ether extract (EE, method 920.39), ADF and lignin (method 973.18), and ash (method 942.05) according to AOAC International (2000). Samples were analyzed for NDF content according to Mertens (2002), using thermostable α-amylase with no sodium sulfite in the detergent. Insoluble N in NDF and insoluble N in ADF were analyzed according to Licitra et al. (1996). Organic matter was calculated subtracting ash content from DM. Nonfiber carbohydrate content was

calculated as follows: NFC = 100 – (NDF% + CP% + EE% + ash%). Total digestible nutrient and NE_L were calculated according to NRC (2001).

For analyses of dietary FA profile, samples of ingredients were lyophilized and ground in a 1-mm-screen mill. Then, lipids were extracted according to Folch et al. (1957) and FAME formed according to Kramer et al. (1997). The FAME were quantified using a gas chromatograph (GC-2010, Shimadzu, Kyoto, Japan) equipped with flame ionization detector, automatic split injector (AOC-20i, Shimadzu), and capillary column of fused silica (100 m × 0.25 mm diameter) surrounded by 0.2 μm of cyanopropyl polysiloxane pellicle (Rt-2560, Resetek, Bellefonte, PA). The temperature cycle in the gas chromatograph was programmed with the following steps: initial temperature of 70°C for 4 min, an increase in temperature until it reached 175°C (13°C/min) maintained for 27 min, and a second increase in temperature until it reached 215°C (4°C/min), which was maintained for 31 min. Hydrogen was used as the carrier gas with a constant flow (40 cm/s). The FAME were identified based on the retention time of FA standards [Supelco 37 Component FAME Mix, Bellefonte, PA; vaccenic acid, code V038-1G, Sigma-Aldrich, St. Louis, MO; *trans*-10, *cis*-12 CLA, code UC-61M, Nu-Chek Prep, Elysian, MN; and *cis*-9, *trans*-11 CLA, code UC-60M, Nu-Chek Prep]. Nonadecanoic acid was added as free FA to the samples to correct losses during the methylation process.

Nutrient Intake and Total-Tract Apparent Digestibility

Amounts of forage and concentrate provided, and refusals of each cow were weighed daily to calculate feed intake. Samples of diet ingredients and refusals were collected daily from d 16 to 23 of each period to form a composited sample per period per cow. Total-tract apparent digestibility of nutrients was determined after total feces collection from the 8 rumen-cannulated cows. Feces were collected from d 21 to 23 of each period from the pen floor, immediately after cows defecated, and were stored in plastic bags (50 L). During the total feces collection periods, the floor was constantly cleaned to avoid sand or feed particles in fecal samples. Total fecal output was weighed every 24 h, and a representative sample (10% of total weight on a wet-basis) was frozen. Fecal samples were dried in a forced-air oven (60°C for 72 h) and analyzed for DM, ash, CP, EE, and NDF, as described earlier. Fecal DM and nutrient excretion were estimated based on the chemical composition and fecal output from the 72 h-collection period. Digestibility coefficients were calculated as {[intake (kg/d) – fecal output (kg/d)] ÷ intake (kg/d)} × 100.

Ruminal Fermentation and Bacterial Quantification

Ruminal digesta samples (~500 g from each site) were collected on d 19 of each experimental period from 5 different sites within the rumen (cranial sac – dorsal portion, cranial sac – ventral portion, ventral sac, dorsal sac, and ventral sac – caudal portion), pooled, and strained in 4 layers of cheese cloth to obtain ruminal fluid. Digesta samples were collected every 2 h relative to the morning feeding (0800 h – time point 0) until 12 h after. Ruminal fluid pH was measured immediately after the digesta collection (MB-10, Marte Científica, Santa Rita do Sapucaí, Brazil). Digesta was returned into the rumen after ruminal fluid was sampled. Fifty milliliters of ruminal fluid were centrifuged ($7,000 \times g$ at 4°C for 15 min), and 1.6 mL of supernatant was collected for VFA analysis. Another aliquot of the supernatant (0.8 mL) was collected and transferred to 2.0-mL centrifuge tubes containing 0.4 mL of sulfuric acid (H_2SO_4 , 0.5 M) for $\text{NH}_3\text{-N}$ analysis. The phenol-hypochlorite colorimetric method was used to measure $\text{NH}_3\text{-N}$ concentration in samples (Broderick and Kang, 1980). Volatile fatty acid concentrations were measured using a gas chromatograph (GC-2104, Shimadzu) with splitless injector, capillary column at 145°C (Stabilwax, Restek), and double flame ionization detector at 250°C , according to methods described by Erwin et al. (1961) and adapted by Getachew et al. (2005). Samples for VFA analysis were thawed at room temperature, centrifuged ($14,500 \times g$ at 4°C for 10 min), and 1 mL of supernatant transferred to chromatograph vials containing 0.1 mL of the internal standard (2-ethylbutyric acid 100 mM, Chem Service, West Chester, PA). External standards were prepared (acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid; Chem Service) to aid in the identification of peaks, and calculations of VFA concentration were performed by GCSolution software (Shimadzu).

On d 19, the ruminal digesta collected 6 h after the morning feeding was also analyzed for bacterial quantification. Twenty-five grams from the solid portion and 25 mL from the liquid portion were processed according to Stevenson and Weimer (2007), then the bacterial pellet was resuspended in 700 μL of buffering solution (Tris 100 mM, 10 mM EDTA, and 0.15 M NaCl; pH 8.0) and stored at -80°C until DNA extraction. Two hundred microliters from the resuspended sample was used in the DNA extraction process using a commercial kit (Qiagen QIAamp DNA stool, Qiagen, Valencia, CA) according to the manufacturer's recommendations. An ethanol precipitation was performed as the last step of DNA extraction (Sambrook and Russell, 2001). Relative bacterial quantification was estimated by real-time PCR utilizing the ABI Prism 7500 detection system

(Applied Biosystems, Lennik, Belgium) and SYBR technology (Invitrogen, Carlsbad, CA) in 96-well microplates. Samples were analyzed in duplicate with a negative control for each bacterium assessed. Each well was filled with 10 μL of $2\times$ SYBR Green Master Mix (Life Technologies, Foster City, CA), 0.30 μM of primers for each bacterium specie, 6.6 μL of nuclease-free water, and 1 μL of extracted DNA, totaling a final volume of 20 μL per well. Five bacterial taxa were quantified: *Butyrivibrio* group, *Butyrivibrio proteoclasticus*, *Anaerovibrio lipolytica*, *Streptococcus bovis*, and *Fibrobacter succinogenes*, whereas primers were selected based on GenBank (EU714406.1, AM039827.1, NR_114696.1, M58835.1, and EU606019.1, respectively). Forward (**F**) and reverse (**R**) sequences of bacterial primers were F: GYGAAGAAGTATTTTCGGTAT and R: CCAACACCTAGTATTCATC for the *Butyrivibrio* group (Boeckaert et al., 2008; 418 bp); F: TCCG-GTGGTATGAGATGGGC and R: GTCGCTGCAT-CAGAGTTTCCT for *B. proteoclasticus* (Paillard et al., 2007; 185 bp); F: TGGGTGTTAGAAATGGATTC-TAGTG and R: GCACGTCCATTCGGTATTAGCAT for *A. lipolytica* (Khafipour et al., 2009; 109 bp); F: TTCCTAGAGATAGGAAGTTTCTTCGG and R: ATGATGGCAACTAACAATAGGGGT for *S. bovis*; (Stevenson and Weimer, 2007; 127 bp); and F: GG-TATGGGATGAGCTTGC and R: GCCTGCCCCCT-GAACTATC for *F. succinogenes* (Tajima et al., 2001; 445 bp). A general oligonucleotide (*Eubacteria universal*; F: GTGSTGCAYGGYTGTCTGTC and R: AC-GTCRTCCMCACCTTCCTC; Maeda et al., 2003; 147 bp) was used to quantify total bacteria. Five bacterial targets were relative quantified to *Eubacteria* (16S ribosomal RNA gene). This universal primer was used for quantification of total bacteria for each ruminal sample individually, to standardize the amount of DNA added to the reactions. The standard curves were generated from a serial dilution ($n = 5$; 100, 60, 30, 15, and 7.5%) from a pool of samples used in this study with the same DNA concentration. The quantitative PCR reactions of all bacteria were run in duplicate, and a negative control was included in each assay to assess the specificity of quantitative PCR reaction. No positive controls were designed. The PCR amplification protocol included an initial denaturation (95°C for 10 min) followed by 44 cycles of heating (95°C for 15 s) and cooling (55°C for 30 s). A standard curve was designed for bacterial species and presented similar reaction efficiency of *E. universal*. The Ct method assumes that the efficiency of the PCR is close to 1 and the PCR efficiency of the target gene is similar to the internal control gene (Schmittgen and Livak, 2008). The relative quantification of bacteria was performed using the $2^{-\Delta\text{Ct}}$ method (Livak and Schmittgen, 2001).

Microbial Protein Synthesis and N Utilization

Microbial N synthesis (**micN**) was estimated based on purine derivatives (**PD**) excretion through urine and milk as described by Chen and Gomes (1992). Total urine collection was performed during the last 3 d of each experimental period (Ahvenjärvi and Huhtanen, 2002; González-Ronquillo et al., 2003) from the 8 rumen cannulated cows concomitantly with total feces collection. Urine was collected via a Foley catheter inserted into the urinary bladder (12 h before collections) connected into a nontoxic polyvinyl chloride tube (1.2 cm diameter) plugged in a urine drainage bag (2L Free-Bac closed system; Changshy Senlin Medical Appliance Ltda., Changshu, China). The urine drainage bag was attached to a thoracic elastic belt (80 mm width) so that cows had free movement inside the pen during collections.

After every spontaneous urination, urine was transferred to containers (30 L) containing 500 mL of sulfuric acid (40%), which maintained the urine pH below 3 while avoiding PD microbial degradation (Chen and Gomes, 1992) and ammonia volatilization (Plaizier et al., 2000). By the end of the 24-h periods, urine production was measured and a representative sample was collected. Twenty milliliters of urine was diluted in 80 mL of distilled water to avoid uric acid crystals and precipitation in samples. These samples were filtered in a Whatman filter and analyzed for PD; allantoin was analyzed according to Fujihara et al. (1987) using a microplate reader (Expert Plus, Asys Hitech GmbH, Eugendorf, Austria) and uric acid was analyzed using a commercial kit (K139, Bioclin, Belo Horizonte, Brazil), absorbances were measured in a spectrophotometer (SBA 200, CELM, São Caetano do Sul, Brazil). Milk samples were collected during 4 consecutive milkings on d 17 and 18 of each period. Samples were deproteinized with trichloroacetic acid solution (25%), filtered in Whatman filter, and analyzed for allantoin concentration as described earlier.

Daily PD excretion was calculated as the sum of allantoin and uric acid excreted in urine and the amounts of allantoin secreted in milk (mmol/d). Absorbed microbial purines (**absP**) was calculated from PD excretion, where $\text{absP} = (\text{PD} - 0.236 \times \text{BW}^{0.75}) \div 0.84$, in which 0.236 mmol/kg of $\text{BW}^{0.75}$ is the PD endogenous contribution and 0.84 is the recovery coefficient of absP as PD (Orellana Boero et al., 2001). The synthesis of micN (g/d) was estimated according to adapted equation of Chen and Gomes (1992), as follows: $\text{micN} = (\text{absP} \times 70) \div (0.83 \times 0.103 \times 1,000)$ where 70 is the N content of purines (mg of N/mmol), 0.83 is the intestinal digestibility coefficient of microbial purines, and 0.103 is the N in purines to N in ruminal bacteria

ratio obtained in this study. The N in purines to total N in bacteria ratio values were obtained after the bacteria extraction from ruminal digesta samples. The bacterial pellet was lyophilized, macerated, and analyzed for DM and total N (AOAC International, 2000; methods 930.15 and 990.3, respectively), and total purines (Makkar and Becker, 1999). The efficiency of N utilization for micN synthesis was calculated as the $[\text{micN} (\text{g/d}) \div \text{N intake} (\text{g/d})] \times 100$. The apparent OM degradation in rumen was estimated as $\{[\text{OM intake} (\text{kg/d}) \div \text{total apparent digestibility of OM} (\%)] \times 100\} \times 0.65$, according to ARC (1984). Utilization efficiency of N intake to milk production was calculated as $[\text{milk N} (\text{g/d}) \div 0.93] \div \text{N intake} (\text{g/d})$, according to Dschaaka et al. (2010) wherein the milk N secretion was calculated as $[\text{milk yield} (\text{g/d}) \times \text{milk protein content} (\%)] \div 6.38$.

Milk Yield and Composition, and Blood Metabolites

Cows were milked twice daily (0600 and 1600 h) and milk yield electronically recorded (DeLaval, Tumba, Sweden). Milk samples were collected from d 16 to 19 in all milkings and analyzed for fat, protein, and lactose in an ultrasonic milk analyzer (Lactoscan MCC, Milkotronic Company, Nova Zagora, Bulgaria). Samples collected on d 17 and 18 were deproteinized as described earlier and analyzed for urea concentration using a commercial kit (K056, Bioclin) and absorbance measured in a spectrophotometer (SBA 200, CELM). Milk yield was corrected for 3.5% fat (FCM) according to the equation proposed by Sklan et al. (1992) where $\text{FCM} = [0.432 + 0.1625 \times \text{milk fat content} (\%)] \times \text{milk yield} (\text{kg/d})$. Energy-corrected milk was calculated as $\text{ECM} = \text{milk yield} (\text{kg/d}) \times 0.327 + \text{milk fat content} \times 12.86 + \text{milk protein} (\text{kg/d}) \times 7.65$. Milk samples collected on d 17 and 18 were analyzed for FA profile. Milk sample aliquots (40 mL) were centrifuged at $17,800 \times g$ (30 min at 4°C) followed by another centrifugation of $19,300 \times g$ (20 min at 4°C) to collect the fat supernatant (Feng et al., 2004). Lipids extracted from milk samples were methylated according to Kramer et al. (1997). The FAME were quantified by GC as described earlier.

On d 18 of each experimental period, blood samples were collected by venipuncture from the coccygeal vein in vacutainer tubes with no anticoagulants before and 4 h after the morning feeding. Tubes rested on ice until clot formation, then were centrifuged ($3,000 \times g$, 15 min at room temperature), serum was collected and frozen. Commercial kits (Bioclin) were used to analyze serum glucose (K082) and total cholesterol (K083), and absorbances were measured in a spectrophotometer (SBA 200, CELM).

Statistical Analysis

Data were analyzed for normality of residuals (Shapiro-Wilk method) and variance homogeneity (Hartley method) using UNIVARIATE and GLM procedures of SAS 9.2 (SAS Institute Inc., Cary, NC), respectively. The ANOVA of nutrient intake and digestibility, ruminal bacteria population, micN synthesis, blood metabolites, and milk yield and composition data were performed using the MIXED procedure of SAS following the model below:

$$Y_{ijklm} = \mu + \text{CHI}_i + \text{WRS}_j + \text{CHI}_i \times \text{WRS}_j + P_k + S_l + c_m(S_l) + e_{ijklm},$$

where Y_{ijklm} is the dependent variable, μ is the intercept, CHI_i is the fixed effect of CHI, WRS_j is the fixed effect of WRS, $\text{CHI}_i \times \text{WRS}_j$ is the fixed interaction effect of CHI by WRS, P_k is the fixed effect of period, S_l is the fixed effect of Latin square, $c_m(S_l)$ is the random effect of cow nested in Latin square, and e_{ijklm} is the residual.

Ruminal fermentation results were generated from data of 8 cows analyzed as repeated measures by the MIXED procedure of SAS according to the following model:

$$Y_{ijklmn} = \mu + \text{CHI}_i + \text{WRS}_j + \text{CHI}_i \times \text{WRS}_j + P_k + S_l + c_m(S_l) + T_n + T_n \times \text{CHI}_i + T_n \times \text{WRS}_j + T_n \times \text{CHI}_i \times \text{WRS}_j + e_{ijklmn},$$

where T_n is the fixed effect of time (h relative to the morning feeding); $T_n \times \text{CHI}_i$ is the fixed interaction effect of time by CHI; $T_n \times \text{WRS}_j$ is the fixed interaction effect of time by WRS; $T_n \times \text{CHI}_i \times \text{WRS}_j$ is the fixed interaction effect among time, CHI, and WRS; and e_{ijklmn} is the residual. The interaction between period and cow within Latin square was used as the error term. Data were submitted to 10 covariance structures for residuals [CS, CSH, UN(1), AR(1), ARH(1), HF, TOEP, TOEPH, and VC] and Akaike method was used to choose the best covariance structure, based on the lowest corrected Akaike information criterion value. Means were adjusted by LSMEANS statement. When the $\text{CHI} \times \text{WRS}$ interaction effect was $P \leq 0.05$, the PDIFF option was used to produce a protected least-significant difference. Fermentation variables data were submitted to F test each time using the SLICE option from LSMEANS. Significance level was set at 0.05, and tendencies were considered when $0.05 < P \leq 0.10$.

RESULTS

Diets containing WRS increased ($P < 0.001$) EE intake, but decreased ($P < 0.001$) CP total-tract digestibility in cows (Table 2). An interaction effect between CHI and WRS was observed on intake of DM, OM, CP, NDF, and NFC, as well as on digestibility of DM, OM, NDF and EE of cows. In general, the combination of WRS and CHI decreased nutrient intake (DM, OM, CP, NDF, and NFC) and digestibility (DM, OM, NDF,

Table 2. Influence of whole raw soybeans (WRS) and chitosan (CHI; 0 or 4 g/kg of DM) on nutrient intake and total-tract digestibility of lactating cows

Item	Treatment ¹				SEM	P-value ²		
	CON		WRS			CHI	WRS	CHI × WRS
	0	4	0	4				
Intake, kg/d								
DM	23.7 ^{ab}	23.8 ^a	23.2 ^b	22.6 ^c	0.476	0.181	<0.001	0.028
OM	22.1 ^{ab}	22.2 ^a	21.7 ^b	21.1 ^c	0.444	0.182	<0.001	0.030
CP	4.31 ^a	4.33 ^a	4.29 ^a	4.16 ^b	0.090	0.124	0.007	0.031
NDF	6.81 ^a	6.88 ^a	6.66 ^b	6.51 ^c	0.136	0.404	<0.001	0.022
Ether extract	0.599	0.603	1.19	1.17	0.023	0.550	<0.001	0.370
NFC	10.7 ^a	10.8 ^a	9.71 ^b	9.45 ^c	0.200	0.345	<0.001	0.029
NE _L , Mcal/d	36.7	36.9	37.0	36.3	0.607	0.222	0.419	0.070
Total-tract digestibility, %								
DM	69.6 ^a	69.2 ^a	67.0 ^b	64.4 ^c	0.683	0.010	<0.001	0.047
OM	71.8 ^a	71.4 ^a	69.3 ^b	66.7 ^c	0.700	0.007	<0.001	0.043
CP	73.8	75.5	67.2	66.6	1.574	0.570	<0.001	0.267
NDF	46.7 ^a	45.2 ^a	42.7 ^b	38.0 ^c	1.288	<0.001	<0.001	0.013
Ether extract	83.4 ^a	81.7 ^a	82.6 ^a	76.8 ^b	1.061	<0.001	0.004	0.028

^{a-c}Values in the same row with a different superscript differ significantly (LSD, $P < 0.05$).

¹Control (CON), WRS added at 14% diet DM, and CHI at 0 or 4 g/kg of DM.

²Probabilities for effects of CHI, WRS, or the interaction of CHI and WRS (CHI × WRS).

Table 3. Influence of whole raw soybeans (WRS) and chitosan (CHI; 0 or 4 g/kg of DM) on ruminal fermentation of lactating cows

Item	Treatment ¹				SEM	P-value ²			
	CON		WRS			CHI	WRS	CHI × WRS	T
	0	4	0	4					
pH	6.26	6.34	6.28	6.33	0.039	0.005	0.761	0.610	<0.001
NH ₃ -N, mg/dL	24.9	26.0	23.3	22.7	1.373	0.780	0.013	0.339	<0.001
VFA, mM									
Acetate	79.0	76.9	77.7	74.3	1.616	0.026	0.114	0.573	<0.001
Propionate	25.6	26.6	27.6	28.2	0.813	0.280	0.020	0.798	<0.001
Butyrate	15.5	15.2	13.7	13.5	0.394	0.384	<0.001	0.801	<0.001
Valerate	1.97	1.87	2.00	1.88	0.045	0.013	0.721	0.904	<0.001
Branched-chain fatty acids	4.28	4.02	4.52	4.56	0.110	0.171	<0.001	0.072	<0.001
Total	126	125	126	122	2.492	0.159	0.291	0.631	<0.001
Acetate-to-propionate ratio	3.10	2.91	2.83	2.66	0.047	<0.001	<0.001	0.586	<0.001
VFA, mol/100 mol									
Acetate	62.6	61.6	61.8	60.9	0.454	0.005	0.027	0.942	<0.001
Propionate	20.3	21.2	22.0	23.1	0.409	0.016	<0.001	0.888	<0.001
Butyrate	12.3	12.2	10.9	11.1	0.341	0.869	<0.001	0.309	<0.001

¹Control (CON), WRS added at 14% diet DM, and CHI at 0 or 4 g/kg of DM.

²Probabilities for effects of CHI, WRS, the interaction of CHI and WRS (CHI × WRS), and hours relative to the morning feeding (T).

and EE) of cows when compared with other treatments. However, cows fed CHI with no WRS in diets exhibited similar nutrient intake and digestibility in comparison with CON.

Regardless of the negative effects of CHI and WRS combination on DMI and nutrient digestibility, no interaction effects were observed in rumen fermentation variables (Table 3). Cows fed CHI had higher ($P \leq 0.016$) ruminal pH and propionate molar proportion than cows fed no CHI. However, CHI decreased acetate and valerate ruminal concentrations, acetate to propionate ratio, and acetate molar proportion. Diets containing WRS decreased ($P \leq 0.027$) ruminal NH₃-N and butyrate concentrations, and the molar proportions of acetate and butyrate while increasing ($P < 0.001$) the molar proportion of propionate in cows. Interaction effect between WRS and time was observed ($P = 0.012$) on ruminal NH₃-N concentration (Supplemental Figure S1; <https://doi.org/10.3168/jds.2018-14675>). No other interaction effect between treatment and time were

observed in ruminal fermentation variables (data not shown).

No CHI by WRS interaction effects were observed on ruminal bacterial populations assessed in this experiment (Table 4). Chitosan decreased ($P = 0.001$) the relative population of *Butyrivibrio* group in comparison with the other treatments. Diets containing WRS negatively affected ($P \leq 0.031$) the rumen bacterial populations from the *Butyrivibrio* group and *F. succinogenes*; however, WRS increased ($P < 0.001$) the relative rumen bacterial population of *S. bovis*.

A negative interaction effect between CHI and WRS was detected ($P = 0.018$) on micN (Table 5). The CHI alone had no effect on N utilization of cows. On the other hand, WRS decreased ($P = 0.001$) urinary N excretion and increased ($P = 0.001$) fecal N output. The combination of CHI and WRS negatively affected ($P \leq 0.04$) the N intake and milk N secretion of cows.

Diets containing WRS increased ($P = 0.008$) milk fat concentration and decreased ($P \leq 0.012$) the urea

Table 4. Influence of whole raw soybeans (WRS) and chitosan (CHI; 0 or 4 g/kg of DM) on bacterial population in the rumen of lactating cows

Item	Treatment ¹				SEM	P-value ²		
	CON		WRS			CHI	WRS	CHI × WRS
	0	4	0	4				
<i>Streptococcus bovis</i>	1.00	0.790	55.3	52.5	1.267	0.686	<0.001	0.796
<i>Butyrivibrio</i> group	1.00	0.748	0.822	0.594	0.029	0.001	0.011	0.815
<i>Butyrivibrio proteoclasticus</i>	1.00	0.698	0.824	0.778	0.028	0.082	0.709	0.192
<i>Anaerovibrio lipolytica</i>	1.00	1.37	1.07	1.26	0.030	0.455	0.978	0.808
<i>Fibrobacter succinogenes</i>	1.00	1.02	0.701	0.801	0.009	0.600	0.031	0.684

¹Control (CON), WRS added at 14% diet DM, and CHI at 0 or 4 g/kg of DM. Means expressed as fold change in relation to CON.

²Probabilities for effects of CHI, WRS, or the interaction of CHI and WRS (CHI × WRS).

Table 5. Influence of whole raw soybeans (WRS) and chitosan (CHI; 0 or 4 g/kg of DM) on microbial protein synthesis and N use in lactating cows

Item	Treatment ¹				SEM	P-value ²		
	CON		WRS			CHI	WRS	CHI × WRS
	0	4	0	4				
Microbial N, g/d	317 ^a	331 ^a	305 ^a	263 ^b	22.8	0.202	0.002	0.018
Microbial N ÷ rumen degradable OM, g/kg	32.6	34.1	33.3	31.0	1.88	0.753	0.358	0.142
Microbial N ÷ N intake, g/g	0.493	0.515	0.471	0.433	0.0241	0.604	0.004	0.068
N intake, g/d	643 ^a	644 ^a	645 ^a	604 ^b	30.1	0.064	0.068	0.040
Milk N, g/d	150 ^a	153 ^a	148 ^a	138 ^b	8.43	0.099	0.001	0.009
Urinary N, g/d	252	246	218	213	17.7	0.488	0.001	0.928
Fecal N, g/d	169	159	213	203	15.2	0.205	<0.001	0.980

^{a-c}Values in the same row with a different superscript differ significantly (LSD, $P < 0.05$).

¹Control (CON), WRS added at 14% diet DM, and CHI at 0 or 4 g/kg of DM.

²Probabilities for effects of CHI, WRS, or the interaction of CHI and WRS (CHI × WRS).

concentration in milk, and efficiency of milk N production of cows (Table 6). The combination of CHI and WRS negatively affected ($P \leq 0.017$) yields of milk, FCM, ECM, protein, and lactose of cows. In terms of milk synthesis efficiency, cows fed diets with CHI and without WRS had the highest values of feed efficiency (FCM ÷ DMI and ECM ÷ DMI), whereas cows fed diets with WRS had intermediate values, and CON ex-

hibited the lowest values of feed efficiency. The CHI had no effect on blood metabolites, but WRS decreased ($P = 0.010$) blood concentrations of glucose and increased ($P < 0.001$) blood concentration of total cholesterol.

Chitosan decreased milk concentration of 4:0, 6:0, 8:0, total SFA, SFA to UFA ratio, and 18:0 to UFA ratio (Table 7). On the other hand, CHI increased milk concentration of 18:1 *trans*-11, 18:2 *cis*-9,*cis*-12, 18:3

Table 6. Influence of whole raw soybeans (WRS) and chitosan (CHI; 0 or 4 g/kg of DM) on efficiency of milk production and blood metabolites of lactating cows

Item	Treatment ¹				SEM	P-value ²		
	CON		WRS			CHI	WRS	CHI × WRS
	0	4	0	4				
Yield, kg/d								
Milk	34.6 ^{ab}	35.4 ^a	34.2 ^b	33.1 ^c	0.932	0.628	<0.001	0.014
3.5% FCM	36.0 ^a	37.1 ^a	36.3 ^a	34.7 ^b	0.866	0.568	0.023	0.005
ECM	35.9 ^a	36.9 ^a	36.0 ^a	34.5 ^b	0.855	0.562	0.008	0.004
Fat	1.25 ^a	1.30 ^a	1.29 ^a	1.24 ^b	0.038	0.963	0.735	0.025
Protein	1.07 ^{ab}	1.09 ^a	1.05 ^b	1.02 ^c	0.028	0.577	<0.001	0.012
Lactose	1.60 ^{ab}	1.63 ^a	1.59 ^b	1.53 ^c	0.042	0.488	0.001	0.017
Milk composition, %								
Fat	3.67	3.72	3.86	3.77	0.106	0.683	0.008	0.083
Protein	3.08	3.08	3.08	3.08	0.024	0.428	0.849	0.560
Lactose	4.63	4.61	4.63	4.62	0.035	0.165	0.665	0.791
Urea, mg/dL	18.8	18.7	17.2	17.6	0.514	0.838	0.003	0.549
Milk synthesis efficiency, kg/kg								
Milk ÷ DMI	1.47	1.50	1.48	1.47	0.036	0.287	0.353	0.058
FCM ÷ DMI	1.52 ^b	1.57 ^a	1.56 ^{ab}	1.53 ^{ab}	0.027	0.523	0.827	0.020
ECM ÷ DMI	1.51 ^b	1.56 ^a	1.55 ^{ab}	1.52 ^{ab}	0.027	0.480	0.977	0.016
N efficiency ³	0.253	0.260	0.249	0.248	0.012	0.312	0.012	0.224
NE _L efficiency ⁴	0.649 ^a	0.661 ^a	0.648 ^a	0.627 ^b	0.014	0.569	0.019	0.022
Blood glucose, mg/dL	71.4	72.2	70.2	70.3	1.199	0.410	0.010	0.557
Blood total cholesterol, mg/dL	150	154	185	190	7.390	0.062	<0.001	0.876

^{a-c}Values in the same row with a different superscript differ significantly (LSD, $P < 0.05$).

¹Control (CON), WRS added at 14% diet DM, and CHI at 0 or 4 g/kg of DM.

²Probabilities for effects of CHI, WRS, or the interaction of CHI and WRS (CHI × WRS).

³Efficiency of N = {[milk protein (kg/d) ÷ 0.93] ÷ 6.38} ÷ N intake (kg/d), according to Dschaaka et al. (2010).

⁴Net energy of lactation efficiency = NE_L milk ÷ NE_L intake.

cis-9,*cis*-12,*cis*-15, 18:1 *cis*-9,*trans*-11, total MUFA, and total PUFA. In addition, CHI increased the product to substrate ratio of 18:1 *cis*-9 to 18:0. Diets containing WRS decreased FA concentration in milk of all FA up to 16 carbon chain length. Furthermore, WRS increased 18:0, 18:1 *cis*-9, 18:2 *cis*-9,*cis*-12, 18:3 *cis*-9,*cis*-12,*cis*-15, 20:0, and 20:3 *cis*-8,*cis*-11,*cis*-14 FA concentrations in milk of cows. In summary, WRS increased long-chain FA (>C16), total MUFA, PUFA, and 18:0 to UFA ratio in milk of cows. The WRS decreased total SFA, FA with odd carbon chain, and total SFA to UFA ratio in milk of cows. Finally, WRS decreased the product-to-

substrate ratios of 14:1 *cis*-9 to 14:0, 16:1 *cis*-9 to 16:0, and 18:1 *cis*-9 to 18:0.

DISCUSSION

To the best of our knowledge, no data are available regarding the effects of CHI and WRS on nutrient digestibility, metabolic profile, and performance of lactating dairy cows. Gandra et al. (2016b) evaluated the combination of CHI and WRS in diets of Jersey heifers and did not report interaction effects on nutrient digestion, microbial protein synthesis, and blood metabo-

Table 7. Influence of whole raw soybeans (WRS) and chitosan (CHI; 0 or 4 g/kg of DM) on milk fatty acid (FA) profile of lactating cows (g/100 g of FA)

Item	Treatment ¹				SEM	P-value ²		
	CON		WRS			CHI	WRS	CHI × WRS
	0	4	0	4				
4:0	1.44	1.43	1.43	1.39	0.018	0.044	0.042	0.237
6:0	1.59	1.56	1.50	1.47	0.020	0.030	<0.001	0.777
8:0	1.17	1.13	1.04	1.02	0.024	0.026	<0.001	0.450
10:0	3.03	2.92	2.55	2.52	0.090	0.105	<0.001	0.371
11:0	0.087	0.083	0.067	0.064	0.008	0.495	<0.001	0.929
12:0	3.79	3.64	3.04	3.03	0.120	0.175	<0.001	0.261
13:0	0.235	0.246	0.197	0.191	0.013	0.670	<0.001	0.215
14:0	11.8	11.5	10.1	10.2	0.200	0.310	<0.001	0.225
<i>cis</i> -9 14:1	1.00	0.98	0.74	0.77	0.036	0.674	<0.001	0.119
15:0	1.10	1.12	0.97	0.96	0.044	0.733	<0.001	0.691
16:0	33.0	32.6	29.1	29.2	0.511	0.497	<0.001	0.256
<i>cis</i> -9 16:1	1.44	1.46	1.15	1.22	0.047	0.056	<0.001	0.347
17:0	0.626	0.626	0.538	0.533	0.011	0.717	<0.001	0.679
18:0	9.64	9.44	13.4	12.5	0.275	0.014	<0.001	0.124
<i>cis</i> -9 18:1	22.0	22.7	25.5	25.8	0.591	0.092	<0.001	0.418
<i>trans</i> -9 18:1	0.192	0.193	0.185	0.190	0.006	0.350	0.139	0.482
<i>trans</i> -11 18:1	0.623	0.681	0.500	0.549	0.020	<0.001	<0.001	0.743
<i>cis</i> -9, <i>cis</i> -12 18:2	2.31	2.38	3.06	3.17	0.060	0.048	<0.001	0.633
<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15 18:3	0.160	0.173	0.283	0.311	0.008	0.003	<0.001	0.246
<i>cis</i> -9, <i>trans</i> -11 CLA	0.354	0.394	0.284	0.324	0.016	<0.001	<0.001	0.951
20:00	0.109	0.108	0.133	0.128	0.003	0.085	<0.001	0.243
<i>cis</i> -11 20:1	0.007	0.010	0.007	0.007	0.004	0.275	0.335	0.335
<i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14 20:3	0.107	0.108	0.117	0.112	0.005	0.551	0.023	0.322
<i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14 20:4	0.157	0.161	0.160	0.160	0.005	0.400	0.646	0.463
Σ not identified	4.19 ^a	4.16 ^a	3.96 ^b	4.11 ^a	0.087	0.147	0.002	0.036
Summary								
Σ 4C to 14C	23.8	23.2	20.3	20.2	0.445	0.149	<0.001	0.367
Σ more than 16C	36.5	37.2	44.2	43.6	0.726	0.991	<0.001	0.107
Σ SFA	67.5	66.6	63.8	63.2	0.685	0.022	<0.001	0.650
Σ MUFA	25.2	26.1	28.1	28.6	0.586	0.043	<0.001	0.494
Σ PUFA	3.09	3.23	3.92	4.09	0.084	0.006	<0.001	0.715
Σ Odd carbon chain FA	2.07	2.08	1.75	1.75	0.074	0.937	<0.001	0.985
SFA-to-UFA ratio	2.41	2.29	2.00	1.95	0.065	0.012	<0.001	0.273
C18 SFA-to-UFA ratio	0.379	0.357	0.452	0.415	0.011	<0.001	<0.001	0.268
Product-to-substrate ratio								
<i>cis</i> -9 14:1 to 14:0	0.086	0.087	0.073	0.077	0.003	0.068	<0.001	0.205
<i>cis</i> -9 16:1 to 16:0	0.044	0.045	0.040	0.042	0.001	0.121	0.001	0.270
<i>cis</i> -9 18:1 to 18:0	2.30	2.43	1.92	2.07	0.062	<0.001	<0.001	0.946
<i>cis</i> -9, <i>trans</i> -11 CLA to <i>trans</i> -11 18:1	0.581	0.590	0.568	0.599	0.020	0.089	0.852	0.347

^{a-c}Values in the same row with a different superscript differ significantly (LSD, $P < 0.05$).

¹Control (CON), WRS added at 14% diet DM, and CHI at 0 or 4 g/kg of DM.

²Probabilities for effects of CHI, WRS, or the interaction of CHI and WRS (CHI × WRS).

lites. However, the DMI level (5.98 kg/d in average) and WRS dietary inclusion (16.3% diet DM), besides physiological state, make the comparisons between experiments not reliable. Our research group evaluated CHI provision to cows fed a diet with soybean oil and we found similar interaction effects observed in the current study, including decreased DMI and milk yield (Del Valle et al., 2017). Thus, the hypothesis that supplementing WRS instead of soybean oil could eliminate the negative effects of CHI and UFA supplementation on performance was not accepted.

Provision of CHI in a diet with no WRS and relatively low EE has not altered nutrient intake in ruminants (Goiri et al., 2010a; Araújo et al., 2015; Paiva et al., 2016). On the other hand, CHI provision to cows fed a diet containing soybean oil decreases nutrient intake (Del Valle et al., 2017). In agreement with the latter study, the combination of CHI and WRS decreased the intake of nutrients except for EE. In this experiment, the reduced feed intake of cows fed CHI+WRS is likely related to changes in ruminal fermentation and negative effects on nutrient digestibility. Although the exact mechanism of CHI on the gastrointestinal tract of cows is not fully understood, changes in ruminal fermentation and nutrient utilization (Goiri et al., 2010a; Araújo et al., 2015; Paiva et al., 2016) are very similar to those observed when feeding ionophores (Ipharraguerre and Clark, 2003; Duffield et al., 2008). Similar to ionophores and CHI, UFA affect ruminal metabolism by altering ruminal microbial population (Jenkins, 1993; Lourenço et al., 2010). In the current study, changes in ruminal fermentation were observed when providing to cows either CHI or WRS including an increase in ruminal molar proportion of propionate and a reduction in acetate proportion and acetate to propionate ratio. Regardless of the improvements in fermentation efficiency in terms of energy when CHI was fed with no WRS, nutrient digestibility was not affected. However, when CHI was provided with a diet containing WRS, fiber digestion drastically decreased in comparison with the control treatment (46.7 vs. 38.0%, respectively).

Changes in rumen microbial population (e.g., decreases in gram-positive bacteria and ciliate protozoa and an increase in gram-negative bacteria) and decreased OM and NDF digestibility can be observed when lipid supplementation exceeds 5% of dietary FA (Lock and Shingfield, 2004). In addition, the inclusion of lipids in diets increases the release of intestinal peptides regulators that suppress intestinal motility and feed intake of cows (Relling and Reynolds, 2007). Although diets did not exceed 5% diet EE, decreases in digestibility of OM and NDF, and reduced NDF intake were observed in the current experiment. The WRS have relatively

lower ruminal availability of FA (Barletta et al., 2016), but seemed to affect fiber digestion. According to NRC (2001), the adverse effects of oilseeds on ruminal fermentation depend on diet composition besides the inclusion level, whereas the negative effects of oilseeds are more evident in corn-silage-based diets with low forage proportion. Specific PUFA, such as linoleic acid (present in high amounts in WRS), have toxic effects on cellulolytic and butyrate producing bacteria (Maia et al., 2007, 2010; Yang et al., 2009).

According to Lourenço et al. (2010), the manipulation of a specific microbial activity, such as reduction of biohydrogenation, will always influence other microbial activities in the rumen, particularly the cellulolytic activity. For instance, *Butyrivibrio fibrisolvens*, which is part of the *Butyrivibrio* group (major group related to biohydrogenation), has a critical role in fiber digestion, and some species within this group have high proteolytic capacity (Maia et al., 2010). However, *B. fibrisolvens* is susceptible to the bacteriostatic effects of UFA (Maia et al., 2010). Our results suggest that *B. fibrisolvens* was affected by the inclusion of WRS in diets, resulting in decreased CP digestibility. Changes in ruminal bacteria population of cows fed WRS are likely the main cause of decreases in CP digestibility and ruminal $\text{NH}_3\text{-N}$ concentration. According to Ferme et al. (2004), the major ammonia-producing bacteria are *Prevotella ruminantium* and *Prevotella bryantii*, and the latter have low tolerance to PUFA (Maia et al., 2007). In addition, UFA exert toxic effects on protozoa ruminal population, decreasing their predatory and proteolytic activities with consequent reduction of $\text{NH}_3\text{-N}$ production (Doreau and Ferlay, 1995; Eugène et al., 2004). However, measures of rumen population of *P. bryantii* and protozoa were not performed in this study. The reduction of $\text{NH}_3\text{-N}$ in rumen of cows fed WRS may also be related to the lower urea dietary inclusion in diets with WRS in comparison with the other treatments. Although CHI presented a similar negative effect of WRS on *Butyrivibrio* group population, it did not affect CP digestibility and ruminal $\text{NH}_3\text{-N}$ concentration, suggesting that *B. fibrisolvens* was not the major bacteria affected within the *Butyrivibrio* group. In addition, improvements in CP digestibility have been reported when feeding CHI to lactating cows (Paiva et al., 2016; Vendramini et al., 2016; Del Valle et al., 2017).

The combination of CHI and WRS decreased total-tract digestibility of EE. Earlier studies evaluating CHI dietary supplementation did not report differences in EE digestibility of lactating cows (Paiva et al., 2016; Del Valle et al., 2017). Nevertheless, Zhang et al. (2008) observed that CHI has hypolipidemic activity

in high-fat diet fed mice with reduction in plasmatic and hepatic levels of lipids and increase in lipids fecal excretion—effects attributed to a decrease in intestinal fat absorption. Chitosan has high adherence capacity to fat *in vitro* due to its polycationic structure (Cho et al., 1998). Similar response to CHI was observed in broilers, which exhibited lower ileal digestion of fat, and no changes on CP and starch digestibility (Razdan and Petterson, 1994). Authors from the latter study speculated possible effects of CHI on digesta viscosity that could impair the activity of intestinal lipases in feed particles or glucosamine groups in CHI acting as a chelator of lipid micelles.

Regarding ruminal fermentation, this study demonstrated that CHI maintained pH at higher values when compared with the other treatments, but the increase of ruminal pH (+0.06) due to CHI provision is of minimal biological significance. Although previous studies did not find differences in rumen pH (Goiri et al., 2010a; Paiva et al., 2016; Del Valle et al., 2017), an *in vitro* study demonstrated higher pH values when CHI was added to the batch cultures (Goiri et al., 2009). Whole raw soybeans had no influence on ruminal pH, despite the unexpected increase of *S. bovis* in cows fed WRS. *Streptococcus bovis* (*Firmicutes*) have high amylolytic activity and are the main lactate producers in the rumen, often associated with ruminal acidosis (Russell and Strobel, 1989). We expected that the inclusion of WRS in diets would decrease the relative population of *S. bovis* because the inclusion of WRS implies in a reduction of dietary NFC (main substrate for *S. bovis*). In agreement with the current study, Rico et al. (2015) reported an increase (380%) in the relative population of *S. bovis* when the dietary content of FA was increased by 2%. The positive effects of UFA on *S. bovis* may be related to their tolerance to PUFA, despite their susceptibility to ionophores (Russell and Strobel, 1989).

The negative effects of CHI and WRS combination were also observed on micN production. Several factors can be related to micN production reduction including changes in ruminal fermentation and bacterial populations, and decreased feed intake and nutrient digestibility of cows fed WRS+CHI. In addition, evidence indicates that CHI can inhibit ciliate protozoal populations in a lower degree in comparison with vegetable oils (Wencelová et al., 2014). The inhibitory effects of lipids on protozoa can improve the efficiency of microbial protein synthesis even with a reduction of OM and NDF digestibility (Doreau and Ferlay, 1995; Eugène et al., 2004). In terms of N utilization, the combination of WRS and CHI reduced milk N secretion due to lower N intake, CP digestibility, and micN synthesis of cows. Cows fed WRS exhibited greater N excretion in feces

and lower N excretion in urine, which are related to the lower CP digestibility of cows fed WRS in comparison with other treatments.

The negative influence of WRS+CHI on DMI, digestion, and metabolism of cows decreased the production of milk, FCM, ECM, and milk solids in relation to the other treatments. The increase in milk fat content of cows fed WRS can be attributed to a decrease in milk yield rather than an increase of fat synthesis in the mammary gland because the total milk fat production was similar in comparison with the control group. Agreeing with the current experiment, Venturelli et al. (2015) reported a linear increase in milk fat content and a linear decrease in milk yield when cows were fed increasing dietary levels of WRS. Cows fed WRS exhibited lower milk urea concentration than other treatments due to the lower CP digestibility, $\text{NH}_3\text{-N}$ ruminal concentration, and blood urea concentration. Interestingly, cows fed CHI and no WRS exhibited the highest values of milk yield and similar total fat production, and consequently exhibited the highest values of feed efficiency in terms of FCM and ECM. Paiva et al. (2016) reported a linear increase on milk yield of cows fed CHI, but the authors did not calculate feed efficiency of cows considering fat or energy production. The increase in blood cholesterol concentrations when feeding WRS to cows is related to an increase of EE intake. Dietary fat supplementation often increases the lipoprotein cholesterol exportation by the intestine, the major site of cholesterol synthesis in ruminants (Noble, 1981). In agreement with the current study, Naves et al. (2016) fed WRS to lactating cows and reported a trend to decreased blood glucose concentration and an increase in blood cholesterol concentration.

In the current study, feeding CHI to cows fed a diet with no WRS increased the concentrations of MUFA and PUFA in milk, whereas similar results were reported by Del Valle et al. (2017). Among the positive effects of CHI on milk FA profile observed in the current study, we highlight the increase (12.9%) of *cis-9,trans-11* CLA concentration in milk, which has been related to health benefits in humans (Parodi, 2001; Benjamin and Spener, 2009). Although literature lacks sufficient data regarding the effects of CHI on biohydrogenation, Goiri et al. (2010b) reported that CHI inhibited the complete biohydrogenation of PUFA from sunflower oil and canola meal and increased the concentrations of *trans-11* 18:1 and CLA in an *in vitro* study. In the current study, we quantified 2 bacterial species involved in ruminal lipolysis (*A. lipolytica*) and lipid transformation (*S. bovis*) and bacteria involved with FA biohydrogenation (*Butyrivibrio* group and *B. proteoclasticus*). In addition, we quantified *F. succino-*

genes, the major cellulolytic bacteria in rumen that is susceptible to antimicrobial effect of CHI (Belanche et al., 2016).

Chitosan decreased or tended to decrease bacterial populations related to ruminal biohydrogenation (*Butyrivibrio* group and *B. proteoclasticus*), whereas WRS supplementation decreased *F. succinogenes* and *Butyrivibrio* group, which corroborates with the decreased digestibility of NDF in cows fed WRS. Feeding WRS increased *S. bovis* population. Although the latter specie is often associated with lactate production in rumen, some studies (Hudson et al., 1998; Hudson et al., 2000) indicated the *S. bovis* easily hydrate linoleic acid to 13-hydroxy-9-octadecenoic acid. The FA hydration competes with biohydrogenation processes in the rumen (Hudson et al., 1998; Hudson et al., 2000), which could be related to the relative low concentration of intermediates of biohydrogenation in milk of cows fed WRS.

CONCLUSIONS

Provision of CHI to dairy cows fed a diet supplemented with WRS alters the ruminal fermentation and bacterial population, increasing the milk content of UFA, but decreases nutrient intake and digestibility, microbial protein synthesis, and milk yield. However, evidence indicates that CHI provision in diets with no lipid supplementation improves feed efficiency of lactating cows, besides increasing the milk content of UFA and *cis*-9,*trans*-11 CLA. Feeding WRS instead of soybean oil as a fat source did not avoid the negative interaction effects with CHI on performance of cows and unchanged milk FA profile.

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