p-ISSN 2615-787X e-ISSN 2615-790X
Accredited by Directorate General of Higher Education, Research, and Technology, Republic of Indonesia, No. 225/E/KPT/2022

Tropical Animal Science Journal, June 2024, 47(2):225-234
DOI: https://doi.org/10.5398/tasj.2024.47.2.225
Available online at https://journal.ipb.ac.id/index.php/tasj

Effects of Replacing Dietary Antibiotic Supplementation with Chitosan Levels on Rumen Metabolism and Nitrogen Use in Finishing Steers Fed Forage-Free Diets

T. I. S. Silva[a], R. H. T. B. de Goes[a,*], N. R. B. Cônsolo[a], J. R. Gandra[b], M. P. Osmari[c], C. C. Silva[a], L. E. F. Oliveira[a], T. M. Souza[a], L. C. G. S. Barbosa[a], D. G. Anschau[a], G. K. R. Silva[a], L. B. Lopes[a], & J. W. S. Fernandes[a]

[a]Department of Animal Science, School of Agricultural Sciences, Federal University of Grande Dourados, Dourados, State of Mato Grosso do Sul, Brazil
[b]Department of Animal Science, Federal University of the South and Southeast of Pará, Xinguara, State of Pará, Brazil
[c]Department of Animal Science, Federal University of Santa Catarina, Florianópolis, State of Santa Catarina, Brazil
*Corresponding author: rafaelgoes@ufgd.edu.br
(Received 13-11-2023; Revised 07-03-2024; Accepted 08-03-2024)

ABSTRACT

The aim of this study was to evaluate the effects of replacing antibiotics with increasing doses of chitosan (CHI) on nutrient intake and digestibility, corn grain excretion, ruminal fermentation, nitrogen metabolism, and feeding behavior in finishing steers fed forage-free diets. Five ruminally cannulated crossbred steers were assigned to a 5 x 5 Latin square experimental design and given the following diets: C0= basal diet with no additives, ANT= basal diet with inclusion of virginiamycin 30 mg/kg DM, C375= basal diet with inclusion of chitosan 375 mg/kg DM, C750= basal diet with inclusion of chitosan 750 mg/kg DM, and C1500= basal diet with inclusion of chitosan 1500 mg/kg DM. Supplementation with CHI did not affect ruminal pH and ammonia nitrogen ($N\text{-}NH_3$) concentration. Chitosan quadratically affected corn grain excretion and molar proportion of ruminal butyrate, with greater values for C750. Animals fed C375 showed greater dry matter intake and neutral detergent fiber intake; and greater digestibility of DM, starch, and NDF. Animals fed C750 and C1500 diets presented greater absorbed N compared to ANT. Animals fed ANT, C750, and C1500 spent more time eating and chewing than animals fed CON. More specifically, the dose of 375 mg/kg DM of diet was very promising for steers fed free-forage diet due to its ability to improve nutrient digestibility, with only slight changes in N metabolism.

Keywords: chitosan; forage-free diet; replacing antibiotics; ruminal fermentation; steers

INTRODUCTION

In the last decade, there has been a reduction of forage concentration in finishing diets in Brazilian feedlots. In 2009, the average of using forage on a total dry matter basis was 28.8% and it dropped to 16.7% in 2019 (Silvestre & Millen, 2021). The benefits of increasing concentrate levels in cattle finishing diets and the use of forage-free diets are not only related to the increase in dietary net energy for gain, improving feed efficiency, reduction of the cost per unit of metabolizable energy, and facilitating the feedlot management by the easiness of diet handling but also because of the demand for property space for crops, storing and distributing roughage in confinement (Rivaroli *et al.*, 2016).

The concept of a forage-free diet has been applied to diets without neutral detergent fiber (NDF) from forage. Still, when supplied to ruminants, the nutritionist must take into consideration several key points, such as i) the level of starch and type of corn grain processing, ii) the use of non-forage NDF, iii) adaptation of animals to the diet, iv) the diet particle size, v) the use of some additives to prevent ruminal metabolic problems, and vi) the precise control of animal feed intake. Otherwise, as explained by Paula *et al.* (2019), the rumen epithelium can be susceptible to injury, resulting in translocation of endotoxin and bacteria into the bloodstream (Emmanuel *et al.*, 2008), with a further cascade of non-specific immune responses, infection, and abscess formation in the liver and the foot, bloat, and others disorders correlated with greater ruminal production of lactate and lower pH (Ametaj *et al.*, 2009; González *et al.*, 2012; Owens *et al.*, 1998). All these alterations are associated with a decrease in animal performance and damage to animal welfare and health, impacting the system's profitability (Paula *et al.*, 2019).

Antibiotics have been used to manipulate ruminal fermentation, preventing those problems and increasing animal performance (Tiseo *et al.*, 2020). As a result, worries about the potential harm these compounds might do to human health and the spread of bacterial multidrug resistance have grown (Cheng *et al.*, 2014).

Because of this, resistant bacteria are now a major threat to global health, and one of the biggest problems facing scientists today is halting their spread (Ferri *et al.,* 2017; Van Boeckel *et al.,* 2015).

In this sense, chitosan (CHI), a natural biopolymer extracted from exoskeletons of crustaceans and insects, has been extensively investigated. Senel & McClure (2004) explained that chitosan exhibits various biochemical properties resulting in numerous applications in diverse fields of knowledge, such as pharmaceutical, medical, agriculture, cosmetics, nutritional enhancement, textile, and food processing in livestock industry and veterinary medicine. In animal production, testing CHI in animal diets has gained enormous interest due to its properties as a natural feed additive to replace traditional antibiotics and improve growth, performance, and meat production because of its antimicrobial, anti-inflammatory, antioxidant, and digestive modulatory activity on ruminal metabolism (Dias *et al.,* 2017; Goiri *et al.,* 2010; Harahap *et al.,* 2022; Jiménez-Ocampo *et al.,* 2019). Ruminant nutritionists are very interested in CHI because it has a mode of action similar to ionophores as a ruminal modulator, altering ruminal fermentation to a more energy-efficient pathway (Seankamsorn *et al.,* 2021, 2020) by inhibiting Gram-positive bacteria growth and decreasing acidosis events (Goiri *et al.,* 2010).

Several authors have reported consistent results, including a significant increase in ruminal propionate concentration and consequently improvement in DM, CP, and NDF digestibility when beef steers and dairy cows received CHI in the diet (Araújo *et al.,* 2015; Dias *et al.,* 2017; Vendramini *et al.,* 2016), but none of those studies had included CHI in the ruminant forage-free diet. Thus, this study hypothesized that animal-fed forage-free diets have beneficial changes in ruminal pattern fermentation by receiving CHI in the diet and that greater levels of CHI promote better results. The technological innovation in this study is based on using an active organic ruminal fermentation modulator in diets without forage, highlighting the great metabolic challenge for animals without antibiotics to guarantee favorable conditions for ruminal fermentation. Therefore, this study aimed to evaluate the effects of replacing ANT with CHI, ranging from 0 to 1,500 mg/kg on nutrient intake and digestibility, ruminal fermentation, and nitrogen use of finishing steers fed forage-free diets.

MATERIALS AND METHODS

All animal care procedures adopted in this study were conducted following the Institutional Animal Care and Use Committee Guidelines of the Federal University of Grande Dourados (approval protocol: 023/2015 CEUA/UFGD).

Experimental Site, Animals, and Diet

This study was conducted at the Ruminant Nutrition facility and Animal Nutrition Laboratory, School of Agrarian Sciences of the Federal University of Grande Dourados, Dourados, Brazil. Five crossbred steers, fitted with rumen cannulas, 2 years of age (350 ± 20 kg body weight) were randomly distributed to a 5 x 5 Latin square experimental design. Prior to the beginning of this study, animals received a step-up adaptation diet for 30 days with a progressive inclusion of the concentrate in the diet, 50%, 60%, and 80%, until they reached the level of 100% concentrate based on corn grain. Later, animals were housed in individual concreted pens (8 m^2, 2 x 4 m) with individual feed bunks of 0.5 linear m for 19 days during each experimental period (10 days for adaptation and 9 days for data collection), totaling 95 days. Animals were randomly assigned to the following treatments: C0= basal diet with no additives, ANT= basal diet with inclusion of virginiamycin 30 mg/kg DM, C375= basal diet with inclusion of chitosan 375 mg/kg DM, C750= basal diet with inclusion of chitosan 750 mg/kg DM, and C1500= basal diet with inclusion of chitosan 1500 mg/kg DM. Chitosan doses were selected for their similarity to those already tested by our research group with animals fed on pasture (Dias *et al.,* 2017). The chitosan used in the present study has the following technical specifications: ≥ 850 g/kg deacetylation degree, 0.32 g/mL density, pH 7.90, viscosity < 200 cPs, total ash 1.35 g/100 g, and loss on drying 9.3 g/100 g (Polymar Indústria e Cia. Imp. and Exp. LTDA, Fortaleza, Brazil).

Animals were fed *ad libitum* twice a day at 8 AM and 3 PM with a diet containing 85% corn grain and 15% pellet composed of protein, minerals, and vitamins (Table 1), with no forage inclusion. Ingredients and orts of each diet were weighed every day to estimate individual dry matter intake (DMI); feed was adjusted daily to allow for a minimum of 5%-10% orts.

Sample Collection and Chemical Analysis

Dietary ingredients and orts were sampled weekly, pooled according to the period and animal, and stored at -20 °C for chemical composition and nutrient intake estimation. Fecal samples were collected from day 11 to day 15 of each experimental period directly

Table 1. Composition and analyzed nutrient content (dry matter basis) of the finishing steers diet

Ingredients	g/kg of DM		
Corn grain	850		
Pellet[1]	150		
Analyzed composition	Corn grain	Pellet	Whole diet
Dry matter	850.0	822.0	845.8
Organic matter	957.0	799.0	933.2
Crude protein	95.0	380.0	137.0
Starch	668.0	58.2	576.6
Neutral detergent fiber	150.0	757.0	241.0
Acid detergent fiber	18.0	321.0	63.5

Note: [1]Engordim Grão Inteiro 38® - Protein, mineral e vitamin supplementation (Agrocria Comércio e Indústria LTDA) following components: Ca, 43 g/kg; P, 10 g/kg; S, 4 g/kg; Mg, 0,7 g/kg; K, 2,7 g/kg; Na, 9,7 g/kg; Co, 5 mg/kg; Cu, 175 mg/kg; Cr, 1,4 mg/kg; F, 130 mg/kg; I, 5 mg/kg; Mn, 182 mg/kg; Mo, 0,35 mg/kg; Ni, 0,3 mg/kg; Zn, 421 mg/kg; Vitamin A, 21.000 U.I; Vitamin D, 3.000 U.I; Vitamin E, 140 U.I; Virginiamycin, 150 mg/kg.

from the rectum at 8 AM, 10 AM, 12 PM, 2 PM, and 4 PM. Ingredients, orts, and feces samples were dried in a forced air oven at 65 °C for 72 h, ground to pass through a 1-mm screen, and then pooled according to the fecal excretion, chemical composition, and nutrient digestibility period.

Diet ingredients, orts, and feces samples were analyzed according to AOAC (Official Methods of Analysis, 2020) for DM (method 930.15), crude protein (N x 6.25; method 984.13), and ash (method 942.05). The neutral detergent fiber was analyzed using α-amylase without sodium sulfite (Mertens, 2002). Acid detergent fiber was analyzed according to Van Soest *et al.* (1991). Starch concentration was analyzed following the methodology proposed by Gandra *et al.* (2016). Fecal excretion was estimated using titanium dioxide (TiO_2) as an external marker added daily to the diet between day 5 and day 15 of each experimental period, being the first five days for adaptation of the animals to the TiO_2 on diet and the last five for fecal collection, following the methodology described by Dias *et al*. (2017). Fecal TiO_2 was analyzed by UV/Vis spectrophotometry using the methodology previously described by Myers *et al*. (2004). Total DM fecal excretion was calculated as follows:

$$FE = TIS \div TICF$$

where FE is the daily DM fecal excretion (g/d), TIS is the titanium dioxide supplied (g/d), and TICF is the titanium dioxide content in feces (g/g DM).

Corn Grain Excretion

Feces samples were collected directly from the animal's rectum on days 15, 16, and 17 of each experimental period, before the morning and afternoon meals, to evaluate the corn grain excretion. Approximately 400 g of feces were weighed, and then samples were washed on a 4-mm sieve as described by Rennó *et al*. (2015). Corn grain particles were manually collected and weighed to evaluate the corn grain excretion.

Nitrogen Balance and Microbial Protein Synthesis

Urine samples (100 mL) were collected by preputial stimulation on day 16 of each experimental period, four hours after the concentrate supply. Urine aliquots (10 mL) were diluted in sulfuric acid (40 mL; 0.036 N H_2SO_4) to prevent purine derivative degradation and uric acid precipitation; then, diluted urine samples were analyzed for creatinine, urea, uric acid, and allantoin. Allantoin was assessed according to Vendamini *et al.* (2016), creatinine and uric acid were determined by colorimetric methods using commercial kits (Labtest, Lagoa Santa, Brazil; Gold Analisa Diagnostica Ltda, Belo Horizonte, Brazil), and readings were taken using a semi-automatic biochemical analyzer (BIO-200, Bioplus, Barueri, SP, Brazil). Daily urinary excretion was estimated as follows:

Urinary excretion= (27.36 * BW) ÷ creatinine concentration in urine (Paiva *et al.*, 2016).

The total excretion of purine derivatives (PD) was calculated as the sum of allantoin and uric acid excreted in urine. The absorbed microbial purines (Pabs, mmol/day) were calculated using the equation:

$$Pabs = (PD-0.236*BW^{0.75}) / 0.84$$

where 0.84 is the recovery of purines absorbed as purine derivatives and $0.236*BW^{0.75}$ is the endogenous excretion of purine derivatives (Paiva *et al.*, 2016).

Ruminal synthesis of nitrogen compounds (Nmic, gN/day) was calculated based on the absorbed purines (Pabs, mmol/day) using the equation (Chen & Gomes, 1992):

$$Nmic = (70*Pabs) / (0.83*0.134*1.000)$$

where 70 is the N content in purines (mgN/mol), 0.134 is the purine N: total N ratio in bacteria (Paiva *et al.*, 2016), and 0.83 is the intestinal digestibility of microbial purines.

Urea and Creatinine Metabolism

On day 16 of each experimental period, blood samples (20 mL) were collected by puncturing coccygeal vessels four hours after the diet supply. Then, samples were centrifuged at 2,700 x g for 20 min (4 °C), serum supernatant was harvested and analyzed for urea and creatinine by the colorimetric method using commercial kits (Gold Analisa Diagnostica Ltda, Belo Horizonte, MG, Brazil) and readings were taken by a semi-automatic biochemical analyzer (BIO-200, Bioplus, Barueri, SP, Brazil). Plasma depuration or clearance of creatinine and urea was obtained by the ratio of the urinary excretion for 24 hours to the plasmatic concentration of each substance. Urea nitrogen and creatinine nitrogen excretion were analyzed by their total urinary concentration multiplied by 0.466 or 0.3715, respectively.

Ruminal Fermentation

Samples of ruminal fluid (around 50 mL) were collected through the ruminal cannula and filtered on day 17 of each period before the morning feed and 2 h, 4 h, 6 h, and 8 h after the morning meal. Immediately after collection, the ruminal fluid pH was determined using a potentiometer (pH 1500, Instrutherm, São Paulo, SP, Brazil). Short-chain fatty acid (SCFA) were determined by mixing 2 mL ruminal fluid aliquots, collected from all animals, with methanolic acid (400 μL; 98%–100% H_2CO_2), and then centrifuging at 7.000 x g at 4 °C for 15 min. The supernatant was collected and stored at −20 °C for posterior analysis of ruminal SCFA by gas chromatography (Shimadzu GC 2010 with automatic injection) as previously explained by Dias *et al.* (2017). Briefly, the equipment was equipped with a split injector, dual flame ionization detector (temperature at 250 °C), and capillary column (30 m length and 0.53 mm internal diameter; Stabilwax, Restek, Bellefonte, PA, USA) at 145 °C. The start temperature was 40 °C followed by 40 °C/min increments until 120 °C was reached. In sequence, the temperature increments

were 10 °C/min until a temperature of 180 °C was reached, and from this temperature to 240 °C, the increment was 120 °C/min, which was maintained for 3 min. Two standards were used to identify fatty acids: WSFA-2 (Ref. 47056, Supelco, Bellefonte, PA, USA) and glacial acetic acid (Ref. 33209, Sigma-Aldrich©). SCFA concentration was calculated using the GC solution v. 2.42.00 software (Shimadzu©).

For ammonia nitrogen (NH_3-N) concentration measurements, 2 mL ruminal fluid was mixed with sulfuric acid (0.5 mol/L H_2SO_4) and stored at −20 °C for posterior analysis following the method of Paiva *et al*. (2016).

Feeding Behavior

The feeding behavior of animals was evaluated on day 18 of each experimental period by visual observations at 5-min intervals of the variables: time spent on feeding (h/d), ruminating (h/d), feed intake frequency, water intake, and idleness (Johnson & Combs, 1991); beginning at 8 AM, during 24 hours as previously described by Ferrari *et al.* (2019). For better data collection, steers were adapted to artificial light at night for 3 days before the evaluation.

Statistical Analysis

Statistical analysis tests for normality and homogeneity were performed using PROC UNIVARIATE in SAS 9.2 software (SAS Institute Inc., Cary, NC, USA, 2009). Differences between ANT and all other treatments individually (CON, C375, C750, and C1500) were tested by the Dunnett test, and differences between CHI treatments (CON, C375, C750, and C1500) were tested by polynomial regression using the PROC MIXED procedure. Results are presented as LSMEANS and significance was declared p≤0.05. Data were analyzed according to the model:

$$Y_{ijl} = \mu + D_i + P_j + A_l + e_{ijl;}$$

where, Yijk is the dependent variable, μ is the overall mean, Di is the fixed effect of diet, P_j is the random effect of the experimental period, A_l is the random effect of the animal, and e_{ij} is the residual error.

Ruminal fermentation data were analyzed as repeated measures using the REPEATED from PROC MIXED following the model below:

$$Y_{ij} = \mu + D_i + t_j + D_i(t_j) + e_{ij;}$$

where, Y_{ijk} is the dependent variable; μ is the overall mean, D_i is the fixed effect of diet, t_j is the fixed effect of time of measurement, $D_i(t_i)$ is interaction, and e_{ij} is residual error. N stands for Gaussian distribution, s2v is the variance associated with experimental diets, MVN stands for multivariate normal, and R is the variance–covariance matrices of residuals due to the repeated measurements. It was evaluated the following variance–covariance matrix (CS, CSH, AR(1), ARH(1), TOEP, TOEPH, UN, FA(1), and ANTE(1)). The matrix was chosen using a Bayesian method. Experimental period and animals were used as random effects.

RESULTS

There was no linear or quadratic effect (p>0.05) according to CHI levels included in the diet on nutrient intake and digestibility, and fecal pH (Table 2). However, a quadratic effect (p= 0.037) was found for corn grain excretion, with the highest values for animals fed C750 (Table 2). When CHI treatments were compared to ANT, the C375 diet showed greater DM and NDF intakes (p<0.05), 480 g/day and 90 g/day, respectively.

Regarding nutrient digestibility, animals fed C375 had greater DM, starch, and NDF digestibility compared to the ANT, while the treatments C750 and C1500 presented similar intake and digestibility to the ANT diet. Fecal pHs were greater (p<0.05) for C0, C375, and C750 diets compared to the ANT. Animals fed ANT and C1500 had lesser (p<0.05) fecal pHs compared to the other treatments (Table 2).

Analyzing ruminal fermentation, CHI did not affect ruminal pH, NH_3-N, and total SCFA molar proportions, but CHI linearly affected (p= 0.038) the molar proportion of ruminal acetate and quadratically affected butyrate (p= 0.005), being the highest values found for the C750 diet (Table 3). In addition, the molar proportion of isovalerate was significant with a quadratic effect (p=0.002), with the highest value found for the ANT group (Table 3).

No differences existed in the molar proportion of ruminal propionate, butyrate, and isobutyrate when CHI treatments were compared to animals fed ANT. Nonetheless, compared to animals fed ANT, the molar proportions of ruminal acetate were lower for animals fed C0 and C375 (p<0.05), while molar proportions of ruminal valerate were lower for animals fed C0 and C1500 (p<0.05). The molar proportions of isovalerate presented lower values for the treatments C375 and C750 (Table 3).

Neither linear nor quadratic effects (p>0.05) were detected for nitrogen (N) intake, excretion, balance, or microbial protein synthesis (Table 4). However, compared to the ANT diet, animals fed C0 diet had greater (p<0.05) fecal N excretion and lower absorbable N, while animals fed C375 presented a greater (p<0.05) urine N excretion, resulting in lower retained N (p<0.05), but no changes on microbial N and protein synthesis compared to the ANT (Table 4). On the contrary, C750 diet decreased (p<0.05) urine N excretion and consequently improved the N retained compared to animals fed ANT. Despite these changes in N excretion and balance among treatments, microbial protein synthesis parameters were only greater for C1500, compared to ANT (Table 4). However, the other CHI inclusion levels presented similar results compared to the ANT group (Table 4).

Chitosan linearly increased urine creatinine (p=0.044) and N-creatinine (p=0.045); in addition, CHI quadratically affected clearance of urea (p=0.050), creatinine (p=0.030), and urea excretion (p=0.042) (Table 5). Comparing animals fed ANT to steers fed other diets, animals fed C0 had greater (p<0.05) urine concentrations of urea, and urea-N, as a consequence, lower (p<0.05)

Table 2. Nutrient intake, total tract digestion, fecal pH, and corn grain output of steers fed forage-free diets with increasing levels of chitosan

Variables	ANT	Chitosan levels				SEM	P-value[1]	
		C0	C375	C750	C1500		L	Q
Intake (kg/day)								
Dry matter	7.14[b]	7.26[b]	7.62[a]	7.27[b]	7.23[b]	0.32	0.624	0.347
Crude protein	0.98	1.00	1.05	1.00	0.99	0.04	0.604	0.352
Starch	5.26	5.41	5.32	5.52	5.32	0.24	0.822	0.401
Neutral detergent fiber	1.23[b]	1.26[a]	1.32[a]	1.26[b]	1.25[b]	0.56	0.612	0.370
Apparent digestibility (g/kg)								
Dry matter	901.51[b]	923.46[b]	942.06[a]	921.15[b]	929.89[b]	4.56	0.990	0.869
Crude protein	933.88[a]	900.62[b]	955.63[a]	950.86[a]	953.17[a]	5.03	0.233	0.350
Starch	909.15[b]	931.07[b]	958.62[a]	959.05[a]	962.75[a]	4.77	0.446	0.666
Neutral detergent fiber	736.13[b]	761.14[b]	766.04[a]	711.38[b]	720.01[b]	4.87	0.698	0.955
Fecal pH	6.28[b]	6.38[b]	6.40[a]	6.39[a]	6.31[b]	0.05	0.731	0.724
Corn grain excretion[2]	284.62[b]	253.89[b]	289.93[b]	390.00[a]	285.24[b]	4.57	0.047	0.037

Note: ANT= basal diet with the inclusion of virginiamycin 30 mg/kg DM; C0= basal diet with no additives; C375= basal diet with the inclusion of chitosan 375 mg/kg DM; C750= basal diet with the inclusion of chitosan 750 mg/kg DM; and C1500= basal diet with the inclusion of chitosan 1500 mg/kg DM.
[1]Linear (L) and quadratic (Q) effect;
[2]$Y=237.26 +30.22X – 0.017X^2$; $r^2=0.15$.
Dunnet test: means in the same row with different superscript differ significantly ($p<0.05$).

Table 3. Ruminal pH, $N\text{-}NH_3$, and short chain fatty acids molar proportions of steers fed forage-free diets with increasing levels of chitosan

Variables	ANT	Chitosan levels				SEM	P-value[1]	
		C0	C375	C750	C1500		L	Q
pH	6.26	6.38	6.41	6.27	6.39	0.32	0.431	0.321
$N\text{-}NH_3$	8.27	9.10	9.74	8.47	9.05	0.04	0.342	0.223
Short chain fatty acids (mmol/L)								
Total	115.52	96.89	93.31	116.68	106.80	0.13	0.231	0.431
Acetate[2]	56.95[a]	45.41[b]	47.34[b]	52.29[a]	53.84[a]	1.60	0.038	0.951
Propionate	34.66	30.24	27.12	34.95	29.29	2.30	0.990	0.869
Butyrate[3]	15.30	14.55	11.13	21.31	16.38	1.32	0.254	0.005
Isobutyrate[4]	1.11	1.13	1.04	1.09	1.17	0.06	0.446	0.666
Valerate	3.54[a]	1.82[b]	4.60[a]	4.32[a]	2.39[b]	0.57	0.698	0.955
Isovalerate	3.93[a]	3.73[a]	2.06[b]	2.68[b]	3.70[a]	0.28	0.652	0.002
Branched chain fatty acid	8.59	6.68	7.71	8.10	7.27	0.54	0.117	0.817
C2:C3	1.64	1.50	1.74	1.49	1.80	1.45	0.188	0.205

Note: ANT= basal diet with the inclusion of virginiamycin 30 mg/kg DM; C0= basal diet with no additives; C375= basal diet with the inclusion of chitosan 375 mg/kg DM; C750= basal diet with the inclusion of chitosan 750 mg/kg DM; and C1500= basal diet with the inclusion of chitosan 1500 mg/kg DM.
[1]Linear (L) and quadratic (Q) effect;
[2]$Y= 49.726 + 2.1015X$; $r^2=0.22$;
[3]$Y= 15.8425 + 0.4575X - 0.000302X^2$; $r^2=0.14$;
[4]$Y= 3.2825 - 0.1425X + 0.000188X^2$; $r^2=0.16$
Dunnet test: means in the same row with different superscript differ significantly ($p<0.05$).

serum concentrations of those compounds but greater ($p<0.05$) serum concentrations of creatinine and N-creatinine, higher ($p<0.05$) urea excretion, and lower urea clearance compared to the ANT (Table 5).

Animals fed C375 presented greater ($p<0.05$) values of serum urea, creatinine, N-urea, and N-creatinine, lower urea excretion, clearance of urea and creatinine, and lower urea excretion, compared to the ANT. Additionally, compared to the ANT, animals fed C750 had lower ($p<0.05$) urine concentrations of urea and urea-N, while the C1500 presented lower ($p<0.05$) urine concentrations of creatinine and N-creatinine but similar responses to the serum concentrations were observed between C750 and C1500 vs ANT, where creatinine and creatinine-N were higher ($p<0.05$) compared to the ANT, with lower ($p<0.05$) urea excretion and clearance (Table 5).

Chitosan quadratically affected water intake ($p=0.039$), with a lower value for animals fed C750, and frequency of visits to the feeder ($p=0.027$), with a greater value for animals fed ANT (Table 6). Compared to the ANT, animals fed C375 spent less time ($p<0.05$) feeding, chewing, and lower frequency of visits to the feeder, but longer time ruminating. Animals fed C750 and C1500 diets spent a longer ($p<0.05$) time ruminating and a lower ($p<0.05$) frequency of visits to the feeder compared to the ANT (Table 6).

Table 4. Nitrogen intake, output, balance, and rumen microbial protein synthesis of steers fed forage-free diets with increasing levels of chitosan

Variables	ANT	Chitosan levels				SEM	P-value[1]	
		C0	C375	C750	C1500		L	Q
N intake (g/day)	157.97	162.4	159.83	165.65	159.83	5.32	0.819	0.397
N output (g/day)								
Feces	9.43[b]	14.02[a]	7.86[b]	7.50[b]	7.27[b]	1.13	0.200	0.395
Urine	27.61[b]	23.93[b]	41.73[a]	20.65[b]	29.67[b]	1.56	0.918	0.610
N output (% TN)								
Feces	5.97[b]	8.63[a]	4.92[b]	4.53[b]	4.55[b]	1.64	0.225	0.345
Urine	17.48[b]	14.74[b]	26.11[a]	12.47[a]	18.56[a]	1.13	0.842	0.620
N balance (g/day)								
Absorbable	148.53[b]	148.38[b]	151.96[b]	158.14[a]	152.55[a]	3.56	0.69	0.969
Retained	120.92[b]	124.44[b]	110.24[a]	137.49[a]	122.88[b]	6.03	0.639	0.986
N balance (% TN)								
Absorbable	94.03[a]	91.36[b]	95.08[a]	95.47[a]	95.45[a]	1.45	0.233	0.350
Retained	76.55[b]	76.63[b]	68.97[a]	83.00[a]	76.88[b]	1.54	0.699	0.907
Microbial protein synthesis mmol/L								
Allantoin	3.17[a]	3.27[b]	2.91[a]	3.27[a]	2.90[a]	0.14	0.490	0.855
Uric acid	0.35[a]	0.40[a]	0.56[b]	0.42[a]	0.44[a]	0.03	0.912	0.323
Total purines mmol/day	3.52[a]	3.68[a]	3.48[a]	3.69[a]	3.34[b]	0.13	0.547	0.786
Allantoin	46.75[b]	47.49[b]	54.13[b]	47.54[b]	103.92[a]	2.56	0.612	0.870
Uric acid	5.64[b]	5.12[b]	7.82[b]	6.04[b]	16.62[a]	1.56	0.679	0.639
Total purines	52.38[b]	52.62[b]	61.95[b]	53.59[b]	120.54[a]	2.87	0.321	0.344
Absorbed purines	46.15[b]	46.37[b]	57.44[b]	47.58[b]	126.99[a]	6.54	0.234	0.399
Microbial nitrogen (g/day)	33.55[b]	33.71[b]	41.76[b]	34.59[b]	92.33[a]	8.77	0.200	0.385
Microbial protein (g/day)	209.72[b]	210.71[b]	261.02[b]	216.24[b]	577.04[a]	12.67	0.200	0.385

Note: ANT= basal diet with the inclusion of virginiamycin 30 mg/kg DM; C0= basal diet with no additives; C375= basal diet with the inclusion of chitosan 375 mg/kg DM; C750= basal diet with the inclusion of chitosan 750 mg/kg DM; and C1500= basal diet with the inclusion of chitosan 1500 mg/kg DM.
[1]Linear (L) and quadratic (Q) effect.
Dunnet test: means in the same row with different superscript differ significantly ($p<0.05$).

Table 5. Urea and creatinine metabolism, output, and clearance of steers fed forage-free diets with increasing levels of chitosan

Variables	ANT	Chitosan levels				SEM	P-value[1]	
		C0	C375	C750	C1500		L	Q
Urine (mg/dL)								
Urea	34.90[b]	48.90[a]	31.56[b]	22.80[a]	34.09[b]	4.00	0.207	0.137
Creatinine[2]	2.94[a]	2.94[a]	2.30[a]	1.98[a]	1.43[b]	0.37	0.044	0.918
N-Ureic	16.26[a]	22.78[b]	14.70[a]	10.62[b]	15.88[a]	1.18	0.547	0.786
N-Creatinine[3]	1.09[a]	1.09[a]	0.85[a]	0.73[a]	0.53[b]	0.10	0.045	0.904
Serum (mg/dL)								
Urea	45.99[b]	29.58[a]	62.16[a]	34.15[b]	39.44[b]	4.86	0.967	0.138
Creatinine	1.04[b]	1.31[a]	1.37[a]	1.22[a]	1.31[a]	0.17	0.887	0.942
N-Ureic	21.43[b]	13.78[a]	28.96[a]	15.91[b]	18.38[b]	2.25	0.967	0.138
N-Creatinine	0.38[b]	0.48[a]	0.51[a]	0.45[a]	0.48[a]	0.06	0.886	0.963
Excretion (mg/kg of body weight)								
Urea	35.32[b]	40.00[a]	18.01[a]	9.03[a]	19.43[a]	4.54	0.200	0.385
Creatinine	28.45	28.44	28.42	28.46	28.37	0.20	0.654	0.670
Clearance (24 hours)								
Urea[4]	2.68[a]	1.59[b]	0.27[b]	0.37[b]	0.61[b]	0.46	0.095	0.050
Creatinine[5]	67.65[a]	30.58[b]	27.93[b]	27.35[b]	38.38[b]	2.35	0.305	0.030
Fraction excretion (%)								
Urea[6]	4.42[a]	5.10[a]	1.51[b]	1.73[b]	4.92[a]	1.09	0.972	0.042

Note: ANT= basal diet with the inclusion of virginiamycin 30 mg/kg DM; C0= basal diet with no additives; C375= basal diet with the inclusion of chitosan 375 mg/kg DM; C750= basal diet with the inclusion of chitosan 750 mg/kg DM; and C1500= basal diet with the inclusion of chitosan 1500 mg/kg DM.
[1]Linear (L) and quadratic (Q) effect;
[2]$Y=2.798-0.00096X$; $r^2=0.18$;
[3]$Y= 1.037 - 0.00035X$; $r^2=0.18$;
[4]$Y= 1.48 - 3{,}01X + 0.00162X^2$; $r^2=0.31$;
[5]$Y= 30.77 - 1.32X + 0.0012X^2$; $r^2= 0.23$;
[6]$Y= 4.86 - 9.71X + 0.00653X^2$; $r^2=0.33$.
Dunnet test: means in the same row with different superscript differ significantly ($p<0.05$).

Table 6. Feeding behavior and frequency of steers fed forage-free diets with increasing levels of chitosan

Variables	ANT	Chitosan levels				SEM	P-value[1]	
		C0	C375	C750	C1500		L	Q
Min/day								
Eating	239.75[a]	183.99[b]	172.57[b]	224.54[a]	201.09[a]	0.32	0.490	0.855
Chewing	313.40[a]	259.36[b]	264.86[b]	311.32[a]	283.14[a]	0.04	0.674	0.852
Ruminating	73.65[b]	75.37[b]	92.28[a]	86.78[a]	82.04[a]	0.24	0.622	0.801
Idle	1051.05	1097.05	1112.72	1101.14	1084.77	0.56	0.612	0.870
Water intake[2]	75.54[a]	83.58[a]	62.42[a]	27.53[b]	72.08[a]	4.56	0.679	0.039
Frequency/day								
Feed bunk[3]	20.20[a]	16.00[b]	16.80[b]	14.06[b]	17.60[b]	4.77	0.865	0.027

Note: ANT= basal diet with the inclusion of virginiamycin 30 mg/kg DM; C0= basal diet with no additives; C375= basal diet with the inclusion of chitosan 375 mg/kg DM; C750= basal diet with the inclusion of chitosan 750 mg/kg DM; and C1500= basal diet with the inclusion of chitosan 1500 mg/kg DM.
[1]Linear (L) and quadratic (Q) effect.
[2]$Y = 87.83 - 12.68X + 0.0077X^2$; $r^2=0.24$.
[3]$Y = 16.44 - 3.21X + 0.00259X^2$; $r^2=0.30$.
Dunnet test: means in the same row with different superscript differ significantly ($p<0.05$).

DISCUSSION

To the best of our knowledge, no data are available regarding the effect of increasing levels of CHI and comparing the animals fed antibiotics to finishing steers fed forage-free diets on nutrient intake, ruminal digestibility, fermentation patterns, and nitrogen metabolism.

Forage-free diets can decrease feed intake, resulting from the decrease of saliva flow and ruminal motility (Nagaraja & Titgemeyer, 2007), leading to some metabolic disorders. However, in the present study, animals showed similar DMI compared to the other studies that also fed beef cattle forage-free diets (Contadini *et al.*, 2017; Paula *et al.*, 2019).

The fact that steers were fed whole corn grain, which has a slower rate and less ruminal starch digestion than processed maize, may indicate that they should have acclimated to diets devoid of forage. Furthermore, according to Owens & Soderlund (2006), the pellet can offer some non-forage NDF that stimulates ruminal motility and rumination to some extent, avoiding a significant ruminal pH decrease.

In general, our data showed that steers fed 375 mg/kg DM presented greater DM, starch, and apparent total digestibility of NDF compared to animals fed ANT, which might be due to the greater intakes of DM and NDF for the C375 diet since up to some point the DMI drives the digestibility. This improvement in nutrient use by animals fed the C375 diet may lead to benefits in animal performance. However, even presenting a greater starch digestibility, animals fed the C375 diet had no improvement in ruminal propionate concentration and similar results regarding ruminal fermentation, urea metabolism, and nitrogen balance.

Several lines of evidence suggest the beneficial effects of CHI-supplemented diet on nutrient digestibility in cattle. In general, some authors suggested that the increased digestibility provided by chitosan addition to ruminant diets is related to its positive effect on ruminal microbiota and fermentation processes since chitosan has a strong antimicrobial effect against Gram-positive rather than Gram-negative bacteria, avoiding pH drops and decreasing ruminal protein deamination, preventing nitrogen losses, but without effect on protein total-tract digestibility (Araújo *et al.*, 2015; Dias *et al.*, 2017; Mingoti *et al.*, 2016; Pereira *et al.*, 2018). Dias *et al.* (2017) also evaluated the increasing levels of chitosan but feeding grazing beef and reported greater DM, CP, and NDF intakes in steers fed intermediate levels (400 mg/kg and 800 mg/kg of DM) and observed a linear increase in DM and CP digestibility. Gandra *et al.* (2016) supplemented confined heifers and reported an increase in DM digestibility and decrease in DM and NDF intakes. In turn, Araújo *et al.* (2015) studied steers fed CHI and reported an increase in DM digestibility, which was unrelated to changes in feed intake. All the changes in ruminal microbiota can lead to the described changes in nutrient apparent digestibility due to CHI supplementation, which further results in beneficial changes in ruminal pH, ammonia N, SCFA production, and improves microbial protein synthesis. Especially because the basal diet used in the present study (forage-free) could lead to greater lactate production in the ruminal, pH drop, inflammation of ruminal papillae, and consequently, imbalance in ruminal fermentation, and impair the N balance and microbial protein synthesis. Nevertheless, those variables were not affected, showing that animals fed ANT or CHI (regardless of the dose) presented similar ruminal metabolism, suggesting that ANT can be replaced with CHI in the forage-free diet for steers.

According to the literature, responses to CHI supplementation can range widely from no effect on ruminal metabolism (Kirwan *et al.*, 2021; Vendramini *et al.*, 2016) to important changes in ruminal fermentation parameters (Araújo *et al.*, 2015; Del Valle *et al.*, 2017; Dias *et al.*, 2017; Goiri *et al.*, 2010). Araújo *et al.* (2015) fed Nellore steers a 60:40 roughage:concentrate ratio, and Dias *et al.* (2017) supplemented cattle on pasture system, and found a linear increase in molar proportion of ruminal propionate by increasing doses of CHI. Paiva *et al.* (2016) fed Holstein cows in lactation with increasing doses of CHI and reported a linear increase in ruminal propionate and consequently a decrease in the acetate:propionate ratio. The same authors also found a linear reduction in the ruminal isobutyrate and isovalerate by CHI inclusion, which indicates a

reduction in ruminal amino acid deamination (Paiva *et al*., 2016).

Those differences between studies are probably related to diet composition, which plays a key role in ruminal fermentation and metabolism. Despite no changes in the ruminal NH_3-N production, slight changes in N metabolism were observed between CHI and ANT diets. In general, animals that were given C375 diet presented greater urine N excretion, and lower retained N, showing less efficiency in regulating N balance when compared to animals fed ANT. Nevertheless, no changes in microbial N and protein synthesis were observed in these two treatments, showing that N metabolism did not disadvantage ruminal fermentation patterns and protein synthesis.

Kirwan *et al.* (2021) explained that when consumed N is not used by ruminal microorganisms for protein synthesis, it is degraded to NH_3-N, metabolized to urea in the liver and excreted via urine, which is an economic loss to the system with environmental impacts. The same authors explained that the lower use of dietary CP by CHI supplementation might be due to the defaunation of protozoa caused by its antimicrobial properties since protozoa play an important role in ruminal protein metabolism. However, the present study found no difference in CP digestibility or ruminal NH3-N concentration among treatments. Although this process is not fully understood, it is important to highlight that the diet used in this study does not include forage, which might be responsible for some differences in ruminal metabolism compared to the other studies.

Del Valle *et al*. (2017), Araújo *et al*. (2015), and Mingoti *et al.* (2016) also did not detect an influence of CHI levels on microbial protein synthesis. N metabolism data are still inconsistent in the literature regarding increasing CHI supplementation. Pereira *et al*. (2018) added increasing levels of CHI to the lamb diet and reported that the use of 272 mg chitosan/kg BW showed to be less efficient in regulating N balance, damaging microbial protein synthesis compared to the lower level of CHI (136 mg chitosan/kg BW). The authors explained that with higher levels, CHI tend to impair the development of not only Gram-positive, but also Gram-negative bacteria specialized in ruminal ammonia production, leading to defaunation, and decreasing N balance and microbial protein synthesis.

Dias *et al.* (2017) supplied increasing levels of CHI to beef cattle and reported a quadratic effect on the absorbed purines and microbial nitrogen production. The authors explained that CHI antimicrobial properties may impair ruminal fermentation at a level of 1,600 mg/kg DM concentrate. Nevertheless, in the present study, the highest level of CHI increased the microbial N and protein synthesis compared to animals fed the ANT diet, but with no difference from the other diets with CHI inclusion at different levels. Those inconsistencies among studies might be due to the differences in the basal diet composition, responsible for several changes in ruminal metabolism, and as previously commented, no other study had evaluated CHI supplementation in beef cattle fed a forage-free diets.

The excretion (mg/kg body weight) and urea and creatinine clearance were quadratically affected, with lower values in the C375 and C750 diets. These changes are generally attributed to changes in N intake or CP digestibility (Garcia-Rodriguez *et al*., 2015) since urea excretion in kidneys is influenced by its concentration in blood, which in turn is influenced by animal dietary conditions. However, in the present study, none of these variables were affected by CHI increasing levels. Dias *et al*. (2017) also observed that increasing levels of CHI lead to a quadratic effect on urea clearance and fractional excretion.

Not surprisingly, nitrogen and creatinine metabolisms were different between animals fed ANT and CON since animals fed a diet with no additive presented greater fecal N excretion and greater urinary concentrations of urea and urea-N, which can be explained by lower CP digestibility for animals fed C0 and consequently, presenting a lower ruminal efficiency on N metabolism, resulting in N loss through feces and later through urine. Araújo *et al*. (2015) explained that ANT and CHI treatments are expected to decrease N excretion via feces through greater CP digestibility and better N use.

Feeding behavior is extremely important when animals are given a forage-free diet since different feed additives might not stimulate chewing and rumination to the same extent, which in turn are correlated with ruminal pH and fermentation patterns, keeping animals healthy. In the present study, animals fed the ANT diet presented longer rumination time and higher frequency of visits to the feeder, which could suggest better patterns of ruminal fermentation and motility; however, those changes did not affect ruminal health, as indicated by ruminal pH and SCFA concentration. Despite improving salivation and keeping high ruminal pH, ruminating is important to control the utilization of roughage and limit the use of low-quality feed ingredients, which comprise the productive performance of animals (Huzzey *et al*., 2013). However, in the present study, no forage was included in the diet, so the corn pericarp and non-forage NDF of the pellet might have stimulated salivation and rumination, keeping the rumen healthy (Paula *et al*., 2019). Haraki *et al*. (2018) reported that heifers fed CHI had lower feeding efficiency of DM and NDF and chewing efficiency of NDF; the authors explained that it might be due to the lower DM intake combined with the shortest time spent ruminating standing for the CHI treatment compared to the other treatments.

CONCLUSION

In general, this study provides evidence that CHI can replace ANT in a forage-free cattle diet since, despite different responses among these treatments, CHI provides a positive result in rumen and N metabolism. More specifically, the dose of 375 mg/kg DM of diet was very promising for steers fed free-forage diet due to its ability to improve nutrient digestibility, with only slight changes in N metabolism.

CONFLICT OF INTEREST

The authors declare no conflict of interest with any organization regarding the material discussed in this manuscript.

ACKNOWLEDGEMENT

The authors would like to thank CAPES, CNPq (Process: 307871/2017-7; 310837/2020-0) and FUNDECT – MS (Process: 71/700.187/2017, SIAFEN 27876; and - Process: 71/700.134/2018 - No. TO: 051/2018 - SIAFEM: 028997) for the financial support.

REFERENCES

Ametaj, B. N., K. M. Koenig, S. M. Dunn, W. Z. Yang, Q. Zebeli, & K. A. Beauchemin. 2009. Backgrounding and finishing diets are associated with inflammatory responses in feedlot steers. J. Anim. Sci. 87:1314–1320. https://doi.org/10.2527/jas.2008-1196

AOAC. 2020. Official Methods of Analysis, 15th ed. 2020. Association of Official Analytical Chemists, Arlington, VA.

Araújo, A. P. C., B. C. Venturelli, M. C. B. Santos, R. Gardinal, N. R. B. Cônsolo, G. D. Calomeni, J. E. Freitas, R. V. Barletta, J. R. Gandra, P. G. Paiva, & F. P. Rennó. 2015. Chitosan affects total nutrient digestion and ruminal fermentation in Nellore steers. Anim. Feed Sci. Technol. 206:114–118. https://doi.org/10.1016/j.anifeedsci.2015.05.016

Chen, X. B. & M. J. Gomes. 1992. Estimation of Microbial Protein Supply to Sheep and Cattle Based on Urinary Excretion of Purine Derivatives -an Overview of the Technical Details. 20. Kluwer Academic Publishers, Austria.

Cheng, G., H. Hao, S. Xie, X. Wang, M. Dai, L. Huang, & Z. Yuan. 2014. Antibiotic alternatives: The substitution of antibiotics in animal husbandry ? Front. Microbiol. 5:217. https://doi.org/10.3389/fmicb.2014.00217

Contadini, M. A., F. A. Ferreira, R. R. S. Corte, D. S. Antonelo, J. F. M. Gómez, & S. da L. e Silva. 2017. Roughage levels impact on performance and carcass traits of finishing Nellore cattle fed whole corn grain diets. Trop. Anim. Health Prod. 49:1709-1713. https://doi.org/10.1007/s11250-017-1381-x

Del Valle, T. A., P. G. de Paiva, E. F. de Jesus, G. F. de Almeida, F. Zanferari, A. G. B. V. B. Costa, I. C. S. Bueno, & F. P. Rennó. 2017. Dietary chitosan improves nitrogen use and feed conversion in diets for mid-lactation dairy cows. Livest. Sci. 201:22–29. https://doi.org/10.1016/j.livsci.2017.04.003

Dias, A. O. C., R. H. T. B. Goes, J. R. Gandra, C. S. Takiya, A. F. Branco, A. G. Jacaúna, R. T. Oliveira, C. J. S. Souza, & M. S. M. Vaz. 2017. Increasing doses of chitosan to grazing beef steers: Nutrient intake and digestibility, ruminal fermentation, and nitrogen utilization. Anim. Feed Sci. Technol. 225:73–80. https://doi.org/10.1016/j.anifeedsci.2017.01.015

Emmanuel, D. G. V., S. M. Dunn, & B. N. Ametaj. 2008. Feeding high proportions of barley grain stimulates an inflammatory response in dairy cows. J. Dairy Sci. 91:606–614. https://doi.org/10.3168/jds.2007-0256

Ferrari, V. B., N. R. B. Cônsolo, R. T. Sousa, J. M. Souza, M. H. A. Santana, & L. F. P. Silva. 2019. Effects of energy sources and inclusion levels of concentrate in sugarcane silage-based diets of finishing Nellore young bulls: Feeding behaviour, performance and blood parameters. J. Agric. Sci. 157:350-356. https://doi.org/10.1017/S0021859619000583

Ferri, M., E. Ranucci, P. Romagnoli, & V. Giaccone. 2017. Antimicrobial resistance: A global emerging threat to public health systems. Crit. Rev. Food Sci. Nutr. 57:2857–2876. https://doi.org/10.1080/10408398.2015.1077192

Gandra, J. R., C. S. Takiya, E. R. de Oliveira, P. G. de Paiva, R. H. de T. e B. Goes, E. R. de S. Gandra, & H. M. C. Araki. 2016. Nutrient digestion, microbial protein synthesis, and blood metabolites of Jersey heifers fed chitosan and whole raw soybeans. Revista Brasileira Zootecnia 45:130–137. https://doi.org/10.1590/S1806-92902016000300007

Garcia-Rodriguez, A., J. Arranz, N. Mandaluniz, I. B. de Heredia, R. Ruiz, & I. Goiri. 2015. Production performance and plasma metabolites of dairy ewes in early lactation as affected by chitosan. Span. J. Agric. Res. 13:e06SC04. https://doi.org/10.5424/sjar/2015134-7683

Goiri, I., L. M. Oregui, & A. Garcia-Rodriguez. 2010. Use of chitosans to modulate ruminal fermentation of a 50:50 forage-to-concentrate diet in sheep. J. Anim. Sci. 88:749–755. https://doi.org/10.2527/jas.2009-2377

González, L. A., X. Manteca, S. Calsamiglia, K. S. Schwartzkopf-Genswein, & A. Ferret. 2012. Ruminal acidosis in feedlot cattle: Interplay between feed ingredients, rumen function and feeding behavior (a review). Anim. Feed Sci. Technol. 172:66–79. https://doi.org/10.1016/j.anifeedsci.2011.12.009

Harahap, R. P., S. Suharti, M. Ridla, E. B. Laconi, N. Nahrowi, A. Irawan, M. Kondo, T. Obitsu, & A. Jayanegara. 2022. Meta-analysis of dietary chitosan effects on performance nutrient utilization. Anim. Sci. J. 93:e13676. https://doi.org/10.1111/asj.13676

Haraki, H. M. C., J. R. Gandra, E. R. Oliveira, C. S. Takiya, R. H. T. B. Goes, A. M. A. Gabriel, G. C. G. Rodrigues, E. R. S. Gandra, T. L. Pereira, J. Damiani, & J. D. O. Batisa. 2018. Effects of chitosan and whole raw soybeans on feeding behavior and heat losses of Jersey heifers. Iran. J. Appl. Anim. Sci. 8:397–405.

Huzzey, J. M., J. A. Fregonesi, M. A. G. von Keyserlingk, & D. M. Weary. 2013. Sampling behavior of dairy cattle: Effects of variation in dietary energy density on behavior at the feed bunk. J. Dairy Sci. 96:247–256. https://doi.org/10.3168/jds.2012-5796

Jiménez-Ocampo, R., S. Valencia-Salazar, C. E. Pinzón-Díaz, E. Herrera-Torres, C. F. Aguilar-Pérez, J. Arango, & J. C. Ku-Vera. 2019. The role of chitosan as a possible agent for enteric methane mitigation in ruminants. Animals 9:942. https://doi.org/10.3390/ani9110942

Kirwan, S. F., K. M. Pierce, E. Serra, M. McDonald, G. Rajauria, & T. M. Boland. 2021. Effect of chitosan inclusion and dietary crude protein level on nutrient intake and digestibility, ruminal fermentation, and n excretion in beef heifers offered a grass silage based diet. Animals 11:771. https://doi.org/10.3390/ani11030771

Mertens, D. R. 2002. Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beaker or crucibles: collaborative study. Journal of AOAC International 85:1217-1240. https://doi.org/10.1093/jaoac/85.6.1217

Mingoti, R. D., J. E. Freitas Jr, J. R. Gandra, R. Gardinal, G. D. Calomeni, R. V. Barletta, T. H. A. Vendramini, P. G. Paiva, & F. P. Rennó. 2016. Dose response of chitosan on nutrient digestibility, blood metabolites and lactation performance in holstein dairy cows. Livest. Sci. 187:35–39. https://doi.org/10.1016/j.livsci.2016.02.008

Myers, W. D., P. A. Ludden, V. Nayigihugu, & B. W. Hess. 2004. Technical note: A procedure for the preparation and quantitative analysis of samples for titanium dioxide. J. Anim. Sci. 82:179–183. https://doi.org/10.2527/2004.821179x

Nagaraja, T. G. & E. C. Titgemeyer. 2007. Ruminal acidosis in beef cattle: The current microbiological and nutritional outlook. J. Dairy Sci. 90:E17–E38. https://doi.org/10.3168/jds.2006-478

Owens, F. & S. Soderlund. 2006. Ruminal and Postruminal Starch Digestion by Cattle, in: Cattle Grain Processing Symposium. pp. 116–128. https://doi.org/10.1017/CBO9781107415324.004

Owens, F. N., D. S. Secrist, W. J. Hill, & D. R. Gill. 1998. Acidosis in cattle: A Review. J. Anim. Sci. 76:275–286. https://doi.org/10.2527/1998.761275x

Paiva, P. G. de., E. F. de Jesus, T. A. Del Valle, G. F. de Almeida, A. G. B. V. B. Costa, C. E. C. Consentini, F. Zanferari, C. S. Takiya, I. C. da S. Bueno, & F. P. Renno. 2016. Effects of chitosan on ruminal fermentation, nutrient digestibility, and milk yield and composition of dairy cows. Anim. Prod. Sci. 57:301-307. https://doi.org/10.1071/AN15329

Paula, R. M. de., C. A. Zotti, L. F. D'Abreu, N. R. B. Cônsolo, P. R. Leme, S. da Luz e Silva, & A. S. Netto. 2019. Roughage-free finishing diet based on whole corn grain and a mixture of additives for Nellore heifers. Revista Brasileira Zootecnia 48:e20180004. https://doi.org/10.1590/rbz4820180004

Pereira, F. M., G. G. P. Carvalho, T. S. Magalhães, J. E. F. Júnior, L. F. B. Pinto, G. B. Mourao, A. J. V. Pires, C. E. Eiras, D. Nobais-Eiras, J. A. G. Azevêdo, & A. E. Filho. 2018. Effect of chitosan on production performance of feedlot lambs. J. Agric. Sci. 156:1138–1144. https://doi.org/10.1017/S0021859619000017

Rennó, F. P., N. R. B. Cônsolo, R. V. Barletta, B. Ventureli, R. Gardinal, C. S. Takiya, J. R. Gandra, & A. S. C. Pereira. 2015. Grão de soja cru e inteiro na alimentação de bovinos: Excreção de grão de soja nas fezes. Archivos Zootecnia 64:331–338. https://doi.org/10.21071/az.v64i248.417

Rivaroli, D. C., A. Guerrero, M. V. Valero, F. Zawadzki, C. E. Eiras, M. del M. Campo, C. Sañudo, A. M. Jorge, & I. N. do Prado. 2016. Effect of essential oils on meat and fat qualities of crossbred young bulls fi nished in feedlots. Meat Sci. 121:278–284. https://doi.org/10.1016/j.meatsci.2016.06.017

Seankamsorn, A., A. Cherdthong, S. So, & M. Wanapat. 2021. Influence of chitosan sources on intake, digestibility, rumen fermentation, and milk production in tropical lactating dairy cows. Trop. Anim. Health Prod. 53:241. https://doi.org/10.1007/s11250-021-02697-0

Seankamsorn, A., A. Cherdthong, & M. Wanapat. 2020. Combining crude glycerin with chitosan can manipulate *in vitro* ruminal efficiency and inhibit methane synthesis. Animals 10:37. https://doi.org/10.3390/ani10010037

Senel, S. & S. J. McClure. 2004. Potential applications of chitosan in veterinary medicine Sevda S. Adv. Drug Deliv. Rev. 56:1467–1480. https://doi.org/10.1016/j.addr.2004.02.007

Silvestre, A. M. & D. D. Millen. 2021. The 2019 Brazilian survey on nutritional practices provided by feedlot cattle consulting nutritionists. Revista Brasileira Zootecnia 50:e20200189. https://doi.org/10.37496/rbz5020200189

Tiseo, K., L. Huber, M. Gilbert, T. P. Robinson, & T. P. Van Boeckel. 2020. Global trends in antimicrobial use in food animals from 2017 to 2030. Antibiotics 9:918. https://doi.org/10.3390/antibiotics9120918

Van Boeckel, T. P., C. Brower, M. Gilbert, B. T. Grenfell, S. A. Levin, T. P. Robinson, A. Teillant, & R. Laxminarayan. 2015. Global trends in antimicrobial use in food animals. Proc. Natl. Acad. Sci 112:5649–5654. https://doi.org/10.1073/pnas.1503141112

Van Soest, P. J., J. B. Robertson, & B. A. Lewis. 1991. Methods of dietary fiber, neutral detergent fiber, and nonstarch polysaccha- rides in relation to animal nutrition. J. Dairy Sci. 74:3583–3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2

Vendramini, T. H. A., C. S. Takiya, T. H. Silva, F. Zanferari, M. F. Rentas, J. C. Bertoni, C. E. C. Consentini, R. Gardinal, T. S. Acedo, & F. P. Rennó. 2016. Effects of a blend of essentials oils, chitosan or monensin on nutrient intake and digestibility of lactating dairy cows. Anim. Feed Sci. Technol. 214:12-21. https://doi.org/10.1016/j.anifeedsci.2016.01.015