Degradability, in vitro fermentation parameters, and kinetic degradation of diets with increasing levels of forage and chitosan

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ABSTRACT: Chitosan is the second most important natural biopolymer in the world, extracted from crustaceans, shrimps, and crabs and can modulate rumen fermentation. Our hypothesis is that the addition of chitosan alters the fermentation patterns of different diets for ruminants. This study aimed to evaluate the effects of different levels of chitosan and forage on in vitro dry degradation kinetics and fermentation in a gas production system. The chitosan levels (0, 1625, 3,500, or 7,500 mg/kg of dry matter [**DM**]) were arranged in a completely randomized block design, and for in vitro ruminal fermentation assay, we used a *split splot* arrangement. Into the incubator, all chitosan levels were distributed in the four jars, and the forage levels varying on 100, 65, 50, 35, and 20 on DM basis. There was an interaction effect for chitosan and forage levels ($P \leq 0.05$) on IVDMD; IVOMD. IVDCP and IVDNDF. Chitosan negatively affected IVDMD in all roughage levels evaluated. The pH and ammonia concentration present effect only for roughage levels and incubation hours. The chitosan did not change ($P = 0.3631$) the total short-chain fatty acid concentration (overall mean = 21.19 mmol/L) and the C2:C3 ratio (overall mean = 5.85). The IVDCP showed the same decreasing quadratic behavior ($P < 0.0001$). The increasing chitosan addition increases ($P < 0.0001$) the gas production and decreases ($P < 0.0001$) the lag time (parameter C) of diets with greater concentrate participation, characterizing greater efficiency in the degradability of the diet, confirming its potential use in diets for ruminants. Chitosan changes in vitro dry degradation kinetics and fermentation at the minimum dose of 1,722 mg/kg DM for all diets. The roughage level influenced the in vitro nutrients degradability and cumulative gas production.

Key words: digestibility, ruminal digestion, ruminant nutrition

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INTRODUCTION

Chitosan is a natural polysaccharide derived from the deacetylation of chitin which is a component of the invertebrate exoskeletons (insects, crustaceans, and mollusks) and cell walls of some fungi and algae (Senel et al., 2004). It is a nontoxic, biodegradable biopolymer with great potential for applications in medicine and food preservation and for its antimicrobial property (Shahidi et al., 1999; Jeon et al., 2002) against bacteria, fungi, and yeasts (Sudarshan et al., 1992; Fang et al., 1994). In the last decades, the use of this polymer has grown significantly due to its bioactivity and biocompatibility, and because it is a renewable and biodegradable source, representing great opportunity for the scientific and industrial community (Belanche et al., 2016a). However, it is necessary to know the levels of chitosan to be inserted in the diet of animals (Hirano et al., 1990).

Goiri et al. (2009a) and Li et al. (2013) evaluating chitosan in vitro and indicated its possible use as a modulatory additive to ruminal fermentation in order to improve propionate and altered the feed efficiency of ruminant animals. Chitosan could cause a reduction of the fibrolytic bacteria (predominantly Gram-positive) along with the increase of the amylolytic (predominantly Gram-negative) bacteria and the amylolytic activity, thus supporting the idea that the mode of action of chitosan is based on the electrostatic interaction with the cell wall of bacteria (Sudarshan et al., 1992).

In vitro studies show negative effects of chitosan on the degradability of DM and NDF in forage-based diets (Wencelova et al., 2014), this effect is associated with the adverse effects of chitosan on cellulolytic protozoa, but Belanche et al. (2016b) have demonstrated negative effects on cellulolytic bacteria such as *Fibrobacter*; *Butyrivibrio*, and *Ruminococcus* and *Eubacterium*. The hydrolysis of chitosan by bacterial amylases may favor its use as a source of energy, altering the bacterial population and the final products of the fermentation (Wu, 2011).

Chitosan is a nontoxic and a biodegradable biopolymer, therefore, we hypothesized that addition of chitosan alters the fermentation patterns of different diets for ruminants. The objective of this work is to evaluate the effect of chitosan levels as a modulatory additive of in vitro, fermentation and degradability of nutrients in diets with different roughage levels.

MATERIALS AND METHODS

Ethical Considerations and Study Location

The in vitro degradability experiment (trials 1 and 2) were conducted at the Laboratory for the evaluation of oilseed by-products, at the Center of Research Laboratories in Agroenergy and Environmental Conservation (LAPAC/FINEP) and the chemical analyses were conducted at Laboratory of Animal Nutrition, Faculty of Agrarian Sciences, Federal University of Grande Dourados, Dourados, Mato Grosso do Sul. The total gas production and ruminal fermentation kinetics (trial 3), were conducted in a Laboratory of Applied Nutrition of the Federal University of Mato Grosso do Sul, Faculty of Veterinary Medicine and Animal Science, Campo Grande, Mato Grosso do Sul. These trials were conducted in accordance with the recommendation's the Ethics Committee on Animal Experimentation in this institution, (approval protocol: 023/2015 CEUA/UFGD).

Chitosan and Diets

Chitosan (**CHI**) (> 85% deacetylation, viscosity: 50 cPs at 20°C, LVt 31,11.7% torque) was used from Polymar Company (Ceará State, Brazil), presents a 91.60%DM, 0.27% of ash and 39,41%CP; and added in the concentrations of 0, 1,625, 3,500, or 7,500 mg/kg of DM. Tifton 85 hay (*Cynodon* spp) was used as the only forage, being evaluated five different diets consisting of different levels (100, 65, 50, 35, and 20%); and concentrated (maize, 60%, soybean meal, 35% and mineral mixture, 5%; Table 1). Mineral mixture contained per kg active elements: 120 g Ca, 88 g P, 75 mg I, 1,300 mg Mn, 126 g Na, 15 mg Se, 12 mg Se, 3,630 mg Zn, 55 mg Co, 1,530 mg Cu, and 1,800 mg Fe.

Animals and Rumen Fluid

The ruminal fluid (4.0 L) was collected at morning from two Jersey cattle (350 ± 6.86 kg), provided with ruminal cannula, grazing *Urochloa brizantha* (*syn Brachiaria*), and receiving only mineral supplementation. Collected rumen digesta was processed in a blender and filtered through four layers of cheesecloth into a warm (39 °C) insulated flask, and purged with CO_2.

The buffer solution, consisting of solution A and B, was prepared with the following reagents: Solution A (g L-1) was composed of 10.0 g potassium dihydrogen phosphate (KH_2P0_4); 0.5 g

Table 1. DM, CP, and NDF contents of diets

		CP	NDF[2]
Roughage levels[1]	DM, g/kg as fed	g/kg DM	
100:0	895	97.2	762
80:20	889	126	637
65:35	883	147	544
50:50	878	168	450
35:65	872	189	357
20:80	867	211	263

[1]Roughage:concentrated ratio at 1,000 g/kg DM.

magnesium sulfate ($MgSO_4.H_2O$); 0.5 g Sodium chloride (NaCl); 0.1 g calcium chloride dehydrate ($CaCl_2.2H_2O$); and 0.5 g urea. Solution B (g/100 mL) was composed of 15.0 g sodium carbonate (Na_2CO3) and 1.0 g sodium sulfide ($Na_2S.9H_2O$). The solutions were mixed in the ratio 1: 5 reaching pH 6.8 at the constant temperature of 39 °C (Silva and Queiroz, 2002; Camacho et al., 2019).

In Vitro Degradability Experiment (trial 1)

In vitro degradability was determined according to a methodology described by Tilley and Terry (1963) modified by Holden et al. (1999); using two in vitro incubators (TE-150-Tecnal, São Paulo, Brazil). The treatments evaluated were arranged in a randomized complete block design (three blocks), were each block have two repetition, in subdivided factorial arrangements with roughage levels (Roughage: concentrated – R:C) at 100:0, 65:35, 50:50, 35:65, 20:80, and chitosan were added in the concentrations of 0, 1,625, 3,500, or 7,500 mg/kg of DM, in the jar. Each jar contained a dose of chitosan and all R:C in a total of 22 bags, four replicates for each R:C and two blank bags.

Preparation of the non-woven bags (TNT-100 g/cm^2), 5.0 × 5.0 cm (0.5 g DM) was performed as described by Casali et al. (2008). The jars remained in the artificial rumen for 48 h under continuous agitation. After that period, the fermentative phase was stopped and 40 mL of hydrochloric acid (6N HCl) and eight grams of pepsin (Sigma 1:10,000) were added, as described by Holden (1999).

Samples were predried in a forced-ventilation oven at 55 °C for 72 h. Then, samples of ingredients were ground in a Wiley knife mill with a sieve size of 3 mm. The samples were stored in plastic jars with lids, labeled, and subjected to analyses to determine their dry matter (**DM**; method 967.03), Ash (method 942.05, ignition at 600 °C for 2 h), organic matter (**OM** = 100-ash), crude protein (**CP**, Nx6.25; method 981.10) content according to AOAC (1990). Fractions of neutral detergent fiber (**NDF**) were determined according to Van Soest et al. (1991), using a fiber determiner (TE-149 - Tecnal Piracicaba – Brazil).

In vitro dry matter degradability (**IVDMD**), organic matter (**IVOMD**), neutral detergent fiber (**IVNDFD**), and crude protein (**IVCPD**) were obtained by calculating the difference between the nutrient concentration in the sample before and after incubation. The degradability coefficients (**DC**) were determined from the equation: CD = [P1 – (P2 – B)]/P1 × (100), where: P1 = initial weight of the sample; P2 = Sample weight after in vitro degradability; B = correction of the blank bag.

pH, Ruminal Ammoniacal Nitrogen, and Short-Chain Fatty Acids (trial 2)

To determine ruminal fermentation parameters: pH, ruminal ammoniacal nitrogen ($N\text{-}NH_3$) and short-chain fatty acids (**SCFA**), caps were fitted with a three-way system to allow the collection of buffered rumen fluid and a Büssen valve to release gases produced during fermentation. In each vial was weighed 10 g sample from each diet, together with 1,600 mL buffer solution and 400 mL rumen inoculum. Jars were kept in an environment at 39 °C under continuous stirring for 10 h incubation.

The rumen fluid (40 mL) of collections were performed, in triplicate, shortly after incubation (time "0"), and at the time 2, 4, 6, 8, and 10 h, using a syringe and the three-way tap installed in the cap of each jar. Values of pH were measured immediately after the collection by means of a portable digital pH meter (Instrutherm, pH-1500, São Paulo, Brazil), and for the determination of ammonia nitrogen, a 20-mL aliquot was separated, which was fixed with 1 mL H_2SO_4 1: 1, stored in a glass with polyethylene lid, identified for further analysis. Determination of the levels of NH3-N was carried

out according to the INCT-CA N-007/1 method, described by Detmann et al. (2012).

For the determination of SCFA, another 20 mL aliquot was used, in which the samples were allocated in test tubes and destined to centrifugation for 10 min, 10 °C and 3,000 × *g*. From these samples, 2.0 mL of the supernatant was removed and transferred to other test vials, where 400 µL of formic acid (88%) was added per tube and again centrifuged for 10 min, 10 °C, and 3,000 rpm.

The samples were analyzed in a Gas Chromatograph (Mark SHIMADZU, model GC-2014) with automatic injector model AOC-20i. T Injector = 200 °C; T Column = 80 °C/3 min to 240 °C (20 °C/min); Column = HP INNOwax - 19091N (30 m long, 0.32 mm ID, 0.50 m film); T Detector = 250 °C; Detector = Flame Ionization; Injected volume = 1 µL; Drag gas = Nitrogen; Drag gas flow = 3.18 mL/min; Split rate = 15.

Total Gas Production and Ruminal Fermentation Kinetics (trial 3)

For the determination of the total gas production and the parameters of the kinetics of rumen fermentation, we used chitosan levels according to Dias et al. (2017). An experiment was carried out in a completely randomized design with a factorial arrangement 6 × 5, considering roughage levels (100:0, 80:20, 65:35, 50:50, 35:65, and 20:80), and five doses of chitosan (0, 400, 800, 1,200, and 1,600 mg/kg DM), in triplicates.

The in vitro automatic technique was conducted using the same procedures as described in trial 1; using flasks with a capacity of 250 mL, with addition of 1.0 g of the volumetric sample: concentrate in 100 mL of the buffer solution, 25 mL of ruminal inoculum and purged with CO_2. For each incubation, two flasks were used as blank, containing only ruminal inoculum and buffer solution, in order to adjust the pressure values.

The increase in pressure produced inside the flasks during incubation was measured in pounds per square inch (psi) using the automatic RF - Gas Production System (Ankom Technology Corp., Fairport, NY) system. The gas pressure inside the vials was recorded by pressure sensors located on the bottle caps or modules, which transferred the information from each vial by means of a coordinating base connected to a computer, at 5 min intervals, totaling 216 readings during 48 h incubation.

The data obtained from gas production were measured in psi and transformed to moles of gas by means of the ideal gas equation: n = VP/RT, where n = amount of gas in moles; V = volume of gas occupied in liters; P = pascal pressure (KPa); T = Kelvin temperature (°K); R = gas constant (8.314472 kPa × L × K^{-1} mol^{-1}).

Subsequently, the moles were converted in mL of gas produced (V) under normal conditions of temperature and pressure (**CTP**) using the following equation: V = n RT/P. The following reference values of the CTP conditions were used: 273.15°K (0 °C) and 101 325 Pa (1 atm = 760 mmHg). To calculate the gas production in mL, the corrected pressure of the flasks, the atmospheric pressure of the region (96,538 kPa), and the atmospheric pressure under normal conditions (101,325 kPa) were used, this being the value of P.

In determining the extent and rate of gas production due to food degradation, we used an exponential bicompartmental logistic model proposed by Pell et al. (1994): $y = [A/(1 + Exp.^{[2 + 4 \times B \times (C - T)]}) + D/(1 + Exp.^{[2 + 4 \times E \times (C - T)]})]$, where y = Total volume of gas at time T (extent of degradation); A and D = gas volume (mL) of the rapid degradation fractions (soluble sugars and starch) and slow digestion (cellulose, hemicellulose), respectively; B and E = rates of degradation of the fast and slow digestion fractions (/h), respectively; and C = time of colonization of the bacteria.

Statistical Analysis

The computer program R (R Core Team 2014) was used to analyze the data obtained in the experimental tests. The data related to the in vitro degradability variables of DM, CP, OM, and NDF were adjusted by means of covariance analysis for the incubation effect. After the adjustments, the data were submitted to exploratory analyses to eliminate the existence of outliers and the bases of analysis of variance (linearity, homocessance, and error normality). After the preliminary analysis, analyzes of variance, using $P > 0{,}005$, were performed following the statistical model:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + e_{ij} + \gamma_k + (\alpha\beta)_{ij} + e_{ijk}$$

where i = 1, ..., a; j = 1, ..., b; k = 1 ..., r, where Yijk = variables studied (DM, CP, OM, and NDF); µ = general mean of the response variable, αi = effect of the i^{th} chitosan concentration; βj = effect of j^{th} block (incubation effect); e_{ij} = effect of the error associated with the parcel (ij); γk = effect of the k^{th} roughage level; (αβ) ij = effect of the interaction of the i^{th} chitosan concentration with the k^{th}

roughage level; eijk = effect of the error associated with the subplot (ijk). For the analysis of variance the procedure, we used a psub2.dbc of the ExpDes.pt package (Ferreira et al., 2011); in the computational environment R (R Core Team, 2014). The means were compared by the Tukey test.

Significant interactions (CHI: RC) have been dismembered and isolated effects were evaluated through polynomial regressions models of chitosan within each forage levels, and forage levels within each chitosan level.

Ruminal parameters (pH, N-NH_3, and SCFA), trial 2, were collected for each experimental unit, following a sequence of measurements over time. In the case of this study, the assumption of the use of analysis of variance (**ANOVA**) was verified by means of the Mauchly sphericity test, in which the covariance matrix satisfies the HF condition (nonsignificant sphericity test) subdivided parcel form. Thus, the following statistical model was adopted:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_k + \omega_{l+}(\alpha\omega)_{il} + (\beta\omega)_{jl} + (\alpha\beta\omega)_{ijl} + e_{ijkl}$$

where i = 1, ..., a; j = 1, ..., b; k = 1 ..., ni; 1), where Y_{ijkl} = the ruminal variables studied (pH, N-NH_3, and SCFA); μ = general mean of the response variable; αi = effect of the i^{th} chitosan concentration; ßj = effect of the j^{th} roughage level; (αβ) ij = effect of the interaction of the i^{th} chitosan concentration with the j^{th} roughage level; γk = effect of the error associated with the plots; (ωl) = effect of l^{th} time of collection; (αω)il = effect of the interaction of i^{th} chitosan level with l^{th} of collection time; (βω) jl = effect of the interaction of the j-th roughage level with l^{th} of collection time; (αβω) ijl = effect of triple interaction of the i^{th} chitosan concentration with the j^{th} roughage level, and l^{th} of collection time, eijkl = effect of errors associated with any observation.

The statistic used to test the sphericity of the matrix model was the Mauchly - W test (Mauchly, 1940), as well as the corrections of the number of degrees of freedom, GG—Geisser and Greenhouse (1958) and HF—Huynh and Feldt (1970). The statistics to test the hypothesis of absence of the effects of chitosan levels, volumetric ratio levels:-concentrate, time, and their interactions, for the multivariate case were Lambda de Wilks, Pillai Trait, Lawley-Hotelling Trait, and Larger Root characteristic of Roy. All analyzes were performed using the ANOVA procedure of the computational car package (Fox and Weisberg, 2011), where the parameters i data and i design were used to specify the time factor in the model.

For the trial 3, the kinetic parameters of the ruminal fermentation through the gas production technique, were submitted to preliminary analyzes, followed by the analysis of variance following the statistical model:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ij}$$

where Yijk = variables responses (kinetic parameters of ruminal fermentation), μ = average overall response variable; αi = effect of the i^{th} chitosan concentration; βj = effect of the j^{th} roughage level; (αβ) ij = effect of the interaction i^{th} chitosan concentration with the j^{th} roughage level; eij = error effect associated with the sub-portion (ij). For the analysis of variance was used the procedure of fat2.dic ExpDes.pt package (Ferreira et al. 2011); in the computational environment R (R Core Team, 2014).

RESULTS

There was an interaction between the chitosan and roughage level (CHI × RC; $P < 0.001$); for dry matter (Figure 1a and b) and crude protein (Figure 1c and d) degradability.

There was effect ($P < 0.001$), and a linear decrease in IVDMD (Y = 80.31 - 2.7x, r^2 = 0,81) for chitosan doses and an increase, as the levels of roughage were reduced (Y = 52.91 + 6.88x; r^2 = 0.93). For the forage levels (R:C) 100:0 and 65:35, the maximum degradability of 59.7 and 72.8 was obtained at the chitosan concentration of 1,578 and 1,867 mg/kg of DM, respectively, according to the equations derived from the data.

There was a quadratic function of the DIVCP in the R:C of 50:50, 35:65, and 20:80, with a minimum degradability point of 35.27, 29.45, and 24.75, at the levels of 3,945, 1,933, and 4,415 mg/kg of DMS, respectively, according to the equations derived from the data. At the 100:0 and 65:35 R:C, the degradability averages were statistically equal.

There was no interaction effect, but the effect of the main factors in the IVDMO and IVDNDF was observed, in which the R:C presented a linear increasing effect for IVDMO (P = 0.047; Y = 51.91 + 7.88x; r^2 = 0.92) and quadratic effect for chitosan doses ($P < 0.001$) by IVDNDF (Y = 52.44 + 11.36x – 3.03 x^2; r^2 = 0.97).

pH was effect by time and time × R:C interaction, were both effects rejected by all tests (Table

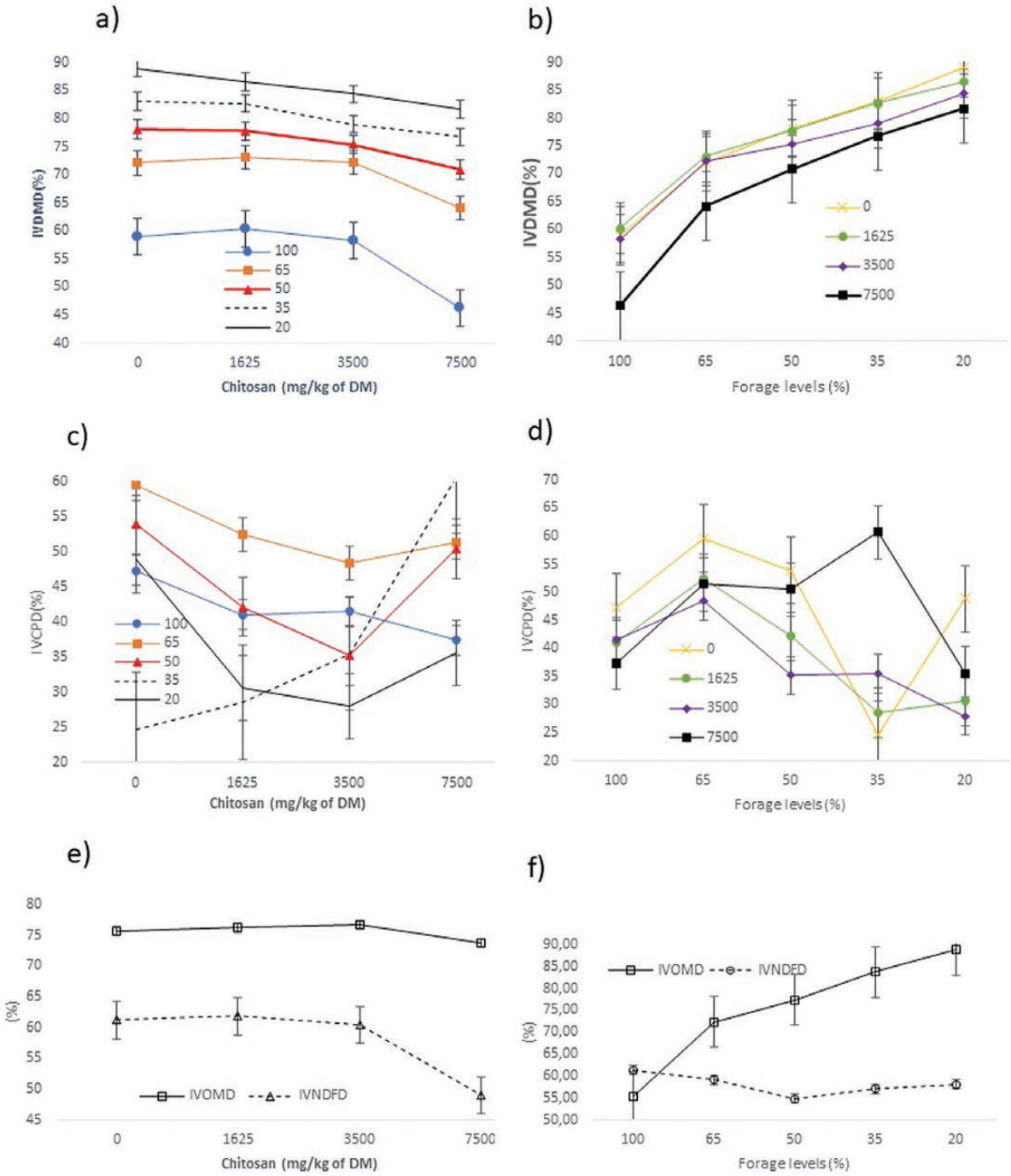

Figure 1. In vitro degradability ($P < 0.0001$) of dry matter (IVDMD – a, b), crude protein (IVCPD c, d), organic matter (IVOMD $P = 0.0047$, e, -□) and neutral detergent fiber (IVNDFD $P < 0.0001$, f, -○) in relation to the chitosan (mg/kg of DM) concentration (0 -x; 1,625 -●; 3,500 -◆; 7,500 -■) and roughage levels (R:C; 100 -●; 65 -■; 50 -▲, 35 --; 20-).

2), and at time × CHI was only rejected by the Roy Maximum Root test; for the parameter N-NH_3, only the hypothesis that tests time was rejected by all tests, with time × R: C and time × R:C × CHI only being rejected by Roy's maximum root. This rejection indicates, by the hypothesis of parallelism, that at least a combination of R: C ratio and time interacts in a dependent way for the parameters.

There was a significant effect of time ($P < 0.0001$) and the forage levels (R: C) ($P = 0.0067$) for ruminal pH data (Table 3). Thus, we compared the means of the R:C within each time. The R:C 65:35 showed pH values closer to neutrality (pH 7), while the 100:0 R:C presented the lowest values range to neutrality.

Significant effect was observed for the time ($P < 0.0001$) and the time × R:C interaction ($P = 0.0010$) for the N-NH_3 data. The concentrations of ruminal ammonia presented a quadratic increase for all R:C ratios (Figure 2).

Table 2. Result of multivariate analysis of the variables pH and N-NH3, considering the completely randomized design and testing the effects of time, roughage levels (R:C), chitosan levels (CHI), and R:C × CHI

Estatistic	Values			
pH	Time	Time × R:C	Time × CHI	Time × R:C × CHI
Lambda de Wilks	0.1128***	0.2968***	0.5772ns[1]	0.3466ns
Pillai trace	0.8872***	0.9881***	0.4821ns	0.9310ns
Hotelling – Lawley trace	7.8624***	1.5325**	0.6310ns	1.2159ns
Roy maximum root	7.8624***	0.7991***	0.3752*	0.4427ns
NH_3-N[2]				
Lambda de Wilks	0.0761***	0.5382ns	0.6592ns	0.3249ns
Pillai trace	0.9238***	0.5337ns	0.3798ns	0.9410ns
Hotelling – Lawley trace	12.1329***	0.7288ns	0.4588ns	1.3722ns
Roy maximum root	12.1329***	0.4944**	0.2386ns	0.6983*

[1]ns: no significance.

[2]ammonia nitrogen in the rumen fluid.

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 3. Mean ruminal pH to roughage levels within each incubation time in rumen

	Time, h						
Roughage levels[1]	0	2	4	6	8	10	SEM
100:0	6.52[b]	6.60[b]	6.60[b]	6.66[b]	6.71[b]	6.74[a]	0.021
65:35	6.81[a]	6.82[a]	6.82[a]	6.89[a]	6.92[a]	6.96[a]	0.025
50:50	6.69[a]	6.75[ab]	6.75[ab]	6.81[ab]	6.83[ab]	6.96[a]	0.035
35:65	6.71[a]	6.72[ab]	6.72[ab]	6.79[ab]	6.88[ab]	6.92[a]	0.054
20:80	6.71[a]	6.75[a]	6.75[a]	6.80[ab]	6.83[ab]	6.85[a]	0.032

[1]Roughage:concentrated ratio at 1,000 g/kg DM.

[a,b]Means followed by equal letters in the columns do not differ by Tukey test at the 5% probability level.

There was a nonsignificant effect of chitosan and roughage levels (R:C) for molar concentrations of SCFA acetic (C2), propionic (C3), butyric, isobutyric, isovaleric, valeric, C2:C3 ratio, and total short fatty acids in mmol/L (Table 4; $P = 0.3631$).

There was a significant effect of chitosan levels ($P < 0.001$) for production of fast gas fraction (parameter A), lag time (parameter C), and a production of slow gas fraction (D) and cumulative gas production (A + D). The R:C levels affect ($P < 0.001$) the parameters A, C, D, E, and cumulative gas production, and the interaction of the factors ($P < 0.001$) presents significance for parameters A, C, D, E, and A + D (Table 5). Chitosan ($P < 0.001$) affects the cumulative gas production for different R:C (100:0; 35:65, and 20:80) and linearly the 50:50 R: C.

DISCUSSION

Diets composed exclusively of roughage tend to have a lower IVDMD with the use of chitosan, compared to diets with a higher proportion of concentrate. Thus, the present study is in agreement with the studies conducted by Wencelová et al. (2014), who found that chitosan tends to decrease IVDMD in high forage diets.

Chitosan has an adverse effect on the rumen cellulolytic population, affecting the microbial ecosystem of the rumen through cellulolytic bacteria for high diets forage (Wencelová et al., 2014). Belanche et al. (2016a) demonstrated the effects of chitosan on cellulolytic bacteria such as *Fibrobacter*; *Butyrivibrio*, and *Ruminococcus* and *Hemicellulolytic* bacteria (*Eubacterium*).

Changes in the composition of the diets, mainly in NDF and CP, as the increasing inclusion of concentrate reduces NDF and increases CP, improving nutrient degradability. The same reduction was also observed in previous studies with chitosan levels (1,625, 3,500, and 7,500 mg/kg DM) and diets with corn silage and diets with different R:C (Goiri et al., 2009a, 2009b).

Chitosan could cause a reduction of the fibrolytic bacteria (predominantly Gram-positive) along with the increase of the amylolytic (predominantly

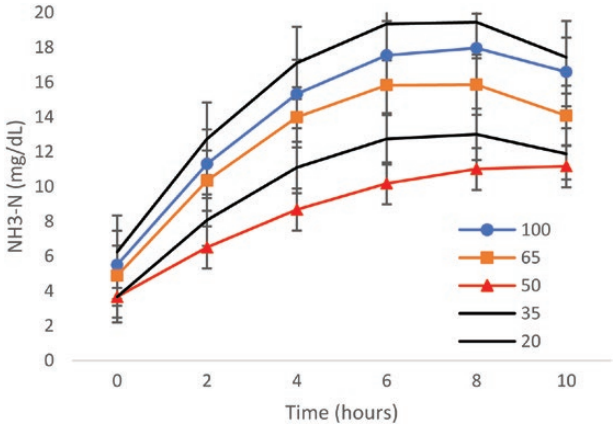

Figure 2. Mean values of N-NH_3 (mg/ L) in vitro of the ruminal liquid (P = 0.0010) of the different roughage levels (R:C) ratios in time. (100:0 -x-) $\hat{y}$ = 5.4957 + 3.3544*T – 0.2245*T2; R^2 = 0.95; (65:35 ---) $\hat{y}$ = 4.8816 + 3.1802*T – 0.2261*T2; R^2 = 0.94; (50:50 ···) $\hat{y}$ = 3.6658 + 1.5866*T – 0.0836*T2; R^2 = 0.95; (35:65 –) $\hat{y}$ = 3.6672 + 2.5464*T – 0.1725*T2; R^2 = 0.93; and (20:80 -■-) 5$\hat{y}$ = 6.2453 + 3.7786*T – 0.2660*T2; R^2 = 0.83.

Gram-negative) bacteria and the amylolytic activity, thus supporting the idea that the mode of action of chitosan is based on the electrostatic interaction with the cell wall of bacteria (Sudarshan et al., 1992). Alternatively, the potential for hydrolysis of chitosan by amylases (Wu, 2011) could also promote proliferation of these bacteria capable of using chitosan as an energy source (e.g., amylolytic bacteria) leading to changes in the structure of the bacterial community and the products of fermentation. Belanche et al. (2016a) demonstrated these effects are correlated with the total of bacteria and can be compensated by the low abundance of cellulolytic bacteria. Thus, it was observed a more evident action in the IVDMD of diets containing a greater proportion of concentrate, when compared with diets containing only roughage.

The inclusion of chitosan leads to a reduction in the IVCPD regardless of the use of concentrate, however, with the maximum dose of 7,500 mg/ kg DM of chitosan, the IVCPD tends to increase. The inclusion of chitosan had no effect on N-NH_3; however, Belanche et al. (2016b) pointed out that chitosan increases ammonia concentration 2 h after feeding. However, degradation of the amine group (R-NH_2) in ammonia may explain the higher concentrations of ammonia (Beier and Bertilsson, 2013) in diets with chitosan. Belanche et al. (2016b) emphasized that the extra supply of N provided by chitosan deamination and low ammonia retention by ruminal microorganisms led to higher rumen ammonia peaks, rather than increasing proteolysis of feed.

The structure of chitosan also undergoes losses of nitrogen (amide and amino) groups, which may be indicative of its possible use as non-protein nitrogen for protein synthesis (Fadel El-Seed et al., 2003). Goiri et al. (2010) identified the reduction in NH_3-N concentrations by the inclusion of chitosan (136 mg/kg of BW of CHI). The fact that NH_3-N decrease may be indicative of a lower rate of deamination of the CP of the diet by the ruminal microbiota and, consequently, a greater flow of amino acids to the small intestine and better utilization of nitrogen by the tissues (Schelling, 1984). The impact of lower ruminal ammonia rates may cause changes in the population of cellulolytic bacteria, providing negative effects on diets with a higher proportion of forages, which is in line with what occurred in this work.

However, it is important to note that this reduction in ammonia concentration the reduction of amino acid degradation, due to the microbial properties associated with chitosan, or an increase in the use of microbial protein synthesis.

According to Kong et al. (2010), when the pH is below the pka of chitosan, the electrostatic interaction between the polycationic and anionic structures of the surface components of the microorganisms plays an important role in the antibacterial activity. This is because the chitosan molecules become polycationic at a pH below the pka of the molecules (range 6.3 at 6.5) according to Lim and Hudson (2004).

In contrast, chelating and hydrophobic effects are responsible for the antibacterial activity of chitosan when the pH of the environment is above the pka of the molecules (Kong et al., 2010). In the ruminal environment with pH close to neutrality, the -NH_2+ groups of chitosan may interact electrostatically with the negative charge of the carboxyl group of AA, protecting against ruminal degradation (Chiang et al., 2009). In the present study, it was observed that pH values remained high (average 6.7), values igher than the minimum limit of 6.2 proposed by Russell and Wilson (1996), so that the maximum activity, as well as microbial growth, ruminal fermentation and degradation of NDF.

In this study, chitosan did not alter the concentrations of SCFA, different from diets. Some studies with chitosan have shown results that vary from no effect on ruminal parameters and increases in propionate concentrations (Goiri et al., 2010; Araújo et al., 2015, Paiva et al., 2016). Dias et al. (2017) observed that chitosan caused a linear increase in propionate concentrations, for cattle supplemented (0.3%BW), at pasture. Belanche et al.

Table 4. Means and SEM of the kinetic parameters of ruminal fermentation of diets with chitosan concentration of the diets, roughage levels (R:C), and interaction between the two factors (Chitosan × R:C)

	Chitosan levels mg/kg of DM					*P*-value[2]		
R:C[1]	0	1,635	3,500	7,500	SEM	CHIT	R:C	CH×R:C
	Acetic, mmol/L							
100:0	13.85	14.11	14.13	14.08	0.010	0.3326	0.3868	0.7989
65:35	15.15	15.00	14.98	15.05	1.575			
50:50	15.32	14.82	34.66	14.80	4.797			
35:65	14.56	15.85	14.34	15.19	1.502			
20:80	15.64	15.24	15.68	15.89	2,595			
	Propionic, mmol/L							
100:0	2.70	2.59	2.46	2.57	0.055	0.1519	0.0981	0.9693
65:35	2.90	2.94	2.92	2.92	0.035			
50:50	2.97	2.91	2.86	2.78	0.075			
35:65	2.67	2.67	2.69	2.81	0.050			
20:80	2.84	2.59	2.68	2.62	0.067			
	Butyric, mmol/L							
100:0	1.96	1.90	1.82	1.91	0.895	0.7989	0.0793	0.8911
65:35	1.87	1.96	1.97	1.86	1.012			
50:50	1.90	1.95	1.95	1.82	0.622			
35:65	1.76	1.82	1.86	2.00	3.155			
20:80	2.10	2.10	2.05	2.04	1.600			
	Isobutyric, mmol/L							
100:0	0.17	0.19	0.18	0.17	0.505	0.7225	0.0504	0.8900
65:35	0.24	0.28	0.26	0.26	0.855			
50:50	0.26	0.25	0.27	0.21	0.722			
35:65	0.18	0.18	0.20	0.20	3.737			
20:80	0.26	0.26	0.24	0.22	6.782			
	Isovaleric, mmol/L							
100:0	0.21	0.20	0.18	0.20	0.010	0.2990	0.1099	0.4017
65:35	0.24	0.25	0.26	0.24	0.010			
50:50	0.22	0.23	0.22	0.22	0.025			
35:65	0.19	0.20	0.22	0.37	0.010			
20:80	0.26	0.28	0.27	0.27	0.011			
	Valeric, mmol/L							
100:0	0.13	0.13	0.15	0.12	0.400	0.6010	0.1902	0.8407
65:35	0.11	0.11	0.12	0.11	0.832			
50:50	0.09	0.09	0.11	0.10	1.750			
35:65	0.11	0.12	0.11	0.12	0.530			
20:80	0.13	0.14	0.13	0.13	1.685			
	Total, mmol/L							
100:0	19.04	19.12	18.91	19.11	0.400	0.3631	0.3526	0.3632
65:35	20.53	20.61	20.53	20.37	0.833			
50:50	20.78	20.24	20.24	39.80	1.750			
35:65	19.50	20.12	19.44	21.37	0.531			
20:80	21.25	21.25	20.61	20.96	1.699			
	C2:C3							
100:0	5.19	5.49	5.78	5.54	0.255	0.3294	0.4701	0.4083
65:35	5.25	5.15	5.18	5.19	0.835			
50:50	5.15	5.07	5.19	5.32	1.750			
35:65	5.54	6.26	5.55	5.91	0.212			
20:80	5.49	6.24	5.69	5.97	1.685			

[1]Roughage: concentrated ratio at 1,000 g/kg DM.

[2]Significance at $P < 0.05$.

Table 5. Means and standard error of the mean (± SEM) of the kinetic parameters of ruminal fermentation of diets with chitosan concentration of the diets, roughage levels (R:C), and interaction between the two factors (Chitosan × R:C)

	Chitosan concentration, mg/kg of DM						*P*-value[2]		
R:C[1]	0	400	800	1,200	1,600	SEM	CHI	R:C	CH × R:C
	Parameter A—Production of fast gas fraction, mL/100 mg of DM								
100:0	1.06	0.54	0.34	1.42	2.45	0.048	<0.001	<0.001	<0.001
80:20	0.78	0.61	0.33	2.17	0.65	0.206			
65:35	0.33	4.16	1.34	2.03	0.74	1.278			
50:50	2.76	7.61	1.83	2.08	1.70	4.002			
35:65	2.64	1.97	1.53	1.621	1.97	1.196			
20:80	4.33	5.12	0.64	2.78	5.84	2.480			
	Parameter B—Production rate of fraction A, h								
100:0	0.71	0.66	0.63	0.66	0.39	0.052	0.130	0.197	0.750
80:20	0.74	0.99	0.76	0.54	0.98	0.030			
65:35	0.65	0.57	0.87	0.51	0.67	0,058			
50:50	0.49	0.66	0.71	0.62	0.58	0.076			
35:65	0.70	0.72	0.82	0.53	0.87	0.042			
20:80	0.68	0.46	0.84	0.42	0.61	0.070			
	Parameter C – *Lag time*, h								
100:0	5.04	4.25	9.66	4.82	5.81	0,926	<0.001	<0.001	<0.001
80:20	4.52	7.44	11.7	6.53	7.34	0.376			
65:35	8.01	0.86	6.44	4.08	2.14	2.023			
50:50	10.7	0.75	0.63	4.11	9.66	1.174			
35:65	3.51	3.61	6.02	6.19	0.57	2.543			
20:80	5.22	0.34	9.65	4.59	2.63	1.762			
	Parameter D—Production of slow gas fraction, mL/100 mg of DM								
100:0	5.51	3.76	4.08	7.52	7.37	0.408	<0.001	<0.001	<0.001
80:20	4.92	6.71	5.93	5.69	8.88	2.260			
65:35	4.04	4.62	2.53	3.84	5.74	0.692			
50:50	5.41	1.51	2.01	4.07	2.48	1.744			
35:65	3.77	6.81	7.13	9.35	6.5	2.996			
20:80	6.03	3.72	8.38	4.32	11.3	4.207			
	Parameter E—Production rate of fraction D, h								
100:0	0.08	0.04	0.06	0.06	0.04	0.010	0.54	0.013	0.002
80:20	0.05	0.02	0.02	0.05	0.02	0.010			
65:35	0.04	0.13	0.05	0.11	0.09	0.012			
50:50	0.03	0.06	0.18	0.04	0.02	0.016			
35:65	0.09	0.03	0.02	0.04	0.04	0.010			
20:80	0.02	0.05	0.02	0.07	0.02	0.012			
	Cumulative (A + D) gas production, mL/100 mg of DM								
100:0	6.56	4.31	4.42	8.94	9.82	0.322	<0.001	<0.001	<0.001
80:20	5.71	7.32	6.26	7.86	9.53	1.706			
65:35	4.38	8.76	3.88	5.87	6.48	1.402			
50:50	8.17	9.10	3.83	6.15	4.17	0.760			
35:65	6.41	8.77	8.66	10.9	8.47	2.950			
20:80	10.36	8.81	9.02	7.11	17.25	1.978			

[1]Roughage:concentrated ratio at 1,000 g/kg DM.

[2]Significance at $P < 0.05$.

(2016a) in a dose–response assessment of chitosan in culture reported a quadratic effect of chitosan on propionate concentrations, where the highest value was observed when the 2 g/L dose of chitosan was added. This effect was further confirmed in a study using the Rusitec system, where the addition of chitosan increased the propionate concentration to 36.8% (Belanche et al., 2016b).

From the observed results, it was verified that diets containing more forage present a greater production of gas of the slow fraction (parameter D), in a time (parameter C-lag time) greater of colonization and degradation by the microorganisms, showing a low rate of gas production per hour, characteristic behavior of more fibrous feeds.

In diets with higher concentrate proportions, the degradability of nutrients increases, increasing the fast fraction gas production (parameter A), with the shortest fermentation time, characteristic of more soluble diets. With the increasing addition of chitosan, the gas production increases and the fermentation time decreases, resulting in a greater efficiency of the degradability of the diet.

In the cumulative production (A + D) of gases, the diets containing the highest amount of concentrate combined with chitosan showed higher yields compared to diets with high volume, confirming the efficiency of chitosan use in more energetic diets.

CONCLUSION

Chitosan altered the in vitro ruminal degradability and increased the total cumulative gas production of the diets. Chitosan changes the most efficiently evaluated parameters at the minimum dose of 1,722 mg/kg DM for all diets. The roughage level influenced the in vitro nutrients degradability and cumulative gas production.

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