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Chitosan combined with technical cashew nut shell liquid improves *in vitro* ruminal parameters and gas production kinetics

Elis Regina de Queiroz Vieira[1], Rafael Henrique de Tonissi e Buschinelli de Goes[2]*, Tatiana Garcia Diaz[3], Milene Puntel Osmari[4], Leonardo de Oliveira Seno[2], Luís Carlos Vinhas Ítavo[5], Jefferson Rodrigues Gandra[6], Douglas Gabriel Anschau[2], Raquel Tenório de Oliveira[2], Nayara Gonçalves da Silva[2], Amanna Gonzaga Jacaúna[7]

[1] Universidade Federal do Tocantins, Escola de Medicina Veterinária e Zootecnia, Araguaína, TO, Brasil.
[2] Universidade Federal da Grande Dourados, Faculdade de Ciências Agrárias, Dourados, MS, Brasil.
[3] Universidade Estadual de Maringá, Departamento de Zootecnia, Maringá, PR, Brasil.
[4] Universidade Federal de Santa Catarina, Departamento de Zootecnia e Desenvolvimento Rural, Florianópolis, SC, Brasil.
[5] Universidade Federal de Mato Grosso do Sul, Faculdade de Medicina Veterinária e Zootecnia, Campo Grande, MS, Brasil.
[6] Universidade Federal do Sul e Sudeste do Pará, Instituto de Estudos em Desenvolvimento Agrário e Regional, Marabá, PA, Brasil.
[7] Universidade Estadual Paulista "Júlio de Mesquita Filho", Faculdade de Medicina Veterinária e Zootecnia, Botucatu, SP, Brasil.

***Corresponding author:**
rafaelgoes@ufgd.edu.br

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ABSTRACT - The objective was to evaluate the inclusion of chitosan (CHI) and technical cashew nut shell liquid (CNSLt) as natural feed additives in cattle diets on nutrient digestibility, ruminal fermentation, and *in vitro* gas production kinetics. We conducted a completely randomized design with 5×4 factorial arrangement, with 20, 35, 50, 65, and 100% Tifton 85 hay and four additives, monensin (200 mg/kg DM), CNSLt (500 mg/kg DM), CHI (500 mg/kg DM), and CNSLt+CHI (500 mg/kg DM/each). Dry matter (DM) and organic matter (OM) digestibility showed a linear reduction according to forage levels. The highest DM digestibility was observed with CHI on cattle diets. Inclusion of CHI increased DM digestibility. The highest *in vitro* organic matter and crude protein (CP) digestibilities were observed for CNSLt+CHI. The *in vitro* dry matter digestibility increased linearly with concentrate in the diet. There was interaction of forage:concentrate ratio and the additives for neutral detergent fiber, acid detergent fiber and hemicellulose digestibility. Chitosan, CNSLt, and CNSLt+CHI promoted the lowest acetate:propionate ratio compared with monensin. Total gas production showed interaction of the forage:concentrate ratio and additives. Lag time was lowest with CNSLt+CHI. Chitosan and CNSLt can be considered alternative fermentation modulators to ionophores by improving nutrient digestibility and increasing ruminal propionate concentrations.

Keywords: gas production, natural feed additives, rumen fermentation, short-chain fatty acids

1. Introduction

The use of additives in cattle nutrition is becoming increasingly important because they are substances with the ability to enhance animal performance and improve rumen function, which reflect on the use of dietary nutrients. Besides, they can reduce energy losses resulting from excess methane emissions

(Belanche et al., 2016a). Ionophore antibiotics are commonly used in ruminant production to improve animal performance and decrease energy and protein losses. However, researchers have been working in search of natural additives that, besides improving production efficiency, promote animal health without leaving residues in the carcass.

Plant extracts and natural compounds have been the focus of studies as alternatives to ionophores because they have antimicrobial properties and could, therefore, be used to manipulate the rumen microbial ecosystem (Belanche et al., 2016b). Among these products, chitosan (CHI) and technical cashew nut shell liquid (CNSLt) have been investigated for antimicrobial properties (Pedro et al., 2020; Konda et al., 2019).

Chitosan is the most important derivative of chitin, the second most important natural biopolymer in the world, extracted from crustaceans, shrimps, and crabs (Pedro et al., 2020). It has been shown to have the ability to decrease methane emission by up to 42% (*in vitro* study; Belanche et al., 2016a; Harahap et al., 2020), increase propionate concentration (Dias et al., 2020; Dias et al., 2017), and act as a ruminal fermentation modulator (Goiri et al., 2009).

Technical cashew nut shell liquid is a functional oil obtained from processing cashew nut (*Anacardium occidentale* L), considered a natural source of phenolic lipids such as anacardic acid, cardol, and cardanol (Konda et al., 2019). Among the effects found in the literature with the addition of CNSLt to cattle diets, changes in the bacterial species of the rumen stand out, inhibiting growth of Gram-positive bacteria, favoring increased propionate production and reduction of acetic acid, lactic acid, and methane concentrations (Branco et al., 2015); and affecting the metabolic hydrogen flow (Mitsumori et al., 2014).

Chitosan and CNSLt are non-toxic and biodegradable biopolymers; therefore, we hypothesized that addition of CHI and CNSLt alters the fermentation patterns of different diets for ruminants. Thus, the present experiment aimed to evaluate the effects of the inclusion of CHI and CNSLt as natural feed additives in cattle diets on digestibility, ruminal fermentation, and *in vitro* gas production kinetics.

2. Material and Methods

The experiments were conducted in Dourados, Mato Grosso do Sul State, Brazil (latitude 22°14' S and longitude 54°49' W); according to the recommendations of the Ethics Committee on Animal Experimentation Guide (approval protocol: 023/2015).

2.1. Experimental design, and treatments

The experimental design was a 5×4 completely randomized factorial, with five forage:concentrate ratios (20, 35, 50, 65 and 100% of Tifton 85 hay) and four additives, monensin (MON, 200 mg/kg DM), CNSLt (500 mg/kg DM), CHI (500 mg/kg DM), and CNSLt+CHI (500 mg/kg DM/each), totaling 20 treatments.

Chitosan deacetylation increases its solubility and presumably its activity (Rhoades and Roller, 2000). We used CHI with deacetylation degree> 86.30%, viscosity <200 cPs, pH 7.9, 1.35% ashes, and 0.32 g/mL apparent density (Polymar Indústria e Comércio Importação e Exportação LTDA, Fortaleza, state of Ceará, Brazil). The CNSLt was provided by Usibras Company (Aquiraz, state of Ceará, Brazil) and contained 10.03 mg/g anacardic acid, 540.77 mg/g cardanol, 102.34 mg/g cardol, and 19.17 mg/g 2-methylcardol. Chemical analysis of CNSLt was performed by High-Performance Liquid Chromatograph (Varian 210 model), Diode Arrangement Detector (DAD), and software Star WS (workstation 2.0). The column used was C18 reverse phase (25 cm × 4.6 mm × 5 µm) (Phenomenex). Elution was performed using acetonitrile/water/acetic acid gradient system (66/33/2 v:v:v) (A) and tetrahydrofuran (B), which started elution with 10% B and in 40 minutes reached 100% B. The pump flow rate was 1 mL/min and the injected volume was 20 µL. The analysis was conducted at 22 °C, both in the preparation of the analytical curve and in the product analysis, and injections were performed in triplicate. The product was solubilized in acetonitrile/water (66/35 v:v) providing a final concentration of 1000 µg/mL. The

external standard curves employed to quantify anacardic acid, cardanol, 2-methylcardol, and cardol in the CNSLt product were prepared employing compounds of 97% purity at concentrations 10-100 µg/mL. Results were expressed in mg/g sample obtained from an external standardization curve with a correlation coefficient of 0.9992 for all compounds analyzed.

Experimental diets consisted of Tifton 85 hay (*Cynodon* spp.) as forage, and corn, soybean meal, and mineral supplement as concentrate ingredients. Percentages for feed formulation and chemical composition are listed in Table 1.

Table 1 - Chemical composition of experimental feed (g/kg DM)

Item	Forage level (%)				
	100	65	50	35	20
Ingredients					
Tifton 85 (*Cynodon* spp.)	1000.0	650.0	500.0	350.0	200.0
Ground corn	0.0	174.0	248.6	323.2	397.8
Soybean meal	0.0	140.2	200.3	260.3	320.4
Mineral mix[1]	0.0	35.8	51.2	66.5	81.8
Chemical composition					
Dry matter	899.0	883.0	866.0	848.0	845.0
Mineral matter	101.0	983.0	975.0	966.0	956.0
Crude protein	129.0	163.0	178.0	193.0	207.0
Neutral detergent fiber	762.0	535.0	438.0	342.0	244.0
Acid detergent fiber	306.0	229.0	197.0	164.0	132.0

[1] Mineral mix: product of minerals contained per kg: 120 g Ca, 88 g P, 75 mg I, 1300 mg Mn, 126 g Na, 15 mg Se, 12 mg Se, 3630 mg Zn, 55 mg Co, 1530 mg Cu, and 1800 mg Fe.

2.2. Preparation of ruminal inoculum and artificial saliva

Two castrated male Holstein cattle, with a mean body weight of 380 kg±4 kg and with a permanent ruminal cannula, were used as donors for collection of the ruminal inoculum. Animals were fed twice a day, at 08:00 and 16:00 h, with a basal diet containing Tifton 85 hay (*Cynodon* sp.) and mineral supplementation. Ruminal fluid was collected in the morning before the first meal with a ruminal cannula, using a vacuum pump and a vacuum flask with a capacity of 2,000 mL. Ruminal fluid was kept in water bath at 39 °C, and the container purged with CO_2 before and after collection. Extracts were filtered through four layers of cotton cloth and used in the incubations.

A buffer solution, consisting of solutions A and B, was prepared with the following reagents: solution A (g/L) composed of 10.0 g potassium dihydrogen phosphate (KH_2P0_4), 0.5 g magnesium sulfate ($MgSO_4.H_2O$), 0.5 g sodium chloride (NaCl), 0.1 g calcium chloride dihydrate ($CaCl_2.2H_2O$), and 0.5 g urea. Solution B (g/100 mL) was composed of 15.0 g sodium carbonate (Na_2CO_3) and 1.0 g sodium sulfide ($Na_2S.9H_2O$). Solutions were mixed in the ratio of 1:5 reaching pH 6.8 at constant temperature of 39 °C (Camacho et al., 2019).

2.3. *In vitro* digestibility

The *in vitro* digestibility of dry matter (IVDMD), organic matter (IVOMD), crude protein (IVCPD), neutral detergent fiber (IVNDFD), acid detergent fiber (IVADFD), and hemicellulose (IVHCELD) of diets was determined according to the methodology described by Tilley and Terry (1963) and modified by Holden et al. (1999), using two artificial rumens (Tecnal®, Piracicaba, Brazil), in a completely randomized block design (four blocks and two repetitions (jars) per block).

Samples were weighed (0.5 g) and placed inside 5.0 × 5.0 cm TNT bags (100 g/cm^2), according to Casali et al. (2009). Bags with samples were uniformly distributed among the jars of the artificial rumen (four jars/artificial rumen - totaling eight jar), with 22 bags/jar (20 bags with samples, two blank bags). Blank bags (without sample) were used to correct the data. Each jar received one additive, and five forage:concentrate ratios (two jar/additive). Then, 1,600 mL buffer solution and 400 mL rumen inoculum were added. The jars remained in the artificial rumen TE-150 (Tecnal®) at 39 °C for 48 h under continuous stirring.

Incubation was stopped after 48 h, and the second stage of the *in vitro* method was initiated by adding 40 mL 6 N hydrochloric acid (HCl) and 8 g pepsin (Sigma 1: 10.000) to each jar. Incubation was continued for another 24 h at 39 °C under continuous stirring. After 24 h incubation, jars were drained and rinsed, the bags were pre-dried in a forced-air oven at 55 °C for 12 h, at 105 °C oven for additional 24 h, and finally weighed. The IVDMD was calculated using the weight of the residue after incubation. Nutrient digestibility was calculated by the difference between the concentration of the nutrient in the sample before and after incubation.

2.4. Ammonia, pH, and volatile fatty acids (VFA) in the artificial rumen

To determine ammonia, pH, and VFA *in vitro*, 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, 5 g sample from each diet was weighed, in duplicate, together with 1,600 mL buffer solution and 400 mL rumen inoculum.

Jars were kept under continuous stirring at 39 °C for 10 h incubation. Thirty milliliters of rumen fluid were collected at 2-h intervals for 8 h, using a syringe and the three-way tap installed in the cap of each jar for pH and ammonia analysis (Diaz et al., 2018). At times 0, 2, 4, 8 h after the beginning of incubation, a sample was taken to determine VFA. The pH was measured immediately after each collection in 10 mL rumen fluid, using a digital potentiometer Digimed DM20. For ammonia determination, 10 mL rumen fluid was acidified with 1 mL sulfuric acid (H_2SO_4 50%) to stop the microbial activity and prevent loss of ammonia from the ruminal fluid, and 10 mL rumen fluid for VFA analysis. The collected material was stored at −20 °C for further analysis.

2.5. Rumen fermentation kinetics

The automated *in vitro* gas production technique was used to determine the rumen fermentation kinetics parameters. Samples with 0.5 g of each diet were weighed in duplicate in glass vials, with a capacity of 250 mL. Each flask was added with 100 mL buffer solution, 25 mL rumen inoculum, and CO_2. For each incubation, two flasks were used as blank, containing only rumen inoculum and buffer solution, to adjust the pressure values. Flasks remained at 39 °C under constant agitation. Pressure values were measured using the automated system RF: Gas Production System (ANKOM®). Gas pressure values were recorded in pounds per square inch (psi), through pressure sensors on the bottle caps (modules), which sent the information from each vial to the coordinating base connected to a computer. Readings were recorded at 5-min intervals for 24-h incubation.

Gas pressure data were transformed into moles of gas through the ideal gas equation. Subsequently, data in moles were converted into mL of gas produced under standard conditions of temperature and pressure (STP) using 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). The logistic bicompartmental model proposed by Schofield et al. (1994) was used to determine the kinetic parameters of rumen fermentation.

2.6. Chemical analysis

Feed samples were pre-dried in a forced-air oven at 55 °C for 72 h and ground individually in a Wiley mill equipped with a 1-mm screen. Subsequently, samples were analyzed for DM (#934.01; 105 °C

for 16 h), ash (#942.05; ignition at 600 °C for 2 h), organic matter (100-ash), CP (#984.13; N×6.25), and ether extract (EE; #920.39), according to the techniques described by AOAC (2000). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) was determined according to Van Soest et al. (1991), using a TECNAL® TE-149 fiber analyzer (Piracicaba, SP, Brazil) and Hemicellulose (HCEL = FDN − FDA). In the determination of NDF, heat-stable α-amylase was used, and no sodium sulfite addition was added. Determination of the ammonia content in the rumen liquid was performed according to the INCT-Animal Science method and described by Detmann et al. (2012). To determine the molar concentrations of VFA in rumen fluid, the samples were centrifuged at 30.000 × *g* for 20 min at 4 °C and analyzed by gas chromatograph (SHIMADZU, model GC-2014) equipped with an automatic injector (model AOC-20); the injector temperature was 200 °C, and the column temperature was raised at a rate of 80°C/3 min to 240 °C. The column used was HP INNOwax - 19091N (30 m long, 0.32 mm ID, 0.50 µm film), and the detector was flame ionization.

2.7. Statistical analysis

Data analyses were run in SAS program (Statistical Analysis System, version 9.2). Data were subjected to preliminary exploratory analyses to check for normality and outliers. Data for IVDMD, IVNDFD, IVADFD, IVCPD, and IVHCELD were adjusted by analysis of covariance for the effect of incubation. After adjustment, data were subjected to exploratory analyses to remove outliers and the bases of analysis of variance (linearity, homoscedasticity, and error normality). Subsequently, analyses of variance were run following the statistical model:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + e_{ij} + \gamma_k + (\alpha\beta)_{ij} + e_{ijk}, \quad (1)$$

in which i = 1, ..., a; j = 1, ..., b; k = 1 ..., r; wherein Y_{ijk} = variables studied (DM, CP, OM, and NDF), μ = overall mean of the response variable, α_i = effect of i-th additive concentration, β_j = effect of j-th block (incubation effect), e_{ij} = effect of the error associated with the plot (ij), γ_k = effect of k-th forage level, $(\alpha\beta)_{ij}$ = effect of the interaction of i-th additive concentration with the k-th forage level, and e_{ijk} = error effect associated with the subplot (ijk).

Ruminal parameters (pH, N-NH_3, and VFA) were collected from each experimental unit, following a sequence of measurements over time. Thus, the following statistical model was adopted:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_k + \omega_l + (\alpha\omega)_{il} + (\beta\omega)_{jl} + (\alpha\beta\omega)_{ijl} + e_{ijkl}, \quad (2)$$

in which i = 1, ..., a; j = 1, ..., b; k = 1 ..., ni; wherein Y_{ijkl} = ruminal variables studied (pH, N-NH_3, and VFA); μ = overall mean of the response variable; α_i = effect of i-th additive concentration; β_j = effect of j-th forage level; $(\alpha\beta)_{ij}$ = effect of the interaction of the i-th additives concentration with the j-th forage level; γ_k = effect of the error associated with the plots; ω_l = effect of l-th time of collection; $(\alpha\omega)_{il}$ = effect of the interaction of i-th additive level with l-th collection time; $(\beta\omega)_{jl}$ = effect of the interaction of j-th forage level with l-th collection time; $(\alpha\beta\omega)_{ijl}$ = effect of triple interaction of i-th additive concentration with j-th forage level and l-th collection time, and e_{ijkl} = effect of errors associated with any observation.

Mauchly's Test of Sphericity (1940) was applied to test the sphericity of the matrix model, as well as the correction 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 no effects of additives, forage level:concentrate ratio, time, and their interactions, for the multivariate case were Wilks Lambda, Pillai Trace, Lawley-Hotelling Trace, and Roy's Largest Root. All analyses described above were performed using the REPEATED command included in the SAS PROC GLM.

Data for VFA (acetate, propionate, butyrate, and C2:C3 ratio) were subjected to MIXED procedure, considering repeated measurement effect by REPEATED procedure, indicating the combination of additive effects and forage:concentrate ratio (id) as subject (via the SUBJECT = id command). The restricted maximum likelihood method was used for estimating the variance components. The better time-series covariance structures were selected based on the lowest Akaike and Bayesian information criteria. Time-series covariance structures were modeled using the options of unstructured order (UN).

Kinetic parameters of ruminal fermentation obtained by the gas production technique were subjected to preliminary analyses, followed by the analysis of variance following the statistical model:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ij}, \quad (3)$$

in which Y_{ijk} = response variables (kinetic parameters of ruminal fermentation), μ = overall mean of the response variable, α_i = effect of *i*-th additive concentration, β_j = effect of *j*-th forage level, $(\alpha\beta)_{ij}$ = effect of the interaction *i*-th additive concentration with *j*-th forage level, and e_{ij} = error effect associated with the sub-plot (*ij*).

The fit of the curves and parameter estimates of biological interest used iterative Gauss-Newton processes through the procedure for non-linear models (PROC NLIN) of SAS software. Then, data were subjected to PROC GLM. The effects were considered significant at α = 0.05.

3. Results

Inclusion of CHI in diets increased IVDMD (P<0.0001) compared with the other additives evaluated. The IVOMD increased (P = 0.0024) with the inclusion of CHI and the combination CNSLt+CHI. The highest IVCPD (P = 0.0024) was observed in diets with CNSLt+CHI. The lowest IVDMD, IVOMD, and IVCPD were found with the inclusion of CNSLt. The IVDMD ($\hat{Y}$ = 0.893552 − 0.00422977x; R^2 = 0.98) and IVOMD ($\hat{Y}$ = 0.825427 − 0.004159x; R^2 = 0.99) increased linearly (P<0.001) with the inclusion of concentrate in the diet. There was a quadratic effect (P<0.001) of the concentrate in the diet on IVCPD ($\hat{Y}$ = 0.589255 + 0.00647997x − 0.00006240x^2; R^2 = 0.94), being estimated the highest IVCPD with 48.1% concentrate (Table 2). There was effect for inclusion of concentrate (Table 3) in the diet (P<0.0001), presenting a quadratic effect for IVNDFD ($\hat{Y}$ = 0.3037 + 0.00037x − 0.00003x^2; R^2 = 0.33), IVADFD ($\hat{Y}$ = 0.1073 + 0.0049x − 0.00004x^2; R^2 = 0.48), and IVHCELD ($\hat{Y}$ = 0.0269 + 0.00126x − 0.00009x^2; R^2 = 0.98).

Diurnal changes in the *in vitro* fermentation parameters with the inclusion of additives in diets for ruminants were observed. All experimental diets presented similar diurnal changes in the fermentation

Table 2 - *In vitro* dry matter (IVDMD), organic matter (IVOMD), and crude protein (IVCPD) digestibility from diets with different forage levels (%) and inclusion of monensin (positive control, MON), technical cashew nut shell liquid (CNSLt), chitosan (CHI) and the combination CNSLt+CHI

	Forage level (FL; %)	Additive (A)				Mean	SEM	P-value		
		MON	CNSLt	CHI	CNSLt+CHI			A	FL	A×FL
IVDMD	20	0.762	0.775	0.686	0.716	0.735a	0.008	<0.0001	<0.0001	0.802
	35	0.684	0.736	0.654	0.650	0.681ab				
	50	0.618	0.667	0.605	0.580	0.618b				
	65	0.541	0.616	0.533	0.541	0.571c				
	100	0.413	0.475	0.357	0.358	0.401c				
	Mean	0.604b	0.567c	0.654a	0.569c					
IVOMD	20	0.859	0.842	0.759	0.785	0.809a	0.017	0.0024	<0.001	0.143
	35	0.759	0.828	0.687	0.727	0.751a				
	50	0.744	0.746	0.625	0.668	0.695ab				
	65	0.595	0.662	0.547	0.599	0.589b				
	100	0.433	0.479	0.385	0.629	0.482c				
	Mean	0.666ab	0.601b	0.712a	0.682a					
IVCPD	20	0.756	0.693	0.489	0.876	0.704ab	0.016	0.0024	<0.001	0.143
	35	0.750	0.714	0.671	0.729	0.716a				
	50	0.814	0.759	0.695	0.818	0.771a				
	65	0.758	0.754	0.655	0.825	0.748a				
	100	0.698	0.625	0.366	0.758	0.612b				
	Mean	0.755ab	0.575C	0.709b	0.801a					

SEM - standard error of the mean; A×FL - interaction between additives and forage levels.
abc - Lowercase letters in the same row indicate significant differences by Tukey's test (P<0.05).

parameters, consisting of a progressive decline in pH with increasing VFA and increasing ammonia concentrations after feeding. Values of pH ($\hat{Y} = 6.7736 - 0.0078x + 0.00007x^2$; $R^2 = 0.92$; P = 0.003) and ammonia concentrations ($\hat{Y} = 7.1713 + 0.2714x - 0.0018x^2$; $R^2 = 0.87$; P = 0.002) in the ruminal fluid showed a quadratic effect for the forage:concentrate ratio. The minimum pH point was verified for the inclusion of 55.71% forage in the diet and the maximum ammonia concentration point was observed for the inclusion of 75.38% forage in the diet. Ammonia concentration and ruminal pH were not affected by the inclusion of additives in the diet (P>0.05; Table 4).

The molar proportion of VFA in the *in vitro* fermentation was affected by the forage:concentrate ratio and showed an interaction with the inclusion of additives on acetate (P<0.001), propionate (P<0.001), and butyrate (P<0.020) concentrations. Chitosan promoted the production of acetate and propionate when added to the diet with 50 and 65% concentrate, respectively. On the other hand, MON promoted the highest values of butyrate using diets with 65% forage. The inclusion of CHI, CNSLt, and CNSLt+CHI resulted in the lowest butyrate concentrations with a 50% concentrate diet. Additionally, the inclusion of CHI, CNSLt, and CNSLt+CHI had the lowest acetate:propionate ratio (C2:C3; P<0.001), indicating higher propionate concentrations with the inclusion of these additives in diets compared with MON (Table 5).

Table 3 - *In vitro* neutral detergent fiber (IVNDFD), acid detergent fiber (IVADFD), and hemicellulose (IVHCELD) digestibility of diets with different forage levels (%) and inclusion of monensin (positive control, MON), technical cashew nut shell liquid (CNSLt), chitosan (CHI), and the combination CNSLt+CHI

	Forage level (FL; %)	Additive (A)				Mean	SEM	P-value		
		MON	CNSLt	CHI	CNSLt+CHI			A	FL	A×FL
IVNDFD	20	0.323AB	0.399AB	0.404A	0.417A	0.386	0.008	0.151	<0.0001	<0.0001
	35	0.389AB	0.345B	0.338B	0.423A	0.374				
	50	0.289C	0.370B	0.459A	0.421AB	0.385				
	65	0.604A	0.387B	0.451B	0.433B	0.469				
	100	0.346B	0.425A	0.403AB	0.341B	0.379				
	Mean	0.391	0.411	0.385	0.407					
IVADFD	20	0.216A	0.195A	0.185A	0.250A	0.211	0.010	0.045	<0.0001	<0.0001
	35	0.213A	0.235A	0.159A	0.194A	0.200				
	50	0.188A	0.225A	0.288A	0.208A	0.227				
	65	0.506A	0.224B	0.243B	0.236B	0.302				
	100	0.302A	0.139B	0.191B	0.141B	0.193				
	Mean	0.252	0.235	0.216	0.204					
IVHCELD	20	0.196AB	0.157A	0.182A	0.210B	0.187	0.015	0.032	<0.0001	0.0002
	35	0.347A	0.321A	0.288A	0.322B	0.322				
	50	0.328A	0.442A	0.429A	0.303B	0.376				
	65	0.452A	0.451A	0.442A	0.402B	0.437				
	100	0.147B	0.363B	0.531A	0.479A	0.380				
	Mean	0.294	0.256	0.374	0.343					

SEM - standard error of the mean; A×FL - interaction between additives and forage levels.
ABC - Uppercase letters in the same row indicate significant differences by Tukey's test (P<0.05).

Table 4 - Effect of different forage:concentrate ratio and inclusion of monensin (MON), technical cashew nut shell liquid (CNSLt), chitosan (CHI), and CNSLt+CHI on rumen fluid pH and ammonia concentrations *in vitro*

Item	Forage level (FL)					SEM	P-value		
	20	35	50	65	100		Additive (A)	FL	A×FL
pH	6.66	6.56	6.55	6.58	6.68	0.01	0.851	0.003	0.687
Ammonia	10.67	17.56	13.71	17.42	16.04	1.43	0.575	0.002	0.988

SEM - standard error of the mean; A×FL: interaction between additives and forage levels.

The inclusion of concentrate affected the fractions V_{F1} ($\hat{Y} = 10.108 - 0.1132x + 0.0005x^2$; $R^2 = 0.96$; $P = 0.002$), V_{F2} ($\hat{Y} = 2.1938 + 0.3128x - 0.0023x^2$; $R^2 = 0.87$; $P = 0.007$), and total gas production ($\hat{Y} = 4.8437 + 0.2938x - 0.0025x^2$; $R^2 = 0.65$; $P<0.001$). The lowest values of fraction V_{F2} was found for high-concentrate diets (80%). The highest values for total gas production occurred with diets containing 65 and 50% concentrate (Table 6). Total gas production showed interaction ($P = 0.007$) of the forage:concentrate ratio and additives in the diets, indicating that the diets with CNSLt had the highest gas production. Lag time (fraction L) was lower ($P = 0.010$) with CHI+CNSLt. Inclusion of concentrate above 50% presented the shortest lag time.

Table 5 - Acetate, propionate, and butyrate concentrations (mmol/100 mL) in ruminal fluid *in vitro* using diets with different forage levels (%) and inclusion of monensin (MON), technical cashew nut shell liquid (CNSLt), chitosan (CHI), and the combination CNSLt+CHI

	Forage level (FL; %)	Additive (A)				SEM	P-value		
		MON	CNSLt	CHI	CSNLt+CHI		A	FL	A×FL
Acetate	20	9.29Abc	8.34Ac	8.99Ab	8.98Ab	0.191	0.377	<0.001	<0.001
	35	10.2Babc	11.6Aab	9.86Bab	7.60Cc				
	50	9.35ABbc	6.87Cd	8.18ABCb	6.95BCc				
	65	11.2ABab	11.1ABab	10.2Bab	11.4ABab				
	100	8.81Bc	10.1Ab	10.1Aab	10.2Aab				
Propionate	20	5.81Ba	7.98ABcd	7.92ABbc	8.715ABbc	0.313	0.421	<0.001	<0.001
	35	7.16Ca	14.7Aa	11.644Bab	10.1Babc				
	50	5.32Aa	5.65Ac	6.07Ac	6.06Ac				
	65	7.46Ba	10.2Abcd	9.81Aabc	11.9Aab				
	100	7.08Ba	10.8Aabc	11.4Aab	9.93Aabc				
Butyrate	20	0.91Aab	0.69Aab	0.77Aa	0.76Aa	0.020	0.532	0.009	0.020
	35	0.94ABab	0.86ABab	0.72ABa	0.58Ba				
	50	0.96Aab	0.57Bb	0.60Ba	0.57Ba				
	65	1.09Aab	0.82Bab	0.71Ba	0.81Ba				
	100	0.78Ab	0.74Aab	0.66Aa	0.80Aa				
C2:C3 ratio	20	2.15	1.10	1.26	1.08	0.095	<0.001	0.983	0.996
	35	2.80	0.86	0.92	0.79				
	50	2.41	1.31	1.35	1.18				
	65	2.38	1.11	1.040	0.99				
	100	2.80	0.97	0.88	1.05				
	Mean	2.50A	1.07B	1.09B	1.02B				

SEM - standard error of the mean; A×FL: interaction between additives and forage levels.
abc - Lowercase letters in the same row indicate significant differences by Tukey's test ($P<0.05$).
ABC - Uppercase letters in the same row indicate significant differences by Tukey's test ($P<0.05$).

4. Discussion

There is an increasing interest in the use of natural additives for promoting changes in the fermentation pattern and improving the digestibility of feeds. Considering the antimicrobial properties of CHI and CNSLt, these have been studied as possible alternative additives to ionophores in ruminant nutrition. The results on IVDMD and IVOMD with the inclusion of CHI are possibly explained by changes in the bacterial community. According to Belanche et al. (2016b), changes in rumen fermentation patterns with CHI (>85% deacetylation) tend to reduce protozoan activity by up to 56%, which favors bacterial growth and, consequently, nutrient digestion. Increased digestibility of DM and OM of 21 and 19%, respectively, was also described by Henry et al. (2015) in heifers fed diets with low concentrate (36%) and 1% CHI (DM basis). In contrast to these results, Goiri et al. (2009) observed a reduction in nutrient digestibility with the inclusion of CHI in the diet evaluated *in vitro*. These differences in results may be due to the diets, forage types, and the different methods used to evaluate digestibility.

Table 6 - *In vitro* ruminal fermentation kinetics parameters using diets with different forage levels (%) and inclusion of monensin (MON), technical cashew nut shell liquid (CNSLt), chitosan (CHI), and the combination CNSLt+CHI

	Forage level (FL; %)	Additive (A)				Mean	SEM	P-value		
		MON	CNSLt	CHI	CSNLt+CHI			A	FL	A×FL
V_{F1} (mL/gas)	20	8.29	6.87	9.43	6.41	7.75A	0.482	0.583	0.002	0.105
	35	9.72	5.88	7.43	5.63	7.16A				
	50	6.49	3.96	6.10	6.41	5.74B				
	65	4.80	4.39	3.59	4.26	4.26B				
	100	3.22	3.45	3.54	3.66	3.46C				
	Mean	6.50	4.91	6.02	5.27					
μ_1 (h^{-1})	20	0.153	0.183	0.066	0.109	0.12	0.010	0.698	0.848	0.342
	35	0.055	0.097	0.150	0.237	0.13				
	50	0.145	0.119	0.047	0.143	0.11				
	65	0.093	0.106	0.141	0.098	0.11				
	100	0.153	0.148	0.138	0.123	0.14				
	Mean	0.120	0.130	0.108	0.142					
L (h)	20	3.240	3.320	6.442	5.242	4.05AB	0.508	0.010	<0.001	0.091
	35	3.463	5.162	2.904	3.018	3.63BC				
	50	2.182	2.188	2.002	2.002	2.09C				
	65	8.775	6.073	8.483	5.931	7.32A				
	100	7.569	7.463	7.014	6.168	7.05A				
	Mean	4.65b	4.64b	4.97a	4.07b					
V_{F2} (mL/gas)	20	1.086	0.453	0.958	0.548	2.51B	0.475	0.599	0.007	0.088
	35	5.920	7.689	6.998	6.998	6.90A				
	50	6.995	4.549	9.999	9.999	7.87A				
	65	7.112	7.079	8.290	6.746	7.31A				
	100	5.425	5.936	5.867	6.040	5.82AB				
	Mean	5.31	6.56	6.42	6.06					
μ_2 (h^{-1})	20	0.001	0.050	0.003	0.047	0.025	0.002	0.052	0.272	0.079
	35	0.031	0.024	0.034	0.028	0.029				
	50	0.026	0.037	0.006	0.044	0.028				
	65	0.035	0.034	0.033	0.032	0.034				
	100	0.042	0.039	0.039	0.038	0.040				
	Mean	0.027b	0.036a	0.023b	0.037a					
V(t) (mL/gas)	20	9.37Bb	7.3Ba	10.3Bbc	6.95Cb	8.94B	0.808	0.516	<0.001	0.007
	35	15.6Aab	13.5Aa	14.4Aab	12.62Aa	14.1A				
	50	13.4ABab	8.5Ba	16.1Aab	16.4Aa	13.6A				
	65	11.9Aab	11.4Aa	11.8ABab	11.0ABa	11.5AB				
	100	8.65Aab	9.39Ba	9.41Bb	9.69Ba	9.28B				
	Mean	11.8	10.1	12.4	11.3					

SEM - standard error of the mean; A×FL: interaction between additives and forage levels.
ABC - Uppercase letters in the same row indicate significant differences by Tukey's test (P<0.05).
abc - Lowercase letters in the same row indicate significant differences by Tukey's test (P<0.05).
Bicompartmental model to describe the fermentation for all experimental diets. The kinetic parameters obtained from the fermentation of gas production *in vitro* were analyzed on 100 mg substrate according to the model $V(t) = V_{F1} / \{1 + \exp[2 + 4\ \mu_1(L-t)]\} + V_{F2} / \{1 + \exp[2 + 4\ \mu_2(L-t)]\}$, in which V(t) is the total volume of gas (mL); V_{F1} and V_{F2} are the gas volume (mL) from rapid (soluble sugars and starch) and slow digestion (cellulose and hemicellulose), respectively; μ_1 and μ_2 correspond to the rate of degradation of fractions of fast and slow degradation (h^{-1}), respectively; L is the lag time (h) of bacterial colonization.

The combination of CHI with CNSLt appear to have a beneficial effect on IVCPD, which may contribute to increased nitrogen supply to microbial growth, which is responsible for nutrient degradation (Vendramini et al., 2016). Increased IVCPD has been observed with the inclusion of CHI in animal experiments, and although the mechanism of action is not fully understood, the authors attribute this effect to the absorption of peptides in the duodenum or the number of amino acids that escape from rumen fermentation, without effects on ammonia concentrations (Paiva et al., 2016; Vendramini et al., 2016). In the case of CNSLt, this effect on protein digestibility may be due to increased nitrogen flow

to the small intestine and, consequently, a decrease in peptide and amino acid fermentation due to less deamination (Osmari et al., 2017). On the other hand, Patra (2011) suggested that functional oils may inhibit ammonia-producing bacteria involved in the deamination process.

The lowest IVDMD, IVOMD, and IVCPD values were observed with CNSLt compared with the other additives used. Studies on sheep by Kang et al. (2018) and on ruminants in Thailand by Konda et al. (2019) showed negative effects on feed digestion. However, according to Diaz et al. (2018), the inclusion of 0.5 g CNSLt/kg DM increased IVDMD. Higher doses resulted in reduced digestibility.

The forage level in ruminant diet is a factor that affects the balance among fermentation rate, passage of carbohydrates, and gas production (fermentation end products, such as VFA; Diaz et al., 2018). Higher dietary concentrate levels provide more energy available for rumen microorganism growth from readily fermentable carbohydrates (Diaz et al., 2018), favoring increased IVDMD and increased gas production, as observed in this experiment. Also, lag time is shorter in diets with higher inclusion of concentrate because it facilitates the adherence of ruminal microorganisms to food particles, allowing a faster onset of feed degradation (Mertens, 1997).

Ruminal VFA concentrations are also directly related to the forage level. In general, fermentation of the fiber present in the cell wall results in higher C2:C3 ratio, (higher acetate concentrations), as well as greater losses in the form of methane (Mitsumori et al., 2014). The use of CHI, CNSL, and their combination was more efficient than MON in reducing the C2:C3 ratio, which indicates higher propionate concentrations (the most important substrate for hepatic gluconeogenesis). Most of the effects described in the literature on MON are related to changes in the VFA profile, mainly decreasing acetic acid and increasing propionic acid (Quinn et al., 2009). According to Goodrich et al. (1984), MON can reduce the C2:C3 ratio by 5 to 6%, as well as methane losses. However, these changes appear to be associated with a reduction in animal feed intake rather than a direct effect on ruminal microorganisms.

Increasing propionate concentrations with the inclusion of CHI and CNSLt in the ruminant diet has been described by several authors (Mitsumori et al., 2014; Branco et al., 2015; Henry et al., 2015; Dias et al., 2017; Dias et al., 2020). This increase in ruminal propionate concentration is attributed to the antimicrobial characteristics of CHI and CNSLt (Henry et al., 2015; Konda et al., 2019).

The main antimicrobial mode of action of CHI has been described to be based on a change in cell permeability due to interactions between the polycationic chitosan ($R\text{-}NH_3$ +), and the electronegative charges on the microbial surfaces causing the cell lysis (Belanche et al., 2016a). On the other hand, the antimicrobial action on CNSLt is due to the amphipathic properties of phenolic lipids (anacardic acid, cardol, and cardanol) present in its composition, which increase the membrane cell permeability, causing the leakage of cytoplasmic components, consequently lysing the cell (Kubo et al., 1993). As a result, there is a reduction in the number of bacteria such as *Fibrobacter* and an increase in *Bacteroidetes* and *Proteobacteria*, which include most amylolytic bacteria (Henry et al., 2015; Konda et al., 2019), explaining the increase in propionate concentrations as fermentation products. Additionally, the effect of additives on cellulolytic bacteria was also reflected in IVNDFD, IVADFD, and IVHELD, mainly in diets with higher forage contents.

The change in gas production presented by diets with the addition of CHI and CNSLt are related to the reduction in the production of greenhouse gases. Belanche et al. (2016b) pointed out that the addition of chitosan reduces the production of H_2 due to less protozoan activity or changes in the bacterial community, which may alter the synthesis of non-methanogenic compounds, such as succinate, propionate, and lactate; or even provide specific action on methanogenic microorganisms. Mitsumori et al. (2014) and Branco et al. (2015), highlighted that CNSLt increases the concentrations of propionate, drastically reducing methane production, with no detrimental effects on total VFA production. Danielsson et al. (2014), reported that the decrease in CH_4 production may be due to a change in the bacterial population, possibly resulting in a reduction in H_2 or format, which are substrates for methanogens.

5. Conclusions

Chitosan and technical cashew nut shell liquid can be considered to alter rumen fermentation, improving nutrient digestibility, and increasing ruminal propionate concentrations. Besides, their combination may potentiate the modulating effects of rumen fermentation. Forage levels may influence the effects of additives.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: R.H.T.B. Goes. Data curation: E.R.Q. Vieira and L.O. Seno. Formal analysis: E.R.Q. Vieira and J.R. Gandra. Investigation: E.R.Q. Vieira, L.C.V. Ítavo, D.G. Anschau, R.T. Oliveira and N.G. Silva. Methodology: J.R. Gandra, R.T. Oliveira and A.G. Jacaúna. Project administration: R.H.T.B. Goes. Software: L.O. Seno. Writing – original draft: T.G. Diaz. Writing – review & editing: M.P. Osmari.

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