Rev. Bras. Saúde Prod. Anim., Salvador, v.25, 01 - 12, 20220034, 2024
http://dx.doi.org/10.1590/S1519-994020220034

Animal Reproduction
Received on: 14/11/2022
Accepted on: 23/10/2023

Digestibility, in vitro fermentation parameters *and kinetic degradation* of diets with crambe crushed

Digestibilidade, parâmetros de fermentação in vitro e Cinética de degradação de dietas em dietas contendo torta de crambe

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ABSTRACT

Aimed with this study was evaluate the increasing levels of crambe crushed, on in vitro digestibility of dry matter (IVDDM), organic matter (IVDOM) and crude protein (IVDCP), parameters of fermentation and kinetics of the cumulative gas production. The crushed crambe were included in the diets at 0; 50; 100; 150 g/kg of DM. There was a quadratic effect of the proportions of crambe crushed in the concentrated supplements on IVDDM and IVDOM (maximum point at 0.25 and 0.91 g Kg^{-1} DM, respectively). Total gas production (A + D) was influenced by the presence of crambe crushed with quadratic effect on minimal point on 0.02 g Kg^{-1} DM. There was a quadratic effect for pH in the collection times in all diets, the lowest pH values were observed between 4.8 to 8.7 hours. There was a quadratic effect for ammoniacal nitrogen in the collection times in all diets with peaks of NH_3-N production occurred between 4.8 and 6.3 hours after the beginning of the incubation. Concluded that the inclusion of crambe crushed up to 150 g kg^{-1} DM in diets for ruminants does not impair the ruminal degradation kinetics.

Key words: byproduct, *Crambe abyssinica*, *in vitro* digestibility, ruminal parameters

RESUMO

O objetivo deste estudo foi avaliar a inclusão de níveis crescentes de torta de crambe, sobre a digestibilidade in vitro da matéria seca (DIVMS), matéria orgânica (DIVMO) e proteína bruta, cinética da produção cumulativa de gás e parâmetros de fermentação. Foram avaliadas as inclusões de 0; 50; 100; 150 g / kg de MS em dietas. Houve efeito quadrático das proporções de torta de crambe nos suplementos concentrados para DIVMS e DIVMO (ponto máximo em 0,25 e 0,91 g Kg-1 DM, respectivamente). A produção total de gás (A + D) foi influenciada por a presença de torta de crambe com efeito quadrático tendo como ponto mínimo em 0,02 g kg^{-1} DM. Houve um efeito quadrático do pH e NH_3-N nos tempos de coleta em todas as dietas, os menores valores de pH foram observados entre 4,8 a 8,7 e os picos de produção de NH_3-N entre 4,8 e 6,3 horas após o início da incubação. Conclui-se que a substituição do farelo de soja por crambe triturado até 150 g kg^{-1} em suplementos concentrados para ruminantes não prejudica a cinética de degradação ruminal.
Palavras chaves: coproduto, *Crambe abyssinica*, digestibilidade in vitro, parâmetros ruminais

INTRODUCTION

Crambe is an important feedstock in the oleochemical industry as an industrial oil crop, crambe has clear advantage over the rapeseed as it does not cross over with other food oilseed crops (Zhu, 2016), also due to its potential industrial application, crambe attracted wide interests from many companies to invest more resources on production, research and oil extraction.

The high protein and fiber levels make crambe seed cake a potential valuable byproduct as potential nutritional feeds for animals. Goes et al. (2010) evaluated *in situ* degradability of by-products and found that crambe crushed and crambe meal had lower ruminal degradation compared to soybean crushed, can then be characterized as a source of non-degradable protein in the rumen.

Carrera et al. (2012) evaluated the crude protein of crambe crushed and found 43.35% of non-protein nitrogen values (NPN); with ruminal degradability of 92.44%, with a undegradable fraction of 7.56. Mizubuti, et al. (2011), evaluated the *in vitro* kinetics of ruminal fermentation of crambe byproducts and found most fibrous carbohydrates degradation rate (CF) for crambe pie crushed (0.1350% / h) less than soybean cake (0,288% / h), which is due to the lower concentration of lignin present in these foods. But the biggest gas production was obtained by crambe meal, which demonstrates good rumen fermentation depending on the balance found between energy and nitrogen compounds provided to rumen microorganisms.

However, when evaluating the potential use of unconventional by-products, studies that are capable of determining the digestive capacity of this potential food are necessary. So, the use of rumen fermentation in vitro techniques is considered less costly and allowing controlling the experimental conditions; is effective since they are readily repeatable (Getachew et al., 1998). Nutrient digestibility by gas production technique is directly proportional to the microbial fermentation of food and can be measured at frequent intervals in order to evaluate the microbial attack on food degradation in the rumen (Neiva Junior et al., 2010).

In this context, the objective with this study was evaluate the increasing levels of crambe crushed, on the in vitro digestibility of dry matter; organic matter; crude protein, fermentation parameters and the kinetics of the cumulative gas production.

MATERIAL AND METHODS

Local and diets

The experiment was carried out at the Laboratory of Animal Nutrition from Federal University of Grande Dourados (UFGD) and State University of Maringa (UEM), between september of 2012 and may of 2013. This experiment was conducted in accordance with guidelines of the Ethics Committee on Animal Use of this institution, under opinion number 021/2012 - CEUA / UFGD.

It was adopted a completely randomized experimental design, and evaluated four diets with different proportions of crambe crushed (0; 50; 100; 150 g/kg of DM) in rations, in a 70:30 concentrated: roughage; and corn silage were used as forage. Diets (Tables 1 and 2) were formulated according to NRC (2007) to be isonitrogenous (150 g kg-^{1}) and isoenergetic with 700 g kg^{-1} total digestible nutrients (TDN).

Table 1. Chemical composition of ingredients used in the experimental diets.

Ingredients	Components						
	DM*	CP*	EE*	NDF*	ADF*	MM*	TC
Crambe crushed	943.40	261.90	188.40	549.80	243.50	57.80	467.3
Soybean meal	892.10	485.10	17.80	149.00	70.00	72.30	424.8
Corn grain	877.00	105.70	31.90	182.10	43.70	16.70	845.7
Corn silage	287.30	59.60	26.10	446.40	245.30	54.10	860.2

DM= dry matter, CP= crude protein, EE= ether extract, NDF= neutral detergent fiber, ADF= acid detergent fiber, MM= mineral matter. TC= total carbohydrates (Sniffen et al., 1992)

Table 2. Concentrates and chemical compositions of experimental diets fed to ewes.

	g kg^{-1} dry matter crambe crushed			
	0	50	100	150
Corn silage	150.0	150.0	150.0	150.0
Corn grain	659.1	666.2	673.3	668.5
Soybean meal	150.0	100.0	50.0	00.0
Crambe crushed	00.0	50.0	100.0	150.0
Urea	05.0	05.0	0.5.0	05.0
Dicalcium phosphate	18.3	17.3	16.3	15.3
Mineral mixture (1)	17.6	11.5	05.4	11.2
Chemical composition (g kg-1 DM)				
Dry matter	700.1	701.9	700.5	720.8
Crude protein	149.3	150.0	149.1	148.8
Ether extract	17.1	21.1	23.6	41.2
Neutral detergent fiber	294.1	307.0	295.9	294.2
Acid detergent fiber	100.0	111.1	105.6	113.2
Mineral matter	49.6	50.2	48.7	69.8

Lignin	25.9	27.8	30.9	34.9
TDN	742.2	734.8	741.1	742.1

[1]Mineral Supplement (nutrients per kilogram of product): 80g phosphorus; 140g calcium; 7g magnesium; 12g sulfur; 133g sodium; 4,200 mg zinc; 300 mg copper; 800 mg manganese; 1,500 mg iron; 100 mg cobalt; 150 mg iodine; 15 mg selenium; 800 mg fluoride (max); 2% citric acid soluble phosphorus (min) 95%.

Inoculum

Inoculum was obtained from two rumen fistulated Holstein steers, with body weight of 380 kg; receiving a 800 g kg^{-1} of corn silage and 200 g kg^{-1} concentrate (corn, soybean meal and mineral supplement). The rumen fluid was collected in the morning, before the first meal, from 4 distinct sites in the rumen, filtered through three layers of cheesecloth, combined in equal portions from each animal and transported to a thermos flask preheated previously purged with CO_2 and twisted tightly closed. The rumen fluid was kept in a water bath at 39 °C in the recipient purged with CO2 before and after collection.

The buffer solution, consisting of solution A and B, was prepared with the following reagents: Solution A (g L-1) composed of: 10.0 g potassium dihydrogen phosphate (KH_2P0_4); 0.5 g magnesium sulfate ($MgSO_4 7.H_2O$); 0.5 g Sodium chloride (NaCl); 0.1 g calcium chloride dehydrate ($CaCl_2.2H_2O$); 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 digestibility

The in vitro digestibility (IVD) of dry matter (IVDDM), organic matter (IVDOM) and crude protein (IVDCP) was determined according to the methodology described by Tilley and Terry (1963) modified by Goering and Van Soest (1970), using an artificial rumen (Daisy II Fermenter®, Ankom).

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). Two blank bags were used in each jar for data correction. The bags with samples were placed in the jars, evenly distributed 10 bags/pitcher (8 bags with sample and two blank bags, totaling 40 bags. Then, they were added 307.7 ml of buffer solution and 77 ml of rumen inoculum and added CO_2 to maintain anaerobic conditions. Upon this procedure, the jars remained in the artificial rumen Daisy II Fermenter® (Ankom) at 39 ° C for 48 hours with continuous stirring.

After 48h, incubation was stopped, and the bags were washed and treated with neutral detergent solution, resulting in a residue consisting of only indigestible cell wall, as described by Goering and Van Soest (1970), allowing to determine the true in vitro dry matter digestibility. The bags obtained at the end of the incubation were used to determine the *in vitro* dry matter digestibility of crude protein (IVDCP) and organic matter (IVDOM). The CP and ash in the residues were determined as described by Association Official Analytical Chemist (AOAC, 2006). 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.

Chemical analysis

Samples of forage and foods of experimental diets were analyzed for dry matter (DM, 934.01), ash (942.05), organic matter (100-ash), crude protein (CP, Nx6.25; 984.13), ether extract (EE; 920.39) according to the techniques described by AOAC (2005). Neutral detergent fiber (NDF), acid detergent fiber (ADF). were determined according to Van Soest et al. (1991). In the determination of NDF, heat-stable α-amylase and no sodium sulfite addition were used. Cellulose was solubilized in 72% sulfuric acid, and the lignin content was obtained from the resulting weight difference (Goering and Van Soest, 1970).

Total carbohydrates (CHO), were calculated by the following equation (Sniffen et al., 1992): CHO=100−(%CP+%EE+%ash).

Rumen fermentation kinetics

For the total gas production and kinetic parameters of the ruminal fermentation determinations, it was used in vitro automatic technique. Eight glass bottles were used, with a capacity of 250 mL, in which were added 0.5 grams of diets samples in duplicate, 100 ml of buffer solution, 25 mL of ruminal innocuous and CO_2. For each incubation performed, two bottles were used as white, containing only ruminal innocuous and buffer, with the aim of adjusting the pressure values.

The increased pressure inside the bottles produced during the incubation was measured in pounds per square inch (psi) using an automated system RF: Gas Production System® (ANKOM). The gas pressure inside the bottles was registered by pressure sensors located in bottle caps or modules, which transferred the information of each flask with a base connected to a computer coordinator, in 5 minutes intervals, totaling 288 readings for 24 hours of incubation.

The data obtained for gas productions were measured in psi, 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}).

Thereafter, the moles were converted in ml of gas produced at normal temperature and pressure conditions (STP) using the following equation: $V = n\ RT/P$. The following reference values of the conditions of STP are used: 273,15ºK (0ºC) and 101 325 Pa (1 atm = 760 mmHg). To calculate the gas production in mL, they were used the bottles corrected pressure, the atmospheric pressure region (96.538 kPa) and the atmospheric pressure at normal conditions (101.325 kPa), this being the value of P.

In determining the extent and rate of gas production caused by food degradation, it was used a bicompartimental exponential logistic model proposed by Pell et al. (1994).

In vitro ruminal parameters (ammonia and pH)

An adaptation was performed on the glass jars, lids used to simulate the rumen conditions in an in vitro digestibility, as described by Díaz et al. (2018) to determine the concentration of ammoniacal-N and the ruminal fluid pH. The jars were provided with lids with valves and a three pathways system to allow the collect of buffered rumen fluid, as well as a valve type Bussen allowed the release of the gases generated during the fermentation.

It was weighed 10 g of sample in each jar and incubated in duplicate with 1600 mL of buffer solution and 400 mL of rumen

fluid. The jars were maintained in an environment at 39 ° C under continuous stirring.

During incubation, samples of 20 ml of buffered rumen fluid were collected using a syringe, and a faucet of three routes installed in each jar lid. The samples were collected at the times: 0, 1, 2, 3, 4, 5, 6, 7 and 8 hours after the start of incubation.

At each time, 10 ml of buffered rumen fluid were used to measure the pH of samples in duplicate with a digital pH meter (Instrutherm®, pH-1500, São Paulo, Brazil). The remaining ruminal fluid (10 mL) was stored in plastic pots containing 1 ml of sulfuric acid (H_2SO_4) 1:1, stopping the fermentation process and reducing the pH, thus preventing the volatilization of the ammoniacal nitrogen (NH_3-N). The determination of the ammonia content in the rumen liquid (NH3) were performed according to the INCT-CA N-007/1 method, described by Detmann et al. (2012).

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, and OM were adjusted by means of covariance analysis for the incubation effect. After the adjustments, the data were submitted to exploratory analyzes 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:

$Yij=\mu+Ti+eij$

where: Yi = observed variable i; μ = overall mean; Ti = effect crambe crushed proportion, ranging from (0, 50, 100 and 150 g kg^{-1}); eij = experimental error.

Data obtained from each parameter were broken down into orthogonal polynomials in order to allow the analysis of variance and regression, according to their distributions.

Ruminal parameters (pH and N-NH_3), 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 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:

$Y_{ijkl} = \mu + \alpha_i + \omega_{j+} (\alpha\omega)_{ij} + e_{ij}$

where: i = 1, ..., a; j = 1, ..., b), where Y_{ij} = the ruminal variables studied (pH and N-NH_3); μ = general mean of the response variable; αi = effect of the i^{th} crambe crushed level; (ωj) = effect of l^{th} time of collection; (αω)ij = effect of the interaction of i^{th} crambe crushed level with l^{th} of collection time; eij = effect of errors associated with any observation.

The statistic used to test the sphericity of the matrix model was the Mauchly - W test (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 cramb crushed levels, 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.

RESULTS AND DISCUSSION

There was a quadratic effect of the proportions of crambe crushed in the

concentrated supplements on IVDDM (maximum point at 0.25 g of crambe crushed Kg^{-1} DM) and IVDOM (maximum point at 0.91 g of crambe crushed Kg^{-1} DM) (Table 2). Similar results were reported by Goes et al. (2018) when evaluating digestibility by ewes fed crambe replacing soybean meal in the diet.

Table 2. In vitro digestibility coefficient of dry matter (IVDDM), organic matter (IVDOM) and crude protein (IVDCP) in g/g.

Variable (g/g)	Diets (g/kg DM)				Mean	SEM	P<0,05
	0	50	100	150			Q
IVDDM	0,90	0,89	0,89	0,88	0,89	0,01	0,0329*
IVDOM	0,92	0,91	0,91	0,90	0,91	0,01	0,0024**
IVDCP	0,52	0,33	0,49	0,23	0,39	0,05	0,0928^{ns}

- Q - quadratic effect; ns – not significant; SEM – Standard Error of the Mean.
- Quadratic effect equations = *Y = 0,900711+0,000548249x-0,00109848x^2; r^2 = 0,21; **Y = 0,927796-0,00199039x-0,00109644x^2; r^2 = 0,60.

The presence of lignin can increase the fraction of indigestible food, and thus potentially reduce the digestible fraction. It was verified an increase of lignin with increasing levels proportion with crambe crush (Table 1), and this certainly was the fact that interfered in reduction of organic matter and dry matter digestibility. and will inhibit biomass digestion in several ways, mainly by preventing microbes and enzymes from gaining access to cellulose (Halpin, 2019).

No effect of proportions of crambe crushed in IVDCP was observed, with mean 0,39 g g^{-1} (Table 2). Given this result is possible to state that crambe crushed has better protein profile than soybeans, because even having a lower CP content of soybean meal (Table 1) could have similar digestibility. Carlson et al. (1996) claim that crambe crush is a source of cysteine, methionine, lysine and threonine may allow the arrival of these amino acids to the duodenum, this protein sources with of low degradability enable the manipulation of aminoacid profile in the duodenum.

There was not effect of the proportions of crambe crushed on the kinetic parameters of digestion (Table 3).

Table 3. Kinetics parameters of *in vitro* cumulative gas production in diets with increasing proportions of crambe crushed inclusion concentrated

Parameters	Diets (g kg^{-1} MS)				Mean	SEM	P<0.05
	0	50	100	150			Q
A (mL gas^{-1})	7.97	6.80	7.82	7.46	7.46	1.76	ns
B (h^{-1})	0.05	0.01	0.04	0.08	0.08	0.12	ns
C (hours)	0.07	0.05	0.09	0.05	0.06	0.13	ns
D (mL gas^{-1})	5.52	4.66	5.14	5.72	5.26	2.90	ns
E (h^{-1})	0.05	0.03	0.04	0.05	0.04	0.02	ns
A+D (mL gas^{-1})	13.50	11.47	12.38	13.55	12.72	3.00	0.0393*

- Q - quadratic effect; ns – not significant; SEM – Standard Error of the Mean, A and D are volume of gas from rapid digestion (soluble carbohydrates and starch) and slow digestion (cellulose and hemicellulose), respectively; B and E correspond to the degradation rates of the fractions of rapid and slow degradation, respectively; C is lag time, time of bacterial colonization, A + D total volume of gas
- Quadratic effect equations = *Y: 0.00164533-0.0389042x+0.799839x^2 - r^2: 0.72.

The reason that may have led to such an effect is due to the similar proportions of carbohydrates (NFC, TC, NDF and ADF) in diets (Table 1). Opposite to the results found here Souza (2011) noted highest gas production in the fast fraction (A), however, the rate of gas production derived the fast fraction did not differ for the different levels of crambe meal in replacement the soybean meal. For slow fraction, the same author observed that gas production was lower when the crambe meal was part of the composition of the concentrates.

Total gas production (A + D) was influenced by the presence of crambe crushed with quadratic effect on minimal point on 0,02 g of crambe crushed Kg^{-1} DM. This fact allows us to state that crambe has a greater extent of degradation than soybean meal, possibly due to the smaller particle size of the crambe crushed than soybean meal. Beran et al. (2005) claimed that the pressing process of the grain causes compaction, which after grinding, may lead to small particles that facilitates the solubilization, directly influencing the gas production.

The pH behavior referring to the in vitro digestibility (Picture 1), evidenced that the presence of crambe crush reduced values as the incubation hours went by, with variation being observed along the analyzed period, different from of the control diet, which showed higher stabilization. The greater pH decreasing was observed at hour 2 and soon after tented to increase, however, at hour 8 eight the diets with crambe crush showed a second decrease.

There was no interaction effect between the collection time and the diets for the pH values of the solution. Just as there was no isolated effect of diets on the same parameter. However, there was a quadratic effect for pH in the collection times in all diets (Figure 1).

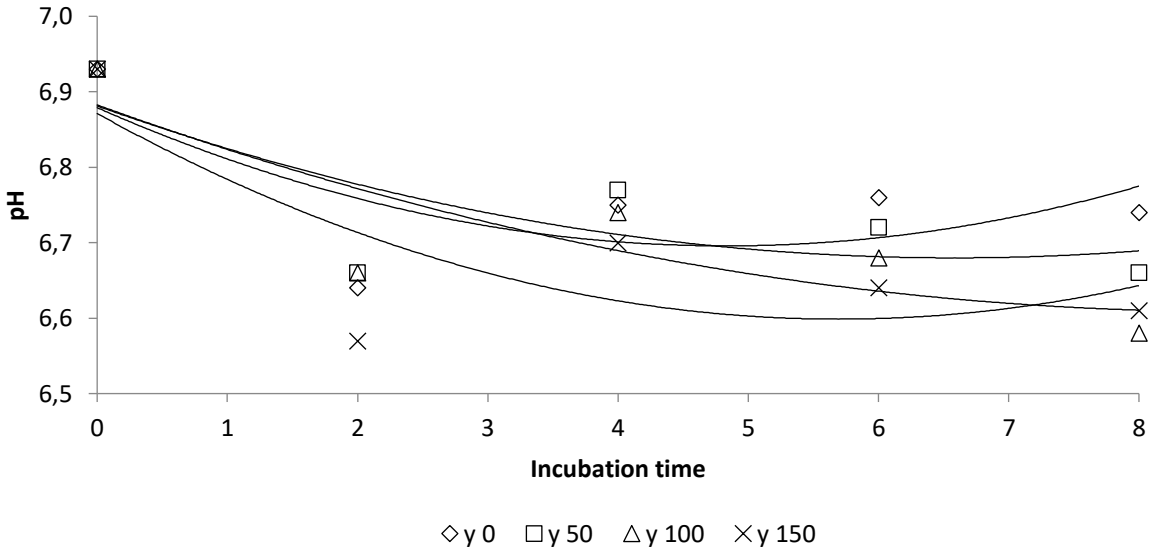

Figure 1. Ruminal liquid pH of in vitro digestibility of diets with different levels of crambe crushed at various times of collection.

Rev. Bras. Saúde Prod. Anim., Salvador, v.25, 01 - 12, 20220034, 2024
http://dx.doi.org/10.1590/S1519-994020220034

DM – dry matter; #0 = treatment without inclusion of crambe crush; 50 = Treatment with 50 g Kg^{-1} crambe crushed included; 100 = treatment with 100 g Kg^{-1} crambe crushed included; 150 = Treatment with 150 g Kg^{-1} crambe crushed included. $^{A}y= 0.0079x^2-0.0759x+6.8789$ (R^2: 0.47; minimum point= 4.80 h); $^{B}y= 0.0046x^2-0.0611x+6.8811$ (R^2: 0.56; minimum point= 6.64 h); $^{C}y= 0.0036x^2-0.0626x+6.8826$ (R^2: 0.71; minimum point= 8.69 h); $^{D}y= 0.0084x^2-0.0956x+6.8711$ (R^2: 0.59; minimum point= 5.69 h).

The lowest pH values were observed between 4.8 to 8.7 hours after start incubation. Although there were differences in the collection times, the crambe was not able to cause a reduction in pH that could damage ruminal health, being within the limits considered ideal for the maximum development of ruminal microorganisms (Van Soest, 1994; Orskov, 1988). The factor that allowed the pH to remain within the optimum limits for microbial development is the high participation of fibrous components in diets (NDF and ADF, table 1).

There was no interaction effect between the collection time and the diets for the ammoniacal nitrogen values on solution. Just as there was no isolated effect of diets on the same parameter. However, there was a quadratic effect for ammoniacal nitrogen in the collection times in all diets (Figure 2).

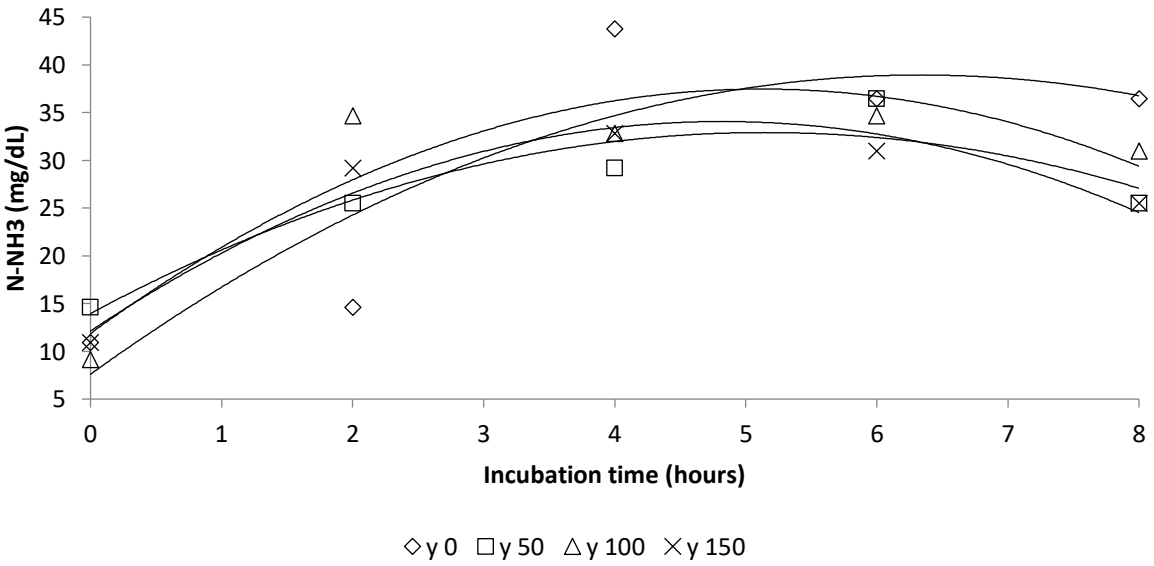

Figure 2. Concentration of ammoniacal nitrogen of in vitro digestibility ruminal liquid of diets with different levels of crushed crambe at various times of collection. #0 = treatment without inclusion of crambe crush; 50 = Treatment with 50 g Kg^{-1} crambe crushed included; 100 = treatment with 100 g Kg^{-1} crambe crushed included; 150 = Treatment with 150 g Kg^{-1} crambe crushed included. y^0: $0.7811x^2+9.8946x+7.6054$ (R^2: 0.77; maximum point = 6.34 h); y^{50}: $0.9764x^2+9.9994x+11.871$ (R^2: 0.85; maximum point = 5.12 h); y^{100}: $0.7161x^2+7.3686x+13.963$ (R^2: 0.89; maximum point = 5.14 h); y^{150}: $0.9439x^2+9.1004x+12.139$ – R^2: 0.96; maximum point = 4.8 h).

The peaks of NH_3-N production occurred between 4.8 and 6.3 hours after the beginning of the incubation. The increase in NH3-N concentrations indicates the maximum fermentation activity, which can occur 3 to 5 hours after feeding when true protein sources are provided, varying with ruminal

degradability and passage rate (Santos and Pedroso, 2011). In all diets the NH_3-N value were sufficient for bacterial growth, according to the minimum value cited by Preston (1986), of 5 mg N-NH_3 100 mL^{-1}. However, the level of ammonia must be greater than 10 mg N-NH_3 100 mL^{-1} in order to increase ruminal digestion of dry matter (Leng, 1990).

CONCLUSION

In view of the above, it is concluded that the replacement of soybean meal with crambe crushed up to 150 g kg^{-1} DM in diets for ruminants is a viable alternative protein source, as it does not impair the ruminal degradation kinetics in addition to improving the energy value of the diets given the highest concentration of ether extract.

ACKNOWLEDGMENT

The Coordination for the Improvement of Higher Education Personnel - CAPES; The Federal University of Grande Dourados, for financial support and scholarships. To State University of Maringá in person of professors Geraldo Tadeu dos Santos e Antonio Ferriani Branco; Support Foundation for the Development of Education, Science and Technology of the State of Mato Grosso do Sul (FUNDECT) and the National Council for Scientific and Technological Development - CNPq; for funding part of this work. Besides, the authors express thanks to teacher Luís Carlos Ferreira de Souza, for the donation of the Crambe used.

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