

## Fractionation of carbohydrates and proteins and in vitro rumen kinetics of corn silage under various storage durations

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### Abstract

The experiment evaluated the effects on the chemical composition (fermentation, kinetic parameters, and protein and carbohydrate fractions) of corn silage after various storage durations (45, 90, 180, and 360 days). Experimental mini silos of the corn plants were made and opened after various storage periods. The experimental design was completely randomized with four treatments and four replications per treatment. Variables such as pH, lactic acid and acetic acid showed a quadratic effect, whereas effluent and gas losses increased linearly with prolonged storage. Proteolysis was observed with an increase in storage, resulting in a rise in ammonia-N, soluble N, and non-protein N concentration and reduction in true protein. The soluble carbohydrate fractions were reduced, and digestible and indigestible fibre were increased. For the kinetic degradation parameters of the silages obtained through in vitro gas methodology, the highest fermentation rate occurred in the first 12 hours of incubation, and the highest volume of gas produced within 96 hours was obtained for silages with shorter storage (45 days). The increase in storage resulted in greater losses and lower ruminal degradability in vitro, causing a decrease in protein quality and nutritional value.

**Keywords:** fermentation, gas production, nitrogen fraction

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### Introduction

Corn silage has low buffering capacity and adequate levels of soluble carbohydrates that stimulate the growth and development of lactic acid-producing bacteria (Kung Jr *et al.*, 2018), which allows a marked reduction in pH during the initial phase of the fermentation process. Maize silages, as a major part of ruminant rations, are usually stored anaerobically for 1 to 12 months before being fed. A stable phase of the ensiling process was reported in literature (Pahlow *et al.*, 2003), meaning that most active metabolic processes in silos ceased within two to six weeks of fermentation. However, studies have shown that certain chemical components and fermentative parameters of silages change even after the onset of the stable phase (Der Bedrosian *et al.*, 2012; Arcari *et al.*, 2016).

Other processes also appear to continue after active fermentation ceases, as reported by Hoffman *et al.* (2011) and Ferraretto *et al.* (2015), who evaluated corn silage with storage of up to 240 days. In both studies, they observed that ammonia-N and soluble N increased with storage time. Although not fermented directly by lactic acid bacteria, fibrous fractions of silages can be altered with increased storage, according to Der Bedrosian *et al.* (2012), who observed partial hydrolysis of hemicellulose during ensiling. Hoffman *et al.* (2011) reported that longer storage contributed to the degradation of proteins such as prolamins, which involve the protein matrix of the grain, thus allowing greater access to starch by ruminal microorganisms. Increasing the silage storage time may be a beneficial strategy for milk producers to increase rumen starch hydrolysis (Kung Jr. *et al.*, 2018).

The in vitro gas production technique has been used widely to evaluate the kinetic degradation of ruminal microbial fermentation processes (Li *et al.*, 2014). This technique is based on the assumption that gas produced from cultures inoculated with mixed microorganisms is directly related to the amount of substrate that is fermented (López *et al.*, 2007). When food is analysed with the vitro gas production

technique, it is expected that samples that produce more gas would be better used by ruminants, thus being able to increase their production. The application of non-linear models to the quantity of gas produced across time results in the estimation of rates of degradation and volume of gas that is produced from fibrous and non-fibrous carbohydrates and the latency time of the fermentation process (Schofield *et al.*, 1994).

The *in vitro* gas technique enables accurate prediction of the quality of ensiled material on a large scale. Naeini *et al.* (2014) demonstrated changes in *in vitro* digestibility and gas production from sorghum silages stored for a prolonged period. Moreover, evaluating the influence of the length of time corn silage is stored in experimental silos on the fermentation process could provide information about the need for and types of inoculation and additives to be included in corn silages. Therefore, fermentative losses and changes in digestibility and in the chemical and nitrogen composition of the silages may require changes in the formulation of the diet over time. Thus, the objective of this study was to evaluate the effects of storage duration on fermentation, kinetic parameters, and protein and carbohydrate fractions of corn silage.

## Materials and Methods

The experiment was conducted in the city of Santa Maria at latitude 29.71'80' S and longitude 53.73'31' W, with an average height above sea level of 95 m, in the state of Rio Grande do Sul, Brazil, from October 2016 to June 2018. The climate of the region is the Cfa type, that is, humid subtropical (Köppen & Geiger, 1928). The soil is classified as red dystrophic sandy Acrisol (Embrapa, 2006).

The experimental area of 1500 m<sup>2</sup> was sown in October 2016, in rows spaced 0.45 m apart with 3.6 seeds planted per linear metre. Fertilizer was applied at the time of sowing. Corn plants were harvested at an average soil height of 20 cm when most of the grains were at the 50% stage of the milk line, and were fragmented in a shredder with an average particle size of 2 cm.

The treatments consisted of various durations of storage (45, 90, 180, and 360 days after ensiling), with four replications (silos) per treatment. The silages were produced on 8 April 2017, 128 days after cultivation, and the material was ensiled in experimental mini silos, compacted properly, and sealed hermetically in four plastic bags to protect against air ingress and light. The average density of silages was 716 kg/m<sup>3</sup> fresh matter. The silos contained 6 kg of silage and 2 kg of sand for effluent recovery. Playne & McDonald's (1966) methodology was used to evaluate the buffering capacity.

At the time of opening, one sample was collected from each silo to determine the pH (Silva & Queiroz, 2002) and another for extraction by pressing the silage liquid (Carver press). These samples were used for the analysis of ammonia-N in relation to total nitrogen (NH<sub>3</sub>-N/TN) using the colorimetry method (Weatherburn, 1967). For volatile fatty acids (VFA), acetic, propionic and butyric acid samples were prepared according to Tangerman and Nagengast (1996), whereas lactic acid was subjected to the derivatization process with the aid of N-tert-butylidimethylsilyl-N-methyltrifluoroacetamide, according to Oms-Oliu *et al.* (2011). Subsequently, the concentrations of all acids were analysed with a gas chromatograph equipped with a Varian Star 3400 CX flame ionization detector (GC-FID, CA, USA). The separation was performed on a polar capillary column (Chrompack, CP-WAX 52 CB, USA) with a 50 m × 0.25 mm i.d. × 0.25 μm thick film, using hydrogen as a carrier gas at a constant pressure of 15 psi. However, for lactic acid, the SGE-BPX 5 column was used with a 30 m × 0.22 mm i.d. × 0.25 mm thick film.

Soluble sugars were determined using the phenol-sulfuric colorimetric method (Hall, 2000). Subsequently, another silage sample was placed in a forced air circulation oven at an average temperature of 55 °C until it reached a constant and heavy weight to determine the partially dry matter, and then ground in a Wiley mill with a mesh sieve of 1 mm for chemical analysis. Dry matter (DM) was determined in a kiln at 105 °C for a minimum of eight hours, and mineral matter was determined by incineration in a muffle furnace at 550 °C for four hours. Crude protein (CP) determination was performed with the micro-Kjeldahl method (method 960.52, AOAC, 1995) and ether extract (EE) was assessed (method 920.39, AOCS, 2005).

Neutral detergent fibre (NDF) levels were obtained with the use of thermostable α-amylase (Termamyl 120 L, Novozimes Latin America, LTDA) and autoclave equipment, according to the technique described by Senger *et al.* (2008). Acid detergent fibre and lignin contents were obtained according to Van Soest *et al.* (1991), with lignin being extracted with sulfuric acid 72%. Hemicellulose and cellulose content was obtained by considering the differences between the levels of NDF and ADF and those of ADF and lignin.

Neutral detergent insoluble N levels, acid detergent insoluble N, soluble N, non-protein N determination, and the equations used to calculate N fractionation (N) were analysed as described by Licitra *et al.* (1996). The fractions are described as follows: A = non-protein N; B1 = borate phosphate buffer soluble protein, which is precipitated in trichloroacetic acid and is rapidly degradable in the rumen; B2 = borate buffer insoluble protein, which is present in cellular content and at the intermediate degradation stage; B3 = insoluble protein, which is degrading slowly in the rumen; C = protein, which is rumen insoluble and indigestible in the gastrointestinal tract.

Carbohydrate fractionation was performed according to Lanzas *et al.* (2007). The fractions were divided as CHO = total carbohydrate, NFC = non-fibrous carbohydrate, A1 = sum of volatile fatty acids (acetic, propionic, butyric, and isobutyric acids), A2 = lactic acid, A3 = other organic acids, A4 = soluble sugars, B1 = starch, B2 = soluble fibre, B3 = digestible fibre, and C = indigestible fibre.

Starch determination was performed using the method proposed by Walter *et al.* (2005), which includes sequential enzymatic digestion with amylase (Termamyl) (120 L), amyloglucosidase (300 L), and protease (Flavourzyme) (500 L) for starch and protein hydrolysis. The sugars that resulted from this digestion were quantified with a spectrophotometer at an absorbance of  $\lambda = 505$  nm, using the glucose oxidase peroxidase kit as the colour reagent.

In vitro ruminal fermentation kinetics analyses were performed according to techniques adapted from Schofield *et al.* (1994) and Theodorou *et al.* (1994). Samples (0.5 g) were weighed in fermentation flasks with capacity of 160 mL. Forty millilitres of culture medium (buffer) and 10 mL of rumen liquid filtered through two layers of cotton gauze were added under continuous CO<sub>2</sub> injection and kept in a water bath at 39 °C. After inoculation of the ruminal liquid, the vials were placed in a water bath at 39 °C with constant agitation for 96 hours. Gas production was measured at 3, 6, 9, 12, 18, 24, 30, 36, 42, 48, 72, and 96 hours. The calculations of the parameters of gas production kinetics were performed with SAS<sup>®</sup> university edition version.

The ruminal fluid was collected from two fistulated cattle with 10 cm cannulas. The use of this technique was approved by the Animal Use Ethics Committee of the Federal University of Santa Maria (CEUA) under protocol number 5439180417. Ruminal fluid collection was performed with a vacuum pump attached to a Kitazato vial. It was kept at a controlled temperature of 39 °C in a water bath during collection. The animals were adapted to corn silage for ten days before rumen fluid collection.

The gas production data were fit to a logistic regression model (Schofield *et al.*, 1994):

$$V_t = V_f(1 + \exp(2 - 4S(t - L)))^{-1}$$

where:  $V_f$  is the final volume of gas (mL) at time  $t$ ,  $S$  is the rate of degradation per hour, and  $L$  is the colonization time (hours).

The organic matter digestibility was calculated from the 24-hour gas production (GP) following Menke *et al.* (1979) as:

$$ODM = 148.8 + (8.89GP_{24}) + 0.45CP + 0.0651CA$$

where:  $ODM$  is the digestibility of organic matter (g/kg DM),  
 $GP_{24}$  is net gas production (ml/g OM) at 24 hours of incubation,  
 $CP$  is crude protein (g/kg DM), and  
 $CA$  is crude ash (g/kg DM).

The experimental design was completely randomized, with four treatments and four replications each. Thus, an analysis of variance was performed to detect differences among times from ensiling to opening using the model:

$$y_{ij} = \mu + t_i + e_{ij}$$

where:  $y_{ij}$  = value observed in the experimental unit receiving treatment  $i$  in repetition  $j$ ,  
 $\mu$  = is the overall effect of the mean,  
 $t_i$  = effect of treatment  $i$ , and  
 $e_{ij}$  = random error (residual).

This model was fit to the data treating the treatment effects both as a categorical effect to estimate the means and as a continuous effect to determine the shape (linear or quadratic) of the response surface. Significance was established with the critical value  $P < 0.05$ . SAS software (SAS Institute Inc. Cary, North Carolina, USA) was used for the analyses.

## Results and Discussion

As the period of time for which the silages were stored increased, the DM content of the maize silage decreased and the effluent increased (Table 1). This may be the result of having harvested the crop before it reached a dry matter content of less than 300 g/kg (Bueno *et al.*, 2020). Harvesting the crop too soon may have resulted in lower production per area and more intense fermentation.

Ammonia-N and soluble N increased as a function of storage duration, as did the quantities of losses of effluent and gas produced. Because of the increase in ammonia-N in the medium, and possibly CO<sub>2</sub>/CH<sub>4</sub>, the pH values rose with storage. Lactic acid had the highest concentration compared with other VFAs.

Lactic acid and acetic acid showed a quadratic behaviour, with maximum concentrations of 41.80 and 19.57 g / kg<sup>-1</sup> at 162 and 233 days of fermentation, respectively. Propionic acid showed an increasing linear behaviour during storage (Table 1). Butyric acid levels did not fit the regression analysis ( $P > 0.05$ ) with a mean value of 0.10% of DM.

**Table 1** Estimates of fermentation parameters for maize silage when stored for various periods after ensiling

Variables, g/kg DM	Storage period of silage					SE	Equations	R <sup>2</sup>	P-value
	Pre-ensiling	45	90	180	360				
DM	279.71	290.40	286.90	265.90	268.70	0.40	$y = 290.0 - 0.07139840x$	0.24	0.05
NH <sub>3</sub> -N		43.20	47.61	55.31	63.20	0.15	$y = 41.8 + 0.061932370x$	0.62	0.0003
pH		3.35	3.72	3.87	3.90	0.03	$y = 3.14 + 0.006517921x - 0.000012319x^2$	0.90	<0.0001
Lactic acid		19.50	34.20	38.25	33.03	0.12	$y = 11.04 + 0.264381720x - 0.000567170x^2$	0.69	0.0005
Acetic acid		15.70	18.43	18.71	11.30	0.32	$y = 14.00 + 0.068991940x - 0.000213430x^2$	0.54	0.006
Propionic acid		3.90	4.10	4.20	5.00	0.01	$y = 3.70 + 0.003188406x$	0.45	0.004
Effluent		9.40	11.15	22.50	31.81	0.15	$y = 5.00 + 0.082487923x$	0.89	<0.0001
Gas loss		36.03	50.40	71.05	81.31	1.37	$y = 37.90 + 0.097739130x$	0.43	0.005
DMR		964.07	946.61	929.01	918.7	1.50	$y = 959.30 - 0.15805310x$	0.39	0.01

DM: dry matter (g/kg), NH<sub>3</sub>-N: ammonia nitrogen (g/kg total nitrogen), x: time in storage

Despite the increase in ammonia-N concentrations observed in this study, these values did not affect silage quality. However, the increase in non-protein N values might affect the dynamics of silage degradation at rumen level, with a direct effect on the performance of formulated diets, because it difficult to synchronize the rate of carbohydrate and protein breakdown in microbial protein synthesis (Yang *et al.*, 2010). In addition, it may increase the risks considerably of using urea in ruminant diets when the inclusion of silage is high.

Owing to its greater volume, lactate acid proved to be the most important acid produced in the silos. According to the statistical model, the accumulation of lactic acid increased until day 162 of fermentation, showing that a possible stabilization of the fermentation could last much longer than has been described (Gary, 1992; Pahlow *et al.*, 2003; Kung Jr. *et al.*, 2013). Likewise, the acetic acid accumulation curve was similar to that described for lactic acid, with peak accumulation at 233 days of fermentation. Acetate is the most oxidized fermentation product relative to glucose (Kozloski, 2017), which indicates that there are losses related to fermentation, especially by gases, during this period of greater acetic acid accumulation within the silo.

The increasing linear behaviour of losses as effluents ( $P < 0.05$ ) and gases ( $P > 0.05$ ) during storage is seen as a reflection of the fermentation process. Possibly, the fermentation side effects, which cause cell disruption and promote intracellular content extravasation to the external environment, could be attributed to the oxidation process of hexoses, which results in the production of organic acids, carbon gas, and water (Muck, 2010; Weinberg & Chen, 2013). Also, the production of acetic acid and other volatile organic compounds by heterolactic bacteria during fermentation could increase DM losses (Kung *et al.*, 2018). The concentration of propionic acid increased linearly with storage (Table 1). This can be explained by the reduction in acetic and lactic acids after its peak, as bacteria can use this acid in the acrylate route, producing propionate as a fermentation by-product (Kozloski, 2017). Under conditions of low concentration of soluble sugars, certain bacteria can use these acids from fermentation as energy sources, especially lactate (Lindgren *et al.*, 1990).

Butyric acid levels did not fit the regression analysis ( $P > 0.05$ ) with a mean value of 0.10% of DM. High levels of butyric acid indicated deterioration of the material. However, at concentrations below 0.10% DM, they did not compromise the nutritional quality of corn silage (McDonald *et al.*, 1991). Bueno *et al.*

(2020), who evaluated corn silage stored for 180 days, found no effect on the levels of butyric acid, corroborating the findings of the current study.

Ammonia-N and non-protein N (A fraction) concentrations increased linearly, whereas crude protein and soluble true protein (B1 and B2 fractions) decreased linearly with storage (Tables 1 and 2), because of the proteolysis, which causes hydrolysis of protein peptide bonds through the action of enzymes present in plant cells generating non-protein nitrogenous compounds such as ammonia, nitrates, nitrites, free amino acids, and peptides (McDonald *et al.*, 1991). Because soluble N and ammonia-N are protein breakdown products, protein degradation generally increases over time. Ammonia-N is described in the literature as an exclusive protein deamination product. This was demonstrated in studies that evaluated storage periods in corn silages such as 240 days (Ferraretto *et al.*, 2015) and 45 and 150 days of silo fermentation (Young *et al.*, 2012), indicating that the proteolytic mechanisms were active during storage.

**Table 2** Protein fractions predicted by the Cornell net carbohydrate and protein system for maize silage as functions of storage time

Protein fraction (g/kg TN)	Storage time					SE	Equations	R <sup>2</sup>	P-value
	Pre-ensiling	45	90	180	360				
CP	85.10	87.60	83.00	84.20	82.21	0.11	$y = 87.0 - 0.014507250x$	0.42	0.02
A	349.30	477.30	552.83	618.05	631.60	1.90	$y = 482.7 + 0.4548000x$	0.98	0.0006
B1	14.00	27.41	21.97	24.03	15.50	10.72	$y = 27.7 - 0.03241546x$	0.62	0.01
B2	390.80	399.60	399.61	271.50	252.71	4.00	$y = 381.3 - 0.40806760x$	0.97	0.0002
B3	127.20	60.50	60.53	40.60	56.47	77.80	$y = 73.7 - 0.31408065x + 0.00073815x^2$	0.85	0.01
C	118.80	34.91	34.97	45.70	43.63	84.01	$y = 4.27$	0.63	0.08

TN: total nitrogen =  $14.1 - 0.26000310x$ , CP: crude protein (g/kg DM), A: non-protein nitrogen (g/kg TN), B1: protein rapidly degradable in the rumen (g/kg TN), B2: protein of intermediate rumen degradation rate (g kg<sup>-1</sup> TN), B3: protein slowly degraded in the rumen (g/kg TN), C: insoluble and indigestible protein (g/kg TN), x: time in storage

The CP content decreased as the silages were stored longer (Table 2). The fractions of true protein with greater solubility (B1 and B2 fractions) underwent intense proteolysis and decreased progressively with time in storage (Table 2). As a result, the non-protein N content (A fraction) increased with each additional day of storage. The increase in the A fraction mirrors the observed increase in ammonia-N. The B3 fraction presented a quadratic effect with a minimum value of 40.28 g / kg-1 total nitrogen at 213 days. No difference was found in the C fraction, which is the protein associated with lignin (acid detergent insoluble N).

The A1 fraction of carbohydrates, which represents the accumulated gas volume (AGV) produced by fermentation, showed a quadratic behaviour with increasing storage (Table 3). The peak AGV occurred on the 167th day of fermentation, similar to the behaviour described in Table 1 for acetic acid and propionic acid. The A2 fraction, which represents lactic acid, also showed a quadratic behaviour, showing a high correlation ( $r = 0.87$ ;  $P = 0.0004$ ) with lactic acid measured directly by means of gas chromatography. The A3 fraction was not detected in silages because organic acids other than lactic and acetic acids were almost undetectable in the silages. Soluble sugar content, corresponding to the A4 fraction, did not fit the regression model, with an average content of 29.11 g / kg DM. The starch (B1) presented a quadratic effect, reaching its maximum value of 205.20 g / kg at 167 days of storage, whereas the amount of soluble fibre (B2) decreased with increasing storage. There was a reduction in soluble fractions and a consequent increase in fibrous fractions (B3) as the storage period increased. The degradation of the B1 and B2 fractions over time adversely affects the nutritional value of the silage (Russell *et al.*, 1992). The evaluation of fraction B2 is important because its degradation is related directly to passage rate, and the extent to which the protein is degraded in the rumen is important in determining the N supply to microorganisms (Carvalho *et al.*, 2008).

The A1 and A2 carbohydrate fractions as determined by the Cornell Net Carbohydrate and Protein System (CNCPS) expressed behaviour similar to that observed for AGVs with greater participation in silages (lactate and acetate). The A2 fraction showed a high correlation with the lactic acid determined by gas chromatography.

According to the statistical model, the concentration of starch in the silages increased until day 167 of fermentation and then decreased, indicating a quadratic behaviour (Table 3). These results were not

expected, and therefore their analyses were repeated without changes in methods. Two factors may be involved in this response, which often affect the fermentative parameters and nutritional value of silages at farm level, namely the type of grain and grain breakage. The hybrid had semi-toothed grains, which have a glassier endosperm than toothed grains. Also, when the silage was chopped, the grains remained intact. These factors could have prevented hydrolysis inside the grain, preventing microbial fermentation of starch, at least until the 167th day of storage. Thus, the concentration of starch increased owing to the use of other nutrients for fermentation. Hentz *et al.* (2017) observed an average content of 1.8 g/kg DM for fraction A4 in corn silage. However, Hall (2000) reported values from 3 to 47 g/kg as the ideal range for the A4 fraction, and an average of 20 g/kg DM in corn silage, which were consistent with the results obtained in this study.

**Table 3** Total carbohydrate and non-fibrous carbohydrate fractions predicted by the Cornell net carbohydrate and protein system for maize silage as functions of storage time

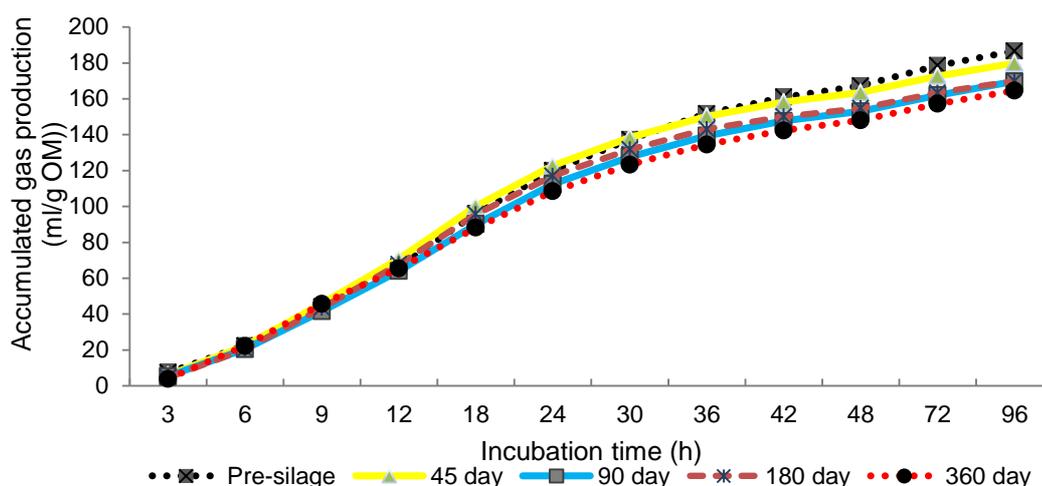
Fraction (g/kg DM)	Storage time					SE	Equations	R <sup>2</sup>	P
	Pre-ensiling	45	90	180	360				
CHO	837.35	834.15	845.12	829.15	834.25	4.14	y = 835.66		0.52
NFC	406.68	468.70	471.34	420.33	332.47	5.78	y = 500.91 - 0.4604435x	0.97	<0.0001
A1	0	20.85	23.68	24.15	17.40	0.95	y = 19.00 + 0.07088710x - 0.0002121x <sup>2</sup>	0.79	0.02
A2	0	19.50	34.20	38.20	33.00	2.59	y = 11.04 + 0.264381720x - 0.00056717x <sup>2</sup>	0.72	0.04
A4	91.97	28.63	32.41	25.41	30.00	1.90	y = 29.11		0.38
B1	174.30	187.90	189.25	205.06	178.75	2.88	y = 173.88 + 0.3715523x - 0.0011018x <sup>2</sup>	0.92	0.001
B2	170.43	211.83	198.80	118.34	63.36	7.77	y = 216.36 - 0.3827720x	0.92	0.0002
B3	341.27	286.56	295.02	327.56	362.95	4.09	y = 276.29 + 0.2473314x	0.94	<0.0001
C	89.39	78.85	78.76	81.24	138.83	3.27	y = 61.07 + 0.19761932x	0.83	0.001

CHO: total carbohydrates, NFC: non-fibrous carbohydrates, A1: sum of volatile fatty acids (acetic + propionic + butyric and isobutyric), A2: lactic acid, A4: soluble sugars, B1: starch, B2: soluble fibre, B3: digestible fibre, C: indigestible fibre, x: time in storage

Pectin (B2 fraction) was the most used nutrient for microbial fermentation after 45 days of fermentation, contributing to the increase in AGV concentration (above) with a linear decrease in its concentration as a function of storage period (Table 3). The reduction in the concentration of carbon nanofibres (CNFs) in the silages showed a high correlation ( $r = 0.87$ ;  $P = 0.0008$ ) with the decrease in pectin values. Although they constitute the cell wall of plants, the fibrous particles (mainly leaves and stem) allowed hydrolysis and microbial fermentation, even if partial, of the primary cell wall, which was represented by the results (Sorrieu *et al.*, 2016).

Cellulose and hemicellulose (B3 fraction) and lignin (C fraction) increased with storage (Table 3), because they are less soluble and potentially less fermentable fractions of carbohydrates (Sniffen *et al.*, 1992). Usually, the increase in these constituents restricts animal consumption because of the filling factor, reducing the animals' performance (Detmann *et al.*, 2014). The increase in these constituents in silages occurs because of the fermentation of non-fibrous carbohydrate components, the soluble fraction, leading to the concentration of the silage fibrous fraction.

The final volume of the gas decreased as the duration increased (Figure 1 and Table 4). The largest volume of gas was produced from the original material. The differences AGV widened as time increased, which suggested that they were associated with slow fermentation carbohydrates. There was a higher concentration of structural carbohydrates (B3 fraction) in silages as they were stored for more time, whereas silages stored for less time had a higher concentration of non-fibrous carbohydrate (Table 2), because of the higher concentration of pectin (B2 fraction), which sustained the greater volume of gas. In addition, silages with longer storage showed greater proteolysis, with a greater amount of ammonia-N and non-protein N (A protein fraction), which possibly contributed to the lower volume of gas (Tables 1 and 2).



**Figure 1** Cumulative gas production profiles for maize silage stored for different periods after ensiling from in vitro fermentation over 96 hours of incubation

The highest fermentation rate ( $S = 5.58 \text{ h}^{-1}$ ) was observed in the first 12 hours of incubation. However, duration did not affect gas production in vitro (Table 4). In the other incubation periods, especially 24 and 48 hours, the volume of gas produced increased as the duration decreased (Table 4). The greatest relative gas accumulation occurred in the first 24 hours of fermentation ( $\approx 65\%$  of the gas produced), with an average fermentation rate of  $4.80 \text{ h}^{-1}$ , and almost all the silage was fermented within 48 hours ( $\approx 90\%$ ) during in vitro incubation. In terms of dietary strategies, in vitro ruminal kinetics evaluations with higher incubation periods would not be necessary or recommended.

**Table 4** Gas production as a function of incubation time and kinetic degradation of maize silage stored for various periods after ensiling

Gas production, ml/g	Storage time					SE	Equations	R <sup>2</sup>	P-value
	Pre-ensiling	45	90	180	360				
Latency time, hours	2.02	2.25	2.14	2.39	1.33	1.01	$y = 2.03$		
12 hours	66.35	70.78	64.01	67.81	65.48	2.66	$y = 67.02$		
24 hours	120.02	122.78	112.46	117.13	108.43	2.55	$y = 120.72 - 0.033267x$	0.42	0.0032
48 hours	167.24	163.52	152.99	154.75	148.02	2.73	$y = 162.90 - 0.044678x$	0.55	0.0005
96 hours	186.65	179.85	169.73	169.89	164.62	2.85	$y = 179.95 - 0.047914x$	0.69	0.0004
S	3.13	3.53	3.32	3.62	3.20	0.95	$y = 3.38$		
OMD	404.06	409.66	389.66	402.12	381.66	5.07	$y = 406.60 - 0.064644x$	0.42	0.0048

S: fermentation rate per hour, latency time: colonization time (hours), OMD: organic matter digestibility (g/kg DM), x: time in storage

A higher fermentation rate was observed in the first 12 hours of incubation (Table 4) because of the fermentation of carbohydrates and highly soluble proteins in the rumen environment, the direct fermentation of pentose and hexoses, and indirectly the neutralization of AGVs by the bicarbonate buffer in the rumen liquid (Getachew *et al.*, 1998).

The differences in the fractions of carbohydrates and proteins apparently complemented each other. On one hand, there was a quadratic increase in the starch content of the silages owing to the storage duration. Therefore, it was expected to increase the volume of gas produced in the first 12 hours of

fermentation, with a ruminal degradation rate of 30% per hour, according to the CNCPS. On the other hand, these silages showed ammonia-N, non-protein N, and soluble protein (with a degradation rate of 315.25%, according to the CNCPS), which may have reduced the volume of gas produced in that incubation period. The degradation of ammonia-N of the protein, related to  $\text{HCO}_3^-$ , intensified the formation of ammonium carbonate, which precipitated in the solution and reduced the formation of  $\text{CO}_2$  (Russell *et al.*, 1992).

After 96 hours of incubation (Figure 1) lower values of total gas production were observed for corn silage samples than for non-ensiled (pre-ensiled) samples, possibly because of fermentation losses during ensiling, as the control samples were not fermented, so there were no fermentation losses (McDonald *et al.*, 1991). A higher gas volume was obtained from silages with shorter storage (24, 48, and 96 hours) (Figure 1 and Table 4), in agreement with Ali *et al.* (2014), who observed an inverse relationship between gas production and silage storage (up to 16 weeks). The higher volume of gas was sustained by the higher concentration of pectin in these silages (Table 3), which has a slower ruminal degradation than starch, contributing to fermentable material beyond the first 12 hours. Furthermore, silages with the longest storage had higher cellulose and hemicellulose content, which have a slow ruminal degradation rate of 5%/h of the B3 fraction, and a higher indigestible fibre content (C fraction), which cannot be degraded in the ruminal environment because it is associated with lignin (Sniffen *et al.*, 1992; Viana *et al.*, 2012).

## Conclusions

The longer silage is stored, the lower its quality, with greater losses of gases and effluents. In addition, silage with lower quality protein and nutritional value has a lower *in vitro* degradability.

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## Authors' Contributions

SNP (conception and design of study; acquisition of data; analysis and/or interpretation of data and drafting the manuscript); JV (conception and design of study; acquisition of data; analysis and/or interpretation of data and drafting the manuscript); TJT (conception and design of study; acquisition of data; analysis and/or interpretation of data and drafting the manuscript), LLS (conception and design of study; acquisition of data; analysis and/or interpretation of data and drafting the manuscript), MBF (acquisition of data and analysis), LTR (acquisition of data and analysis), GRM (acquisition of data and analysis) and Roger Wagner (acquisition of data and analysis).

## Conflicts of Interest Declaration

The authors declare no conflict of interest.

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