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IMPACT OF AN EXOGENOUS PROTEASE ON THE FERMENTATION AND QUALITY OF WET SORGHUM GRAIN SILAGE

Impacto de una proteasa exógena sobre la fermentación y calidad de silaje de grano húmedo de sorgo

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SUMMARY

Sorghum grain structure, along with its accompanying proteins, hinders nutrient availability. The objective was to assess the impact of the addition of a protease on the fermentative and digestive characteristics of ensiled wet sorghum grains. A dual-purpose hybrid sorghum was planted in experimental plots, and panicles were manually harvested in the hard-grain stage. The grains were separated and ground mechanically before being mixed with a protease (PROT; 2 g kg^{-1} of fresh matter) or water (CONTROL), to be subsequently ensiled in PVC microsilos. Grain samples before and after ensiling were analyzed for dry matter (DM), ash, crude protein, alcohol soluble carbohydrates, starch, *in vitro* dry matter digestibility, and net cumulative gas production. At harvest, grains showed 566 g DM kg^{-1} of fresh matter. Ensiling reduced alcohol soluble carbohydrates (*ca.* 50%), and no differences were found between treatments in chemical composition, but the degradation rate increased ("c"; CONTROL= 0.04, PROT= 0.05). Principal component analysis revealed that ensiling was the primary driver of change, rather than protease application; however, 22% of the combined variation in chemical and nutritional variables was attributed to protease addition. Overall, although the digestion rate increased in PROT treatment, changes in nutritional profile were mainly associated with the ensiling process.

Key words. grain processing, silage, stored grain, tropical crops, silage additives.

RESUMEN

La estructura del grano de sorgo, junto con sus proteínas asociadas, dificulta la disponibilidad de los nutrientes. El objetivo de este estudio fue evaluar el impacto de la adición de una proteasa sobre las características fermentativas y digestivas de granos de sorgo húmedos ensilados. Se sembró un sorgo híbrido de doble propósito en parcelas experimentales, y las panojas se cosecharon manualmente en el estado de grano duro. Los granos se separaron y molieron mecánicamente antes de mezclarlos con una proteasa (PROT; 2 g kg^{-1} de materia fresca) o con agua (CONTROL), para luego ser ensilados en microsilos de PVC. Las muestras de grano antes y después del ensilado se analizaron para determinar materia seca (MS), cenizas, proteína bruta, carbohidratos solubles en alcohol, almidón, digestibilidad *in vitro* de la materia seca y producción neta acumulada de gas. Al momento de la cosecha, los granos presentaban 566 g MS kg^{-1} de materia fresca. El ensilado redujo los carbohidratos solubles en alcohol (*ca.* 50%), y no se encontraron diferencias entre tratamientos en la composición química, aunque la tasa de degradación aumentó ("c"; CONTROL = 0,04; PROT = 0,05). El análisis de componentes principales reveló que el ensilado fue el principal factor de cambio, más que la aplicación de la proteasa; sin embargo, el 22% de la variación combinada de las variables químicas y nutricionales se atribuyó a la adición de la enzima. En general, aunque la tasa de digestión aumentó en el tratamiento PROT, los cambios en el perfil nutricional se vincularon principalmente al proceso de ensilado.

Palabras clave. procesamiento de granos, ensilaje, grano almacenado, cultivos tropicales, aditivos para ensilaje.

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Introduction

Ensiled wet sorghum grain (*Sorghum bicolor* L. Moench) is an important feed for many livestock production systems due to its rusticity, plasticity, and capacity to supply rumen-degradable carbohydrates. Early ensiling of harvested sorghum grains affects the chemical and nutritional characteristics, increasing energy availability in the rumen, and facilitating accessibility to harvester contractors' services.

The sorghum grain is formed by the pericarp (3-6% w/w), the embryo (5-10% w/w), and the endosperm (84-90% w/w) (Bean *et al.* 2016). Sorghum grain digestibility is variable and mainly affected by variety, kernel structure, processing (*e.g.* ground, dry-rolled, steam-flaked), protein presence, and amylose/amylopectin ratio. This cereal is mainly used for its contribution of starch (65-85% dry weight), though it also provides protein (6-15%), and fat (0.5-2%), and depending on the variety, it is additionally rich in non-starch polysaccharides, proanthocyanidins, and tannins (Taylor and Emmambux 2010; Zhang *et al.* 2025). Starch is present as granules predominantly in the kernel endosperm. Substantial evidence shows that sorghum starch is less digestible than that of other cereals—including maize—owing to multiple, interacting factors.

The endosperm contains starch distributed in 2 well-differentiated regions: the vitreous (horny) and floury (starchy) zones, both surrounded by the subaleurone layer. Vitreous and floury endosperms differ in starch-granule size and shape and in the proportion of matrix and protein bodies. In the vitreous endosperm, granules are predominantly polygonal and embedded in a continuous, dense protein matrix with more protein bodies and minimal intergranular air spaces. In the floury endosperm, granules are more numerous, rounded and larger; the protein matrix is discontinuous with fewer protein bodies, and intergranular air spaces are larger and more frequent (Montiel and Elizalde 2004).

The nature and composition of associated endosperm proteins strongly affect the physical properties of the endosperm and the accessibility of starch granules to enzymatic hydrolysis. The majority of the protein is found in the endosperm (≈ 80-85%; Bean *et al.* 2016), primarily as prolamines (in sorghum known as Kafirins), serving as reserves within protein bodies that make up 48-70% of total protein on a whole grain flour basis. In addition to the discrete kafirin-rich protein bodies, the sorghum endosperm contains a protein matrix enriched in glutelins that creates a connective framework within which protein bodies and starch granules are embedded (Bean *et al.* 2016). The peripheral endosperm presents several layers of cells with a high amount of prolamins and small starch granules, and it is hard, dense, and resistant to the entry of water. The high concentration of prolamins surrounding the starch granules acts as a barrier to starch gelatinization and hydrolysis (Taylor and Emmambux 2010), and since they are insoluble in ruminal fluid, they can hinder starch accessibility for bacterial degradation, particularly in non-processed grains (Montiel and Elizalde 2004).

Besides the above-mentioned factors, amylose (20-30% of starch) and amylopectin (70-80% of starch) distribution, and proportion influence starch's overall physicochemical characteristics (Gerrano *et al.* 2014). The grain endosperm hardens with the advancement of the phenological stages making it more difficult for ruminal bacteria to access the starch (Isaksen *et al.* 2011). Moreover, the small size and hardness of sorghum grains enable them to remain nearly intact unless they are fractured by the cracker (Montiel *et al.* 2011).

The above mentioned factors account for why sorghum grain usually presents significant responses in its nutritional value after grinding (Offner *et al.* 2003), or altering the integrity of the endosperm protein structures as it eases microorganisms' and digestive enzymes' access to the starch granules (Rooney and Pflugfelder 1986). Considering the barrier that proteins constitute for starch degradation in corn and sorghum grains, it is expected that the addition of proteases before ensiling should improve starch availability. In corn wet grain silages treated with a protease it has been observed an increase in starch digestibility in correspondence with the disruption of the protein matrix cross-linked to starch granules, reduced prolamin content, increased soluble protein and NH_3-N contents (Ferraretto *et al.* 2014; Kung Jr. *et al.* 2014).

In this work, it was hypothesized that exogenous protease enzymes contribute to the degradation of proteinaceous matrix and protein bodies in ensiled wet sorghum grain structures. For this reason, the addition of proteases is expected to increase the degradability rate and the amount of starch and dry matter degraded. The objective was to evaluate the effect of a protease on the fermentation and nutritional quality of wet sorghum grain silage.

Materials and Methods

Experimental site

The trial was set in the Agricultural Experimental Station of Reconquista (INTA, National Institute of Agricultural Technology; 29º15´S 59º44´W), during the 2013/2014 campaign. The experiment was carried out on agricultural land under no-tillage management (Argiudol aquertico, 0-20 cm soil: 12.8 ppm phosphorus; 1.77% organic matter, 27.3 ppm NO3; and pH = 6.7). Rainfall for the 2013/2014 campaign was 242 mm (Nov-Dec) and 632 mm (Jan-Mar) for seeding-flowering and flowering-harvest periods (SMN (Servicio Meteorológico Nacional) 2023; NASA POWER Project 2025).

Treatments and experimental design

The sorghum was planted according to a randomized complete block design (b = 4) in plots of 4 meters wide (8 lines at 0.5 m) by 20 meters long. A dual-purpose hybrid grain sorghum (F2490 – Forratec, Argentina) with a high proportion of grain and tannins was used. Grains were harvested in a hard state (44% humidity, determined by direct assessment of the grain consistency of the middle third of the panicle).

After being manually harvested, the panicles were cut, grains were separated with a static harvester (Pullman, Washington, United States), and samples were passed through a grain grinder (JF2D, Berlato S.A., Buenos Aires, Argentina). Then, the ground material was mixed with the enzymatic treatment (Subtilisin, an alkaline protease produced from *Bacillus subtilis*; EC 3.4.21.62; CAS 9014-01-1; PROT) at 2 g of product (diluted in water) per kg of fresh matter (FM), or an identical volume of water used for the enzyme dilution as control (CONTROL). Subsequently, the treated grain aliquots were ensiled in PVC microsilos (49 cm long and 11 cm in diameter; 280 kg of dry matter [DM] m^{-3}). Grain samples were taken before and after the silage process (65 days).

Laboratory analysis

Chemical analyses were carried out in the Animal Nutrition Laboratory (Department of Animal Science, School of Agriculture, University of Buenos Aires). Samples were stabilized by drying at 65 °C for 48 h in a forced air circulation oven, and afterwards they were ground with a 1 mm mesh (Wiley mill, Thomas Scientific, Swedesboro, NJ, USA). Residual moisture on samples was determined at 105 °C (4 h), ashes (Ash) by ignition (550 °C for 4 h) (AOAC 1990a). Crude protein (CP, i.e., Nitrogen × 6.25) was determined by the Kjeldahl technique (AOAC International 1995), and total starch content (Starch) was measured with the Megazyme enzyme kit (Megazyme, UK). The content of alcohol soluble carbohydrates (ASC) was assessed according to McDonald and Henderson (1964).

In vitro True Dry Matter Digestibility ($ivDMD_t$) was determined along with gas production kinetics using a modification of the technique of Theodorou *et al.* (1994). Briefly, it consisted of incubating *ca.* 400 mg of sample in 100 ml dark brown bottles with bromobutyl septa sealed with aluminium caps. The ruminal liquor (solid-to-liquid ratio 50:50) was collected before morning feeding from 2 rumen-fistulated sheep fed at maintenance (alfalfa pellets and corn grain, respectively, 70: 30 FM). The ruminal liquor was filtered through 4 gauze pads while flushing CO_2 to guarantee an anaerobic environment. The incubation medium was prepared in a 39 °C bath by mixing 4 ml of the ruminal liquor with 38 ml of the carbonate-bicarbonate buffer and nutrient solution. Samples were incubated in duplicate (39 °C), recording the volume (with a syringe) and changes in gas pressure at 2, 4, 6, 9, 12, 17, 20, 24, 30, 36, and 48 h with a pressure transducer (T443A, Bailey and Mackey Ltd., Birmingham, England).

Volume and pressure records were fitted to a linear regression, outlier data were removed (n = 495; r^2 = 0.98), and subsequently, gross gas production was estimated by using pressure records as input. Net cumulative gas production (NCGP) was computed after discounting from the gross cumulated gas production the respective blanks' values (bottles incubated without substrate), and finally it was expressed per gram of DM incubated. In addition, the hourly net gas production rate (HNGPR) was calculated as the difference in NCGP between successive readings (ΔNCGP/Δt, ml g^{-1} DM h^{-1}).

After 48 h, incubation was interrupted by adding 2-3 drops of saturated Thymol solution, and the residue was collected into filter bags (ANKOM #F57, ANKOM Technology, USA). The ivDMDt was calculated as 1 minus the proportion of the 48 h incubation DM residue (105 °C × 4 h), obtained by washing with distilled water until the rinse was clear.

The fermentation profile was characterized by pH, and ammoniacal N content (NH_3-N) (AOAC 1990b). Buffer capacity in the original material, and total acidity in silage were determined according to the procedure proposed by Playne and McDonald (Playne and McDonald 1966).

Statistical analysis

The NCGP (ml g^{-1} DM) data were fitted to a model of the type $y= b\times(1-e^{-c\times(t-Lag)})$ by using NLIN Proc (SAS Institute Inc., 1999), where: "y" is the NCGP, "b" is the NCGP when t0<t<t48, "c" is the rate of NCGP when t0<t<t48, "t" is the time of incubation and "Lag" is the lag time.

Results were analysed according to a randomized complete block design following the model:

$$Y_{ij}: \mu + \tau_i + \beta_j + \varepsilon_{ij}$$

where μ is the common mean, τ_i is the effect of the enzyme treatment, β_j is the effect of the blocks, and ε_{ij} is the residual random error. Data was analyzed by SAS package (SAS Institute Inc. S A S / STAT 1999). Differences were declared significant when $P< 0.05$. Moreover, a principal component analysis (PCA) was performed to explore the main variation in the chemical composition of the silages. This analysis was performed with InfoStat (Di-Rienzo *et al.* 2020).

Results and Discussion

Characterization of pre-ensiled forage

Plants were harvested at the hard-grain stage; however, given the whole-plant DM of 566 g DM kg^{-1} FM (Table 1), the crop likely had not yet reached maximum dry-matter accumulation. This outcome was not unexpected, as the accretion patterns within sorghum panicle make it difficult to establish a clear association between grain humidity and DM accumulation (Gambín and Borrás 2005), as it is in maize. The DM content was lower than that of the grains used by other authors (Hoffman *et al.* 2011; Kung Jr. *et al.* 2014), who used materials with 744 and 728 g DM kg^{-1} FM, respectively. The initial pH of the sorghum grains was 6.3, and the buffer capacity was 42 meq. kg^{-1} DM.

The ivDMDt of the pre-ensiled grain was reasonable (740 g kg^{-1} DM), but slightly higher than that reported for ensiled whole sorghum grains (*in sacco* incubation for 16 h, 694 g kg^{-1} DM) (Aguerre *et al.* 2015).

Ensiling and protease effects

The main change associated with the ensiling process was the *ca.* 50% consumption of the ASC resulting from microorganisms' activity. The rest of the chemical and ruminal degradation

Table 1. Chemical composition, *in vitro* dry matter digestibility, and parameters of gas production kinetics in pre-ensiled wet sorghum grain.
Tabla 1. *Composición química, digestibilidad in vitro de la materia seca, y parámetros de la cinética de producción de gas en los granos de sorgo húmedo previo al ensilado.*

Variables	Mean	SEM[1]
Dry matter (DM), g kg^{-1} fresh matter	566	1.5
Ashes, g kg^{-1} DM	19	0.4
Crude protein, g kg^{-1} DM	104	1.2
Alcohol Soluble Carbohydrates, g kg^{-1} DM	237	8.8
Starch, g kg^{-1} DM	660	17.3
pH initial	6.3	0.02
Buffer capacity, meq kg^{-1} DM	42	1.0
In vitro DM digestibility, g kg^{-1} DM	740	5.2
Parameters of gas production (GP) kinetics[2]		
Lag, hours	3.2	0.18
B, ml g^{-1} DM	303	10.4
c, h^{-1}	0.04	0.002
Maximum Gas Production Rate, ml g^{-1} DM h^{-1}	12.7	0.20
Time to Maximum Gas Production Rate, h	10.5	0.001

[1] Standard Error of the Mean with 3 degrees of freedom.
[2] $Y = B \times (1 - \exp^{(-c \times (time-L))})$

variables showed minimal changes compared to the pre-ensiled material. The ensilage was moderately acidic, and pH numerically identical to the value reported by Aguerre *et al.* (2015) for ensiled ground sorghum grain for 21 days. Besides, the content of NH_3-N was indicative of limited proteolysis and consistent with good conservation.

The protease treatment did not affect the chemical composition and fermentative profile of the ensiled wet grain sorghum (Table 2). In particular, the starch content remained unchanged and was consistent with that of the pre-ensiled grain, indicating that no starch hydrolysis occurred during ensiling via endogenous plant or microbial enzymes (Pflugfelder *et al.* 1986). In a similar work, but with high moisture corn, the addition of a protease at 70 days of ensiling was associated with indicators of protein hydrolysis (lower prolamin content, higher soluble crude protein and NH_3-N), and higher starch concentration and *in vitro* 7-h digestibility (Kung Jr. *et al.* 2014).

Regarding the nutritional quality of the ensiled wet grain sorghum, digestibility was similar to that found in the pre-ensiled material, not showing influences from proteases or the ensiling process. Results that were compatible with those of Aguerre *et al*. (2015) in ensiled sorghum wet grain. However, our results were contrary to those of Kung Jr. *et al.* (2014), who detected increments in starch digestibility in response to exogenous protease in corn grain with a higher DM content (73%). This difference is consistent with the speculation that our sorghum grains had not yet reached maximum DM accumulation or full stabilization of starch and protein structures, thereby reducing the likelihood of a successful response to protease addition.

In addition, gas production showed a similar degradation kinetics for ensiled materials (Figure 1, CONTROL and PROT). But, in ensiled grains, the longer lag phase and lower "B" parameter with respect to the pre-ensiled material were consistent with a lower availability of fermentable carbohydrates in silages. Furthermore, PROT samples showed a higher value of "c" than CONTROL (resp. 0.05 and 0.04 h^{-1}), and pre-ensiled material (c= 0.04 h^{-1}). It resulted in 17 ml g^{-1} DM difference in net gas production in favor of PROT treatment after 48 h of incubation. The increase in rate "c" was consistent with a proteolytic effect on proteins that constrain starch degradation. No difference in the hourly net gas production rate pattern was apparent. In a similar study with sorghum grains reconstituted for 24 hours, the maximum gas production rate was 17.9 ml h^{-1} at 5.66 h, while that of whole sorghum grain ensiled for 21 days was 19.4 ml h^{-1} at 4.96 h (Aguerre *et al.* 2015), showing that the same silage process accelerated and increased the degradation rate of the sorghum grains.

According to the PCA, the ensiling process had a greater effect than the enzyme treatment on the chemical composition of the plant material (Figure 2). The PCA separated the original materials from the silages on principal component 1 (PC1; circles *vs.* squares in Figure 2, ANOVA FM *vs.* CONTROL: $F_{1,11}$=45.3; P< 0.001). This component accounted for 49% of the total variation and was positively associated with pH, DM content, and ASC, while being negatively associated with Ash content (Table 3). The pre-ensiled materials were more

Table 2. Chemical composition, fermentative profile, *in vitro* true dry matter digestibility, and parameters of the gas production kinetics for ensiled wet sorghum grain alone (CONTROL), or with protease (PROT).
Tabla 2. *Composición química, perfil fermentativo, digestibilidad in vitro verdadera de la materia seca, y parámetros de la cinética de producción de gas de los granos de sorgo húmedo ensilados sólos (CONTROL), o con proteasa (PROT).*

Variables	PROT	CONTROL	SEM[1]	p-value
Dry matter (DM), g kg^{-1} fresh matter	556	556	2.1	NS
Ashes, g kg^{-1} DM	21	21	0.2	NS
Crude Protein, g kg^{-1} DM	107	105	0.5	NS
Alcohol Soluble Carbohydrates, g kg^{-1} DM	100	82	37.8	NS
Starch, g kg^{-1} DM	722	649	28.3	NS
pH	5.2	5.3	0.12	NS
Total Acidity, meq kg^{-1} DM	108	91	6.5	NS
NH_3-N, %total Nitrogen	4.9	5.1	0.3	NS
in vitro true DM Digestibility, g kg^{-1} DM	707	759	26.5	NS
Parameters of gas production (GP) kinetics[2]				
Lag, hours	3.5	3.6	0.23	NS
B, ml g^{-1} DM	284	284	7.3	NS
c, h^{-1}	0.05	0.04	0.002	0.02
Maximum Gas Production Rate, ml g^{-1} DM h^{-1}	13.9	12.5	0.30	NS
Time to Maximum Gas Production Rate, h	10.5	12.0	0.70	NS

[1] Standard Error of the Mean with 3 degrees of freedom.
[2] $Y = B \times (1 - \exp^{(-c \times (time-Lag))})$

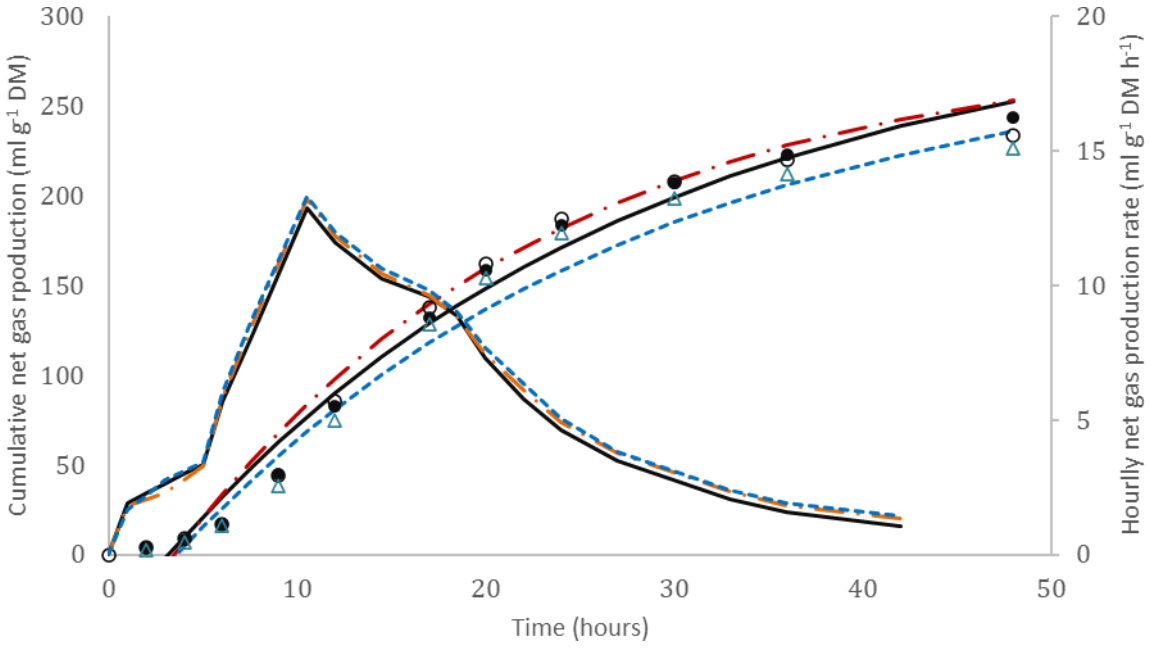

Figure 1. Cumulative net gas production and hourly net gas production rate for original fresh material (FM, —) and ensiled wet sorghum grains with or without proteolytic enzyme (respectively PROT, – · – · –, and CONTROL, ------). Modelled data as $y = b (1 – e^{-c(t-Lag)})$. Mean of original data: ○ FM, Δ CONTROL, ● PROT (blocks = 4).
Figura 1. *Producción de gas neta acumulada y tasa horaria de producción de gas neta del material original fresco (FM, —) y ensilado de granos húmedos de sorgo con o sin enzima proteolítica (respectivamente PROT, – · – · –, y CONTROL, , ------). Datos modelados como $y = b (1 – e^{-c(t-Lag)})$. Promedios de los valores originales: ○ FM, Δ CONTROL, ● PROT (bloques = 4).*

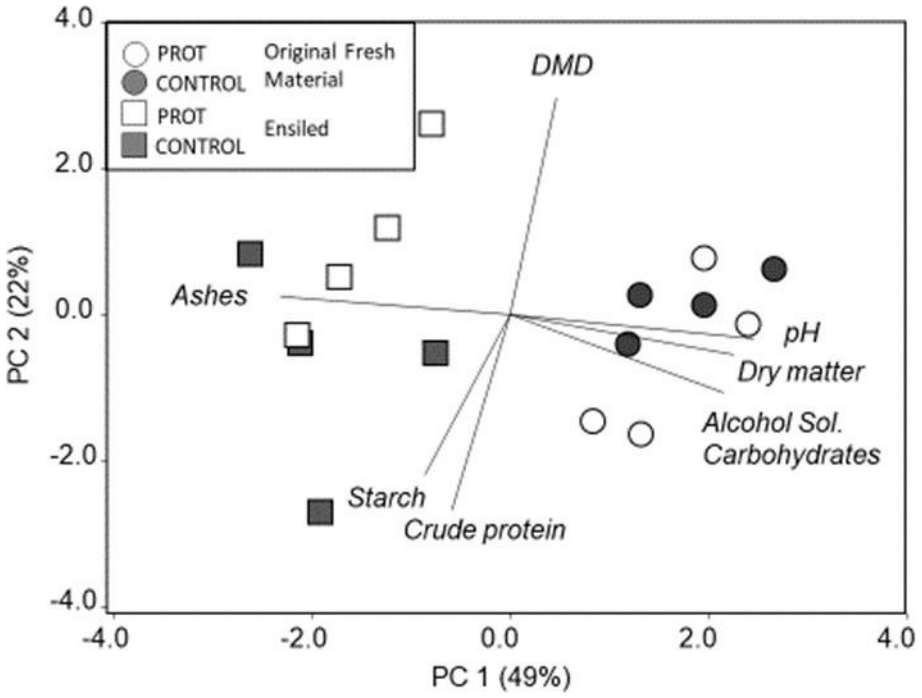

Figure 2. Principal Component Analysis of Protease addition on wet sorghum grain, original fresh material (circles points) and ensiled (square points). Internal lines indicate the intensity and sign of the correlation of the variables with the axis. DMD, *in vitro* true dry matter digestibility.
Figura 2. *Análisis de componentes principales de la adición de una proteasa a grano de sorgo húmedo, material original fresco (círculos) y ensilado (cuadrados). Las líneas internas indican la intensidad y el signo de la correlación de las variables con los ejes. DMD, Digestibilidad in vitro verdadera de la materia seca.*

homogeneous (see dispersion of symbols), with a higher DM content and, as expected, higher ASC and pH than the silages. This differentiation in quality confirmed the differences in composition associated with the silage process (Table 3 and Figure 2).

PC2 (22% of the variance) captured a separation between PROT and CONTROL. An ANOVA on PC2 scores showed a significant treatment effect ($F_{1,3}$ = 35.02; *P* = 0.0096), indicating a multivariate separation between treatments. PC2 loadings were negative for starch and CP, and positive for ivDMDt, indicating their contribution to this displacement. Notably, no significant univariate differences were detected for these variables (Table 2).

The PCA demonstrated integral changes in the materials' chemical composition associated with the silage process and the protease treatment, although no marked changes had been detected through individual variable analyses.

The increase in gas production rate "c" in PROT compared to CONTROL was the sole indicator revealing an effect of the protease addition (2 g kg^{-1} of FM). Neither chemical nor fermentative profile changes were observed as a consequence of this enzyme addition to the ensiled wet sorghum grains. The changes in the characteristics of the ensiled material were primarily attributable to the ensiling process itself, a result consistent with previous observations (Aguerre *et al.* 2015).

As previously noted, these sorghum grains, as suggested by the DM content, may not have reached full starch and protein structure stabilization. The effect of the protease may have been minimized, as the moisture content of the ensiled grain was positively associated with both the rate and extent of fermentation, as well as with proteolysis. Hence, it is speculated that the ensiling process itself solubilized part of the protein, facilitating the bacteria's access to starch (or associated glycans) in similar ways for all treatments.

Table 3. Correlation parameters of the original variables with principal components (PC1, PC2, PC3) of wet sorghum grains ensiled alone or with a protease.

Tabla 3. *Parametros de correlación de las variables originales con los componentes principales (PC1, PC2, PC3) de granos de sorgo húmedo ensilados sólos o con proteasa.*

Variables	PC1	PC2	PC3
Crude Protein	-0.23	-0.71	-0.47
Alcohol Soluble Carbohydrates	0.84	-0.28	0.22
Starch	-0.34	-0.58	0.71
In vitro true Dry Matter Digestibility	0.18	0.78	0.17
pH	0.96	-0.08	0.11
Dry Matter	0.88	-0.14	-0.01
Ashes	-0.91	0.06	0.21

Conclusions

Wet ensiled sorghum grain (dual-purpose grain hybrid; F2490 - Forratec) was harvested at 566 g DM kg^{-1} FM. The protease-treated wet grain silage showed a higher *in vitro* gas-production rate ("c") than the control, consistent with accelerated degradation, although no significant univariate changes were detected in chemical composition or fermentation profile under the conditions of this study. Multivariate analysis indicated that the dominant variation was associated with ensiling and signalled a PROT-associated multivariate shift that was not detected by univariate tests

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Contributions

Jaurena G: Conceptualization, Methodology, Data curation, Formal analysis, Supervision, Statistical analysis, Writing (original draft and review & editing). *Royo LS:* Conceptualization, Methodology, Field work, Chemical and Statistical analysis, Writing (original draft and review & editing). *Tognetti P*: Methodology, Validation, Data curation and Statistical analysis, Writing (original draft and review & editing).

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