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HYBRID TYPE AND PHENOLOGICAL STAGE OUTWIGHED THE EFFECTS OF EXOGENOUS ENZYMES ON SORGHUM SILAGE QUALITY

El tipo de híbrido y el estado fenológico tuvieron mayor influencia que las enzimas exógenas sobre la calidad del silaje de sorgo

Jaurena G[1*], Royo LS[2], Tognetti P[3], Fernández PV[4]

[1]Universidad de Buenos Aires, Facultad de Agronomía, Cátedra de Nutrición Animal. Avenida San Martín 4453, (C1417 DSE) Ciudad Autónoma de Buenos Aires – Argentina. [2] Universidad de Buenos Aires, Maestría en Producción Animal (posgraduate student), Escuela de posgrado A. Soriano. [3] Universidad de Buenos Aires, Facultad de Agronomía, Instituto de Investigaciones Fisiológicas y Ecológicas Vinculadas a la Agricultura (IFEVA), y CONICET, Av. San Martín 4453, C1417DSE, Buenos Aires, Argentina. [4] Universidad de Buenos Aires, Facultad de Agronomía, Centro de Investigación de Hidratos de Carbono (CIHIDECAR), CONICET-, Av. San Martín 4453, C1417DSE, Buenos Aires, Argentina.

* Contact author: gjaurena@agro.uba.ar

SUMMARY

Exogenous enzymes can improve silage fermentation, though results are highly variable. The objective was to assess the effectiveness of a fibrolytic and a proteolytic enzyme on the fermentative and nutritional characteristics of two types of sorghum (forage and double-purpose sorghum) harvested at soft-dough and hard-dough grain stages. Experiment 1 consisted of applying fibrolytic, proteolytic, and control treatments to the two hybrids at the soft-dough and hard-dough grain stages. It was established under a split-plot design at the experimental farm INTA Reconquista (Santa Fe, Argentina). Experiment 2 assessed the susceptibility of structural carbohydrates to enzymatic hydrolysis by treating both sorghum materials at the hard-dough stage with the same enzymatic treatments. In the latter experiment, the two enzymes were equally effective in breaking down sorghum plant structures, although forage sorghum was more susceptible than double-purpose sorghum. In contrast, in the first in-field silage experiment, none of the enzyme treatments significantly affected the chemical, nutritional, or fermentative characteristics of the silages. Hybrids and phenology were found to be more important determinants of changes in digestion parameters than enzymes. Overall, the environmental conditions during the ensiling process seemed to limit enzymatic activity regardless of the ensiling material.

Key word. conserved forages, ensiling, additives, fermentation.

RESUMEN

Las enzimas exógenas pueden favorecer la fermentación de los ensilajes, aunque los resultados reportados en la literatura son muy variables. El objetivo de este estudio fue evaluar la eficacia de una enzima fibrolítica y una proteolítica sobre las características fermentativas y nutricionales de dos tipos de sorgo (forrajero y de doble propósito) cosechados en los estados de grano pastoso y grano duro. En el Experimento 1, se aplicaron los tratamientos fibrolítico, proteolítico y un control a ambos híbridos en las dos etapas fenológicas mencionadas, bajo un diseño de parcelas divididas en la Estación Experimental Agropecuaria INTA Reconquista (Santa Fe, Argentina). El Experimento 2 analizó la susceptibilidad de los carbohidratos estructurales a la hidrólisis enzimática, tratando ambos materiales de sorgo en estado de grano duro con los mismos tratamientos enzimáticos. En este segundo experimento, ambas enzimas mostraron igual capacidad para descomponer las estructuras vegetales del sorgo, aunque el sorgo forrajero presentó mayor susceptibilidad que el de doble propósito. Por el contrario, en el primer experimento de campo, ninguno de los tratamientos enzimáticos afectó significativamente las características químicas, nutricionales o fermentativas de los silajes. Los resultados indicaron que las diferencias entre híbridos y estados fenológicos fueron determinantes más importantes que la acción de las enzimas sobre las variaciones en los parámetros de digestión. En general, las condiciones ambientales durante el proceso de ensilado parecieron limitar la actividad enzimática, independientemente del material ensilado utilizado.

Palabras clave. forrajes conservados, ensilado, aditivos, fermentación.

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Introduction

Sorghum *bicolor* (L. Moench) is widely used as a forage and grain crop due to its good adaptation to environments with edaphic and climatic limitations (Fernandes Perazzo *et al.* 2014; Rodrigues *et al.* 2020). One of the main uses of this crop is as livestock feed, particularly as whole-plant silage. The nutritional quality of silage depends on the characteristics of the original forage and the ensiling process. The species or hybrid type, the phenological stage, or the proportion of stems, leaves, and panicles modify the pre-harvest forage nutritional quality and its fermentative potential (Fernandes Perazzo *et al.* 2014). A successful ensiling process requires sufficient amounts of fermentable substrates and lactic acid bacteria, adequate moisture content, low buffering capacity, and anaerobic conditions (Jaurena 2008). Fermentable sugars can be supplied by the plant material itself or increased by adding molasses, grains (Jaurena and Pichard 2001), or enzymes to hydrolyze plant structures.

The most noticeable change in the crop characteristics with maturity is an increase in dry matter content (DM; Machado *et al.* 2012). Usually, the panicle is the main determinant of silage quality (Cabral *et al.* 2003) due to its high DM content, crude protein (CP) concentration, *in vitro* DM digestibility (ivDMD), and lower cell wall content compared to vegetative components (Neumann *et al.* 2002). In grain sorghum hybrids, neutral detergent fiber (NDF) concentration typically decreases with maturity (Pires *et al.* 2006), while in forage-type hybrids (without panicle) plant NDF content tends to increase (Danley and Vetter 1973). In association with these changes, ivDMD can vary with the proportions of plant parts and the content of tannins (Machado *et al.* 2012). The main contribution of nutrients in the panicle comes from grain starch content, comprising 70 to 80% of DM. The complex interactions between starch granules and grain proteins play an important role in grain hardness and starch ruminal digestion (Montiel and Elizalde 2004; Aguerre *et al.* 2015).

Fibrolytic enzymes have been tested to promote ensiling fermentation via the release of fermentable sugars; however, performance results are contradictory (Muck *et al.* 2018), and animal responses are often erratic (Kung and Muck 1997). The use of proteases to improve the hydrolysis of the grain protein matrix has been less explored (Muck *et al.* 2018). In both corn and sorghum, the grain proteinaceous matrix limits nutrient availability, particularly in late-harvested crops. Treatments of corn plants with experimental proteases at harvest have been shown to increase *in vitro* starch digestibility (Windle *et al.* 2014), which could encourage the harvest of higher-yielding mature crops. Thus, the addition of enzymes can enhance silage fermentation, but their effectiveness depends on forage quality and may vary depending on the phenological stage and hybrid type.

The objective was to determine whether the application of exogenous fibrolytic and proteolytic enzymes at ensiling improves the fermentation profile and nutritional attributes of two sorghum types (forage and dual-purpose) harvested at soft-dough and hard-dough grain stages. We hypothesized that adding exogenous enzymes at ensiling increases the hydrolysis of cell wall and starch fractions, thereby increasing the availability of soluble sugars to lactic acid bacteria and enhancing ruminal utilisation of structural and non-structural carbohydrates.

Material and Methods

Two experiments were developed to assess the effect of plant structure on the action of proteolytic and fibrolytic enzymes (Experiment 1), and a second trial was designed to evaluate the susceptibility of structural carbohydrates to the action of exogenous enzymes under controlled laboratory conditions (Experiment 2).

Experiment 1 – Effect of exogenous enzymes on two sorghum hybrids and phenological stages

Location and treatments

The trial was conducted in Reconquista (Province Santa Fe, Argentina) at the Experimental Station of INTA (National Institute of Agricultural Technology; 29º15´S 59º44´W), during the 2013/2014 campaign. The experiment was carried out on agricultural land in direct drill (*Argiudol aquertico*, 0-20 cm soil: 12.8 ppm P; 1.77% organic matter, 27.3 ppm NO_3; pH=6.7).

In this field experiment, three enzymatic treatments (fibrolytic, proteolytic, and control) were assayed on two sorghum hybrids harvested at two different times. One sorghum hybrid had a low proportion of panicles with a high stem/leaf ratio and sweet stems (Forage Sorghum**;** hybrid F1497 – Forratec, Argentina; FS). The other sorghum was a Dual-Purpose Sorghum (F2490 – Forratec, Argentina; DPS) characterized by a high proportion of panicles, grains, and tannins. Every hybrid was harvested at soft-dough (F1) and hard-dough (F2) grain stages by assessing the grain consistency from the middle third section of the panicle.

Total biomass and composition were measured in F1. Plants were cut at 30 cm above the ground and with a theoretical chopping length of 15 mm with a forage chopping machine (JF2D, Berlato S.A., Buenos Aires, Argentina). The harvested forage from each subplot was mixed separately with an enzymatic treatment: proteases (PROT), fibrolytic (FIBRO), or control (Ctrl), using a different sprayer for each treatment to avoid cross-contamination. The protease treatment consisted of an alkaline protease (No. IUB 3.4.21.62; produced from *Bacillus subtilis*), and was applied at a rate of 2 g kg^{-1} of fresh material (FM). The fibrolytic treatment was a mixture of 70% Xylanase (Dyadic Xylanase Plus E.C. 3.2.1.8) and 30% Cellulase (Dyadic Cellulase CP CONC E.C. 3.2.1.4) and was applied at a rate of 0.35 g kg^{-1} of FM. The control treatment added the same volume of distilled water as used in the two enzymatic treatments. The treatments were allocated in a complete block design with a split-plot arrangement of factors. The main plots were the two hybrids, and subplots were defined by the factorial combination of phenological stage and enzyme treatment (12 treatments: 2 hybrids × 2 phenological stages × 3 enzyme complexes arranged in four blocks – 3.6 × 6.0 m, resulting in 48 mini-silos in total).

The chopped forage mixed with the enzymes was placed in PVC microsilos (49 cm long and 110 mm in diameter), maintaining a similar compaction level across all treatments (*ca.* 260 kg DM m^{-3}), and fermented for 65 d. Forage samples were collected both before and after the ensiling process and were characterized by chemical and nutritional parameters, as described in the "Chemical and nutritional characterization" section.

Experiment 2 *– Laboratory assessment of hydrolysis susceptibility of structural carbohydrates*

The enzymatic hydrolysis of structural carbohydrates was tested under controlled conditions at the Biomolecules chemistry laboratory (School of Agriculture, University of Buenos Aires). The chemical characterization of fresh forage samples of FS and DPS hybrids at stage F2 was conducted before ensiling. The samples were chemically characterized both before (pEF) and after treatment with the same enzymes used in experiment 1 (Ctrl, FIBRO, PROT).

Each sample's residue and supernatant were described by their composition and monosaccharide profile (rhamnose, fucose, arabinose, xylose, xylose/arabinose ratio, mannose, galactose, and glucose) (Morrison 1988). An aliquot of 1 g of each substrate was reconstituted with distilled water carrying the enzymatic dose of FIBRO, PROT, and Ctrl, and 30 ml of distilled water was added to incubate the samples for 24 h at laboratory temperature with magnetic agitation. Subsequently, the samples were centrifuged at 1500 rpm for 15 min and split into "Residue 1" and "Supernatant 1". These residues were re-suspended in 30 ml of distilled water and incubated at 90°C for 3 h with magnetic agitation, and once finished, were centrifuged again (1500 rpm × 15 min) and separated into "Residue 2" and "Supernatant 2". Residues and supernatants were subsequently lyophilized and preserved for analysis. Monosaccharides were measured after hydrolysis and conversion to the respective alditol acetates. Residues 2 samples were dissolved in Trifluoroacetic acid 13M (37°C, 60 min), followed by the acid dilution to 11.5 M (100ºC, 60 min), and finally to 2 M (120ºC, 90 min) to reach the usual hydrolysis conditions for insoluble carbohydrates (Morrison 1988). Monosaccharides were reduced to alditols by treatment with sodium borohydride in NH_3 1 M (24 h at room temperature), and subsequently were passed through a resin ionic exchange column, Amberlite IR-120 (H+) eluting with distilled water. After evaporating the water, the remaining reactive was eliminated by adding methanol and leaving it to dry; this process was repeated three times. The alditols were derivatized by acetylation by reaction with acetic anhydride and pyridine (1:1; 100°C, 45 min). The alditol acetate mixture was extracted with chloroform and then washed with distilled water and saturated sodium bicarbonate solution. Anhydrous sodium sulfate was used to remove any remaining water in the chloroform phase. This phase was dried and brought to -20ºC until analyzed by gas chromatography (GC).

The GC of the derivatized samples was carried out on an Agilent 7890-A gas-liquid chromatograph (Avondale PA, USA) equipped with a flame ionization detector and fitted with a fused silica column (0.25 mm id × 30 m) WCOT-coated with a 0.20 µm film of SP-2330 (Supelco, Bellefonte PA, USA). Flux was set at 1.3 ml min^{-1}, split ratio 25:1, and the injector and detector temperatures were 300ºC. Chromatography was performed as follows: (a) from 200 to 240°C at 2°C min^{-1}, followed by a 15-minute hold for alditol acetates. Molar proportions for each monosaccharide were computed from the respective chromatograph's peaks.

Chemical and nutritional characterization

The fresh forage samples collected before and after the ensiling process in Experiment 1 were first dried in an oven at 65°C. After drying, they were ground using a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA) with a 1 mm mesh size.

All samples' characteristics were expressed on a DM basis by assessing the residual moisture on stabilized samples dried at 105°C in a forced air circulation oven. Ashes (ASH) were determined by ignition in a muffle at 550°C for 4 h (AOAC 1990a). Neutral detergent fiber (NDF; with alpha-amylase and ash free), acid detergent insoluble fiber ash free (ADF), and acid detergent lignin (ADL) were measured with an Ankom 200 fiber analyzer (Göering, and Van Soest 1970; Ankom Technology 2021).

Crude protein (CP, N × 6.25) was determined by Kjeldahl technique (AOAC International 1995). Alcohol-soluble carbohydrates (80% v/v ethanol: water; SolCarb) were assessed by the method proposed by McDonald and Henderson (1964). The samples were also characterized by *in vitro* digestibility at 48 h of DM (ivDMD) and NDF (NDFD), and by *in vitro* gas production kinetics (Theodorou *et al.* 1994), adjusted to a model of the type $y= a+b\times (1-e^{-c(t-L)})$; where "y" is the accumulated gas produced, "a" is the gas produced at t = 0, "b" is the gas produced at t > 0, and "c" is the rate of gas production for "b", a+b is the potential gas production, t is the incubation time and L is the lag time. *In vitro* gas production was also characterized by the maximum hourly net gas production rate (MaxGPR, ml g^{-1} DM h^{-1}) and its time of occurrence (T_{MaxGPR}, hours).

The fermentation profile was characterized by pH (Playne and McDonald 1966) and ammonia nitrogen content [NH3-N] (AOAC 1990b). Volatile fatty acid (VFA) and lactic acid concentrations were determined by chromatography using a Konik 5000B equipment (with Robokrom GC autosampler and a 30 m and 0.32 mm Nukol capillary column; Konik Group, Miami, USA). The fresh forage (before ensiling) was also described by buffer capacity (BC) according to the method proposed by Playne and McDonald (1966).

Statistical analysis

Response variables were analyzed with mixed linear models as a blocked split-plot design (Morris 1999). In Experiment 1, the main plots were the two hybrids (Forage Sorghum and Double Purpose Sorghum) allocated within 4 field blocks, and secondary plots were Harvest stage × Enzyme interaction. On the other hand, in Experiment 2, results were analyzed according to a factorial arrangement of the sorghum hybrid (Forage Sorghum and Double Purpose Sorghum) and enzymatic treatments (pEF, Ctrl, FIBRO, and PROT).

Orthogonal contrasts were conducted according to the following arrangement: C1) pEF *vs.* Ctrl + FIBRO + PROT; C2) Ctrl *vs.* FIBRO + PROT; C3) FIBRO *vs.* PROT. The assumptions of ANOVA regarding homoscedasticity and normality were verified using Levene´s and Shapiro-Wilk tests. All analyses were performed in SAS (SAS Institute Inc. 1999), and differences were considered significant when $P \leq 0.05$.

To study the joint changes in the response variables related to the treatments in both experiments, a principal component analysis (PCA) was performed. The variables selected for this analysis included chemical composition (DM, CP, CSAlc, and cell wall components), nutritional quality (ivDMD and NDFD), and fermentation profile (BC and pH). Data analysis was performed using the statistical package InfoStat (Di Rienzo *et al.* 2020).

Results and Discussion

Differences in plant structure and chemistry

Forage sorghum was taller and had a higher proportion of stem and biomass yield than the DPS (Table 1; $P<0.001$). However, DPS had higher proportions of panicles (FS= 112, DPS= 451 g kg^{-1} DM) and leaves (FS= 100, DPS= 129 g kg^{-1} DM) than the FS. As a consequence, pre- and post-ensiling forages of DPS had higher DM content ($P<0.002$; Table 2 and 3), which is consistent with the lower moisture content typically observed in sorghum panicles (Fernandes Perazzo *et al.* 2014).

The initial analysis of fresh forage revealed that the concentrations of NDF, ADF, and ADL were higher in DPS than in FS ($P<0.01$), and higher in F1 than in F2 ($P<0.01$), while CP did not differ between hybrids but decreased with maturity (F2 vs F1; $P=0.012$) along with BC ($P=0.0283$). A Hybrid×Phenology interaction was detected for ADL and SolCarb in the fresh forage ($P_{Hybrid \times Phenology} \leq 0.005$; Table 2). While for the FS, differences between F1 and F2 were undetected (*i.e.,* 55 g ADL kg^{-1} DM, and 161 g SolCarb kg^{-1} DM), for DPS from F1 to F2, ADL decreased (68 vs 54 g kg^{-1} DM), and SolCarb increased (67 vs 138 g kg^{-1} DM). These patterns indicate that advancing maturity reduced NDF, ADF, CP, and BC ($P<0.03$), probably as a consequence of an increased proportion of panicles (and grains) in the harvested material and the onset of senescence in the vegetative fractions.

Despite the higher panicle contribution in DPS, no significant differences were detected in pre- or post-ensiling CP concentration between hybrids. This finding is consistent with previous research in sorghum (Chaugool *et al.* 2013) and suggests similarities in CP concentration between vegetative and panicle fractions. A general trend in the literature shows that plant CP content decreases with maturity (Machado *et al.* 2012). However, our results and those of Faria Junior *et al.* (2011) also indicate that minimum or unchanged CP levels can be observed under certain genotype × phenology combinations.

After ensiling, differences in NDF, ADF, and ADL between hybrids and phenological stages were attenuated (Tables 2 and 3). The proportion of these cell wall fractions changed during fermentation, reducing the disparity between FS and DPS, and between F1 and F2. This agrees with previous reports in lucerne silages, where partial hydrolysis of structural carbohydrates during ensiling led to smaller differences in fiber concentrations among treatments (Jaurena and Pichard 2001). Overall, these results suggest that differences in cell wall composition before ensiling were partly associated with "labile" NDF fractions that were solubilised during silage fermentation, leading to similar recoverable amounts of structural carbohydrates in the final silages.

The multivariate analysis reinforced the predominant role of genotype and phenology. In the PCA of fresh forage, the first principal component differentiated FS from DPS (41% of total variation, (axis 1; Figure 1), and the second one separated F1 from F2 (26% of total variation; axis 2). The first principal component was positively related to NDF and ADF ($r \geq 0.89$) and negatively associated with SolCarb and ivDMD, revealing contrasting cell wall profiles between hybrids. The second component was negatively associated with DM and positively related to NDFD and CP, differentiating F1 from F2, in agreement with the univariate analysis of DM, CP, and NDFD (Table 2). The CP and NDFD were positively associated with F1, but for FS, the maturity stage did not produce large changes in the multivariate profile (closeness of circles on axis 2; increased variation in DPS group). This pattern indicates that, at the whole-plant level, genotype and phenological stage are the main determinants of the chemical and nutritional profile of sorghum before ensiling.

Regarding digestibility, FS showed higher ivDMD than DPS in the pre-ensiled forage ($P<0.001$), which is consistent with its lower fiber concentrations, and with the typical negative association between structural carbohydrates and digestibility (Jaurena *et al.* 2023). Parameters "B", "c", and TMax of the gas production model were also higher for FS ($P<0.001$), indicating a greater availability and faster fermentation of substrates. Conversely, no differences between hybrids were detected for NDFD, both before and after ensiling, but it declined consistently with advancing maturity (F1 vs F2; $P \leq 0.007$). In the fresh forage, the advance in phenology reduced ivDMD, NDFD, and parameter "B" of the gas production model along with the lag phase ($P<0.005$). But, in silages (Table 3), NDFD was lower than in the original forage and declined from F1 to F2 (F1 = 435, F2 = 397 g NDF kg^{-1} NDF) along with the lag phase (F1 = 0.99, F2 = 0.35 h, $P=0.0008$). However, maturity effects on gas kinetics were hybrid-specific. In FS, parameter "B" declined from 244 to 210 ml g^{-1} DM from F1 to F2 ($P = 0.01$), whereas it did not change in DPS (Hybrid × Phenology, $P \leq 0.05$). Conversely, parameter "c" decreased with maturity only in DPS (F1 = 0.04, F2 = 0.03 h^{-1}, $P = 0.002$), with no corresponding change in FS. Taken together, these findings indicate that changes in plant architecture and cell wall composition with maturity modulate DM and NDF digestion dynamics, and that these effects are strongly genotype-dependent.

The main determinants of the whole-plant DMD are often attributed to panicle and grain contributions at harvest (Neumann *et al.* 2002; Cabral *et al.* 2003). However, our results only partially support this view and instead highlight the predominance of genotype and plant architecture (*e.g.*

Table 1. Plant composition and biomass yield of two sorghum hybrids at soft-dough grain stage.
Tabla 1. *Composición de las plantas y rendimiento de biomasa de dos híbridos de sorgo en estado de grano pastoso.*

Variable	Sorghum Hybrid		SEM[1]	*P*-value
	Forage sorghum (FS)	Double-purpose sorghum (DPS)		
Height (m)	2.25 a	1.77 b	0.02	<0.0001
Composition (g kg^{-1}DM whole plant)				
Leaves	100 b	129 a	4.2	0.0001
Stems	788 a	418 b	16.8	<0.0001
Panicle	112 b	451 a	14.7	<0.0001
Biomass yield (kg DM ha^{-1})	28.829 a	20.723 b	967	<0.0001

[1] Standard error mean

Table 2. Least square means for chemical composition, *in vitro* digestibility, and cumulative gas production parameters of sorghum before ensiling for forage (FS) and double purpose (DPS) sorghum at two phenological stages (F1: soft-dough, F2: hard-dough grain stages).
Tabla 2. *Medias ajustadas por mínimos cuadrados de la composición química, la digestibilidad in vitro, y los parámetros de la producción acumulada de gas del sorgo forrajero (FS) y doble propósito (DPS) previo al ensilado, en dos estados fenológicos (F1: grano pastoso, F2: grano duro)*

Variables[1]	Hybrids (H)				Phenology (Ph)				H × Ph *P*-value
	FS	DPS	SEM[2]	*P*-value	F1	F2	SEM[2]	*P* value	
Chemical characteristics (g kg^{-1} DM except that indicated otherwise)									
Dry matter, g kg^{-1} as fed (DM)	275	341	88.9	0.002	274	343	7.0	<0.0001	NS[3]
Ashes	74	97	1.6	<0.0001	81	90	1.3	<0.0001	NS
Crude Protein	66	66	4.2	NS	70	62	3.3	0.012	NS
Neutral detergent fiber (NDF)	446	499	5.7	<0.0001	483	462	5.7	0.006	NS
Acid detergent fiber	279	309	3.8	0.008	302	286	3.7	0.003	NS
Acid detergent lignin	55	61	1.6	0.003	62	55	1.5	0.001	0.001[4]
Soluble carbohydrates	162	103	20.7	0.001	120	144	20.6	NS	0.005[5]
Buffer capacity, meq kg^{-1} DM	262	234	14.8	NS	257	238	11.3	0.0283	NS
Digestion parameters									
Dry matter digestibility, g kg^{-1} DM	742	709	4.5	0.005	731	719	4.5	0.005	NS
NDFD, g kg^{-1} NDF	454	464	9.2	NS	479	439	9.2	<0.0001	NS
Lag phase, hours	0.34	0.65	0.134	NS	0.82	0.17	0.119	0.0001	0.002[6]
B, ml g^{-1} DM	256	230	3.2	<0.0001	250	236	3.15	0.0007	NS
C, hour^{-1}	0.06	0.04	0.001	<0.0001	0.05	0.05	0.001	NS	NS
MaxGPR, ml g^{-1} DM hour^{-1}	26.7	9.5	9.0	<0.0001	24.2	12.0	9.0	NS	NS
TMaxGPR, hours	2.7	2.5	0.4	NS	2.9	2.3	0.3	0.0287	NS

[1] NDFD, neutral detergent fiber digestibility; cumulative net gas production model parameters y= a+b× (1-e-c$^{[t\text{-}Lag]}$); MaxGPR, maximum hourly gas production rate; TMaxHGPR, time of MaxHGPR.
[2] Standard error of the mean.
[3] No significant (*P*> 0.05).
[4] Hybrids×Phenology, SEM= 2.0, *P*=0.001; FSF1 = FSF2; DPSF1= 68, DPSF2= 54, *P*<0.05.
[5] Hybrids×Phenology, SEM= 23.3, *P*=0.005; FSF1 = FSF2; DPSF1= 67, DPSF2= 138, *P*<0.05.
[6] Hybrids×Phenology, SEM= 0.152, *P*=0.002; FSF1= 0.51 vs FSF2= 0.19, *P*=0.024; DPSF1=1.14 vs DPSF2= 0.17, *P*<0.0001.

panicle proportion, stem/leaf ratio) in modulating the effect of maturity on DMD (Hunt *et al.* 1993). The literature reports reductions (Black *et al.* 1980; Faria Júnior *et al.* 2011; Rodrigues *et al.* 2020), increases (Neumann *et al.* 2002), or negligible changes in DMD (Pires *et al.* 2006) with advancing maturity in different sorghum hybrids, suggesting that the direction and magnitude of the response depend on the interaction between hybrid type and phenology. In our study, the combination of higher panicle contribution and higher fiber concentration in DPS resulted in lower ivDMD and altered gas production kinetics compared with FS, while ensiling tended to reduce the initial differences in fiber content. Discrepancies in NDF physicochemical properties between hybrids may have affected the accessibility of fiber to microbial enzymes, as suggested by the contrasting response in parameters “B” and “c”.

Table 3. Least square means for chemical composition, *in vitro* digestibility, cumulative gas production parameters, and fermentative profile of sorghum silages of forage (FS) and double purpose (DPS) sorghum at two phenological stages (F1: soft-dough, F2: hard-dough grain stages) treated with exogenous enzymes.

Tabla 3. *Medias ajustadas por mínimos cuadrados de la composición química, la digestibilidad in vitro, los parámetros de la producción acumulada de gas, y el perfil fermentativo de silajes de sorgo forrajero (FS) y doble propósito (DPS), en dos estados fenológicos (F1: grano pastoso, F2: grano duro) tratados con enzimas exógenas.*

Variables[1]	Hybrids (H)				Phenology (Ph)				Enzymes (E)[2]					Int. & Contrasts[3]
	FS	DPS	SEM[4]	*P*-value	F1	F2	SEM[4]	*P* -value	PROT	FIBRO	Ctrl	SEM[4]	*P* -value	
Chemical composition (g kg^{-1} DM except that indicated otherwise)														
Dry matter, g kg^{-1} as fed (DM)	241	332	10.6	<0.001	269	304	9.0	0.001	286	283	291	10.1	NS	NS
Ashes	86	100	2.2	0.017	92	94	2.1	NS	93	92	93	2.4	NS	NS
Crude protein	68	65	2.9	NS	69	64	2.5	NS	68	69	63	3.0	NS	NS
Neutral detergent fiber (NDF)	492	503	9.9	NS	494	501	8.1	NS	506	485	501	9.0	NS	C2[5]
Acid detergent fiber	295	284	6.2	NS	290	287	6.2	NS	294	284	288	7.5	NS	NS
Acid detergent lignin	56	48	4.0	NS	51	53	4.0	NS	49	55	52	4.0	NS	H×Ph×E[6]
Soluble carbohydrates	87	65	15.0	NS	80	73	15.0	NS	84	76	68	16.0	NS	NS
igestion parameters														
Dry matter digestibility, g kg^{-1} DM	694	684	6.3	NS	701	677	6.1	0.008	690	690	688	7.6	NS	NS
NDFD, g kg^{-1} NDF	414	419	17.3	NS	435	397	16.1	0.007	427	405	416	17.6	NS	NS
Lag phase, hours	0.64	0.70	0.15	NS	0.99	0.35	0.140	0.0008	0.65	0.78	0.59	0.17	NS	NS
B, ml / g DM	227	216	8.11	NS	226	217	7.4	NS	224	220	221	8.88	NS	H×P[7]
C, hour^{-1}	0.04	0.03	0.002	NS	0.04	0.03	0.001	0.07	0.03	0.04	0.03	0.001	NS	H×P[8]
Fermentation characteristics[9]														
pH	3.76	3.93	0.01	<0.0001	3.84	3.85	0.01	NS	3.83	3.84	3.86	0.0	NS	NS
NH_3-N, % total nitrogen	5.0	4.8	0.7	NS	3.7	6.1	0.7	NS	5.5	4.6	4.6	0.7	NS	NS
Acetic acid,	14	12	1.8	NS	14	11	1.5	0.01	13	13	12	1.6	NS	NS
Propionic acid	9	4	0.1	0.01	8	5	1.2	NS	7	6	6	1.6	NS	NS
Lactic acid (LA)	55	53	8.6	NS	58	50	7.1	NS	55	55	54	7.1	NS	NS
Total fatty acids (TFA)9	88	74	6.6	NS	95	68	6.6	0.007	84	80	81	8.2	NS	NS
LA/TVFA, g g-1	0.73	0.73	0.040	NS	0.72	0.74	0.040	NS	0.73	0.72	0.74	0.040	NS	NS

[1] NDFD, neutral detergent fiber digestibility; cumulative net gas production model parameters y= a+b× (1-e$^{-c[t-Lag]}$).

[2] PROT, protex; FIBRO, fibrolytic enzyme; Ctrl, water as control.

[3] Interactions and Contrasts.

[4] Standard error of the mean

[5] Contrast, C1: PROT+FIBRO vs Ctrl; C2: PROT vs FIBRO; *P*<0.05

[6] H×Ph×E: DPS and F1, Prot= 43, FIBRO= 53, SEM= 4.7, *P*<0.05

[7] Parameter "B", FSF1 vs FSF2 *P*=0.01 (resp. 244 and 210 ml g^{-1} DM; SEM= 11.6); DPSF1 *vs* DPSF2 *P*> 0.10 (resp. 209, 223 ml g^{-1} DM).

[8] Parameter "c", FSF1 vs FSF2 *P*> 0.10 (mean= 0.04 hour^{-1}); DPSF1 vs DPSF2 P=0.002 (resp. 0.04, 0.03 hour^{-1}; SEM= 0.002)

[9] Butyric acid was not detected.

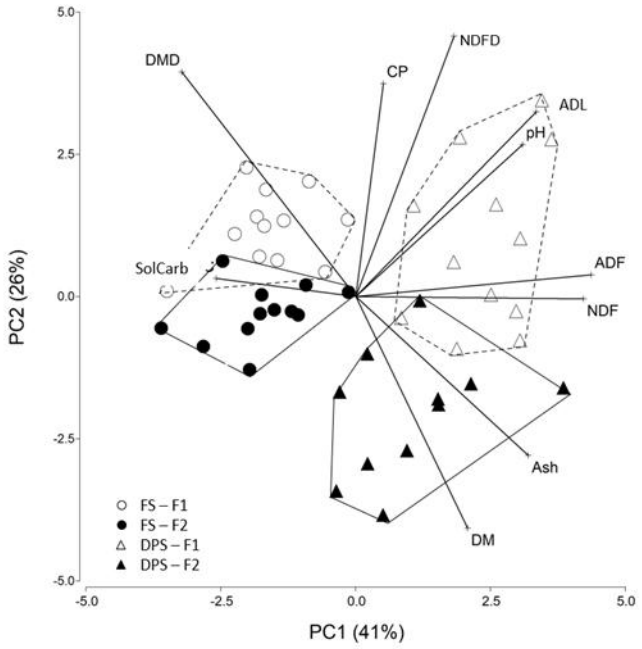

Figure 1. Principal components analysis of chemical composition and nutritional quality of sorghum before ensiling for forage (FS) and double purpose (DPS) sorghum at two phenological stages (F1: soft-dough, F2: hard-dough grain). ADF: acid detergent fiber; NDF: neutral detergent fiber; DM: Dry matter; DMD: DM digestibility; NDFD: NDF digestibility; CP: crude protein; ADL: acid detergent lignin.

Figura 1. *Análisis de componentes principales de la composición química y calidad nutricional de sorgo forrajero (FS) y doble propósito (DPS) antes del ensilado en dos estados fenológicos (F1: grano pastoso, F2: grano duro). ADF: fibra detergente ácido; NDF: fibra detergente neutro; DM: materia seca; NDFD: digestibilidad de la NDF; CP: proteína bruta; ADL: lignina detergente ácido.*

Changes in pH, NH_3-N, fatty acids, and the ratio of lactic to total fatty acids indicate that all silages underwent a good lactic fermentation. However, FS silages showed greater acidification (lower pH, $P<0.001$), which is consistent with their higher SolCarb concentration in the original material. The SolCarb concentration in the silages was not affected by hybrids or maturity stages, but it was considerably lower than in the fresh forage, reflecting its utilization as substrate for bacterial fermentation.

Effect of exogenous enzyme addition

In the field silages, the enzyme treatments had almost no measurable effect on chemical, nutritional, or fermentative characteristics (Table 3). The only significant difference was a slightly lower NDF concentration in silages treated with the fibrolytic enzyme compared with the protease (PROT = 506, FIBRO = 485 g kg^{-1} DM; $P=0.05$), indicating a modest hydrolysis of fiber by FIBRO. A three-way interaction (Hybrid × Phenology × Enzymes) was detected for ADL, but considering the narrow range of variation (34 – 59 g kg^{-1} DM) and the absence of clear pairwise differences within the interaction, this effect was considered biologically negligible and was disregarded.

The multivariate analysis of silage composition and fermentation variables confirmed that hybrid and phenology were more important than the enzyme treatments (Tables 2 and 3). In the PCA, the position of the points changed mostly along the axes associated with hybrid type and maturity (Figure 2), whereas samples did not cluster according to enzyme treatment. Ensiling slightly modified the chemical and nutritional profile but increased the dispersion around the centroid of each Hybrid × Phenology combination, indicating greater variability among microsilos. No consistent trends linked to specific enzymes were observed, and the third principal component (18% variance explained) did not separate enzyme types (data not shown). Together, these results support the view that, under our field conditions, the contribution of exogenous enzymes to the variability in silage composition was small and difficult to predict.

These findings are in line with the heterogeneous responses reported for exogenous enzymes in the literature. Several studies have shown increased fiber degradation or improved silage quality in response to fibrolytic or mixed enzyme products (Sun *et al.* 2009; Xing *et al.* 2009; Thomas *et al.* 2013; Muck *et al.* 2018), whereas others reported no clear effects (Van Vuuren *et al.* 1989). The activity and substrate specificity of commercial enzyme mixtures are often poorly described, and cross-activities, unit definitions, and assay conditions are not always comparable, which makes their performance difficult to anticipate (Muck *et al.* 2018). For instance, enzymes may act on the more labile and digestible components of NDF, but they leave indigestible components intact, lowering the overall digestibility of consumed NDF (Jaurena and Pichard 2001; Dehghani *et al.* 2012; Jin *et al.* 2015; Muck *et al.* 2018).

In our experiment, the limited impact of fibrolytic enzymes on NDF and ADF concentrations in sorghum may be related to the relatively low application rate, similar to doses that have failed to improve fiber degradation in other studies (Zahiroddini *et al.* 2004). Positive effects on NDF and ADF disappearance have been observed with higher doses (Sun *et al.* 2009). Besides, it cannot be discarded that the initial ensiling temperature and pH (relatively low, pH 5.22 – 5-56, data not shown) when enzymes are particularly active may not have been optimal. Consequently, this hindered the distinction between the effects of exogenous enzymes and the natural hydrolytic activity produced by the plant´s enzymes and microorganisms. For proteolytic enzymes, the primary substrate was likely the grain proteinaceous matrix; therefore, their effect was largely constrained by the proportion of grain in the ensiled forage. This variation in response is likely associated with differences among species or hybrids – such as those observed in different sorghum hybrids or in cell wall concentrations (Thomas *et al.* 2013) – as well as with enzyme sources, dosages, and even the specific response variables measured.

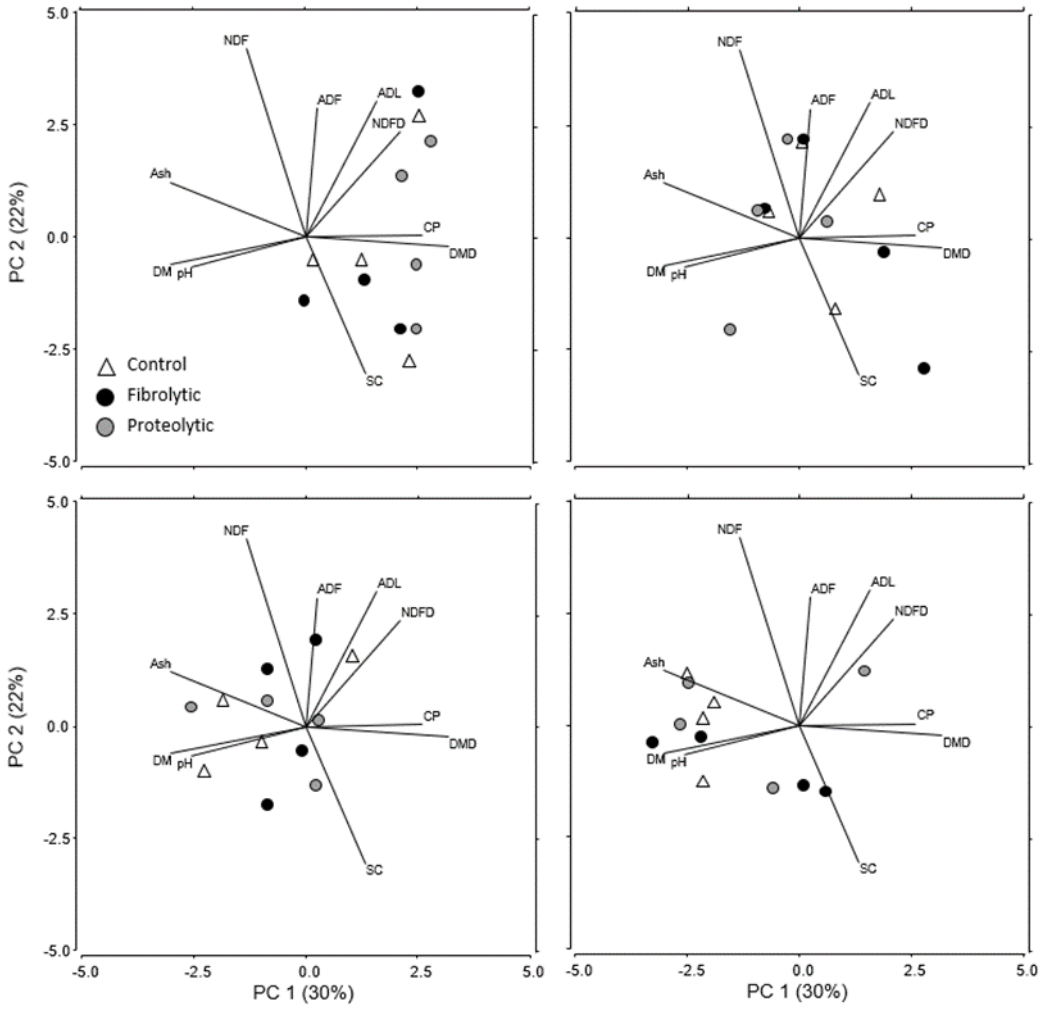

Figure 2. Principal components analysis for the resulting chemical profiles after exogenous enzymes application on fresh forages before ensiling. One unique ordination with the four initial materials was split for clarity. FS-F1 (A), FS-F2 (B), DPS-F1 (C) and DPS-F2 (D). [FS: Forage sorghum; DPS: Double Purpose Sorghum; F1: soft-dough grain; F2: hard-dough grain stages; Protease enz. (light gray circles), Fibrolytic enz. (dark gray circles), Control (triangles).

Figura 2. *Análisis de componentes principales de los perfiles químicos resultantes luego de la aplicación de enzimas exógenas a los forrajes previo al ensilado. Una única ordenación que incluye los cuatro materiales iniciales se dividió para mayor claridad. FS-F1 (A), FS-F2 (B), DPS-F1 (C) y DPS-F2 (D). [FS: sorgo forrajero; DPS: sorgo doble propósito; F1: grano pastoso; F2: grano duro; Proteasa (círculo gris claro), Fibrolítica (círculo gris oscuro), Control (triangulos)]*

Assessment of the enzymes´ activities on sorghum whole plant silage

The second experiment evaluated the effect of the enzymes under laboratory-controlled conditions on both substrates (FS and DPS) at the maturity stage F2, presumably more resistant to enzyme action. In contrast to the field silages, where enzyme effects were minimal, the incubation residues after enzymatic hydrolysis revealed clear responses. Significant differences were detected in the extracted residue between hybrids (FS = 561, DPS = 630 mg g^{-1} DM, SEM = 10.3, P<0.0001) and among enzymatic treatments (Table 4; Figure 3). The fresh original forage differed from the rest of the treatments (P<0.001), and both enzyme treatments (FIBRO and PROT) showed lower recovery rates than Ctrl (P = 0.03). No differences were detected between FIBRO and PROT. This finding contrasts with the in-field silage experiment (Experiment 1), where the same enzyme products did not produce significant differences. The controlled environment settings (pH, temperature, substrate-to-solution ratio) in this experiment likely enhanced hydrolysis efficiency. In addition, the rapid drop in pH and changing environmental conditions in the microsilos may have limited their effective action on the substrates.

Cell wall carbohydrate analyses showed scarce effects of the enzyme treatments on FS substrate and none on DPS. In FS, all the incubation treatments (*i.e.* Ctrl, PROT, and FIBRO) resulted in a lower concentration of mannose compared to the original material (pEF), suggesting the solubilization of this glycan from the cell wall, possibly facilitated by the treatment temperature (90°C) or by the plant's native or added enzymes. Furthermore, both enzyme treatments led to changes in the pectin composition in FS, as indicated by the

Table 4. Monosaccharide composition of incubation residues of whole plant sorghum substrates incubated with enzymes (mg g^{-1} de total neutral sugars).
Tabla 4. *Composición de monosacaridos de los residuos de incubación de sustratos de sorgo de planta entera incubados con enzimas (mg g^{-1} de azúcares neutros totales).*

Monosacáridos	Tratamientos[1]				SEM[2]	*P*-value	Significant Contrasts[3]
	pEF	CTRL	PROT	FIBRO			
Residue, mg g^{-1} DM	645	605	582	550	13.7	<0.0001	C1***; C2**
Forage sorghum							
Rhamnose	3	2	2	6	1.3	NS	C3*
Fucose	5	3	0.2	6	3.8	NS	NS
Arabinosa	102	105	95	113	15.5	NS	NS
Xylose	228	248	228	245	26.5	NS	NS
Xil/Ara	2	2	2	2	0.1	NS	NS
Mannose	9	6	7	6	1.2	NS	C1*
Galactose	20	22	19	20	0.6	0.04	C2**
Glucose	631	614	649	603	40.4	NS	NS
Double-purpose sorghum							
Rhamnose	4	4	5	4	0.9	NS	NS
Fucose	0.8	1.7	8.0	0.4	3.4	NS	NS
Arabinosa	101	95.5	135	101	17.2	NS	NS
Xylose	258	266	258	250	23.8	NS	NS
Xil/Ara	3	3	2	3	0.2	NS	NS
Mannose	11	11	11	9	1.3	NS	NS
Galactose	22	20	26	19	3.3	NS	NS
Glucose	602	602	556	616	37.1	NS	NS

[1] Treatment*:* pEF: fresh forage (prior to ensiling), CTRL: no enzymes; PROT: protease; FIBRO: fibrolytic enzyme.
[2] Standard Error of the mean.
[3] Significant contrasts: C1: pEF vs Ctrl + PROT + FIBRO; C2: Ctrl vs. PROT + FIBRO; C3: PROT vs. FIBRO. *, ≤0.05; **, ≤0.01; ***, ≤0.001.

decreased concentration of galactose observed when comparing PROT and FIBRO to Ctrl. Additionally, the difference in rhamnose concentration between PROT and FIBRO suggests that the enzyme treatments had differential effects on the pectin fraction. The enzymes appeared to act indiscriminately on the entire cell wall, with pectins being the primary components solubilized in FS. Although the enzymes used do not directly target pectins, alterations in other cell wall components may have influenced their solubility due to the intricate interrelationship among cell wall polymers.

Implications

From a practical standpoint, our results suggest that the exogenous fibrolytic and proteolytic enzymes used, and as applied here, should not be considered a primary tool for improving sorghum silage quality. The most effective levers to improve sorghum silages remain hybrid choice, the timing of harvest and overall ensiling conditions. Enzyme-based additives may still warrant evaluation in specific situations (e.*g.* other hybrids, higher doses, other enzyme sources), but such uses should be supported by targeted field trials that include feed quality assessment, nutritional responses and economic outcomes.

Conclusions

Under field conditions (Experiment 1), hybrid type and maturity stage had a much stronger impact on sorghum silage quality than the addition of exogenous enzymes. Enzymes induced negligible effects on chemical, nutritional, and fermentative characteristics (all silages had a good lactic fermentation), except for subtle differences in NDF concentration in silages treated with both enzymes. The impact of hybrid and phenological stage on digestion parameters was significant, as they exhibited notable differences in plant composition (*i.e.,* proportions of leaves, stems, and panicles DM) and in their chemical and nutritional characteristics in pre- and post-ensiling materials. However, under laboratory-controlled conditions (Experiment 2), fibrolytic and proteolytic enzymes hydrolysed sorghum plant structures, although FS was more susceptible than DPS. The enzymatic action on cell wall carbohydrates was greater in FS, with no detectable effects in DPS. This response was mainly associated with changes in the pectin fraction, as evidenced by the apparent solubilization of galactose. A differential effect on the pectin fraction was observed between the fibrolytic and proteolytic enzymes, as suggested by variations in rhamnose concentration. These findings also suggest that further work on enzyme formulation, application rate and timing is needed before recommending exogenous enzymes for routine use in sorghum silages.

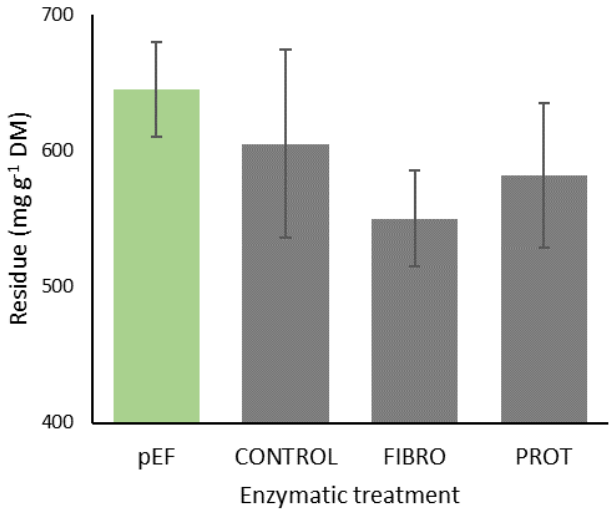

Figure 3. Exogen enzymes' effect on forage and double-purpose sorghum at hard grain stage measured as recovered residue after 24 h of enzyme activity. pEF: original before ensiling fresh forage, Ctrl: control (treatment without enzymes), PROT: Treatment with protease and FIBRO: Treatment with fibrolytic enzyme. Vertical bars indicate standard deviation. Orthogonal contrasts: pEF vs Ctrl+FIBRO+PROT (*P*=0.0005); Ctrl vs FIBRO+PROT (*P*=0.03); FIBRO vs PROT (*P*=0.12).

Figure 3. *Efecto de las enzimas exógenas sobre el sorgo forrajero y doble propósito en estado de grano duro medido como residuo recuperado luego de 24 h de actividad enzimática. pEF: forraje fresco original previo al ensilado, Ctrl: control (tratamiento sin enzimas), PROT: tratamiento con proteasa y FIBRO: tratamiento con enzima fibrolítica. Las barras verticales indican el desvío estándar. Contrastes ortogonales: pEF vs Ctrl+FIBRO+PROT (P=0,0005); Ctrl vs FIBRO+PROT (P=0,03); FIBRO vs PROT (P=0,12).*

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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, 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). *Fernández PV*: Chemical carbohydrate fractionation analysis, Writing (original draft and review & editing).

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