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Effect of Length of Storage and Chemical Additives on the Nutritive Value and Starch Degradability of Reconstituted Corn Grain Silage

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Abstract: The aim of this study was to evaluate the feed quality of reconstituted corn grain silage (RCGS), treated with chemical additives and stored for 15, 30, or 60 d in 5-L plastic buckets. Dry ground corn was rehydrated to 350 g·kg^{-1} and treated with either polysorbate 80 (2 L·t^{-1}) (POL), propionic acid 28% (2 L·t^{-1}) (PRO), Mycoflake™ (2 L·t^{-1}-blend polysorbate 80 and propionic acid) (MYC) or nothing (CON). The effect of the length of storage was combined in a factorial arrangement with the additives. Ammonia-N increased from d-15 of storage. A treatment × storage length interaction was observed for ethanol content at d-60 of storage, and all treatments had lower ethanol concentration than CON. There was an interaction for butyric acid content at d-30 and d-60 of storage; CON showed higher butyric acid concentration than treated silages. Aerobic stability increased from d-15 to d-30. At d-15 of storage, the PRO and MYC treatments decreased the DM losses. The length of storage increased the ruminal in situ degradability of starch, and DM and MYC increased the DM degradability in 3.6 percentage units at 12 h of incubation, compared with POL. In conclusion, increasing the length of storage of the RCGS from d-15 to d-60 improved the starch and DM degradability. Mycoflake increased the availability of nutrients, and the length of storage enhanced the aggregation of particles; further, polysorbate 80 (Tween 80) might be further studied as a potential antimicrobial agent in silages.

Keywords: length of storage; chemical additives; reconstituted corn grain; organic acid; polysorbate 80; Mycoflake; starch degradability

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1. Introduction

Mature corn hybrids with high density and vitreousness have a low ruminal digestibility of starch [1]. The protein matrix in the grain rich in zein-proteins is a physicochemical barrier for starch digestion by ruminal microorganisms [2]. The rehydration and ensiling of corn grain are processing methods capable of improving the starch availability, due to the proteolysis of zein proteins, which could allow for a greater surface area for rumen bacteria [3,4]. Therefore, providing dairy cows with a more efficient feed performance, by decreasing dry matter intake and without altering milk production, is of great importance [5].

Organic acids, such as propionic acid, presents great antifungal properties in its undissociated form, being extremely dependent of the pH [6]. All weak acid-based additives have a common mode of action as antimicrobial agents. The undissociated acid diffuses through the microbial membrane and, once inside of the cytoplasm (pH close to neutrality), changes to its dissociated form; therefore, it acts as an antimicrobial agent by altering the

pumping of protons out of the cell, through the H^+-ATPase pump [6,7]. The accumulation of protons inside the cell can lead to an inhibition of glycolysis, of the active transport and an intervention into the transduction of the signal [7].

Typically, propionic acid is a fermentation product of the metabolism of propionic acid bacteria, and its content in silages is low. However, it can be formed from the reduction in lactic acid under low pH, and by the deamination and decarboxylation of amino acids by *Clostridium propionicum*. Strains of *Lactobacillus diolivorans* have also been associated with the production of propionic acid and 1-propanol from 1,2-propanediol [8]. Previous studies have reported the greater effectiveness of propionic acid base additives, with increasing application rate in corn silage [9] and in high moisture corn silage (HMC); this is utilized by controlling yeast growth, ethanol production and improving the aerobic stability [10].

Surfactants are substances that reduce the surface tension between oil–water or water–oil, increasing emulsification and emulsion stability, and are widely used in the food and pharmaceutical industries [11]. These compounds have lipophilic and hydrophilic properties in the same molecule [12]. The synthesis of polysorbates occurs via the polymerization of ethylene oxide to the sorbitan fatty acid esters and nonionic surfactants; polysorbate 80 (Tween 80) has a hydrophilic group formed by oxyethylene chains, and a hydrophobic group mainly formed by oleic acid [13]. Synthetic surfactants are included in the food and may influence its structure through physical mechanisms; for instance, the interaction between amylose and amylopectin chains in the starch is present in several basic foods [14]. In wheat starch, nonionic surfactants demonstrated the ability to form a strong surfactant–amylose complex that is difficult to dissociate [15]. There are limited studies approaching feed quality in the presence of synthetic surfactants. A survey with lambs feed, comprising reconstituted corn grain silage (RCGS) treated with Mycoflake™ and stored for 50 d, showed changes in the fermentative profile of silages; there was a lower content of lactic acid, butyric acid and ethanol. However no improvement was reported in animal performance or ruminal dry mater digestibility [16].

To shed light on this new potential antimicrobial agent for silage, we evaluate the effect of treatment with propionic acid, polysorbate 80, or a propionic acid and polysorbate 80 mixture on reconstituted corn grain silage stored for either 15, 30 or 60 d. We investigated the chemical composition, fermentation characteristics, the ruminal in situ degradability and the aerobic stability of silages. The hypothesis of our study is that the propionic acid treatment would lead to the enhanced aerobic stability of RCGS, whereas the addition of polysorbate 80 might increase the permeability; this would allow the absorption of moisture earlier by the ground grain and, therefore, increase the ruminal starch degradability at a shorter storage time.

2. Materials and Methods

2.1. Silage Production and Treatments

The experiment was conducted at the Department of Animal Science at the "Luiz de Queiroz" College of Agriculture at the Sao Paulo University (Piracicaba, São Paulo, Brazil). Grains were ground before ensiling, by using a hammer mill with a 5-mm screen. A subsample was dried at 105 °C for 12 h in a forced-air oven for dry matter (DM) estimation. Dry ground corn grain was mixed with distilled water manually for 20 min to achieve a moisture content of 35%.

The rehydrated corn grain (35% of moisture) was split into 4 piles (44.3 kg per pile) per treatment, and each pile was treated with the following: (i) without additive (control); (ii) polysorbate 80 (2 L t^{-1}) (Tween80); (iii) propionic acid 28% (2 L t^{-1}); and (iv) Mycoflake™ (2 L t^{-1}) according to the manufacturer's recommendations. Mycoflake is a blend of polysorbate 80 and propionic acid (Kemin South America, Indaiatuba, SP, Brazil). The effect of the length of storage (15, 30, or 60 d) was also evaluated and combined in a factorial arrangement with the additives. Additives were previously diluted into distilled water used for reconstitution (88.6 mL), and the control received the same amount of

water (88.6 mL). Samples of rehydrated corn grain (n = 4) were collected before ensiling to characterize the dry matter content achieved for treatments (Means DM = 63.6 ± 0.16%).

The experiment consisted of 12 treatments (4 additives and 3 lengths of storage) and 48 mini silos (4 replications per treatment). Silos were weighed after filling and at the opening to determine the DM recovery. After the opening, the silages were mixed and samples were collected for the analysis of the chemical composition, ruminal in situ starch degradability, aerobic stability, and fermentation profile. The chemical composition of the forage before ensiling is presented in Table 1.

Table 1. Dry matter content and chemical composition of fresh reconstituted corn grain (n = 4).

Item	Mean	SD [1]
Dry matter, % as fed	63.62	0.16
Crude protein, % of DM	8.68	0.42
Neutral detergent fiber, % of DM	9.39	1.24
Acid detergent fiber, % of DM	2.57	0.31
Ether extract, % of DM	3.14	0.15
Ash, % of DM	1.20	0.06
Starch, % of DM	66.8	2.89
Soluble protein, % of CP	16.8	3.39

[1] Standard deviation.

2.2. Chemical Composition and Fermentation Profile of RCGS

Silage samples were dried in a forced ventilation oven for 72 h at 55 °C and then ground through a 1-mm screen (Wiley mill, Arthur H. Thomas, Philadelphia, PA, USA). Sub-samples were analyzed for DM (method 934.01), ash (method 942.05), and ether extract (EE; method 920.39), respectively [17]. The ash-free neutral detergent fiber (NDF) was analyzed by filtration in a porous crucible with sodium sulfite and heat stable amylase [18]. The ash-free acid detergent fiber (ADF) was analyzed by filtration in a porous crucible [18]. The starch content was determined via an enzymatic method [19]. The crude protein (CP) was measured by the Dumas method (Leco® FP-2000A nitrogen analyzer; Leco Corp., St. Joseph, MI, USA) [20]. The soluble protein (% of CP) was estimated by submitting the samples to a borate phosphate buffer bath at 39 °C for 1 h and via filtration on a Whatman™ N° 541 filter paper. The soluble protein content was estimated, based on the difference between the silage sample CP content and the residual CP content of the filtered sample.

The aqueous extract was prepared with a fresh subsample of silage (25 g), mixed with 225 mL of distilled water in a stomacher (Lab-Blender Stomacher®, Nova Ética Produtos e Equipamentos Científicos Ltd.a., Vargem Grande Paulista, Brazil) for 4 min. The pH of the extract was measured using a pH meter (TEC-7, Tecnal Equipamentos, Piracicaba, Brazil). The extract was filtered through 2 layers of cheesecloth and centrifuged at 10,000× *g* for 15 min. The supernatant was used to determine the lactic acid [21] and the ammonia-N content [22]. Concentrations of volatile fatty acids, alcohols and esters were determined by gas chromatography with a mass spectrophotometer detector (GCMS QP2010 Plus; Shimadzu, Kyoto, Japan) using a capillary column (Stabilwax; Restek, Bellefonte, PA, USA; 60 m length, 0.25 mm outside diameter, 0.25 µm film thickness). The silage DM content was corrected for volatile compounds (DMcorr) using the equation of Weissbach [23]: DMcorr (% as fed) = oven DM (% as fed) + n-alcohols (% as fed) + 2,3-butanediol (% as fed) + 0.95 × volatile fatty acids (% as fed) + 0.77 × 1,2-propanediol (% as fed) + 0.08 × lactic acid (% as fed).

2.3. Kernel Particle Size Distribution and Ruminal In Situ Starch Degradability of RCGS

The kernel particle size distribution was measured using approximately 400 g of dried sample in a Ro-Tap Shaker (Bertel® Ltd.a., Caieiras, Brazil), equipped with five screens of 4.75, 3.35, 2.36, 1.70, 1.18, 0.59 mm and a bottom pan. The geometric mean particle size (GMPS; µm) and surface area ($cm^2 \cdot g^{-1}$) were determined by log normal distribution [24].

To determine the ruminal in situ starch and DM degradability, 6 g of dried sample was placed in nylon bags (10 cm × 20 cm) with 50 ± 10 µm of porosity (model R1020, Ankon, Macedon, NY, USA). The bags were incubated in 2 rumen-cannulated Holstein dairy cows fed a total mix ration (TMR) containing corn silage (59.1% of DM), citrus pulp (12.6% of DM), dry ground corn (11.2% of DM), soybean meal (13.9% of DM), mineral premix (2.5% of DM) and urea (0.7% of DM). Two replicates from each silo were incubated in each cow for 12 h. After incubation, the bags were removed and put in ice-cold water to stop the fermentation, and then washed in a washing machine. The bags were dried at 55 °C for 72 h in a forced ventilation oven and weighed. After drying, the residues obtained from duplicate samples (two rumen-cannulated cows) were ground through a 1-mm screen mill (Wiley mill, Arthur H. Thomas, Philadelphia, PA, USA) to determine the starch content. Blank bags (without sample) were also incubated, to allow for the correction for particle contamination. The weights of the blank bags were subtracted from the bags containing sample. The ruminal in situ DM degradability was calculated based on the initial DM and the residual DM, and starch degradability was based on initial starch and residual starch. The animal experimental procedure was approved by the ethic committee and animal welfare of the university (protocol: 2018.5.1093.11.4).

2.4. Aerobic Stability Test

One subsample of each silo was used to evaluate aerobic stability (AS). The silage sample (2 kg) was kept in a 20-L bucket and its temperature was measured every 30 min over ten days via dataloggers (Tag Temp, NOVUS Produtos Eletrônicos Ltd., Canoas, Brazil) inserted in the silage mass center. Aerobic stability was calculated as the time needed to achieve 2 °C above the ambient temperature (23 ± 1 °C, basal line) after silo opening [25].

2.5. Statistical Analysis

Data were analyzed using the MIXED procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC, USA), as a completely randomized design with a factorial arrangement of treatments: 4 additives × 3 storage lengths, and 4 replicates per treatment. The model was as follows: $Y_{ij} = \mu + A_i + T_j + AT_{ij} + e_{ij}$, where μ = overall mean, A_i = fixed effect of additive (i = CON, POL, PRO or MYC), T_j = fixed effect of storage length (j = 15, 30, or 60 d) as repeated measure, AT_{ij} = interaction between additive and length of storage, and e_{ij} = error. A mini silo represented the experimental unit. The covariance structure for the repeated measurements was chosen based on Akaike's information criterion among variance components (VC), compound symmetry (CS), first-order autoregressive (AR [1]) or the unstructured covariance structure (UN).

For the ruminal in situ degradability assay at 12 h, two cannulated cows were included as the random effect of blocks; the model was as follows: $Y_{ij} = \mu + A_i + T_j + B_k + A_iT_j + e_{ijk}$, where μ = overall mean, A_i = fixed effect of additive (i = CON, POL, PRO or MYC), T_j = fixed effect of storage length (j = 15, 30 or 60 d) as repeated measure, B_k = random effect of block (k = 1 and 2), A_iT_j = interaction between additive and length of storage, and e_{ijk} = error. All means were compared using Tukey's test at 5% significance. The statistical significance was considered at $p \leq 0.05$.

3. Results

3.1. Chemical Composition of Silage

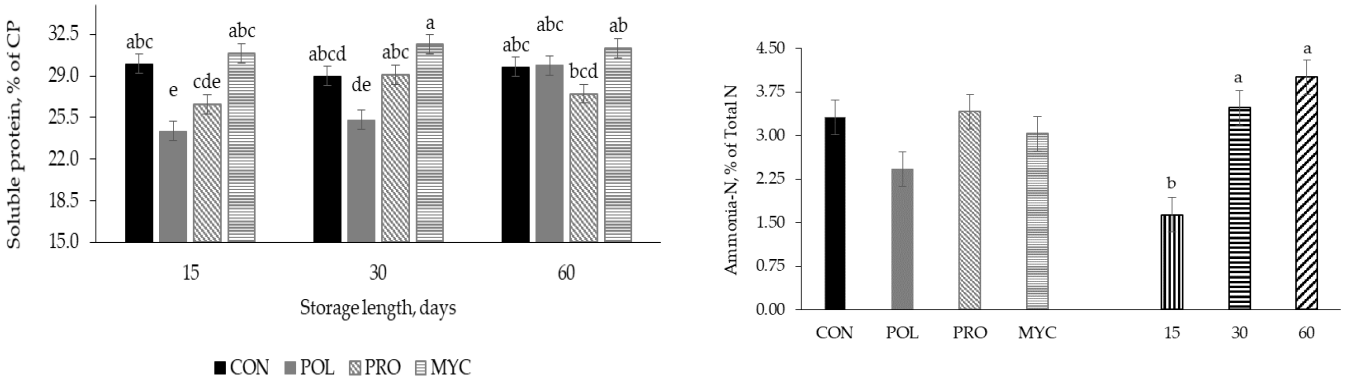
The DM content was higher ($p < 0.05$) at d-15 and d-30 than at d-60 of ensiling (Table 2). The CP content decreased ($p < 0.01$) over time and was lower in silages treated with MYC, compared to the other treatments ($p < 0.05$). The ash content was increased ($p < 0.01$) over time and silages treated with PRO had lower ($p < 0.01$) ash content than the other treatments.

Table 2. Chemical composition of reconstituted corn grain ensiled for 15, 30 or 60 d, without additive treatment (CON), or treated with polysorbate 80 (POL), propionic acid (PRO) or polysorbate 80 and propionic acid (MYC).

	Treatment					Storage Length				*p*-Value [3]		
Item	**CON**	**POL**	**PRO**	**MYC**	**SEM**	**15**	**30**	**60**	**SEM**	**A**	**S**	**A × S**
DM, [1] % as fed	63.1	63.2	63.6	63.3	0.14	63.7 [a]	63.4 [a]	62.9 [b]	0.12	0.12	<0.01	0.65
CP, [2] % of DM	8.46 [a]	8.40 [a]	8.40 [a]	7.88 [b]	0.05	8.53 [a]	8.27 [b]	8.06 [c]	0.04	<0.01	<0.01	0.15
Ash, % of DM	1.16 [a]	1.08 [ab]	0.92 [c]	1.05 [b]	0.03	0.98 [b]	1.06 [a]	1.12 [a]	0.02	<0.01	<0.01	0.09
Starch, % of DM	68.2	68.2	67.5	68.4	1.54	70.7	66.3	67.3	1.32	0.97	0.06	0.79

[1] Oven dry matter content; [2] Crude protein; [3] Effect of additive (A), storage length (S), and the interaction between additive and storage length (A × S). [a–c] Rows with different letters differ by Tukey's test ($p \leq 0.05$).

There was an interaction ($p < 0.01$) between the additive and storage length for the soluble protein content (Figure 1A). At d-15, POL showed a lower ($p < 0.05$) soluble protein content than CON and MYC, and after d-30 it also revealed a lower ($p < 0.05$) soluble protein content than MYC. After 60 d of ensiling, no differences were observed among treatments for soluble protein. Ammonia-N increased ($p < 0.05$) from d-15 of storage through the storage length studied (Figure 1B).



(**A**) (**B**)

Figure 1. Nitrogenous fractions of reconstituted corn grain silage ensiled for 15, 30 or 60 d, without additives treatment (CON) or treated with polysorbate 80 (POL), propionic acid (PRO) or polysorbate 80 and propionic acid (MYC). Statistical effects observed for (**A**) Soluble protein–Additive, $p < 0.01$; Storage, $p = 0.03$; effect of the interaction between additive and storage length (A × S), $p < 0.01$; SEM = 0.81, (**B**) Ammonia-N –Additive, $p = 0.06$, SEM = 0.27; Storage, $p < 0.01$, SEM = 0.28; A × S, $p = 0.08$. Different letters differ by Tukey's test ($p \leq 0.05$).

The length of storage and additives altered the kernel particle size distribution (Table 3). No particle of grain was retained in 4.75 and 3.35-mm sieves. The percentage of particles retained in 2.36 and 1.7-mm sieves increased ($p < 0.05$) for 30 and 60 d of storage. In the 1.18-mm sieve, there was an increase in the particles retained by 2.0 percentage units, for d-15 of storage compared with d-60. There was a rise in the percentage of particles in the 0.59-mm sieve at d-60 of storage, compared with d-15 and d-30 ($p < 0.05$). In the bottom pan, there was a decrease in the material retained at d-60, compared with d-15 of storage ($p < 0.05$). The MYC showed a lower percentage of grain retained in the 1.7 and 1.18-mm sieves, compared to the other treatments. In the 0.59-mm sieve, the PRO and MYC decreased the number of particles retained, regarding CON and POL. However, the percentage of material held in the bottom pan was higher ($p < 0.05$) for MYC than other treatments. An increase ($p < 0.05$) in the GMPS at silages stored for 60 d was observed, and there was a reduction ($p < 0.05$) in the MYC compared with the CON and POL treatments. A decrease in the surface area occurred in silages stored for 60 d, and the MYC presented a higher surface area ($p > 0.05$).

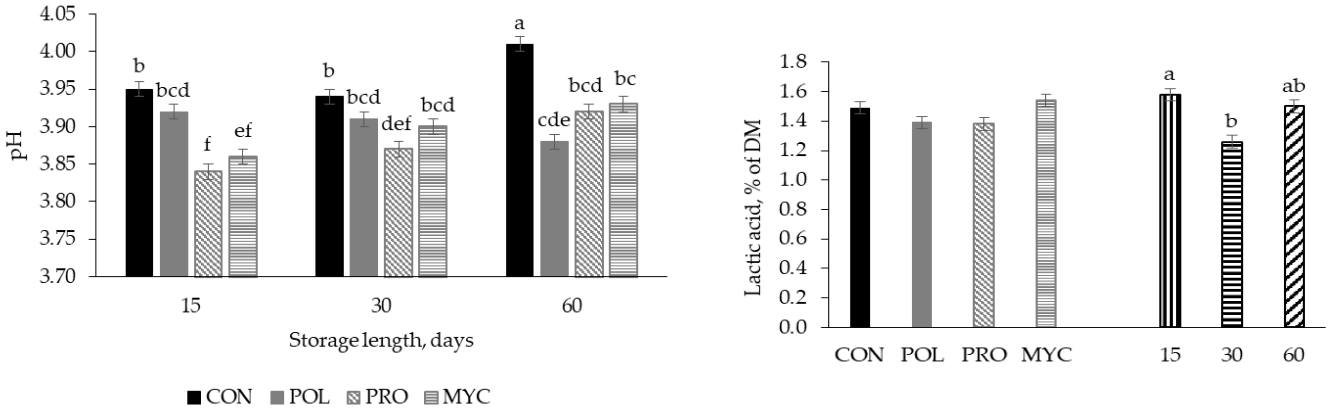
Table 3. Kernel fraction particle size distribution, geometric mean particle size (GMPS) and surface area of reconstituted corn grain ensiled for 15, 30 or 60 d, without additive treatment (CON), or treated with polysorbate 80 (POL), propionic acid (PRO) or polysorbate 80 and propionic acid (MYC).

	Treatment					Storage Length				*p*-Value [2]		
Item	**CON**	**POL**	**PRO**	**MYC**	**SEM**	**15**	**30**	**60**	**SEM**	**A**	**S**	**A × S**
Sieve, [1] mm												
2.36	1.7	1.7	1.7	1.6	0.07	1.5 ^{b}	1.8 ^{a}	1.8 ^{a}	0.06	0.56	<0.01	0.18
1.7	7.0 ^{a}	7.1 ^{a}	7.3 ^{a}	6.2 ^{b}	0.21	6.3 ^{b}	7.0 ^{a}	7.4 ^{a}	0.18	<0.01	<0.01	0.28
1.18	18.9 ^{a}	19.1 ^{a}	18.4 ^{a}	16.5 ^{b}	0.45	17.2 ^{b}	18.3 ^{ab}	19.2 ^{a}	0.39	<0.01	<0.01	0.15
0.59	44.9 ^{a}	44.8 ^{a}	43.3 ^{b}	42.4 ^{b}	0.37	43.2 ^{b}	43.4 ^{b}	44.9 ^{a}	0.32	<0.01	<0.01	0.40
Pan	27.2 ^{b}	26.8 ^{b}	29.4 ^{b}	33.3 ^{a}	0.92	31.6 ^{a}	29.3 ^{ab}	26.6 ^{b}	0.80	<0.01	<0.01	0.15
GMPS, µm	94.5 ^{a}	93.9 ^{a}	80.3 ^{ab}	60.8 ^{b}	5.64	69.1 ^{b}	79.5 ^{b}	98.5 ^{a}	4.89	<0.01	<0.01	0.09
Surface area, $cm^2 \cdot g^{-1}$	106.2 ^{b}	106.2 ^{b}	110.5 ^{ab}	116.5 ^{a}	1.59	113.8 ^{a}	110.6 ^{a}	105.1 ^{b}	1.38	<0.01	<0.01	0.07

[1] Percentage of particles retained in each sieve (DM basis); [2] Effect of additive (A), storage length (S), and the interaction between additive and storage length (A × S). ^{a,b} Rows with different letters differ by the Tukey's test ($p \leq 0.05$).

3.2. Fermentative Profile of Silage

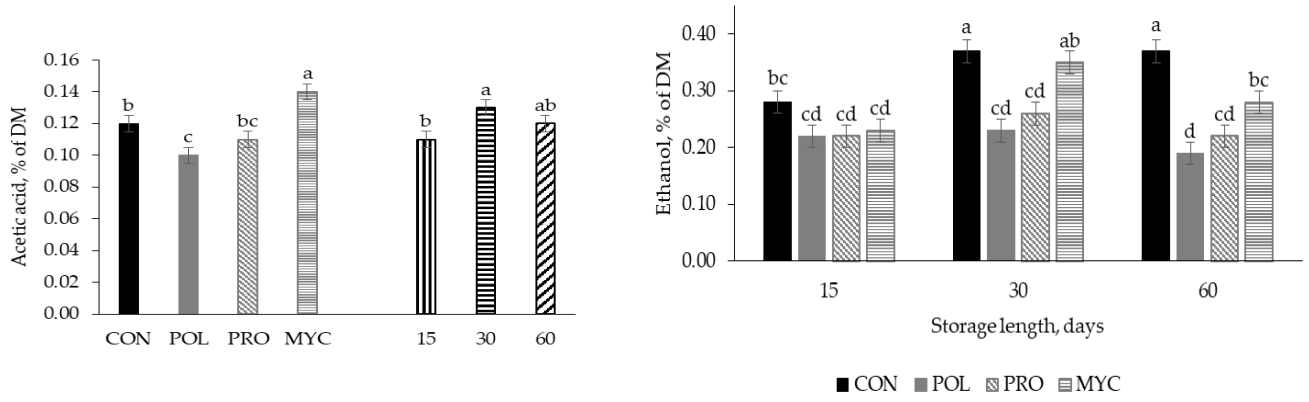
There was an interaction ($p < 0.01$) between the additive and storage length for pH (Figure 2A). At d-30 of ensiling, only PRO had a lower ($p < 0.05$) pH than CON, but after 60 d, all silages treated with additives had a lower ($p < 0.05$) pH than CON. The lactic acid concentration was higher ($p < 0.05$) at d-15 in comparison with d-30 of storage (Figure 2B).



(**A**) (**B**)

Figure 2. pH-value and lactic acid content of reconstituted corn grain silage ensiled for 15, 30 or 60 d, without additives treatment (CON), or treated with polysorbate 80 (POL), propionic acid (PRO) or polysorbate 80 and propionic acid (MYC). Statistical effects observed for (**A**) pH–Additive, $p < 0.01$; Storage, $p < 0.01$; A × S, $p < 0.01$, SEM = 0.01; (**B**) Lactic acid–additive, $p = 0.56$, SEM = 0.09; Storage, $p = 0.02$, SEM = 0.08; A × S, $p = 0.33$. Different letters differ by Tukey's test ($p \leq 0.05$).

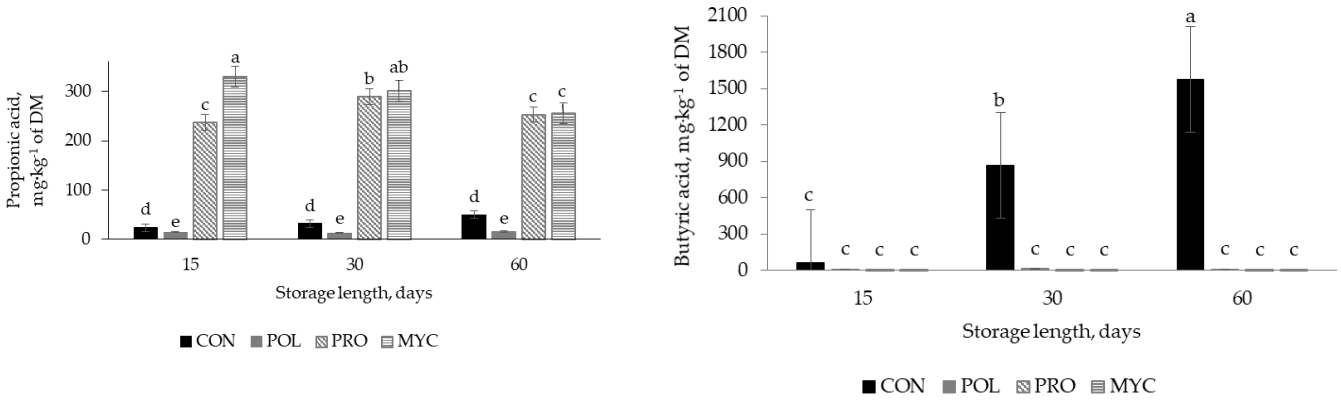
Figure 3 summarizes the values of acetic acid (3A) and ethanol (3B), respectively, an important antifungal agent and a product of yeast metabolism. The POL showed lower values, PRO showed the same and MYC had a higher concentration of acetic acid compared to CON ($p < 0.05$). There was an interaction ($p < 0.01$) between the additive and the storage length for ethanol content. At d-30, only POL and PRO had a lower ($p < 0.05$) ethanol concentration than CON, but at 60 d, all treatments, including MYC, had a lower ($p < 0.05$) ethanol concentration than CON.



(**A**) (**B**)

Figure 3. Acetic acid and ethanol contents of reconstituted corn grain silage ensiled for 15, 30 or 60 d, without additives treatment (CON), or treated with polysorbate 80 (POL), propionic acid (PRO) or polysorbate 80 and propionic acid (MYC). Statistical effects observed for (**A**) Acetic acid–Additive, $p < 0.01$, SEM = 0.004; Storage, $p < 0.01$, SEM = 0.003; A × S, $p = 1.00$; (**B**) Ethanol–Additive, $p < 0.01$; Storage, $p < 0.01$; A × S, $p < 0.01$, SEM = 0.02. Different letters differ by Tukey's test ($p \leq 0.05$).

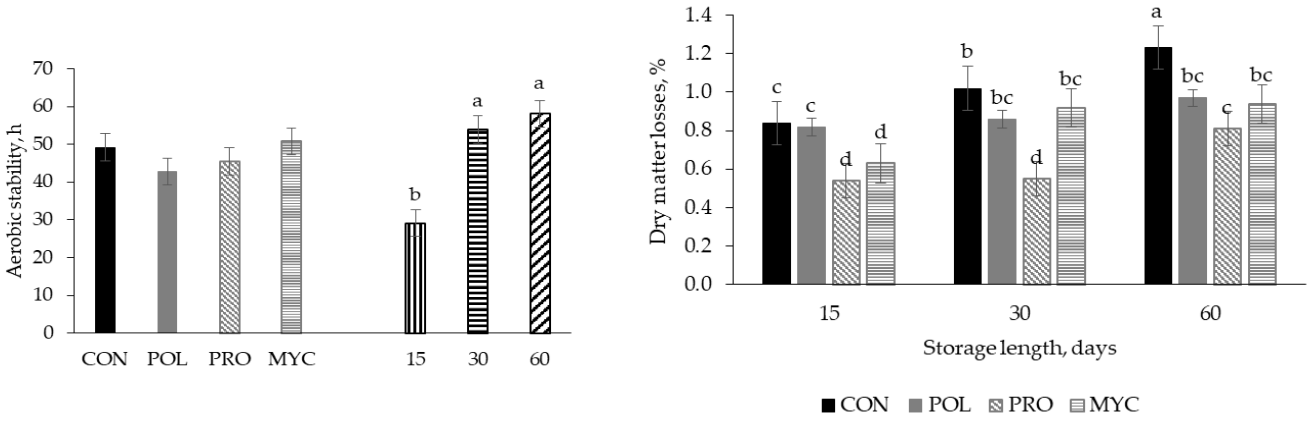
As expected, the propionic acid concentration was higher ($p < 0.05$) in silages treated with additives that contained propionic acid (PRO and MYC) than for those treated with CON and POL, at all lengths of storage analyzed (Figure 4A). A treatment vs. storage length interaction ($p < 0.01$) was observed for the butyric acid content. All silages stored for 15 d had a lower and similar butyric acid concentration, but at d-30 and d-60 of storage, the control silages had a higher butyric acid concentration than treated silages (Figure 4B).



(**A**) (**B**)

Figure 4. Propionic acid and butyric acid contents of reconstituted corn grain silage ensiled for 15, 30 or 60 d, without additives treatment (CON), or treated with polysorbate 80 (POL), propionic acid (PRO) or polysorbate 80 and propionic acid (MYC). Statistical effects observed for (**A**) Propionic acid–Additive, $p < 0.01$; Storage, $p < 0.01$; A × S, $p < 0.01$, SEM = 6.11; (**B**) Butyric acid–Additive, $p < 0.01$; Storage, $p < 0.01$; A × S, $p < 0.01$, SEM = 133.4. Different letters differ by Tukey's test ($p \leq 0.05$).

The aerobic stability of the RCGS increased ($p < 0.05$) from d-15 to d-30 d, but did not change from d-30 to d-60 (Figure 5A). At d-15 of storage, the PRO and MYC treatments decreased the DM losses. At d-30, the propionic acid was more efficient at controlling the losses in the silo, and at d-60 all treated silages presented a lower ($p < 0.05$) DM loss than CON (Figure 5B).

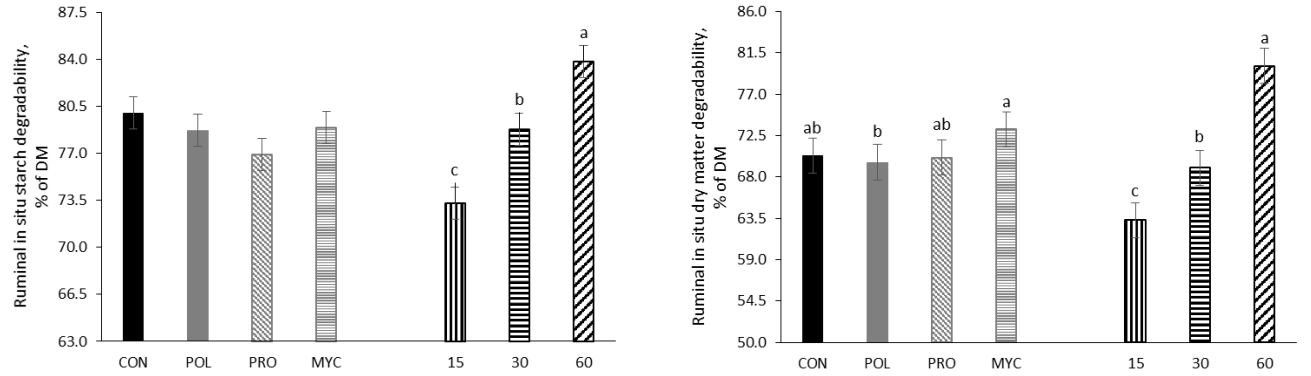


(**A**) (**B**)

Figure 5. Aerobic stability and dry matter losses of reconstituted corn grain silage ensiled for 15, 30 or 60 d, without additives treatment (CON), or treated with polysorbate 80 (POL), propionic acid (PRO) or polysorbate 80 and propionic acid (MYC). Statistical effects observed for (**A**) Aerobic stability–Additive, $p = 0.10$, SEM = 2.35; Storage, $p < 0.01$, SEM = 2.04; A × S, $p = 0.42$; (**B**) Dry matter losses–Additive, $p < 0.01$; Storage, $p < 0.01$; A × S, $p < 0.01$; SEM = 0.04. Different letters differ by Tukey's test ($p \leq 0.05$).

3.3. Ruminal In Situ Degradabitily

Silages stored for 30 and 60 d had a 6.98 and 12.5% higher ruminal in situ degradability of starch ($p < 0.01$), respectively, compared to silages stored for 15 d (Figure 6A). The MYC increased ($p = 0.02$) the DM degradability by 3.6 percentage units in 12 h of incubation, compared with POL (Figure 6B).



(**A**) (**B**)

Figure 6. Ruminal in situ starch degradability in 12 h of reconstituted corn grain silage ensiled for 15, 30 or 60 d, without additives treatment (CON), or treated with polysorbate 80 (POL), propionic acid (PRO) or polysorbate 80 and propionic acid (MYC). Statistical effects observed for (**A**) Ruminal *In situ* starch degradability in 12 h–Additive, $p = 0.07$, SEM = 1.03; Storage, $p < 0.01$, SEM = 0.94; A × S, $p = 0.71$; (**B**) Ruminal *In situ* dry matter degradability–Additive, $p = 0.02$, SEM = 0.89; Storage, $p < 0.01$, SEM = 0.78; A × S, $p = 0.60$. Different letters differ by Tukey's test ($p \leq 0.05$).

There was an increase in the DM degradability ($p < 0.01$) across the length of storage. Silages ensiled for 30 and 60 d improved the DM degradability by 5.7 and 16.8 percentage units when compared to silages ensiled at d 15, respectively.

4. Discussion

4.1. Chemical Composition of Silage

The DM content of silages decreased across the length of storage due to the typical cumulative increase in dry matter losses. The DM decrease is a result of carbon loss due to the oxidation through CO_2, as extensively reported in the literature. The disappearance of the most degradable carbohydrates and proteins explains the increase in ash content over time, being an effect of the concentration of the mineral fraction. The ash and CP fractions of silages can be altered by the composition of the chemical additive used [26]. Mycoflake™ presented ammonium compounds in its composition, despite their concentrations in the product not being disclosed [16]; this may contribute to an increase in the N content. Conversely, silage fermentation is prone to the proteolysis process; therefore, additives, which are mainly propionic-based salts, are supposed to hamper, at least in part, the trend of protein breaking down [2]. The changes in CP in this study need to be further evaluated, considering the N-fractions for a better understanding of this off-set effect.

The main contributors to proteolysis in reconstituted corn grain silage are bacteria (60%) and kernel enzymes (30%) [2]. Several factors affect the breakdown of the protein matrix surrounding the starch granule in the grain endosperm, such as the mechanical processing extent, the moisture content, and the storage length [1,27]. In the present study, the results of the soluble protein content were inconsistent, making it difficult to explain the proteolytic activity only based on the analyzed traits. However, ammonia-N is also an indicator of deamination during the fermentation process [28,29]. In this trial, the length of storage increased ammonia-N from d-15 of storage, as a result of the prolamins' disruption; this probably allowed microorganisms to better access starch granules. In addition, there are reports of a steady increase in ammonia-N content up to 240 d of storage in high moisture corn grain silage, accompanied by a decrease in prolamin-zein subunits [1,30]. In the present study, there was no treatment effect on the concentration of ammonia-N; again, this might be due to the compensation of ammonia compounds carried by additives, hindering the clear detection of the antiproteolytic effect of additives.

In general, the Mycoflake™ reduced the particle size and increased the surface area of the RCGS, while the length of storage increased the GMPS and affected the surface area, significantly resulting in more aggregation. However, we did not find, in the literature related to feed science, a reasonable explanation of how the chemical strategy and storage length changes the structure and the physical traits of grain silage. To the best of our knowledge, this is a significant finding that deserves to be explored further; this is because the changes in the structure of particles may alter the dynamics of conservation in silages, and possess the potential to influence the sorting behavior of animals.

Mature corn kernels typically have a low soluble carbohydrates content because the starch formation in kernel endosperm is completed [31]. The lactic acid bacteria (LAB) ferment soluble sugars, such as hexoses and pentoses, and yield lactic acid, a strong acid able to drop the pH of silages [32]. In the present study, silages were well fermented and pH variations were within the range commonly reported in the literature for well-preserved silages [29].

4.2. Fermentative Profile and Aerobic Stability of Silage

Silages containing propionic acid improved fermentation over CON. The use of propionic acid was previously reported as effective in reducing the pH of high WPCS and high-moisture corn silage, compared to untreated silage [9,10]. Although the acetic acid content was relatively low when compared to the typical data from whole plant corn silage, it is in agreement with the values reported for high-moisture corn silages [10,26]. Noteworthy is the increase in the acetic acid content at d-30 of storage; this is coincident with the lower lactic acid content at the same storage length, demonstrating a mechanism of action that might have converted lactic acid to acetic acid. This can be a defense mechanism of heterofermentative bacteria against a low pH, degrading lactic acid into a fatty acid with a higher pK_a [33].

The clear decrease in ethanol concentration from d-30 of storage in treated silages was most likely due to the effect of the propionic acid and polysorbate on the metabolism of the yeasts; under anaerobic conditions, these can ferment sugars and produce ethanol as the main fermentation product [34]. Previous studies reported that, in acidic conditions (pH 4.5), propionic acid was able to hamper the growth rate and efficiency of yeasts [5,35]. A food-grade study, evaluating the antimicrobial activity of a micro emulsion composed of glycerol monolaurate (GML), propionic acid, polysorbate 80 (Tween 80) and water (3:9:8:12) to prevent *Escherichia coli* and *Staphylococcus aureus*, showed that microbial cells were completely killed after the addition of the micro emulsion. The result was the same even in diluted micro emulsion 10-fold and 100-fold [36]. That experiment proved that bacterial membrane hydrophobicity was altered in the presence of the micro emulsion tested, and that there was extravasation of cytoplasmic cellular material. The micro emulsion altered the phospholipid bilayer, affecting the membrane permeability and leading to its rupture [36].

The same micro emulsion with sodium benzoate combination was evaluated for antimicrobial activity against *Candida albicans* and *Saccharomyces cerevisiae* in another study [37]. The results showed that 1.2 mg mL^{-1} was able to inhibit the growth of *C. albicans* and *S. cerevisiae*. However, it is important to clarify that the antimicrobial activity was only effective in the complete formulation (i.e., propionic acid, GML and sodium benzoate). Polysorbate 80 (Tween 80) did not demonstrate any microbial inhibition [37]. The treatment of RCGS with Mycoflake™ (2 $L{\cdot}t^{-1}$, stored for 50 days) was more effective than in the present study for reducing the ethanol concentration to less than a half [16]. Despite the scarcity of studies on surfactant molecules in feed conservation, the opportunity to further investigate their role in inhibiting microorganisms in silages is evident; this is because their effect on the decrease in ethanol concentration in RCGS is undisputable in the present study.

As expected, treatments that contained propionic acid in the formulation achieved a higher concentration of this acid across all the storage lengths tested. However, there is no reasonable explanation for the difference in the concentration between the PRO and MYC silages at d-15 of storage, since they had the same application rate and showed similar concentrations on d-30 and 60. A significant increase in butyric acid was observed in untreated silages stored for 30 and 60 d. Butyric acid is mainly a product of *Clostridium* fermentation when there is both a low DM and a low water-soluble carbohydrate concentration [38]. A study evaluating different hybrids, maturation and the storage length of reconstituted corn-grain silage, found high levels of butyric acid as the storage period increased from d-0 to d-120 [39]. The microbial profile demonstrated that the *Clostridium* population was already high compared to the *Lactobacillus* population at the ensiling moment (19.5 e 9.1%, respectively), and at d-120 of storage, the *Clostridium* population represented approximately 40% of the total microbial population [39]. The PRO, MYC and POL may have controlled *Clostridium* growth, since the butyric acid concentration was lower than the CON after 15 d of ensiling. It was demonstrated that propionic acid, with a pH range within 5 and 6, can prevent the endospore-forming bacteria [38,40].

The use of chemical additives in high-moisture corn silages (HMCS) has been shown to enhance the aerobic stability of silages, due to the efficient control of yeasts. Apparently, there is a linear increase in the aerobic stability, according to the dose of chemical additive applied within the range of 1 to 4 $g{\cdot}kg^{-1}$ of fresh matter [26]. However, no difference was observed across treatments in the present study. In order to control fungal growth and spoilage in feed with a high concentration of DM, the application of propionic acid equivalents, in a concentration ranging from 12.5 to 30.0 $g{\cdot}kg^{-1}$ of water, was suggested. In addition, as the water concentration increases, more acid is needed to inhibit the development of molds [41]. In the present study, the rate of application, based on the manufacturer recommendations, was 2 $L{\cdot}t^{-1}$ (28%). Considering the concentration of propionic acid found in this present study, the content was equivalent to 862 $mg{\cdot}kg^{-1}$ of DM (approximately 0.22% of DM). The lack of response in the aerobic stability might be attributed, at

least in part, either to the concentration of the acid propionic in the product, or the application rate adopted. Mycoflake™ was developed to treat grains before the flocculation (steam flaking) process and this type of processing requires less moisture than the RCGS. The use of the Mycoflake™ in feed conservation paves the way for future studies with higher acid concentrations or application rates, seeking to increase the stability of ensiled grains upon aerobic exposure. The improvement in aerobic stability after 15 d of storage was probably due to an increase in fermentation end-product levels; this includes acetic acid, which has well-reported antifungal properties.

Overall, the addition of propionic acid was efficient in reducing dry matter losses when compared to CON, probably because of the decreased ethanol content after d-15 of ensiling. The decrease in the DM loss might be a result of preventing sugars from being fermented by yeasts; this produces ethanol and CO_2 as the main fermentation products [32,38], resulting in a loss of DM. Although POL and CON had similar DM losses across all storage lengths studied, at d-60 of storage the untreated silage showed higher DM losses and POL; meanwhile, PRO and MYC had lower losses, suggesting the inhibition of yeasts. Due to the low experimental error and relatively good control of the variation throughout of the trial, DM losses were lower than traditionally reported in the literature [26,42]. For practical application, however, it may be distinguished that the treatments (POL, PRO and MYC) promoted a decrease of 29% (1.2 vs. 0.85%) in DM losses over the CON silages ($p < 0.01$).

4.3. Ruminal In Situ Degradabitily

Proteolysis indicators, such as the soluble protein and ammonia nitrogen content in grain silage, are positively correlated to in vitro starch degradability [2,31]. In the current study, higher DM and starch degradability were also observed over time, possibly as a result of increased proteolytic activity of silages stored for 30 and 60 d. Although many factors influence starch digestibility, the protein matrix breakdown is an important aspect that facilitates the access of enzymes to starch granules [1,2,30]. The ruminal in situ DM degradability is highly correlated to ruminal starch degradability, and may be a parameter to predict starch digestibility, since more than 70% of corn grain is composed of starch [3,43,44]. The treatment with Mycoflake™ increased the DM degradability, with a joint increase in the proportion of particles retained in the bottom pan, a reduction in the GMPS and an increase in the surface area. A negative quadratic relationship between the GMPS and ruminal DM digestibility, at different points of time in the incubation (3, 6, 12, and 24 h), was also reported in unfermented kernels; here, R^2 = 0.97 at 12 h of incubation [24]. The authors also reported a positive quadratic relationship between the surface area and ruminal DM degradability, at the same time as the trend in the incubation [24]. The fragmentation of corn and barley grains increased the enzymatic digestion of starch granules by rumen microorganisms [45]. These data suggest that there was a fragmentation of particles in silage treated with Mycoflake™, resulting in greater ruminal DM degradability. Conversely, silages stored for 60 d revealed a higher aggregation of particles, an increased GMPS and a decrease in the surface area; this was followed by a greater ruminal DM and starch degradability. These data suggest that the traits used to measure particle size do not fully explain the ruminal degradation, implying the need for more sophisticated structural analyses.

In a study evaluating the in vitro digestibility of corn starch gelatinized (normal and waxy starch) with different rates of polysorbate 80 addition, it was shown, by optical microscopy, that the surfactant molecule was adsorbed on the surface of starch particles, and that it possibly formed a surfactant–starch chain complex [14]. The in vitro digestibility analysis demonstrated that the fraction of rapidly digestible starch (RDS) and resistant starch (RS) tended to increase at the expense of a decrease in the fraction of slowly digestible starch (SDS); this was as the rate of polysorbate 80 increased. The effect was greater in the normal corn starch; this has a higher proportion of amylose than the waxy starch, suggesting that amylose may be related to the formation of these indigestible complexes. According to different studies, the increase in the resistant starch fraction can be due to

starch chain–surfactant complexes forming especially with amylose [14,15]. However, it is worth mentioning that both gelatinization and ensiling are processes that makes starch more available for digestion, even though they consist in completely different processes.

Research evaluating the in vitro effect of polysorbate 80 (Tween 80) and monensin on the ruminal fermentation of diets based on barley grain and barley silage, showed that polysorbate 80, applied alone (at 0.5 $\mu L \cdot mL^{-1}$), increased the DM digestibility and the accumulation of reducing sugars at 4 h of incubation [46]. Considering the results of the ruminal in situ DM degradability in the present study, the association between polysorbate 80 and propionic acid in the Mycoflake^{TM} could also have a positive effect on the nutrient availability; this might be studied in the future.

5. Conclusions

Increasing the storage length of reconstituted corn grain silages, from 15 to 60 d, enhanced starch and dry matter degradability. Mycoflake^{TM} increased the availability of nutrients, although it did not efficiently increase the aerobic stability of RCGS; it controlled the ethanol production and reduced DM losses in silages with a more advanced storage length. Additives and the storage length revealed distinct patterns of particle aggregation in grain silages; this needs to be further studied to predict the fate of nutrients in ruminant diets. Polysorbate 80 might be explored as a potential antimicrobial agent in silages. All treatments were effective in reducing DM losses across a 60-d storage-length period in reconstituted corn grain silages.

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