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Different dry matter contents on the fermentation and preservation of wilted ryegrass silage with or without microbial inoculants

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ABSTRACT - The objective of this study was to evaluate the effects of two different dry matter contents at ensiling on the chemical composition, fermentation products, microbial profile, and aerobic stability (AS) of wilted ryegrass (*Lolium multiflorum* L.) silages, with or without microbial inoculants. The ryegrass was wilted in the field until it reached 32 or 45% DM. In addition to DM contents, the effect of adding or not adding microbial inoculants to the forage was evaluated, resulting in the following treatments: Control - no additive (C); commercial inoculant - Magniva Platinum 2 (INO); and commercial inoculant plus enzymes - Magniva Platinum 3 (INO+E). The silos were stored for 62 days. In the 32% DM treatment, the inoculated silages showed higher DM content and lower pH than the control, indicating a more favorable fermentation profile. Silages at 32% DM exhibited lower pH (3.97), higher levels of lactic, acetic, and propionic acids, and a greater population of LAB. The lower DM treatments also showed reduced DM and gas losses, absence of effluent, and lower DM losses during aerobic exposure. The inoculants increased lactic acid production and decreased propionic and butyric acid concentrations. At 32% DM, INO and INO+E inoculants significantly improved *in vitro* estimates of DM, OM, and NDF digestibility. Yeast and mold counts were low (<10-4), with no colony development at the lowest dilution tested. Total DM (8.6 g kg^{-1} DM), gas (8.1 g kg^{-1} DM), and effluent losses (0.51 kg/ton wet basis) were minimal across all treatments. At 32% DM, silages treated with INO presented negative values for gas and DM losses. AS was high for all silages (353 and 328 h for treatments 32 and 45% DM, respectively), with greater stability observed in 32% DM treatments. The inoculants enhanced AS in 45% DM silages and reduced DM losses over 20 days of aerobic exposure. The 32% DM harvest led to better quality silages. Microbial inoculants were effective in improving fermentation and preservation of ryegrass silages by reducing fermentative losses at 32% DM and enhancing aerobic stability at 45% DM.

Keywords: aerobic stability, chemical composition, cool-season forage, forage conservation, pH

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1. Introduction

Ryegrass is a cool-season grass widely used in ruminant feeding in southern Brazil (Cauduro et al., 2007), and in temperate and subtropical regions such as the United States, China, and Korea (Liu et al., 2020). The ensiling of grasses, such as ryegrass, can be a viable alternative for forage preservation

during periods of scarcity or adverse weather conditions (Uslu et al., 2017). However, at the ideal stage of cutting for ensiling, these grasses generally have high moisture content (Borreani et al., 2018). In this context, field wilting emerges as an effective strategy to adjust the dry matter (DM) content of high-moisture forages at the time of ensiling.

The DM content of the forage has a direct influence on the silage fermentation process and, consequently, on its quality. Values below 30% DM, when associated with low concentration of soluble carbohydrates in grass silages, may favor undesirable fermentation such as those caused by microorganisms of the genus *Clostridium* (Muck et al., 2018), resulting in greater fermentative losses, reduced DM content, and excessive effluent production (Queiroz et al., 2018). In this context, wilting forage grasses prior to ensiling may help adjust the DM content and reduce losses. However, after cutting, cellular activities such as respiration continue to occur (Melo et al., 2023). Thus, the time between harvesting and silo sealing can influence the availability of substrates in the plant material, which are essential for proper fermentation.

The application of microbial additives during the ensiling process can enhance fermentation, especially under more challenging conditions (Liu et al., 2020), such as those encountered in wilted forage silages. The use of inoculants containing beneficial microorganisms that promote both fermentation and silage stability (Muck et al., 2018) can be an effective strategy for ensiling wilted ryegrass. Heterofermentative inoculants containing strains of *Lentilactobacillus buchneri* and other heterolactic bacteria, as the recently commercially available *Lentilactobacillus* hilgardii (Ferrero et al., 2021), act on the forage by promoting greater production of lactic and acetic acids, rapid pH decline, and reduced post-harvest proteolysis (Liu et al., 2020), potentially contributing to the prevention of heating and nutrient deterioration in wilted grasses.

Several studies in the literature have reported the use of enzymes in animal feeding with the aim of improving nutrient utilization by animals (Beauchemin et al., 2003; Antonio et al., 2018). Xylanase, for example, hydrolyzes glycosidic bonds, breaking them into simpler sugars and consequently increasing feed digestibility (Beauchemin et al., 2003). The combined use of xylanase and β-glucanase in animal diets has been shown to improve the digestibility of hay from different grasses (Antonio et al., 2018). In the forage preservation process, the use of enzymes such as xylanase and β-glucanase, in combination with the benefits of microbial inoculants, may enhance sugar release from the forage, promoting efficient fermentation, and contributing to greater stability and preservation of the ensiled material.

Thus, the objective of this study was to evaluate the effects of two different DM contents at ensiling, with or without the addition of commercial microbial inoculants, on the fermentation of wilted ryegrass (*Lolium multiflorum* L.) silages.

2. Material and methods

2.1. Ensiling and laboratory analyses

The research was performed by The Center of Forage Research (CPFOR) of Universidade Federal do Paraná (UFPR), in Curitiba (25°25'42" S and 49°16'24" W), located in the state of Paraná, Brazil. Ryegrass (cv. F ABC 1) was seeded on June 14, 2021, at a commercial farm, in Castro (24° 47' 32" S and 50° 0' 42" W), located in the state of Paraná, Brazil. On the evening of August 20, the forage (211 ± 0.12 g kg^{-1} of DM) was manually cut (10 cm above ground; no chopping) and kept in the field for wilting. The forage was manually spread and turned three times per day using metal forks. After 24 (32% DM) or 48 (45% DM) hours of field wilting, forage was manually harvested and immediately chopped using a tractor-powered forage chopper (10-mm particle size). The maximum time elapsed between chopping and sealing for all the silos, at each DM, was three hours.

At each harvesting (32 or 45% DM), chopped forage was sampled (in duplicate) for DM content, chemical composition (Table 1) and microbial counts. The forage was divided into three piles (40 kg each) for treatment application as follows: Control (C - 2 L t^{-1} of distilled water); commercial inoculant (Magniva

Platinum 2) (INO) - *Lentilactobacillus hilgardii* CNCM I-4785 1×10^5 CFU g^{-1} forage, *Lentilactobacillus buchneri* NCIMB 40788 1×10^5 CFU g^{-1} forage, and *Pediococcus pentosaceus* NCIMB 12455 1.0×10^5 CFU g^{-1} forage; and commercial inoculant containing enzymes (Magniva Platinum 3 (INO+E) - *L. hilgardii* 7.5×10^4 CFU g^{-1} forage, *L. buchneri* 7.5×10^4 CFU g^{-1} forage, and *P. pentosaceus* 1×10^5 CFU g^{-1} of forage, plus enzymes: β-glucanase (5.750 UI g^{-1} of product) and xylanase (30.000 UI g^{-1} of product). Thus, the two DM contents were considered the main factor of comparison, combined with the three treatments with or without the addition of inoculants. Therefore, the experimental design consisted of a 3 × 2 factorial arrangement.

Both the inoculants (Lallemand Animal Nutrition®) were weighed (1.00 g) and diluted in 2 L of distilled water, and 80 mL was taken for application. Additives were sprayed onto the forage and carefully homogenized. Piles were kept 2 m apart from each other, and all procedures were followed to avoid cross-contamination (disposable gloves, boots, plastic sheets, buckets, and sprayers; an exclusive team working on each treatment). All the treatments were ensiled simultaneously to avoid the effect of exposure time on the variables.

Treated forage was weighed again to compose each replicate (4.0 or 3.5 kg per silo, for the 32 and 45% DM treatments, respectively). Thirty PVC silos (five silos per treatment) of 8.8 L (15 cm diameter × 50 cm height) equipped with effluent collecting devices (ECD) were filled with 3.94 ± 0.04 kg (32% DM) or 3.45 ± 0.02 kg (45% DM) of forage to reach 145 or 176 kg DM m^{-3}. A pneumatic press was used to compact the forage. The surfaces of the press were cleaned when changing treatments by spraying 70% ethanol. After filling, each silo was hermetically sealed using a silicone ring and flexible sealant glue.

Table 1 - Dry matter, pH, chemical composition and microbial counts of wilted ryegrass silages (*Lolium multiflorum* L) with or without inoculants

Variable	Harvest[1]	
	32% DM	45% DM
pH	6.35	6.29
DM 60 °C (g kg^{-1})	324	450
DM 105 °C (g kg^{-1})	351	485
Crude protein (g kg^{-1} DM)	157	170
NDF (g kg^{-1} DM)	572	585
ADF (g kg^{-1} DM)	318	324
Ash (g kg^{-1} DM)	104	104
IVDMD (%)	76.9	74.3
IVOMD (%)	77.5	74.9
IVNDFD (%)	63.6	58.1
Total LAB (log CFU g^{-1})	3.78	3.70
Enterobacteria (log CFU g^{-1})	7.30	7.30
Yeasts (log CFU g^{-1})	5.17	4.97
Molds (log CFU g^{-1})	3.85	3.78

NDF - neutral detergent fiber; ADF - acid detergent fiber; IVDMD - *in vitro* digestibility of dry matter; IVOMD - *in vitro* digestibility of organic matter; IVNDFD - *in vitro* digestibility of neutral detergent fiber; LAB - lactic acid bacteria; CFU - colony forming units.
[1] DM - Dry matter content at 60 °C.

2.2. Silo opening

The silos were opened after 62 days, on October 23. Each silo was weighed again, and the silage was transferred to a polyethylene bag, thoroughly homogenized, and sampled to assess pH, DM content, chemical composition, volatile organic compounds (VOC), yeast and mold counts, lactic acid bacteria

(LAB), and enterobacteria counts. Dry matter was determined after 72 h in a forced-air oven at 60 °C, and samples were used for further analysis. For pH determination, 25 g of silage was mixed with 225 mL of distilled water for 1 min, and pH was determined using a digital pH meter (PG 1400, Gehaka, Brazil).

For microbial counts, 25 g of silage was mixed with 225 mL of Ringer solution for four minutes at 150 rpm in a Stomacher Lab-Blender, according to Kung and Ranjit (2001). The extracts were filtered through a three-layer gauze. Dilutions were made up to 10^{-7} in MRS broth for LAB, and up to 10^{-6} in Ringer solution for yeasts and molds. One milliliter of the 10^{-4}, 10^{-5}, and 10^{-6} dilutions was plated on Petrifilm® LAB 6461 for LAB counts. One milliliter of the 10^{-2} dilution was plated on Petrifilm® EB for enterobacteria counts. One milliliter of the 10^{-4}, 10^{-5}, and 10^{-6} dilutions was plated on Petrifilm® YM for yeasts and mold counts. According to Petrifilm microbiology manual, enterobacteria counts were performed after 24 h of incubation at 28 °C. LAB counts were performed after 48 h of incubation at 31 °C. Yeast and mold counts were performed after 72 and 120 h, respectively, at 23 °C, in a BOD incubator.

Estimates of fermentative losses were obtained by weighing empty sets (empty silo + lid + ECD), full silos after ensiling, silos right before opening, and wet sets (silo after silage removal + lid + ECD + effluent). Silages were sampled (in duplicate from each silo) to determine the DM content (forced-air oven drying at 60 °C for 72 h), to estimate DM losses (DML), gas losses (GL) and effluent losses (EL) according to Jobim et al. (2007).

The chemical analyses were performed at the Animal Nutrition Laboratory of ESALQ-USP (ESALQlab), in Piracicaba, SP, Brazil. The samples collected before and after the ensiling periods were dried in a forced-air oven at 60 °C for 72 h and then individually ground through a 1-mm screen in a Willey mill (Model No. 2, Arthur H. Thomas Co., Philadelphia, PA). Crude protein (CP) was determined by the Dumas method (FP-528, LECO combustion nitrogen analyzer, LECO Instruments Inc., St. Joseph, MI), according to Wiles et al. (1998), ash was determined according to method 924.05 (AOAC, 2012), and NDF and ADF were analyzed sequentially (without α-amylase) according to Mertens (2002), as modified for use with the Ankom A200 Fiber Analyzer (Ankom Technology, Macedon, NY). The *in vitro* digestibility of dry matter (IVDMD), organic matter (IVOMD), and NDF (IVNDFD) was determined according to Tilley and Terry (1963) and adapted to the ANKOM system (Holden, 1999). The ash correction was obtained after 4 h in the furnace at 600 °C.

The concentrations of ethanol, acetic acid, propionic acid, butyric acid, and 1,2-propanediol in silages were determined by gas chromatography–mass spectrometry (GCMS QP 2010 Plus, Shimadzu, Kyoto, Japan) and a capillary column (Stabilwax, Restek, Bellefonte, PA; 60 m, 0.25 mm ID, 0.25 µm), according to Erwin et al. (1961). Lactic acid was quantified using the colorimetric method according to Pryce (1969). No correction for volatile compounds was performed. Chemical and VOC variables are presented on 105 °C DM corrected basis. Loss-related variables are presented on 60 °C DM basis.

2.3. Aerobic stability

For assessing aerobic stability, 2 kg of silage were loosely stored in a plastic bucket (20 L) for 20 days (480 h) in a temperature-controlled room (24.5 ± 1.4 °C). One data logger (EL-USB, Lascar, England) was placed in the center of each bucket to record temperature every 30 min. Aerobic stability (AS) was considered as the time (h) for silage temperature to reach 26 °C (about 2 °C above the average room temperature). The maximum temperature (Tmax) reached by the silage was also recorded. Side containers (3.6 L) were filled with the remaining silage from each replicate (~700 g) and kept in the same room for pH determination on days 0, 2, 4, 6, 8, 10, and 20 after aerobic exposure. These silages were sampled at the center of the mass. After 10 days, the silos were weighed again and sampled (in duplicate) to assess the DM content to estimate DM losses during AS (DMLas).

2.4. Statistical analysis

Statistical analysis was performed using the ExpDes.pt package of R software, as a completely randomized design in a 3 × 2 factorial arrangement (3 inoculants and 2 DM contents) with five

replicates, totaling six treatments and 30 experimental units (silos). Data were submitted to analysis of variance, and the basic assumptions of normality (Shapiro-Wilk test) and homogeneity of variance (Bartlett's test) were preliminarily tested. The means of those variables that met the basic assumptions and showed significant differences ($P<0.05$) for any factor or interaction were compared by Tukey's test. The statistical model used was

$$Y_{ijk} = \mu + D_i + I_j + (DI)_{ij} + e_{ijk},$$

in which μ = general mean; D_i = effect of the i-th level of the DM; I_j = effect of the j-th level of the inoculant factor, $(DI)_{ij}$ = effect of interaction between in the i-th level of the DM factor and the j-th level of the inoculant factor, e_{ijk} = experimental error.

For the variables that did not meet the basic assumptions (gas losses, DM losses, ADF, ash, IVNDFD, AS, HTmax, pH on days 2, 4, 6, 8 and 10 of aerobic exposure, propionic and butyric acids) a non-parametric test was applied by ranking the data and submitting them to analysis of variance, using the Easynova package of R. The significant factors ($P<0.05$) were also compared by the Tukey's test.

3. Results

The chemical composition and microbiological analyses of the pre-wilted ryegrass before ensiling are presented in Table 1. The population of enterobacteria was high, exceeding 10^7 CFU g^{-1} in both harvests (32 and 45% DM) The chemical composition was similar between both harvests.

The composition of the pre-wilted ryegrass silages is shown in Table 2. Most of the variables analyzed were affected by the treatments (inoculants and harvest stage), as well as by their interaction. Inoculated silages showed higher DM content in the 32% DM harvest and lower pH in both harvests, indicating a more favorable fermentation profile. Silages with higher moisture content (32% DM) presented lower pH (3.97) and higher levels of lactic acid (21.5 g kg^{-1} DM), as well as a greater population of lactic acid bacteria (8.38 log CFU g^{-1}). As a result, these silages had lower DM (8.6 g kg^{-1} DM) and gas losses (8.1 g kg^{-1} DM), no effluent production, and lower DM losses during aerobic exposure (7% DM) compared to silages harvested at 45% DM (Table 3). Acetic acid content was not influenced by the inoculants (Table 2). The INO inoculant treatment resulted in a lower 1,2-propanediol concentration at both DM levels (Table 2).

Ryegrass silages harvested at 45% DM showed crude protein content approximately two percentage points higher than those harvested at 32% DM (Table 2). On the other hand, at 32% DM, the inoculants INO and INO+E significantly increased the *in vitro* estimates of digestibility for DM, OM, and NDF. However, at 45% DM, both inoculants showed similar digestibility coefficients to the control silage (Table 2).

The counts of enterobacteria in 32% DM silages were 37.3% and 49.6% lower for INO and INO+E treatments, respectively, compared with the control silage. However, in 45% DM silages, the INO+E inoculant showed a stronger effect on reducing enterobacteria populations (Table 3). Yeast and mold counts were lower than expected, with no colonies observed at the lowest dilution tested (10^{-4}). Silages treated with INO+E showed a slight tendency toward higher fungal colony numbers; however, no statistics could be performed.

The 32% DM silages presented lower estimates of DM, gas, and effluent losses than the 45% DM treatments (Table 3). In the 32% DM silages, the INO inoculant showed negative values for gas (−21.9 g kg^{-1} DM) and DM losses (−21.6 g kg^{-1} DM). At 45% DM, the treatment INO increased DM losses (60.7, 53.6, and 38.4 g kg^{-1} DM for INO, INO+E, and control, respectively) and gas losses (60.5, 53.6, and 37.3 g kg^{-1} DM, respectively).

Aerobic stability (AS) was high for all samples (Table 3 and Figure 1). At 45% DM, the inoculants significantly enhanced AS. Silages harvested at 32% DM were more stable than those at 45% DM, with no inoculant effect. Control silages heated only after 10 days of aerobic exposure, although visible

Table 2 - Dry matter content, pH, chemical composition, fermentation products and microbial counts of wilted ryegrass silage (*Lolium multiflorum* L.) with or without inoculants

Variable	32% DM			Mean	45% DM			Mean	P-value[1]		
	C	INO	INO+E		C	INO	INO+E		DM	Inoc	D × I
Dry matter – 105 °C (g kg^{-1})	343b	366a	359a	356	475	475	480	477	<0.0001	<0.0001	0.002
pH at opening (day 0)	4.19a	3.86b	3.91b	3.97	4.95a	4.08b	4.12b	4.38	<0.001	<0.001	<0.001
Crude protein (g kg^{-1} DM)	154	150	148	151	173a	162b	174a	170	<0.0001	0.002	0.007
NDF (g kg^{-1} DM)	527	531	523	527	548b	551b	572a	557	<0.0001	0.044	0.001
ADF (g kg^{-1} DM)	337	346	336	340	354	353	352	353	0.006	0.323	0.605
Ash (g kg^{-1} DM)	107a	97b	107a	103	101b	105a	106a	104	0.873	<0.001	<0.001
IVDMD (%)	70.7b	75.0a	75.1a	73.6	71.1ab	68.9b	71.3a	70.4	<0.0001	0.005	0.0001
IVOMD (%)	71.2b	75.5a	75.6a	74.1	71.6	69.9	71.9	71.1	<0.0001	0.003	0.0001
IVNDFD (%)	50.7b	57.2a	55.3a	54.4	52.2a	48.3b	53.7a	51.4	<0.001	<0.001	<0.001
Lactic acid (g kg^{-1} DM)	14.8b	21.8a	28.0a	21.5	5.9b	17.7a	18.8a	14.1	<0.001	<0.001	0.243
Acetic acid (g kg^{-1} DM)	3.5	3.0	3.7	3.4	3.4	3.0	2.7	3.0	0.004	0.723	0.347
Propionic acid (mg kg^{-1} DM)	49a	39b	38b	42	103a	41b	51b	65	<0.001	<0.001	<0.001
Butyric acid (mg kg^{-1} DM)	43a	6b	6b	15	12a	8b	9b	10	<0.001	<0.001	<0.001
Ethanol (g kg^{-1} DM)	0.44	0.69	0.47	0.53	0.99	0.80	0.53	0.77	0.810	0.222	0.046
1,2-Propanediol (mg kg^{-1} DM)	299a	114b	295a	236	160a	70b	114ab	114	<0.001	<0.001	0.008
Total LAB (log CFU g^{-1})	8.73a	7.84b	8.63a	8.38	8.12a	7.27c	7.60b	7.66	<0.001	<0.001	0.216
Enterobacteria (log CFU g^{-1})	4.37a	2.74b	2.20b	3.01	4.34a	3.44b	2.54c	3.44	0.085	<0.001	0.323
Yeasts (log CFU g^{-1})	<10^4	<10^4	<10^4	-	<10^4	<10^4	<10^4	-	-	-	-
Molds (log CFU g^{-1})	<10^4	<10^4	4.58	-	<10^4	<10^4	4.19	-	-	-	-

NDF - neutral detergent fiber; ADF - acid detergent fiber; IVDMD - *in vitro* digestibility of dry matter; IVOMD - *in vitro* digestibility of organic matter; IVNDFD - *in vitro* digestibility of neutral detergent fiber; LAB - lactic acid bacteria; CFU - colony forming units.
C - Wilted ryegrass silage control; INO - Wilted ryegrass silage with *Lentilactobacillus hilgardii* CNCM I-4785 (1×10^5 CFU g^{-1} forage), *Lentilactobacillus buchneri* NCIMB 40788 (1×10^5 CFU g^{-1} forage), and *Pediococcus pentosaceus* NCIMB 12455 (1×10^5 CFU g^{-1} forage); INO+E - Wilted ryegrass silage with *L. hilgardii* (7.5×10^4 CFU g^{-1} forage), *L. buchneri* (7.5×10^4 CFU g^{-1} forage), and *P. pentosaceus* (1×10^5 CFU g^{-1} forage), plus enzymes β-glucanase (5,750 IU g^{-1} of product) and xylanase (30,000 IU g^{-1} of product).
[1] DM - effect of harvest (32 or 45% of dry matter); Inoc - effect of the additives; D × I - interaction between the factors.

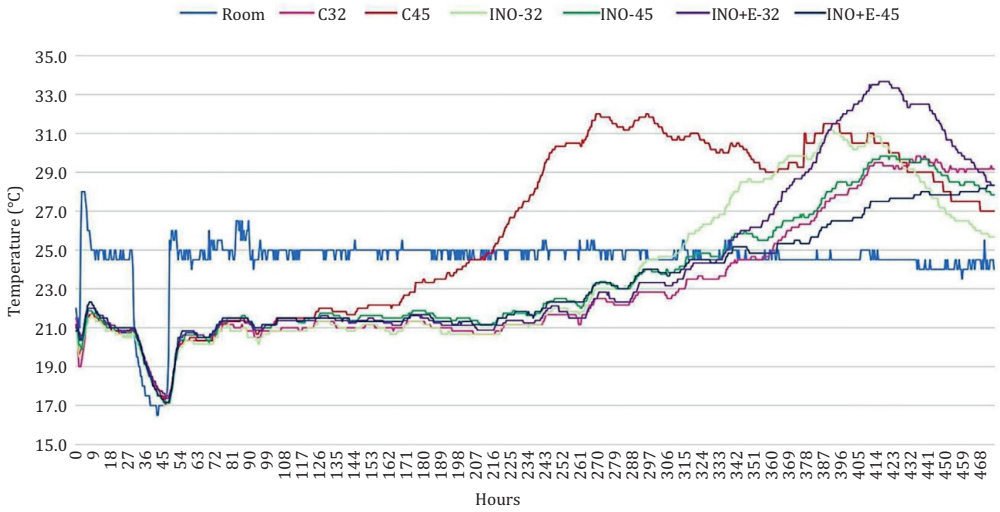
Table 3 - Fermentative losses and aerobic stability of wilted ryegrass silages (*Lolium multiflorum* L.) with or without inoculants

Variable	32% DM			Mean	45% DM			Mean	P-value[1]		
	C	INO	INO+E		C	INO	INO+E		DM	Inoc	D × I
DM losses (g kg^{-1} DM)	37.2a	−21.6c	10.1b	8.6	38.4b	60.7a	53.6ab	50.9	<0.001	0.011	<0.001
Gas losses (g kg^{-1} DM)	36.7a	−21.9c	9.3b	8.1	37.3b	60.5a	53.6ab	50.5	<0.001	0.014	<0.001
Effluent (kg/ton wet basis)	0.51	0.25	0.76	0.51	1.16	0.29	0.00	0.48	0.637	0.194	0.053
Aerobic stability - AS (hour)	386	324	349	353	228b	366a	388a	327	0.119	0.021	<0.001
Max. Temp. (°C)	32.0	31.5	34.0	32.5	33.8	30.3	28.5	30.9	0.179	0.335	0.061
Hour to TMax (hour)	447	405	416	422	315b	431a	460a	402	0.219	0.034	0.001
DMLae (g kg^{-1} DM)	43.2	47.4	114.2	70.0	263.4a	71.6b	70.9b	135.3	0.008	0.011	<0.001
pH day 2 of AS	4.19a	3.84b	3.86b	3.95	5.10a	4.02b	4.06b	4.39	<0.001	<0.001	<0.001
pH day 4 of AS	4.16a	3.88b	3.92b	3.97	4.97a	4.07b	4.16b	4.40	<0.001	<0.001	<0.001
pH day 6 of AS	4.11a	3.86b	3.88b	3.94	4.76a	4.14b	4.10b	4.33	<0.001	<0.001	<0.001
pH day 8 of AS	4.07a	3.87b	3.92b	3.94	4.70a	4.08b	4.13b	4.30	<0.001	<0.001	<0.001
pH day 10 of AS	4.09	3.90	4.14	4.04	5.11a	4.41b	4.17b	4.57	<0.001	<0.001	<0.001
pH day 18 of AS	4.14	6.01	6.65	5.71	7.50	5.77	4.60	5.96	0.440	0.878	<0.001
pH day 20 of AS	7.34	8.19	8.40	8.02	8.65	6.08	6.21	6.98	0.045	0.319	0.009

C - Wilted ryegrass silage control; INO - Wilted ryegrass silage with *Lentilactobacillus hilgardii* CNCM I-4785 (1×10^5 CFU g^{-1} forage), *Lentilactobacillus buchneri* NCIMB 40788 (1×10^5 CFU g^{-1} forage), and *Pediococcus pentosaceus* NCIMB 12455 (1×10^5 CFU g^{-1} forage); INO+E - Wilted ryegrass silage with *L. hilgardii* (7.5×10^4 CFU g^{-1} forage), *L. buchneri* (7.5×10^4 CFU g^{-1} forage), and *P. pentosaceus* (1×10^5 CFU g^{-1} forage), plus enzymes β-glucanase (5,750 IU g^{-1} of product) and xylanase (30,000 IU g^{-1} of product).
[1] DM - effect of harvest (32 or 45% of dry matter); Inoc - effect of the additives; D × I - interaction between the factors.

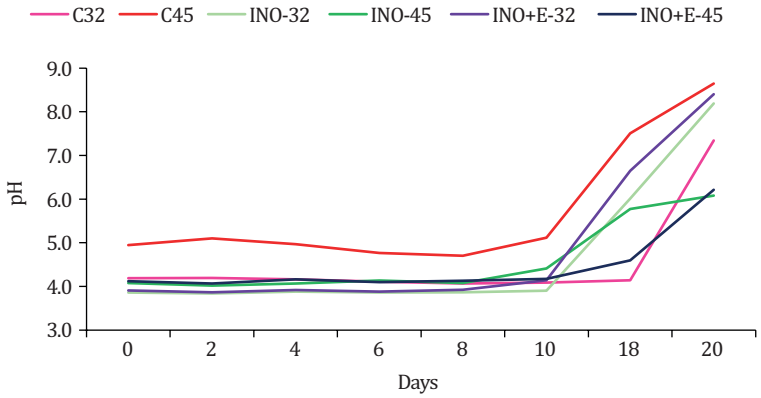
signs of spoilage and unpleasant odor (not evaluated) were observed after six days. In contrast, silages treated with INO and INO+E at 45% DM retained a typical silage odor even after 16 days of exposure at 25 °C (not evaluated). The DM losses under aerobic exposure (DMLae) were higher in 45% DM silages, and the inoculants were effective in reducing them compared with the 32% DM silages.

The pH of the silages remained stable until the 10th day of aerobic exposure. Due to a technical failure, no samples were collected for pH analysis between the 10th and 18th days. After 18 days, pH values showed high variability among replicates, with only the INO-45 and INO+E-45 treatments maintaining average pH values close to 5.0 (Figure 2).



C32 and C45 - Wilted ryegrass silage control with 32% and 45% DM, respectively; INO-32 and INO-45 - Wilted ryegrass silage with *Lentilactobacillus hilgardii* CNCM I-4785 (1×10^5 CFU g^{-1} forage), *Lentilactobacillus buchneri* NCIMB 40788 (1×10^5 CFU g^{-1} forage), and *Pediococcus pentosaceus* NCIMB 12455 (1×10^5 CFU g^{-1} forage) with 32% and 45% DM, respectively; INO+E-32 and INO+E-45 - Wilted ryegrass silage with *L. hilgardii* (7.5×10^4 CFU g^{-1} forage), *L. buchneri* (7.5×10^4 CFU g^{-1} forage), and *P. pentosaceus* (1×10^5 CFU g^{-1} forage), plus enzymes β-glucanase (5,750 IU g^{-1} of product) and xylanase (30,000 IU g^{-1} of product), with 32% and 45% DM, respectively.

Figure 1 - Silage temperature according to treatments through the 20-day period of aerobic exposure.



C32 and C45 - Wilted ryegrass silage control with 32% and 45% DM, respectively; INO-32 and INO-45 - Wilted ryegrass silage with *Lentilactobacillus hilgardii* CNCM I-4785 (1×10^5 CFU g^{-1} forage), *Lentilactobacillus buchneri* NCIMB 40788 (1×10^5 CFU g^{-1} forage), and *Pediococcus pentosaceus* NCIMB 12455 (1×10^5 CFU g^{-1} forage) with 32% and 45% DM, respectively; INO+E-32 and INO+E-45 - Wilted ryegrass silage with *L. hilgardii* (7.5×10^4 CFU g^{-1} forage), *L. buchneri* (7.5×10^4 CFU g^{-1} forage), and *P. pentosaceus* (1×10^5 CFU g^{-1} forage), plus enzymes β-glucanase (5,750 IU g^{-1} of product) and xylanase (30,000 IU g^{-1} of product), with 32% and 45% DM, respectively.

Figure 2 - Silage pH according to treatments through the 20-day period of aerobic exposure.

4. Discussion

Traditionally, farmers wilt ryegrass for silage until 45-50% of DM. Interestingly, few studies have evaluated lower DM content as a goal for wilted silages. In this trial, the 32% DM treatments seem to produce better quality silages over a shorter period.

The chemical composition of the fresh pre-wilted ryegrass evaluated in this study was similar to the values reported by Conaghan et al. (2010). The high population of enterobacteria in the fresh forage may be associated with dust contamination during the wilting process under field conditions. Wang et al. (2020) reported counts of up to 8.16 log CFU g^{-1} of enterobacteria in fresh Italian ryegrass forage. However, despite the high counts of enterobacteria in the fresh forage, the fermentation process contributed to the preservation of the ensiled material, as it reduced the counts of enterobacteria, yeasts, and molds, while increasing the population of lactic acid bacteria, regardless of the treatment.

The higher DM content and lower pH values observed at silo opening in inoculated silages harvested at 32% DM, compared with the control silage, indicate a more favorable fermentative profile of the material. These results are consistent with the higher lactic acid concentrations, increased lactic acid bacteria (LAB) population, and lower butyric acid concentrations observed in these treatments (Table 2).

The benefits of heterolactic LAB on silage fermentation and preservation are well documented in the literature (McDonald et al., 1991; Muck et al., 2018). The main advantage of intensified LAB activity is the production of compounds that enhance silage preservation, resulting in reduced or negligible fermentative losses. Among these compounds, lactic acid stands out due to its low pKa value (3.86), which is the lowest among the acids commonly found in silage, thereby promoting a rapid decline in pH (Muck et al., 2018). pH is one of the key factors influencing silage fermentation quality (Wang et al., 2020). Many undesirable microorganisms in silage, such as certain fungi, exhibit limited activity in low-pH environments (McDonald et al., 1991). Furthermore, higher acetic acid concentrations may contribute to the inhibition of undesirable microorganisms, such as yeasts, due to its well-known antifungal action (Pordeus et al., 2023). Our results demonstrate that the evaluated inoculants were effective in controlling the activity of undesirable microorganisms, such as enterobacteria, and promoted higher concentrations of lactic and acetic acids while reducing butyric acid levels contributing to the preservation of the ensiled material, particularly in wilted ryegrass silages harvested at 32% DM.

As previously mentioned, some cellular activities continue to occur in plants even after cutting, which can influence the concentration of essential compounds for fermentation, such as water-soluble carbohydrates (Melo et al., 2023). The ryegrass used in the 45% DM treatment remained in the field for an additional 24 h to reach the DM content, which led to higher CP and NDF content, likely as result of the loss of soluble carbohydrates plant respiration under field conditions (not evaluated). The 45% DM silages presented higher pH and lower acid production than the 32% DM silages, and the inoculants were less effective in improving fermentation in drier silages, possibly due to reduced substrate availability. Nevertheless, even under these conditions, silages treated with inoculants showed lower pH values than the untreated untreated control, which can be attributed greater lactic acid production. These results indicate that inoculants may also be useful in high-DM forages, which are generally more susceptible to suboptimal fermentation. The lower DM losses, reduced gas production, and absence of effluent during the fermentation process, as well as the lower DM losses during aerobic exposure observed in the 32% DM silages, can be attributed to the greater activity of desirable microorganisms and the metabolites produced by their actions, which play a crucial role both during fermentation and after exposure of the silage to air (Gheller et al., 2021). These results indicate clear advantages for farmers, since keeping the forage in the field for a shorter period represents a lower risk of losses due to adverse weather conditions (Uslu et al., 2017). Moreover, shorter wilting periods contribute to nutrient preservation, which influences the fermentation process and, consequently, the aerobic stability (AS) of the silages, extending the safe utilization period after silo opening.

Higher DM content forages may present compaction difficulties at the time of ensiling, resulting in greater residual oxygen (Kung et al., 2018), which contributes to increased losses both during the

fermentation process and after air exposure. However, this effect was isolated in the present study, as similar bulk densities were used for both DM levels. The 45% DM silages exhibited, on average, sixfold greater DM losses during fermentation compared with the 32% DM silages, as well as higher DM losses during aerobic exposure and a shorter aerobic stability period. A negative interaction between DM content and pH, associated with longer field wilting periods results in a reduction of water-soluble nutrients, increased contamination by undesirable microorganisms, and lower fermentative capacity (Uslu et al., 2017). These factors may explain the higher pH values and greater DM losses observed in 45% DM silages.

Even at 45% DM, the inoculants used extended silage aerobic stability by 138 and 160 h for the INO and INO+E treatments, respectively, and reduced DM losses after air exposure by 19.2%. In addition, up to the 10th day of aerobic exposure no significant changes in silage pH were observed in silages treated with the inoculants, regardless of DM content. From a practical standpoint, these results are highly relevant for farmers, as they indicate an extended utilization window for the preserved feed.

The lower counts of enterobacteria observed in silages treated with INO and INO+E, both at 32 and 45% DM, represent a relevant finding, considering that these microorganisms are potentially pathogenic and compromise silage quality (Queiroz et al., 2018). In addition to directly competing with LAB for water-soluble carbohydrates, enterobacteria are associated with greater DM losses during the fermentation process (McDonald et al., 1991). In a meta-analysis, no significant effect of wilting was detected on LAB, yeast, or aerobic bacteria populations (Ridla et al., 2024). However, the authors report that wilting is related to a general reduction in microbial activity in silages, with a significant decline in undesirable microorganisms. In the present study, the enterobacteria, yeast, and mold counts in wilted ryegrass prior to ensiling were substantially reduced after fermentation, indicating that the ensiling process was effective in suppressing the activity of these undesirable microorganisms.

After aerobic exposure, yeasts and other fungi resume their activity, consuming carbohydrates and nutrients from the silage, which results in gas and heat production and leads to material spoilage (Borreani et al., 2018). However, the observation that control silages exhibited a temperature increase only after 10 days of aerobic exposure despite visible spoilage and unpleasant odor from the sixth day onward, suggests that temperature alone may not be a reliable indicator of aerobic deterioration in wilted grass silages. Diepersloot et al. (2022) 240 h of temperature-based aerobic stability in wilted bermudagrass silages. Gomes et al. (2019) reported pH changes of wilted oat silages after 8 days of air exposure. However, these authors did not report visual or olfactory indicators of silage deterioration.

The main goal of ensiling is to preserve the nutritional value of the conserved forage as much as possible (McDonald et al., 1991). The action of enzymes on cellulose and hemicellulose during the fermentation process can reduce the resistance of the NDF fraction (Muck et al., 2018), making it more digestible. However, the lower NDF contents and higher digestibility of this fraction observed in 32% DM silages, compared to 45% DM silages, can be explained by greater acid hydrolysis of hemicellulose, favored by the lower pH achieved in inoculated silages relative to the control. The inoculated 32% DM silages showed higher NDF, OM, and DM digestibility compared with the control silages, whereas no additional effects of the enzymes present in the INO+E inoculant were observed.

At 45% DM silages treated with INO+E showed higher NDF content, but digestibility coefficients greater than those of INO and similar to the control. These findings suggest that the enzymes used (xylanase and β-glucanase) promoted partial polysaccharide breakdown without increasing solubilization in neutral detergent. The study published by Antonio et al. (2018), which evaluated the use of enzymes in hay conservation (a drier material than wilted forage), demonstrated that xylanase, either alone or in combination with glucanase and cellulases, increased NDF and dry matter digestibility. Together, these results suggest that enzymatic activity may be more pronounced in higher-pH substrates, such as the 45% DM silages compared to the 32% silages.

The results of this study indicate that the traditional DM content applied by farmers (around 45% DM) for ensiling wilted forages may not always represent the most advantageous strategy, especially under unfavorable climatic conditions. It was observed that silage with a lower DM content (32%)

required less time in the field to reach the target DM level, exhibited lower fermentative losses, improved chemical composition, and greater resistance to aerobic deterioration after silo opening. These findings highlight the importance of reassessing established management practices based on scientific evidence. Therefore, dissemination of this information through extension services is essential to support improved decision-making by farmers when producing ryegrass silage or haylage.

5. Conclusions

The treatment with 32% DM resulted in higher-quality silages, requiring less time in the field, with a more favorable fermentation profile and reduced losses during both the fermentation process and after silo opening. The use of inoculants reduced silage pH compared to the control. At 32% DM, inoculants enhanced DM and NDF digestibility, as well as increased lactic acid concentration. In silages with 45% DM, inoculants also increased lactic acid content and significantly improved aerobic stability. These results highlight the importance of setting an appropriate DM content for ensiling, and using additives as key strategies for producing silages with higher nutritional value and improved stability after silo opening.

Data availability

All relevant data are contained within the paper. Contact the corresponding author if further explanation is required.

Author contributions

Conceptualization: Assis, J. A. and Schmidt, P. **Data curation:** Assis, J. A. and Schmidt, P. **Formal analysis:** Assis, J. A.; Carbonare, M. S. D. and Schmidt, P. **Funding acquisition:** Assis, J. A. and Schmidt, P. **Investigation:** Assis, J. A.; Carbonare, M. S. D. and Schmidt, P. **Methodology:** Assis, J. A. and Schmidt, P. **Project administration:** Assis, J. A. and Schmidt, P. **Resources:** Assis, J. A. and Schmidt, P. **Software:** Assis, J. A. **Supervision:** Assis, J. A. and Schmidt, P. **Validation:** Assis, J. A. and Schmidt, P. **Visualization:** Assis, J. A. and Schmidt, P. **Writing – original draft:** Assis, J. A. and Schmidt, P. **Writing – review & editing:** Assis, J. A.

Conflict of interest

The authors declare no conflict of interest.

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