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Bacterial Abundance, Fermentation Pattern, and Chemical Composition of Oat Haylage Are Altered by the Forage Dehydration Method

André Martins de Souza [1,*], Mikael Neumann [2], Odimari Pricila Prado Calixto [1], Admilton Gonçalves de Oliveira Júnior [3], Ellen Baldissera [2], Nicolli Soethe Mokochinski [2], Livia Alessi Ienke [2] and Valter Harry Bumbieris Junior [1]

[1] Department of Animal Science, State University of Londrina, Londrina 86057-970, Brazil; odimari@uel.br (O.P.P.C.); jrbumbieris@uel.br (V.H.B.J.)
[2] Department of Veterinary Medicine, Midwestern Parana State University, Guarapuava 85040-167, Brazil; neumann.mikael@hotmail.com (M.N.); ellen_baldissera@outlook.com (E.B.); nicollisoethemokochinski@gmail.com (N.S.M.); liviaienke@gmail.com (L.A.I.)
[3] Department of Microbiology, State University of Londrina, Londrina 86057-970, Brazil; admilton@uel.br
* Correspondence: andrems_92@hotmail.com

Abstract

The present study aimed to evaluate the aerobic stability, fermentation profile, microbiological diversity, and bromatological composition of the haylage of white oats obtained using three dehydration methods: (1) Mechanical (MEC); (2) Mechanical + Bacterial chemical compound (MEC + BCC); and (3) Chemical (CHE), where Glyphosate was used as a desiccant. The haylage made with the forage dehydrated by the mechanical method showed lower aerobic stability (69.20 h) and a higher cumulative temperature from 0 to 84 h and from 0 to 168 h (12.73 °C and 25.91 °C, respectively). The haylage made with Glyphosate-desiccated forage had higher concentrations of acetic acid (3.96 g kg^{-1}) and isobutyric acid (0.78 g kg^{-1}). The microbiological diversity and richness did not differ among the haylages produced. However, the relative abundance of the genera *Pantoea* and *Lactobacillus* was higher in the haylage made with Glyphosate-desiccated forage. The bacterial chemical compound guaranteed a haylage with lower lignin content (31.97 g kg^{-1}), lower acid detergent nitrogen (7.54 g kg^{-1}), and higher hemicellulose (211.72 g kg^{-1}). The haylage made from dehydrated forage by the Mechanical + Bacterial Chemical Compound methods presented a better fermentation pattern and had lower fermentation losses, and its bromatological quality was superior to the others.

Keywords: aerobic stability; homofermentative bacteria; lactic acid; *Lactobacillus*

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1. Introduction

In the context of food preservation, ensiling oats has emerged as a viable alternative because of their nutritional quality. However, to maximize the nutritional value of the vegetative component, oats must be harvested at the pre-flowering stage. During this stage, the dry matter content of oats is generally low, which may adversely affect the fermentation process [1]. The low dry matter content of the forage favors the proliferation of bacteria of the genus Clostridium, which are producers of butyric acid and consumers of soluble carbohydrates, in addition to favoring the production of effluents, which translocate water-soluble products out of the silo, reducing their quality [2].

Due to these aforementioned issues, dehydrating the plant after cutting is essential in order to generate an environment in the silo that is compatible with the development

of the correct fermentation process, which is characterized by the presence of short-chain fatty acid (lactic acid, acetic acid, and propionic acid), which lower the pH of the medium, function as bactericides and fungicides, preserve the nutrients of the ensiled material, and maintain its preservation over extended periods [3,4]. The forage dehydration period has been shown to alter the soluble carbohydrate content of plants. Specifically, the less efficient the process, the lower the concentration of these carbohydrates, as they are used in the intracellular reactions of the plants after they are harvested [5,6].

Studies comparing dehydration methods are scarce in the literature. Carneiro et al. [7] and Bueno et al. [8], comparing haylage made with forage dehydrated by the chemical and mechanical method, observed lower losses, better bromatological composition and greater aerobic stability when glyphosate was used, which is a reflection of a haylage with a greater population of acetic acid-producing bacteria, which inhibits the proliferation of spoiling bacteria such as *clostridium* [6]. Studies carried out with biological compounds intended for dehydration were not found in the literature.

The microbial community composition is susceptible to manipulation during the haylage-making phase, with the potential to exert a positive or negative influence on the fermentation process [9]. Gene sequencing is a method for evaluating the haylage microbiome and provides a more comprehensive view of the microbial community and its ecology [10].

With the few data found in the literature comparing the Mechanical dehydration method with the use of Glyphosate to dehydrate forage, considering the chemical composition, losses, and aerobic stability, we hypothesize that the use of glyphosate and the Mechanical method + Bacterial chemical compound compared to the Mechanical method will be more efficient, reducing the forage dehydration time and promoting adequate fermentation processes, and with lower losses, which culminates in haylage of better nutritional quality.

In view of the above, the present study sought to evaluate the effect of three different oat forage dehydration methods (Mechanical, Mechanical + Bacterial chemical compound, and Glyphosate as desiccant) on the aerobic stability, fermentation profile through the analysis of organic acids, microbiological diversity at the phylum and genus levels, losses, and bromatological composition in the produced haylages.

2. Materials and Methods

2.1. Experimental Site

The study was conducted at the Animal Production Center (NUPRAN) of the Agricultural and Environmental Sciences Sector, State University of the Midwest (UNICENTRO), located in the municipality of Guarapuava, State of Paraná, in conjunction with the Graduate Program in Animal Science of the State University of Londrina (UEL), located in Londrina, State of Paraná. The climate of the Guarapuava region is humid mesothermal subtropical (Cfb), without a dry season, characterized by cool summers and moderate winters. The climatic data of the experimental period are illustrated in Figure 1.

2.2. Material and Experimental Design

White oat cultivar GMX Tambo was used as the experimental material, which was chosen based on its good agronomic performance and nutritional profile. The forage was planted on 15 April 2021, in a no-till system, with sowing at a row spacing of 0.17 m and an average depth of 0.02 m, in plots of 5 × 5 m, with a total area of 25 m^2 per plot. This was a completely randomized experimental design consisting of three treatments and three forage dehydration methods (MEC + BCC, MEC, and CHE), with five replicates each, where each replicate was represented by one plot.

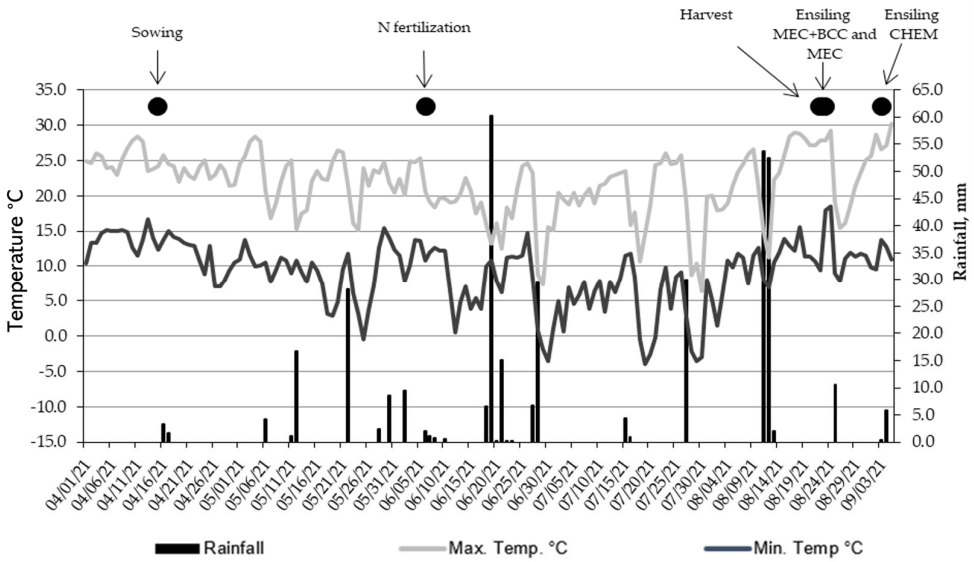


Figure 1. Maximum and minimum temperatures (°C) and average rainfall in mm during the experimental period.

The bacterial chemical compound, consisting of *Bacillus amyloliquefaciens* (7799) 1.0×10^9 CFU g^{-1}, *Bacillus subtilis* (CCT 0089) 1.0×10^9 CFU g^{-1}, *Propionibacterium acidipropionici* (7751) 1.0×10^9 CFU g^{-1}, potassium sulfate, and cellulase (technology owned by the company SLO Biotecnologia e Agropecuária), was applied at a dose of 5 g^{-1} of the commercial product for each 1 L of non-chlorinated water, as recommended by the manufacturer. The bacterial chemical compound was sprayed onto the forage immediately before harvesting. Subsequent to the application, the forage was manually turned over using rakes until the desired dry matter (DM) content for ensiling was achieved.

In the MEC dehydration method, forage was simply mowed and turned over using rakes until the desired dry matter (DM) content for ensiling was achieved.

The herbicide glyphosate was applied at a dose of 0.5 L ha^{-1}; the plants were mowed only when they reached the desired dry matter content for ensiling. The product in question is authorized by the Ministry of Agriculture, Livestock, and Food Supply (MAPA) at this dosage as a desiccant agent for oats for haylage making. The use of glyphosate for this purpose does not have implications for animal or human health because the plant metabolizes it before ensiling and does not leave residues in meat or milk; so, larger doses would not cause harm to health but rather to the nutritional quality of haylage by reducing its digestibility. However, respecting doses recommended by regulatory bodies is necessary to avoid possible damage to the environment. Before the application of glyphosate or the bacterial chemical compound and subsequent harvesting, a total of ten samples, each weighing 500 g, were collected at random. Five samples were kept intact and placed in a forced-air oven to determine the DM of the plants at the time of harvesting. The remaining five samples were separated into their physical components, stems and leaves, to obtain the leaf:stem (L:S) ratio.

In each plot, one linear m was chosen randomly, and the plants within this area were collected. Once a total of 15 linear meters were reached, that is, 15 collection points, the plants were weighed to estimate the average production of green forage mass (kg ha^{-1}). From this value and the DM value of the plants, the production of dry forage mass (kg ha^{-1}) was estimated (Table 1).

Table 1. Characteristics of the white oat crop at the time of harvesting.

Population, plants ha^{-1}	1,930,000
Green forage mass production, kg ha^{-1}	20,707
Dry forage mass production, kg ha^{-1}	4448
DM (%)	21.48
L:S ratio (%)	74.82

ha: hectare.

Upon reaching 45% dry matter, forage from each plot was chopped into a stationary silage harvester and stored in PVC silos measuring 50 cm in height and 10 cm in diameter. The forage was compacted to a density of 240 kg of dry matter m^3 and kept in a covered location for 60 days.

2.3. Dehydration Rate

The forage dehydration rates were determined by collecting samples at specific times. The initial sample was collected at harvest (08:00 h). Subsequent samples were collected every 30 min for the first three hours (08:00, 08:30, 09:00, 09:30, 10:00, 10:30, 11:00 h). After this period, and until reaching the pre-stipulated DM content (45%) for ensiling, samples were taken at three-hour intervals. Upon reaching 45% DM, forage from each plot was chopped in a stationary ensiling machine and stored in PVC silos measuring 50 cm in height and 10 cm in diameter, with a compaction of 240 kg DM m^3, which were sealed with PVC caps and adhesive tape.

2.4. Scanning Electron Microscopy

Before the application of glyphosate or the bacterial chemical compound and subsequent harvesting, two plants were randomly selected within the experimental area, and one leaf was collected, as described by [11]. This leaf was then sent to the laboratory, where it was imaged using scanning electron microscopy (SEM) to evaluate the cuticle structure of the leaf before cutting. This procedure was repeated at the time of ensiling the materials.

2.5. Microbiology

Bacteria were identified by high-throughput sequencing of the V3/V4 region of the 16S rRNA gene. Library preparation was performed following a proprietary protocol (Neoprospecta Microbiome Technologies, Florianópolis, Brazil). Amplification was performed using primers for the V3/V4 region of the 16S rRNA gene, 341F CCTACGGGRSGCAGCAG [12], and 806R GGACTACHVGGGTWTCTAAT [13]. Libraries were sequenced using the MiSeq Sequencing System (Illumina Inc., San Diego, CA, USA), employing paired-end sequencing and V3 and V2 kits, with 600 and 500 cycles, respectively. Subsequently, the sequences were analyzed using the Sentinel pipeline.

In the Sentinel pipeline, fastq files were evaluated for phred quality (PQ) using the FastQC program (v.0.11.8) [14]. Next, the fastq files were trimmed (removal of low-quality sequences or regions that are undesirable because they cause errors in data analysis) of primers and sequences of low quality (PQ < 20). The software used for this purpose was built in Python (v.3.6), which was inspired by the functionalities of the BioPython project [15]. For paired-end data, two file pairs (R1 and R2) were merged into a single file using pandaseq (v.2.11) before trimming [16].

Clusters with an abundance lower than two were excluded from the analyses because such structures are related to chimeric sequences [17]. Taxonomic identification was performed using blastn (v.2.6.0+) [18], using a proprietary database (Neoprospecta Microbiome Technologies, Florianópolis, Brazil) as a reference. Regarding the definition of a species, among the 20 hits retrieved for each cluster, a Python command evaluated

whether the hits met one of three requirements: (1) highest bit-score; (2) lowest e-value; or (3) taxonomies with greater representation. Hits that met any of these criteria were selected as representative species, and these analyses were performed on the Amazon computing platform, where Neoprospecta's bioinformatics structure is hosted.

2.6. Organic Acid Analysis

The concentration of lactic acid was determined according to the methodology described by Pryce [19], and the concentrations of acetic, propionic, butyric, and isobutyric acids were determined by gas chromatography, according to the methodology used in a study developed by Del Valle et al. [20].

2.7. Losses

When the silos were opened, the total dry matter losses (Equation (1)) and gas losses (Equation (2)) were measured, as proposed by Jobim et al. [21].

Equation (1):

$$\mathrm{DML} = \left(\frac{\mathrm{DMi} - \mathrm{DMf}}{\mathrm{DMi}}\right) \times 100 \quad (1)$$

where DML = Total DM loss; DMi = Initial DM amount (silo weight after filling–weight of the empty set, without forage, before filling (dry tare)), multiplied by the DM content of the forage upon ensiling; and DMf = Final DM amount (full silo weight before opening–weight of the empty set, without forage, after opening the silos (wet tare)), multiplied by the forage DM content upon opening. Empty set: silo + cap + sand + screen + cloth.

Equation (2):

$$\mathrm{G} = \left(\frac{(\mathrm{FSwo} - \mathrm{Swe}) * \mathrm{DMuo})}{((\mathrm{SWue} - \mathrm{Swe}) * \mathrm{DMue}) - ((\mathrm{SWue} - \mathrm{Swe}) * \mathrm{DMue}))}\right) \quad (2)$$

where G = Gas losses in % DM; FSwo = Full silo weight upon opening (kg); Swe = Silo weight upon ensiling (silo + lid + sand + screen + cloth) (kg); DMuo = DM content of the haylage upon opening (%); SWue = Silo weight upon ensiling (kg); and DMue = DM content of the forage upon ensiling (%).

2.8. Aerobic Stability Analysis

Immediately after opening the silos, the haylages were tested for aerobic stability. For this purpose, one kilogram of the sample was stored in polypropylene flasks and transferred to a climatic chamber at 25 ± 2 °C. The haylage temperatures were measured with a rod thermometer inserted 10 cm into the center of the mass, at six-hour intervals for seven days.

The loss of aerobic stability was defined as the time required for haylage to rise by 2 °C above room temperature [22]. At the end of the evaluation, pH was measured according to the methodology established by Cherney and Cherney [23]. The cumulative temperature, defined as the sum of the average daily difference in temperatures in haylage exposed to air in relation to room temperature from 0 to 84 and from 0 to 168 h, was calculated according to the methodology described by O'Kiely et al. [24].

2.9. Chemical Analysis

Immediately after opening the silos, pH was measured using a digital potentiometer, according to the methodology established by Cherney and Cherney [23]. Simultaneously, 500 g of haylage from each treatment was collected and placed in a forced-air oven to determine partial DM. After pre-drying, they were ground in a Wiley mill with a 1 mm mesh sieve. These ground samples were then analyzed for neutral detergent fiber (NDF)

content, as proposed by Van Soest et al. [25], using thermostable α-amylase and acid detergent fiber (ADF).

Crude protein (CP) and Ammoniacal nitrogen (NH_3-N) were quantified by the micro Kjeldahl method, and neutral detergent insoluble protein (NDIP), acid detergent insoluble protein (ADIP), ether extract (EE), mineral matter (MM), and dry matter (DM) according to AOAC [26]. Non-fiber carbohydrates (NFCs) were estimated using Equation (3):

$$\mathrm{NFC} = 100 - (\mathrm{CP} + \mathrm{NDFcp} + \mathrm{EE} + \mathrm{MM}) \quad (3)$$

where NDFcp corresponds to NDF, corrected for ash and protein.

2.10. Statistical Analysis

The collected data were subjected to Shapiro–Wilk and Bartlett tests to verify the assumptions of normality and homogeneity of variance, respectively. Once these assumptions were met, the F-test with a 5% confidence level was applied through an analysis of variance (ANOVA), and then the Tukey's test was used to compare multiple means with 5% significance.

The analysis of each variable followed the statistical model:

$$\mathrm{Yi} = \mu + \mathrm{Di} + \varepsilon\mathrm{i}$$

where Yi = dependent variables; μ = overall mean of all observations; Di = effect of forage dehydration methods of order "i"; and εi = residual random effect.

The data related to the rate of forage dehydration were subjected to a polynomial regression analysis, considering the variable hours of evaluation, using the "proc reg" procedure of the SAS software, version 9.4.

The richness and Shannon index data were obtained using the packages (vegan, tidyverse, forcats, and iNEXT) of the statistical software R, version 4.2.1.

The data concerning the relative abundance of phyla and genera with an abundance greater than 2% in at least one of the haylages produced did not meet the assumptions of homogeneity. The Box–Cox transformation was then applied. Following this transformation, the data met assumptions of normality and homogeneity. The F-test at 5% significance was implemented through Analysis of Variance (ANOVA), followed by Tukey's test to compare multiple means at 5% significance. The principal component analysis was performed using the Vegan package of the statistical software R, version 4.3.0, which also made it possible to evaluate the correlations between the variables.

3. Results

The rate of forage dehydration exhibited an increasing linear behavior across all dehydration methods (Figure 2). The MEC + BCC method was the most efficient, increasing the DM content by 0.82% h^{-1} (Green bars), followed by the MEC method, with 0.69% h^{-1} (Blue bars), and the CHE method, which increased the DM content by 0.08% h^{-1} (Orange bars).

Figure 3A (arrow 1) illustrates the structure of the oat leaf cuticle at the tima of harvest (before the start of dehydration and before the application of any substance, be it glyphosate or the bacterial chemical compound evaluated). Larger cracks in the cuticular wax were observed in the leaves of plants dehydrated using the MEC + BCC method (Figure 3C, indicated by arrows 3) compared to the other dehydration methods (Figure 3B, arrows 2 and Figure 3D, arrows 4).

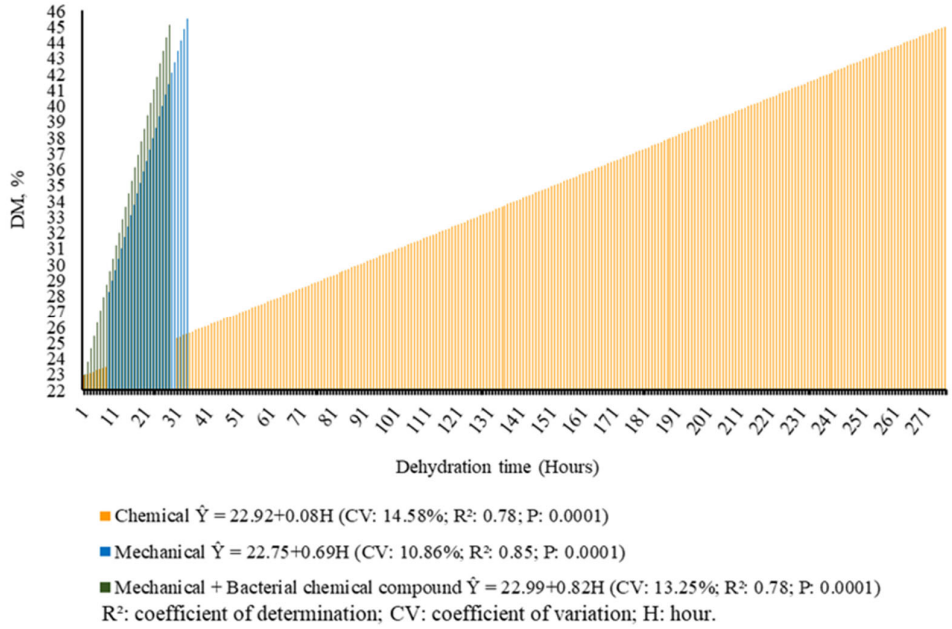


Figure 2. Dehydration rate of white oat forage subjected to three different dehydration methods for making haylage.

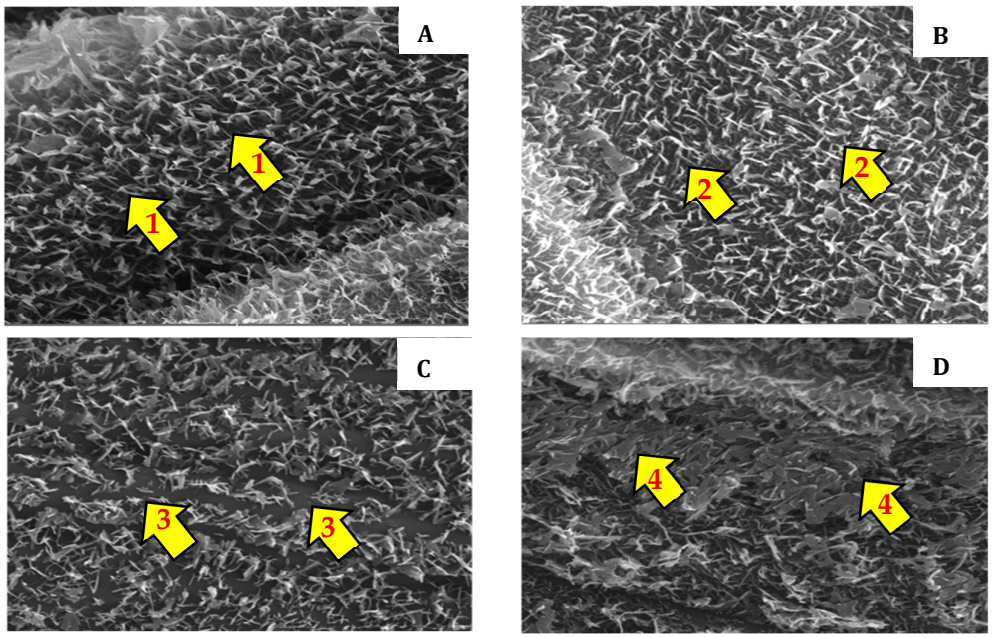


Figure 3. Cuticle structures of white oat leaves subjected to three different dehydration methods. (**A**) Cuticle structure of oat leaf at the time of harvesting; (**B**) Cuticle structure of oat leaf dehydrated by the MEC method at the time of ensiling; (**C**) Cuticle structure of oat leaf dehydrated by the MEC + BCC method at the time of ensiling; (**D**) Cuticle structure of oat leaf dehydrated with Glyphosate at the time of ensiling.

Table 2 presents the microbial diversity of haylages produced at the Phylum and Genus levels, which were not altered by the different dehydration methods tested ($p > 0.05$).

Table 2. Alpha diversity of the microbial community at the phylum and genus levels of white oat haylage subjected to three different dehydration methods.

Parameters	Dehydration Method				
	MEC	MEC + BCC	CHE	SEM	*p*-Value
Phylum					
Richness	4	4	4	-	-
Shannon Diversity	1.07	1.04	1.04	0.0122	0.3816
Genus					
Richness	16	16	17	0.2427	0.1278
Shannon Diversity	2.07	1.97	2.05	0.0258	0.3170

Mean values, in the same row, followed by different lowercases are significantly different by Tukey's test at 5%. SEM: Standard Error of the Mean.

The lack of difference in microbial α-diversity between the haylages produced does not necessarily imply a similarity in microbial abundance, either at the Phylum or Genus level. Figure 4 illustrates the relative abundance of the identified bacterial phyla. Regardless of the haylage produced, Proteobacteria, Bacteroidetes, and Firmicutes were dominant. However, the haylage made from forage dehydrated with glyphosate presented a greater relative abundance of Proteobacteria and Firmicutes but a lower abundance of Bacteroidetes, compared to the others.

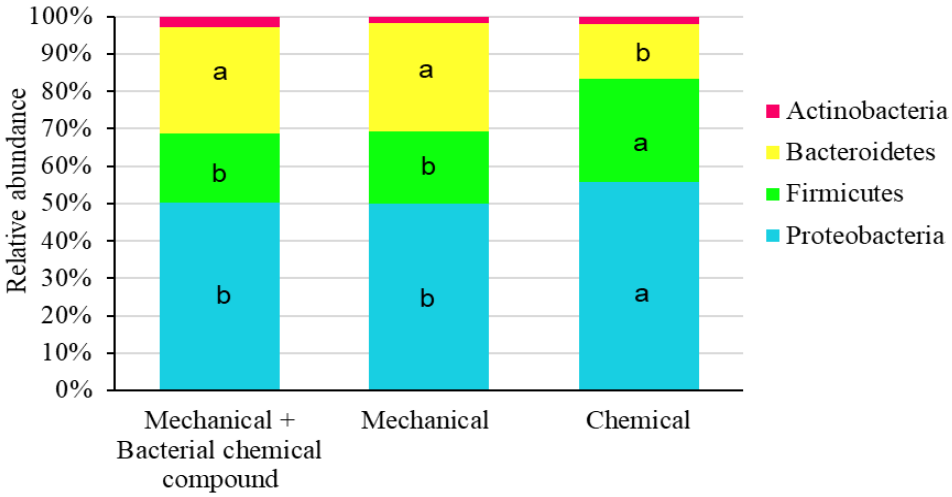


Figure 4. Relative abundance of the bacterial community at the phylum level of white oat haylage made from forage subjected to three different dehydration methods. Bars of the same color with different lowercase letters differ from each other according to Tukey's test at 5%.

The Phylum Proteobacteria was composed of the genera *Pantoea*, *Sphingomonas*, and *Stenotrophomonas*. The Phylum Firmicutes comprised the genera *Aerococcus*, *Enterococcus*, *Lactobacillus*, and *Weissella*. The Phylum Bacteroidetes was constituted by the genus *Sphingobacterium*.

A statistically significant difference ($p < 0.05$) in the relative abundances of the genera *Pantoea*, *Lactobacillus*, *Weissella*, and *Enterococcus* was observed between the haylages evaluated (Figure 5).

The genera *Pantoea* and *Lactobacillus* showed higher relative abundances in the haylage produced from forage desiccated with glyphosate (32.79% ± 2.64 and 10.82% ± 0.51, respectively) than in the other haylages. In contrast, *Weissella* had a greater relative abundance in the haylage obtained from the forage dehydrated by the MEC + BCC method (9.53% ± 0.95).

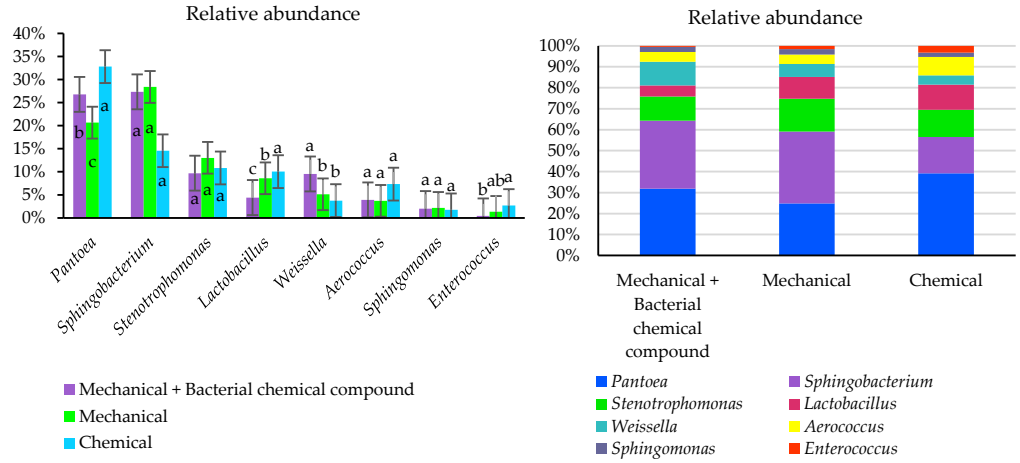


Figure 5. Relative abundance of the bacterial community at the genus level of white oat haylage made from forage subjected to three different dehydration methods. Bars followed by different lowercase letters are significantly different by Tukey's test at 5%.

The genus *Enterococcus*, though present in low abundance, was observed in the haylage made from forage desiccated with glyphosate, exhibiting a higher mean value (2.69% ± 0.71). However, this did not differ from the haylage of the MEC dehydration method (1.32% ± 1.19).

The acetic, propionic, and isobutyric acids produced during the fermentation process were altered by the dehydration method employed ($p < 0.05$, Table 3). These acids had a higher concentration in the haylage obtained from the forage dried with glyphosate and dehydrated by the MEC method (isobutyric acid).

Table 3. Fermentation profile of white oat haylage made from forage subjected to three different dehydration methods.

Parameters	Dehydration Method					
	MEC	MEC + BCC	CHE	Mean	SEM	*p*-Value
Acetic, g kg^{-1}	2.88 c	3.36 b	3.96 a	3.40	0.1188	0.0001
Lactic, g kg^{-1}	28.54	31.17	27.26	28.98	0.8288	0.2309
Butyric, g kg^{-1}	ND	ND	ND	-	-	-
Propionic, g kg^{-1}	ND	ND	0.08 a	0.03	0.0077	0.0001
Isobutyric, g kg^{-1}	0.66 a	0.48 b	0.78 a	0.64	0.0336	0.0003
pH	4.91	4.73	4.62	4.75	0.0826	0.3899

Mean values, in the same row, followed by different lowercases are significantly different by Tukey's test at 5%. ND: not detected; SEM: Standard Error of the Mean.

The aerobic stability of the haylages produced was altered by the dehydration method used ($p < 0.05$, Table 4).

The haylage made from forage desiccated with glyphosate took longer to lose its aerobic stability (127 h), followed by the haylage made from forage dehydrated using the MEC + BCC method (78.20 h) and MEC (69.20 h). On the other hand, the cumulative temperature from 0 to 84 and from 0 to 168 h was lower for the haylage obtained from forage desiccated with glyphosate (6.90 °C and 17.52 °C, respectively), followed by the haylage obtained from forage dehydrated by the MEC + BCC method (8.85 °C and 22.43 °C, respectively).

Table 4. Aerobic stability of white oat haylage made from forage subjected to three different dehydration methods.

Parameters	Dehydration Methods					
	MEC	MEC + BCC	CHE	Mean	SEM	*p*-Value
AE, hours	69.20 c	78.20 b	127.0 a	91.47	6.8190	0.0001
Cumulative temp., °C						
0 to 84 h	12.73 a	8.85 b	6.90 c	9.49	0.6507	0.0001
0 to 168 h	25.91 a	22.43 b	17.52 c	21.95	0.9295	0.0001
pH						
Initial AE	4.91	4.73	4.62	4.75	0.0826	0.3899
Final AE	5.75	5.58	5.72	5.68	0.0413	0.2168

Mean values, in the same row, followed by different lowercases are significantly different by Tukey's test at 5%. AE: Aerobic stability; SEM: Standard Error of the Mean; °C: degrees Celsius.

In addition to evaluating the bacterial genera and products resulting from fermentation separately, it is also important to evaluate them together and the correlation between them. One way to carry out this analysis is through the use of Main Component Analysis (MCA), which aims to summarize the dataset and remove overlaps, as shown in Figure 6.

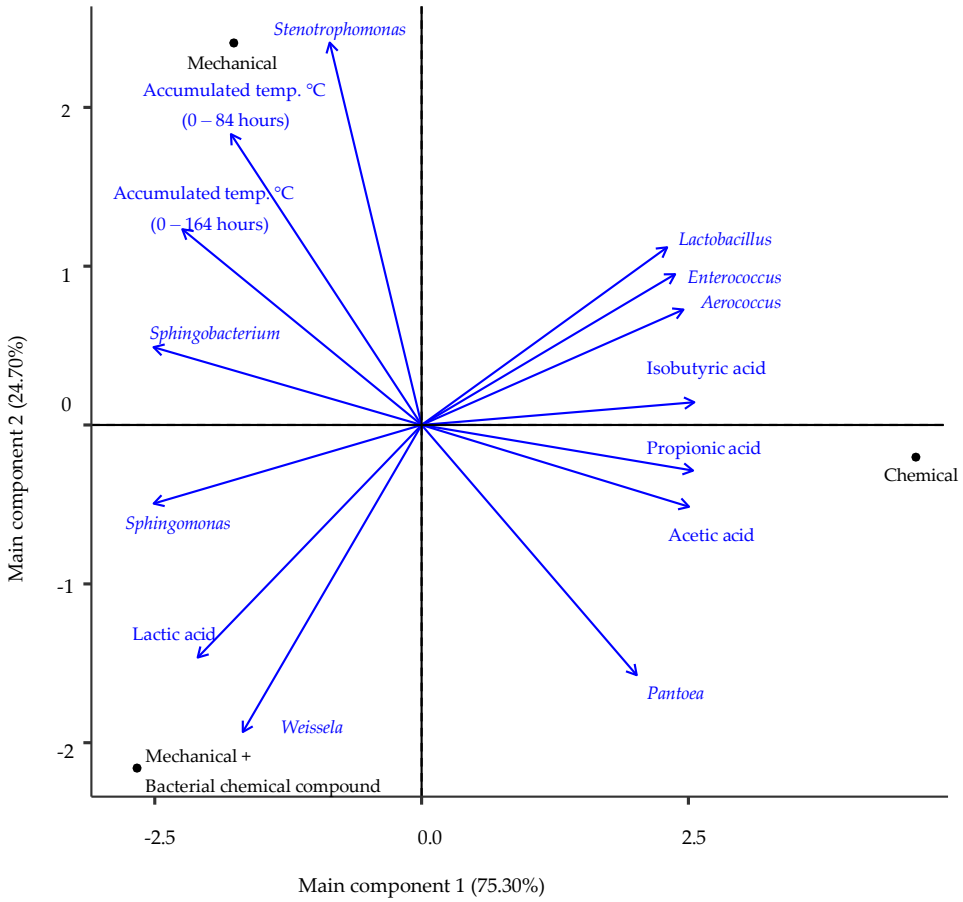


Figure 6. Main component analysis for fermentation parameters and main bacterial genera in white oat haylage made from forage subjected to three different dehydration methods.

The first main component (MC 1) accounted for 75.30% of the data variance, while the second main component (MC 2) explained 24.70%, thereby enabling reliable interpretation of the data. The contribution of the analyzed variables to the formation of main components is defined by the length of the arrows and their proximity to the axis [27]. In this type of analysis, correlation is defined as the angle formed between the variables. When the angle

is acute or close to zero, the correlation is high and positive; when it is close to 180°, the correlation is also high but negative; and when the angle is 90° or close, the variables have little or no correlation [27].

Isobutyric, propionic, and acetic acids contributed the most to the formation of MC 1, whereas the genera *Sphingobacterium* and *Sphingomonas* had a smaller contribution to its formation. The genus *Stenotrophomonas* contributed the most to the formation of MC 2, while *Weissella* had the smallest contribution.

The cumulative temperatures from 0 to 84 h and from 0 to 168 h showed a positive correlation with each other, as well as a positive correlation with the bacterial genera *Stenotrophomonas* and *Sphingobacterium*. There was also a positive correlation between propionic, isobutyric, and acetic acids, and between *Enterococcus* and the genera *Aerococcus* and *Lactobacillus*. A positive correlation exists between the genus *Pantoea* and propionic and acetic acids.

The haylage obtained from the MEC + BCC dehydration method had a higher concentration of lactic acid and bacteria of the genus *Weissella*. When the forage was dehydrated using the MEC method, the obtained haylage had a higher and lower presence of *Stenotrophomonas* and *Pantoea*, respectively. The haylage produced from forage desiccated with glyphosate had a higher concentration of propionic and acetic acids.

When evaluating the chemical composition of the produced haylages and the losses that occurred during the fermentation process, changes ($p < 0.05$, Table 5) were found in NDF, FDA, ADL, hemicellulose, cellulose, CP, ADN, EE, NFC, and NH_3-N, and in the losses that occurred during the fermentation process.

Table 5. Chemical composition of white oat haylage made from forage subjected to three different dehydration methods.

Parameters	Dehydration Methods					
	MEC	MEC + BCC	CHE	Mean	SEM	*p*-Value
			Chemical composition			
DM (g kg^{-1})	445.82	441.78	441.48	443.02	4.1065	0.8915
MM (g kg^{-1})	107.06	103.93	106.07	105.68	1.1815	0.5602
NDF (g kg^{-1})	521.04 b	516.36 b	585.81 a	541.07	2.8639	0.0001
ADF (g kg^{-1})	328.83 a	304.64 b	339.22 a	324.23	2.3691	0.0002
ADL (g kg^{-1})	39.88 a	31.97 b	42.25 a	38.03	0.6520	0.0001
HEM (g kg^{-1})	192.21 b	211.72 b	246.59 a	216.84	4.3223	0.0008
CEL (g kg^{-1})	288.96 a	272.66 b	296.96 a	286.20	2.4754	0.0054
CP (g kg^{-1})	140.41 a	145.33 a	117.64 b	134.46	1.4894	0.0001
NDN (g kg^{-1})	28.56	28.27	29.00	28.61	0.4469	0.8012
ADN (g kg^{-1})	8.13 b	7.54 c	10.68 a	8.78	0.0682	0.0001
EE (g kg^{-1})	40.40 b	45.29 a	34.10 c	30.93	0.4433	0.0001
NFC (g kg^{-1})	318.12 a	322.38 a	278.23 b	306.24	2.7279	0.0001
NH_3-N (% TN)	0.16 b	0.18 b	0.23 a	0.19	0.0051	0.0008
			Fermentation losses			
DM losses (%)	5.33 b	3.04 c	18.18 a	8.85	1.7842	0.0001
Gas losses (%)	2.09 a	1.37 b	2.20 a	1.88	0.1007	0.0001

Mean values, in the same row, followed by different lowercases are significantly different by Tukey's test at 5%. DM: Dry matter; MM: Mineral matter; NDF: Neutral detergent fiber; ADF: Acid detergent fiber; ADL: Acid detergent lignin; HEM: Hemicellulose; CEL: Cellulose; CP: Crude protein; NDN: Neutral detergent nitrogen; ADN: Acid detergent nitrogen; EE: Ether extract; NFC: Non-fiber carbohydrates; NH_3-N: Ammonia nitrogen; TN: total nitrogen; SEM: Standard Error of the Mean.

The haylage made from forage dehydrated by the MEC + BCC method had the lowest mean contents of NDF, ADF, ADL, CEL, and ADN (516.36 g kg^{-1}, 304.64 g kg^{-1}, 31.97 g kg^{-1}, 272.66 g kg^{-1}, and 7.54 g kg^{-1}, respectively) compared to the oth-

ers. The contents of CP, NFC, and EE had higher mean values (145.33 g kg^{-1}, 322.38 g kg^{-1}, and 45.29 g kg^{-1}, respectively) in haylage made from forage dehydrated by the MEC + BCC method.

DM losses were higher in haylage produced from forage dehydrated by the CHE method (18.18%), followed by haylage made using the MEC dehydration method (5.55%) and MEC + BCC method (3.04%). Gas losses were higher in haylage made from forage dehydrated by the CHE and MEC methods (2.20% and 2.09%, respectively), which did not differ from each other.

4. Discussion

The improvement in the dehydration rate promoted by the MEC + BCC method occurred because of the action of potassium sulfate and Bacillus bacteria, which are components of the bacterial chemical compound. Potassium sulfate has been demonstrated to control the osmotic capacity of cells and extend the period of stomatal closure after harvesting. In addition, biosurfactants produced by *Bacillus* bacteria have been shown to reduce surface tension in water, thereby facilitating the degradation of the waxy layer on the leaf surface through their emulsifying and detergent properties [28,29].

Glyphosate alters the selective permeability of cell membranes and impairs the functioning of aquaporins, which are proteins essential for water transport and proper stomatal function, slowing water loss and consequently reducing the dehydration rate of the plant [30,31].

Because evaporation is highly dependent on climatic factors, from the moment the stomata close and the cuticle promotes resistance to dehydration [32], it is important to disrupt this structure so that greater evaporation of water occurs in the plant. These cracks in cuticular wax are generated by metabolites produced by *Bacillus* bacteria, which are present only in the bacterial chemical compound (Biosurfactants).

In this study, the Shannon index represents the bacterial diversity, and if it is above 2.0, it is considered high [33]. When analyzing Table 2, the high diversity at the genus level in the haylages studied is a strong indication that the dehydration methods used did not affect bacterial development. However, the lack of difference in diversity (Phylum and Genus) between the haylages does not indicate similar bacterial abundance between the haylages, as this can be influenced by the concentrations of organic acids and the chemical composition of the ensiled material [10].

The greater abundance of Proteobacteria results from their ability to thrive in environments with relatively low pH and anaerobic conditions, which are established during the fermentation process inherent to haylage production [34]. The significant participation of Bacteroidetes in the bacterial communities of preserved foods (haylages and silages) is of particular significance. These bacteria are capable of hydrolyzing polysaccharides from organic matter and converting them into monosaccharides, which are subsequently available to other microorganisms or animals during feeding [35,36].

The subdominance of the Phylum Firmicutes (26.29% $\pm$ 0.78) in haylage made from forage desiccated with glyphosate is not detrimental, since the bacteria of this phylum, in addition to producing organic acids through fermentative processes, also develop well in anaerobic environments with low pH [37,38].

The role of bacteria in the genus *Pantoea* during fermentation remains to be fully elucidated. Ogunade et al. [35] and Guan et al. [39] have reported that these bacteria can reduce ammonia nitrogen and convert soluble carbohydrates into organic acids during fermentation. Therefore, Li et al. [40] regarded them as undesirable and hypothesized that their existence is limited to competition for the same substrates utilized by lactic acid bacteria. Nevertheless, the higher prevalence of this bacterial genus in the haylages evalu-

ated in the present study did not allow for the determination of losses in the fermentation process. This was because the concentration of lactic acid and the pH values of the haylages evaluated did not differ from each other and were adequate for haylage (Table 3).

Although the genera *Sphingobacterium* and *Stenotrophomonas* do not show any difference between haylages, their relative abundance has attracted attention, and it is important to emphasize their importance. According to Guo et al. [41] and Chen et al. [42], they can sequester carbon dioxide (CO_2) during fermentation, thus contributing to the reduction of gas emissions and nutrient loss. During their autotrophic anaerobic growth, bacteria of these genera convert CO_2 into organic acids via microbial electrosynthesis [43].

The bacterial genus *Lactobacillus* is classified into two distinct categories: homofermentative, which converts glucose into lactic acid, and heterofermentative, which converts glucose into lactic acid, acetic acid, and CO_2 [44]. Bearing this in mind, it is suggested that there was a higher prevalence of heterofermentative bacteria in the haylage obtained from the forage desiccated with glyphosate because its acetic acid content was higher than that of the others (Table 3).

The *Weissella* genus can be substituted by species such as *Lactobacillus*, which are capable of tolerating an acidic pH environment during the fermentation process [38,39]. Most bacterial species within the genus *Weissella* are obligate heterofermentative, meaning that they produce lactate and acetate after metabolizing glucose [38]. The substantial prevalence of this bacterial genus in the haylage obtained from forage dehydrated using the MEC + BCC method (Table 3) justifies its second-highest acetic acid content among the haylages (Table 3).

Some bacteria of the genus *Enterococcus* are considered undesirable by Muck [45] because of their ability to degrade proteins and amino acids and produce ammonia nitrogen. Although their relative abundance was low in the present study, their potential contribution may have been responsible for the production of isobutyric acid in the haylages studied (Table 3), an acid derived from deamination, according to Kung et al. [46].

The products resulting from fermentation (i.e., organic acids) and the maintenance of the aerobic stability of haylage after exposure to oxygen are closely linked to the microorganisms that develop during fermentation. The longer time for the loss of stability in haylage obtained from forage desiccated with glyphosate was due to its higher content of acetic and propionic acids, which have antifungal properties [47,48]. These acids are potentially derived from Lactobacillus, a genus with a higher prevalence in this haylage (Figure 5 and Table 3).

Following exposure of haylage to oxygen, an increase in temperature over time is expected because of the proliferation of fungi and yeasts, marking the onset of food deterioration. However, this process must be controlled as much as possible to mitigate nutrient loss. The cumulative temperature is a metric that quantifies the effectiveness of this control, with lower values indicating lower proliferation of spoilage microorganisms and reduced nutrient loss. It is noteworthy that both haylages exhibiting lower cumulative temperatures attained the highest mean values of acetic acid (3.96 g kg^{-1} and 3.36 g kg^{-1}, respectively). It is known to have significant antifungal properties.

A correlation analysis facilitates the identification of factors that have a direct or inversely proportional influence on each other. The positive correlation between the cumulative temperatures is related to the direct relationship between the variables; that is, once the deterioration process begins, the temperature tends to rise gradually. The positive correlation between the cumulative temperatures and the genera *Stenotrophomonas* and *Sphingobacterium* occurred because of the presence of oxygen, as these are Gram-negative, non-fermenting, aerobic, or facultative anaerobic bacilli [40].

The positive correlation between organic acids is related to the bacterial genera that produces them. According to Kung et al. [46], the production of acetic acid and propionic acid comes from the degradation of glucose and lactic acid, an action carried out by heterofermentative bacteria. *Enterococcus* is one of the main bacterial genera that promotes this action, However, bacteria of the genus *Pantoea* are also capable of performing this action, even though some works in the literature have treated their action as uncertain, Ogunade et al. [35] and Guan et al. [39] report that these bacteria are capable of converting soluble carbohydrates into organic acids, an action that possibly occurred in the present study. The presence of isobutyric acid, which is also produced by *Enterococcus*, is undesirable because it is produced via deamination [45,46]. The observed correlations between the bacterial genera are justified by the fact that they belong to the same phylum.

The negative correlations between cumulative temperatures and acetic and propionic acids are explained by the fact that these acids have antifungal action [49], and the higher their concentrations, the lower the proliferation of fungi and yeasts tends to be after exposure of haylage to oxygen, which is responsible for the temperature rise.

The negative correlation between the genera *Sphingobacterium* and *Stenotrophomonas* and the genus *Pantoea* may be due to the greater conversion of soluble carbohydrates into organic acids, which lowers the pH of the medium and restricts the development of bacteria of the genus *Pantoea* [50]. Negative correlations were also detected between *Lactobacillus*, *Enterococcus*, and *Aerococcus* with *Weissella*, lactic acid, and *Sphingomonas*, which may have arisen due to competition for substrate and pH reduction [39,51].

The haylage obtained from the MEC + BCC dehydration method exhibited a higher content of lactic acid and bacteria of the genus *Weissella*. This was a positive result, as bacteria of this genus have been shown to produce lactate and acetate [38]. However, haylage also had a lower presence of the genera *Lactobacillus*, *Enterococcus*, and *Aerococcus*. In the case of forage subjected to mechanical dehydration, haylage had a higher and lower presence of bacteria of the genera *Stenotrophomonas* and *Pantoea*, respectively. Bacteria of the genus *Stenotrophomonas* have been observed to reduce gas emissions and nutrient losses and enhance the reduction of the pH of the medium during their growth, as they convert CO_2 into organic acids. In contrast, bacteria of the genus *Pantoea* exhibit restricted growth in acidic media, which is consistent with their low prevalence [41,43].

The haylage obtained from forage desiccated with glyphosate had a higher concentration of propionic and acetic acids, suggesting that this is the effect of a higher prevalence of heterofermentative bacteria.

The increase in fiber content was also related to losses during fermentation (Table 2). According to Simionatto et al. [52], fermentation losses reduce the content of non-fiber carbohydrates and concentrate fiber carbohydrates, which is undesirable because it reduces feed intake and digestibility by limiting the action of digestive enzymes produced by ruminal microorganisms [53,54].

The lower CP content of the haylage obtained from CHE dehydration suggests that it is associated with its higher content of NH_3-N (% total nitrogen) (0.23%). NH_3 is a metabolite produced by proteolysis, and this reaction becomes more pronounced when there is a slow decline in pH and intense activity of enterobacteria and *Bacillus* spp. [35,46]. However, it is noteworthy that the values observed in this study are below the threshold established by Kung et al. [46], which is 10%.

The content of nitrogen bound to fiber carbohydrates (NDN and ADN) merits particular consideration, as higher values correspond to reduced utilization by ruminants [55]. Consequently, it can be inferred that when haylage is produced from forage desiccated with glyphosate, the utilization of its protein is relatively low, which may have deleterious effects on animal production, whether it is intended for meat or milk production.

According to Ribas et al. [6], when forage is dehydrated in the field, EE content is reduced. This is because after harvesting, intracellular activities continue, and in an attempt to produce energy, triglycerides are converted into glycerol and pyruvic acid, which are used by the mitochondria. In essence, as the time forage remains in the field increases, EE consumption escalates, validating the observations in Figure 2 and Table 5.

Higher concentrations of non-fiber carbohydrates in feed intended for ruminants are advantageous because these carbohydrates serve as essential substrates for ruminal microorganisms, which are responsible for the fermentation process inside the rumen [56].

The greater DM losses observed during the fermentation process in haylage prepared by the CHE method indicate that these losses are the result of a greater presence of heterofermentative bacteria. These bacteria use one mole of glucose to produce one mole of lactic acid, CO_2, or ethanol and acetic acid, in addition to competing for substrates with homofermentative bacteria, which are responsible for reduced DM losses, as one mole of glucose generates two moles of lactic acid [47,57].

Similarly, gas losses are associated with a higher prevalence of heterofermentative bacteria [58]. Reduced gas loss indicates a lower prevalence of secondary fermentation during the fermentation phase, which is considered undesirable. This phenomenon occurs at a lower intensity, ensuring better preservation of ensiled material [59].

The greater prevalence of heterofermentative bacteria in haylage made from forage desiccated with glyphosate was beneficial in the sense of increasing the aerobic stability of this material due to the greater production of fermentation-inhibiting acids; but, on the other hand, their greater presence culminated in greater losses during the fermentation process, as they were less efficient in producing organic acids than homofermentative bacteria, as mentioned above (they are bacteria that use more glucose and lactic acid molecules to produce acetic and propionic acid).

A comprehensive literature review and data analysis were conducted to identify the most effective fermentation strategy for maximizing nutrient preservation in haylage. Our results indicate that the most successful fermentation was triggered by the increased presence of bacteria from the genera *Sphingobacterium* and *Stenotrophomonas*, which sequester CO_2 during fermentation, leading to a reduction in gas emissions and nutrient loss. In addition, the presence of *Lactobacillus*, which converts glucose into lactic and acetic acids, is critical for successful fermentation. Finally, the inclusion of *Weissella* bacteria, which also produce lactate and acetate, is essential to achieve optimal fermentation results.

5. Conclusions

Haylage made from dehydrated forage using the Mechanical + Bacterial Chemical Compound and Chemical methods presented a better fermentation pattern and provided a greater prevalence of desirable bacterial genera during the fermentation phase.

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