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Dry Matter Content and Additives with Different Modes of Action Modify the Preservation Characteristics of Grass Silage

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Abstract: Two experiments evaluated how grass silage quality can be manipulated by various management options. In Experiment 1, silage characteristics were evaluated at two dry matter (DM) contents and treated with additives presenting different modes of action. Timothy grass was ensiled at low (224 g/kg) and high (534 g/kg) DM contents and five additives were applied: 1. control (C), 2. homofermentative lactic acid bacteria inoculant (HO), 3. heterofermentative lactic acid bacteria inoculant (HE), 4. salt-based additive (SA) and 5. Formic- and propionic-acid-based additive (FPA). A higher DM content and FPA restricted silage fermentation, and additive effects were generally greater in low rather than high DM silages. The chemical additives SA and FPA resulted in the highest aerobic stability, while the HE improved it at a high DM content. In Experiment 2, the low DM content grass was ensiled utilizing resin acids, as follows: 1. C, 2. FPA, 3. Resin acid oil (FOR) at 13 L/t, 4. FOR at 26 L/t, 5. Resin acid soluble in water (ROS) at 13 L/t and 6. ROS at 26 L/t. Wilting combined with additives improved the preservation characteristics of grass silages (Experiment 1), but resin acid products failed to modify silage fermentation compared to the control (Experiment 2).

Keywords: aerobic stability; animal feedstock; formic acid; inoculant; ensiling management; *Phleum pratense*; propionic acid; resin acid; salt; wilting

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1. Introduction

Ensiling is a mainstream technology used to preserve forage that can reduce the shortage of green biomass for ruminants affected by climate seasonality [1]. The ensiling process is characterized by the rapid fermentation of fresh forage caused by the activity of lactic acid bacteria (LAB) under anaerobic conditions, which produces organic acids, mainly lactic acid, and significantly reduces the pH of the forage, suppressing the activities of harmful microorganisms [2–4]. Successful silage production plays an important role in the economics of animal production and helps to overcome the seasonal imbalance between animal feed demand and available high-quality forage by extending the duration of forage storage [5]. The preservation quality of grass silage greatly affects the losses during storage, aerobic stability during the feed-out period and the voluntary feed intake of animals, which emphasizes the need to continuously develop it to ensure the economic performance and safety of the food chain based on ruminants.

Success in ensiling depends on many management factors, such as the dry matter (DM) content of the raw material and the use of silage additives [6]. The DM content can be manipulated by the extent of wilting modified by the prevailing weather conditions. Controlling the DM content of silage may become more challenging in the future due to the increase in extreme weather events related to climate change [7]; it is therefore important to be able to choose the correct additive for grass, which may differ from the targeted DM level.

Guo et al. [8] stated that silage making without the use of inoculants or any other starter is a spontaneous fermentation process in which fermentation mainly relies on the

natural occurrence of epiphytic LAB. However, relying only on them can lead to undesirable fermentation, emphasizing the need to apply exogenous additives, which would accelerate, direct or restrict the ensiling process. The development and use of various silage additives aim to control or modulate the fermentation pattern in silage to ensure a good fermentation quality [9]. Resin acids are the defence chemicals of coniferous trees [10], and it has been hypothesized that they could positively affect animal production, although their effects on ruminants have not been proven [11]. Because of their antimicrobial activity, resin acids may have the potential to improve the silage preservation quality and aerobic stability.

Due to the continuous need to improve silage quality, we aimed to evaluate the fermentation quality, microbial counts and aerobic stability of grass silages produced under different management conditions, such as DM content and silage additive application. In the first experiment, four currently commercially available silage additives with different modes of action were evaluated, because it is important to obtain more direct comparisons of their efficacy when used for biomasses with variable characteristics. In the second experiment, the effects of coniferous resin acids were evaluated for the first time, to the best of our knowledge, as silage additives.

2. Materials and Methods

2.1. Raw Material for Silage Making

Mixed timothy (*Phleum pratense*) and meadow fescue (*Festuca pratensis*) grass was harvested from the first regrowth on 12 August 2020 at the Natural Resources Institute Finland (Luke) in Jokioinen (60°48′ N, 23°29′ E), Finland. A composite sample was obtained from the standing crop before cutting by taking four representative 0.25 m^2 samples, which were manually divided into timothy and meadow fescue to evaluate the botanical composition. The sward was dominated by timothy, with proportions of timothy and meadow fescue being 0.82 and 0.18, respectively, on a DM basis.

The grass was cut using a mower conditioner (JF GMS 3200 Topflex, JF-Fabriken-J Freudendahl A/S, Sonderborg, Denmark and Krone EC 32 CV in front hitch, Maschinefabrik Bernard Krone GmbH, Spelle, Germany), precision chopped using farm-scale machinery (JF FCT 1350, JF-Fabriken-J Freudendahl A/S, Sonderborg, Denmark) and transported to the laboratory without any additives.

2.2. Experimental Treatments and Procedures

2.2.1. Experiment 1—Conventional Silage Additives

The silages were prepared with two distinct DM contents. For the lower DM, grass was ensiled 2 h after harvesting, while for the higher DM, grass was artificially wilted in a forced-air open-circuit drier using an air temperature of 30 °C for 3 h. Raw material samples of both DM contents were taken before treatment application in order to evaluate the chemical composition and microbial counts of the grass before ensiling.

For both DM contents, five additive treatments were applied according to commercial recommendations, including the following:.

1. Control I, as a negative treatment without additive.
2. Homofermentative LAB *Lactobacillus plantarum* (DSM 12836; 1k2078; min. 1×10^{11} cfu/g) and *Pediococcus pentosaceus* (HO; DSM 12834; 1k2103; min. 1×10^{11} cfu/g; Bonsilage, Schaumann Agri International GmbH, Pinneberg, Germany at 1 g/t, resulting in a minimum of 2×10^5 cfu/g of fresh forage).
3. Heterofermentative LAB *Lactobacillus buchneri* (DSM 13573, 1k20733; min. 1×10^{11} cfu/g), combined with homofermentative *Lactobacillus plantarum* (DSM 3676, 1k20731; min. 0.5×10^{11} cfu/g) and *Lactobacillus plantarum* (HE; DSM 3677, 1k20732; min. 0.5×10^{11} cfu/g; Feedtech Silage F600, DeLaval, Tumba, Sweden at 1 g/t, resulting in a minimum of 2×10^5 cfu/g of fresh forage).
4. Salt-based additive (SA; sodium benzoate, potassium sorbate and sodium nitrite; Safesil Pro, Salinity AB, Göteborg, Sweden at 5 L/t).

5. Formic- and propionic-acid-based additive (FPA; formic acid, propionic acid, sodium formate and potassium sorbate; AIV Ässä Na, Eastman, Oulu, Finland at 5 L/t).

After the careful homogenization of the raw material, it was divided into 8 kg batches, in which the additive treatments were applied separately for each replicate. In order to evenly apply the additive to the grass, the additives were diluted in tap water so that the final amount of liquid applied was 10 L per ton of fresh matter for all treatments including the control, which contained solely tap water.

After additive application, the grass was ensiled into cylindrical pilot scale silos with a 12 L capacity using three replicates per treatment. The silos were then covered with a plastic cover, plastic lid, an 8 kg lead plummet, and a water lock. The silos were stored at room temperature with protection from light and opened after an ensiling period of 3 months. The amount of silage in cm and diameter of the cylindrical silos, as well as their weight, were measured right before opening in order to calculate the density after the ensiling period. The resulting densities of the silages for low and high DM contents were 675 kg/m^3 (151 kg DM/m^3) and 464 kg/m^3 (240 kg DM/m^3), respectively. The weight after silage making and the weight right before opening were used to calculate the ensiling losses according to Knicky and Spörndly [12], assuming that the weight loss was CO_2 leaving the silo during fermentation. Visually deteriorated parts on the surface of the silage were discarded, and the silage was carefully mixed and samples were taken and analysed for chemical composition, fermentation quality, microbial counts and aerobic stability.

2.2.2. Experiment 2—Resin Acids Used as Silage Additives

These silages were prepared with the low DM raw material mentioned above and described in Table 1. Six additive treatments were applied using three replicates per treatment, as follows:

1. Control (C), as a negative treatment without additive.
2. Formic- and propionic-acid-based additive (FPA; formic acid, propionic acid, sodium formate and potassium sorbate; AIV Ässä Na, Eastman, Oulu, Finland at 5 L/t).
3. Resin acid oil (FOR; Forchem Ltd., Rauma, Finland at 13 L/t).
4. FOR (at 26 L/t).
5. Resin acid soluble in water (ROS; Forchem Ltd., Rauma, Finland at 13 L/t).
6. ROS (at 26 L/t).

Table 1. Composition, feed values and microbial quality of first regrowth mixed timothy and meadow fescue grass at two dry matter (DM) contents.

	Low DM	**High DM**
DM, g/kg	224	534
Buffering capacity, g lactic acid/100 g DM	5.4	5.6
Fermentation coefficient	39.3	74.3
In DM, g/kg		
Ash	92	92
Crude protein	111	107
Water-soluble carbohydrates	115	145
Neutral detergent fibre	548	527
Organic matter digestibility, g/g OM	0.760	0.781
Microbial counts, colony-forming units/g		
Yeasts	2.6×10^5	6.2×10^5
Moulds	1.4×10^5	3.9×10^5
Total aerobic bacteria	4.5×10^7	9.9×10^7

The resin acid products originated from a northern coniferous tree, Scots pine (*Pinus sylvestris*). FOR is a distilled tall oil fatty acid product containing 2% resin acids, while ROS contains Na salts of tall oil and resin acids, as well as propylene glycol diluted with water containing 2% resin acids in the final product.

To evenly apply the additives to the grass, the additives were diluted in tap water so that the final amount of liquid applied was 26 L per ton of fresh matter for all treatments including the control, which contained solely tap water. After additive application, the 3 kg batch was divided into two plastic vacuum bags (BK3550; 300 mm × 500 mm, 52 µm; Sealed Air Food Care, Duchnice, Poland) and vacuum sealed (sealed for 25 s with a vacuum of 5 mbar, sealed for 1.8 s and cooled for 2.0 s using an industrial vacuum machine; GK187R/2R; Supervac, Vienna, Austria). The silos were stored at room temperature with protection from light and opened after an ensiling period of 3 months.

2.3. Laboratory Analyses

The samples to be used to evaluate the chemical composition and fermentation quality were stored at a temperature of −20 °C prior to analysis, according to standard laboratory methods. Chemical analyses were carried out at Luke Laboratory in Jokioinen. The laboratory has a quality system that follows the SFS-EN ISO/IEC 17025:2005 standards and is accredited by FINAS (the Finnish Accreditation Service) with number T024. The DM content of the silages and the raw material contents were determined by drying the samples at 105 °C for 16 h, and then the silages were corrected for volatile compound losses [13]. The crude protein content was determined according to AOAC [14] method 968.06 (conversion factor 6.25 × N, using Leco FP 428 nitrogen analyser [Leco, St. Joseph, MI, USA]), and the ash content was determined according to AOAC [14] method 942.05. Water-soluble carbohydrates (WSCs) were determined according to Somogyi [15], volatile fatty acids (VFAs) were determined according to Huhtanen et al. [16], and the concentration of propionic acid was corrected for the amount added to the FPA using an 80% recovery rate. The lactic acid content was analysed according to Haacker et al. [17] and ammonia according to McCullough [18]. Lactic acid and ammonia were analysed using the water extract of the samples, and the equipment used was a UV–Vis double-beam UV-1800 spectrophotometer (Shimadzu Co., Kyoto, Japan). The N content of the raw material before ensiling was used to express the ammonia–N proportions in total N after fermentation. The in vitro organic matter digestibility (OMD) was determined according to Nousiainen et al. [19] using a correction equation of pepsin–cellulase solubility to in vivo digestibility by employing data from Finnish in vivo digestibility trials [20]. The neutral detergent fibre (NDF) content was determined by using a ANKOM 220 Fiber Analyzer (ANKOM Technology, Macedon, NY, USA) according to Van Soest et al. [21], using sodium sulphite and being expressed without ash. A spectrophotometric method was used for the determination of ethanol from water–silage extracts (commercial kit cat. No. 10 176 290 035, Boehringer Mannheim GmbH, Mannheim, Germany), according to the manufacturer's instructions. The buffering capacity (BC) of the raw material was analysed according to Weissbach et al. [22]. The fermentation coefficient (FC) was calculated based on the DM, WSCs and BC of the raw material using the following formula: FC = DM (%) + [8 × WSC (%)/BC (g lactic acid/100 g DM)] [23].

The samples to be used to evaluate the microbial quality were immediately sent to the laboratory and analysed from fresh matter. The samples were mixed and 25 g was weighed into stomacher bags and mixed with 225 mL of $\frac{1}{4}$ strength Ringer solution (Merck 1.15525.0001, Merck KGaA, Darmstadt, Germany). The samples were homogenized with stomacher (Stomacher® 400 Circulator, Seward Ltd., Worthing, UK) for 2 min at 230 rpm. Serial decimal dilutions were prepared by mixing 1 mL of sample with 9 mL of Ringer solution. Yeasts and moulds were enumerated via cultivation on Dichloran Rose Bengal Chloramphenicol Agar (Lab217, Lab M Ltd., Lancashire, UK) with 50 µg/mL of oxytetracycline hydrochloride (AppliChem BioChemica A5257, Darmstadt, Germany). The Petri dishes were incubated at 25 °C and visually counted after three and five days. The total aerobic bacteria plate count was determined on Plate Count Agar (Lab M Ltd., Lab010, Lancashire, UK) dishes incubated at 30 °C for 72 h.

For aerobic stability measurement, approximately 600 g of silage for low DM and 400 g of silage for high DM were placed into a polystyrene box with internal dimensions of 13.3 cm × 13.3 cm × 10.3 cm, resulting in 1822 cm^3. Air ingress was allowed into the

box, and a thermocouple wire was inserted into the middle of the sample and connected to a data logger. Temperature was automatically recorded at 10 min intervals for a 336 h (14 days) follow-up period. Aerobic stability was defined as the time taken to increase the temperature of the sample by 2 °C above the ambient temperature. The ambient temperature was 19.0 ± 0.13 °C (min. 18.5 °C and max. 20.0 °C), measured using data logger similar to that employed for the samples.

2.4. Statistical Analyses

Experiment 1 was carried out according to a 2 × 5 factorial scheme (two DM contents and five additive treatments) and the data were analysed using a MIXED procedure of SAS (SAS Inc. 2002–2012, Release 9.4; SAS Inst., Inc., Cary, NC, USA). The DM contents, additive treatments and their interaction were considered as fixed effects, while the replicate was treated as a random effect in the statistical model. Experiment 2 was also analysed using a MIXED procedure of SAS, considering treatment as a fixed effect and replicate as a random effect. Additionally, different contrasts were prepared to evaluate the linear and quadratic effects of the resin acids, the control against the resin acids, the FPA against the resin acids, as well as the FOR vs. the ROS. The Univariate procedure was used to test the normal distribution of data via the Shapiro–Wilk test. Least squares means and the standard errors of the means were reported per treatment and differences among the treatment means were declared significant at 5% of probability. In addition, pairwise comparisons among all treatment means were performed using Tukey's test at a probability level of $p < 0.05$.

3. Results and Discussion

3.1. Raw Material Characteristics

Timothy grass is the most important perennial forage grown for silage and hay production in the cool humid regions of Northern Europe, Asia and North America. The popularity of timothy in these areas is mainly because of its winter hardiness [24]. Timothy typically has a lower WSC concentration than, e.g., ryegrass, but in the current silage raw material, the WSC concentration was high (on average 130 g/kg DM), making the crop relatively easy to ensile.

The raw material DM content of grass is highly dependent on the extent of wilting. In this study, the low and high DM contents (Table 1) position the raw material well below and well above, respectively, the mean values in a Finnish dataset containing more than 110,000 farm silage samples (321 g/kg; [25]). The difference between the high and low DM contents was 2.4-fold; consequently, the likelihood of the FC being directly affected by the DM content was significantly higher for silages produced using a high DM content. The FCs of the low and high DM raw materials were categorized as reflecting an intermediate and high ease of ensilage, respectively [26]. Silages with a DM concentration below 300 g/kg, which was the case in the grass with a low DM content in the current study, are more prone to clostridia proliferation if the silages are prepared without additives [27].

Other parameters related to the chemical composition of the grass raw material were minimally affected by the DM content (Table 1), since the values were expressed on a DM basis. The amount of WSCs in the raw material is of great importance, as it offers a substrate for lactic acid fermentation; therefore, the minimum concentration of WSCs for proper silage fermentation is at least 50 g/kg DM. In our study, the WSC concentration of both DM contents reached the threshold, indicating that the microorganisms had a sufficient amount of substrate to promote lactic acid fermentation. The OMD was slightly higher for the high DM grass raw material; this is somehow unexpected, as plant respiration during wilting should consume digestible nutrients, and typically, the OMD of wilted material is lower than that of fresh grass [28]. The rapid artificial drying may have contributed to the lack of OMD decline. In general, the grass would provide a nutritionally high-quality raw material under Northern European conditions [20].

Low counts of moulds and yeasts in the raw material during harvest facilitate the adequate aerobic stability of the silage when opened and exposed to aerobic conditions

during the feeding period [29]. Kristensen et al. [30] indicated that contamination by yeasts and moulds should be below 10^6 for the raw material to be considered of adequate microbiological quality, which, in the case of this experiment, was achieved (Table 1). The microbial counts were somewhat higher for high rather than low DM raw material, but this is merely an effect of concentrating them, as the counts are expressed per fresh matter. A longer wilting period could modify the epiphytic flora, as evidenced by the case of Franco et al. [28], where a 20 h wilting period under poor wilting conditions resulted in hardly any change in the DM content of the grass material; however, a drastic change in the fermentation pattern was observed for the untreated silages, which probably was a response to the development of epiphytic flora during the wilting period.

3.2. Fermentation Quality of the Experimental Grass Silages

3.2.1. Experiment 1—Conventional Silage Additives

Ensiling is a complex process in which bacterial communities promote fermentation that results in the accumulation of organic acids and consequently a decrease in pH. McDonald et al. [2] established that a pH of 4.2 or less indicates that the silage is well fermented, and in the case of this study (Figure 1), most silages were at this threshold or even below. All pH values found here were below the values established by Kung et al. [4] for grass silages (4.30–4.70), who also indicated that silages produced from raw material with a high DM content have a higher pH than silages produced using low DM raw material; this was found in the current experiment as well. The higher pH of silages produced using raw material with a high DM is caused by the availability of metabolic water for LAB growth becoming gradually limited as the DM increases [31].

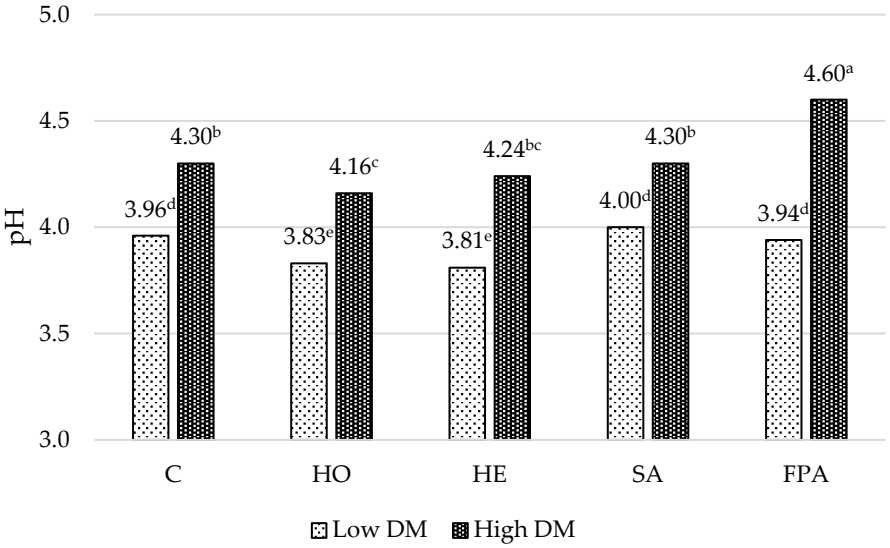

Figure 1. The pH of grass ensiled at two dry matter (DM) contents and treated with additives (Add). Additives: C, control; HO, homofermentative strains of lactic acid bacteria inoculant; HE, heterofermentative and homofermentative strains of lactic acid bacteria inoculant; SA, salt-based additive; FPA, formic- and propionic-acid-based additive. *p*-values: DM, <0.01; Add, <0.01; DM × Add, <0.01. Standard error of the mean: 0.020. Values with the same letter are not significantly different ($p > 0.05$) based on Tukey's test.

During the silage fermentation process, forage nitrogenous compounds undergo vegetal and microbial proteolysis. The restriction of fermentation also typically restricts protein degradation. Kung et al. [4] stated that silages produced using raw material with a low DM content have higher ammonia-N concentrations, which was also detected in the current experiment (Figure 2). A high ammonia-N proportion and butyric acid concentration indicate high clostridial activity, which is a particular risk in low DM silages [2]; however, butyric acid concentrations were low in all silages, even though they were statistically

higher in low rather than high DM silages ($p < 0.05$; Table 2). The rapid drop in the pH of the silage has a direct effect on the inhibition of clostridia since these microorganisms are intolerant to both low pH levels and high osmotic pressures.

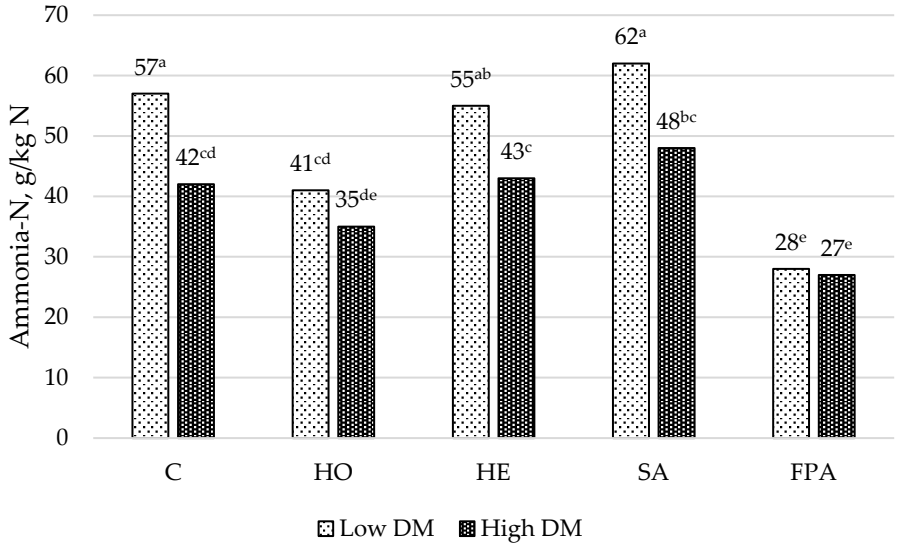

Figure 2. Ammonia-N of grass ensiled at two dry matter (DM) contents and treated with additives (Add). Additives: C, control; HO, homofermentative strains of lactic acid bacteria inoculant; HE, heterofermentative and homofermentative strains of lactic acid bacteria inoculant; SA, salt-based additive; FPA, formic- and propionic-acid-based additive. *p*-values: DM, <0.01; Add, <0.01; DM × Add, <0.01. Standard error of the mean: 1.6 g/kg N. Values with the same letter are not significantly different ($p > 0.05$) based on Tukey's test.

The interaction effect for the silage ammonia-N proportion is visible in Figure 2. The FPA application effectively decreased the silage ammonia-N concentration in both DM contents as only at a high DM content did the HO not statistically differ from the FPA silages. The biological mechanism involved in the ability of the FPA to decrease ammonia-N is due to the rapid pH drop caused by direct acidification and the restricted fermentation, with a lower number of fermenting microorganisms able to cause protein breakdown [2]. The restriction of the fermentation of grass silage via the application of formic acid-based additives is well established in other studies [6,28,32–34]. Wilkinson [35] stated that grass silages with ammonia-N below 50 g/kg total N are extremely well fermented, but those in the range of 50–100 g/kg total N are regarded as well fermented, showing that even if the ammonia-N in the C, HO, HE and SA was higher than in the FPA, they were still well preserved.

The SA has been shown to be effective against the action of clostridia, but in the current material, clostridial fermentation was not evident even in the C; therefore, no effect of the additive ($p = 0.389$; Table 2) was identified regarding the production of butyric acid. Pahlow et al. [36] stated that high ammonia-N in silage may be a result of clostridial activity; however, no elevated ammonia-N concentrations were observed in any of the silages (Figure 2), supporting low clostridial activity in the current material.

The silage ethanol concentration was lower in high rather than low DM silages, but there was a statistically significant ($p < 0.01$) interaction between the silage DM and the additive treatment (Table 2) as, e.g., the FPA-treated silages had the highest ethanol concentration at low DM contents but the lowest concentration at high DM contents. Ethanol is the alcohol most commonly found in silages and can be produced by a wide variety of microorganisms, including heterolactic acid bacteria, enterobacteria and yeasts. Kung et al. [4] indicated that acceptable levels of ethanol in grass silages are around 5 to 10 g/kg DM, which places most of the silages in this study even below the lower value.

Table 2. Chemical composition, fermentation quality, ensiling losses and microbial counts of grass ensiled at two dry matter contents and treated with additives.

Dry Matter (DM)	Low DM					High DM					SEM [2]	*p*-Value [3]		
Additives [1] (Add)	C	HO	HE	SA	FPA	C	HO	HE	SA	FPA		DM	Add	DM × Add
DM, g/kg	221 ^{b}	218 ^{b}	223 ^{b}	226 ^{b}	228 ^{b}	526 ^{a}	512 ^{a}	512 ^{a}	513 ^{a}	521 ^{a}	3.9	<0.001	0.104	0.231
Chemical composition, g/kg DM														
Water-soluble carbohydrates	29 ^{d}	30 ^{d}	23 ^{d}	34 ^{d}	32 ^{d}	82 ^{b}	90 ^{b}	62 ^{c}	82 ^{b}	172 ^{a}	3.6	<0.001	<0.001	<0.001
Ethanol	4.5 ^{b}	4.1 ^{bc}	3.5 ^{bcd}	1.2 ^{e}	7.2 ^{a}	2.0 ^{cde}	1.8 ^{de}	2.0 ^{cde}	1.0 ^{e}	0.7 ^{e}	0.42	<0.001	<0.001	<0.001
Acids, g/kg DM														
Lactic (LA)	93.5 ^{b}	114.0 ^{a}	97.0 ^{b}	86.6 ^{b}	54.8 ^{d}	52.5 ^{d}	68.7 ^{c}	60.2 ^{cd}	51.5 ^{d}	19.1 ^{e}	2.49	<0.001	<0.001	0.245
Acetic (AA)	15.3 ^{d}	12.6 ^{ef}	20.1 ^{b}	17.1 ^{c}	22.0 ^{a}	10.7 ^{g}	8.0 ^{h}	13.8 ^{de}	11.8 ^{fg}	6.3 ^{i}	0.32	<0.001	<0.001	<0.001
Propionic [4]	0.19 ^{ab}	0.21 ^{ab}	0.25 ^{a}	0.15 ^{bcd}	0 ^{e}	0.08 ^{d}	0.08 ^{d}	0.08 ^{d}	0.10 ^{cd}	0.16 ^{bc}	0.014	<0.001	<0.001	<0.001
Butyric	0.05	0.03	0.03	0.01	0.06	0.02	0.02	0.02	0.02	0.01	0.010	0.013	0.398	0.180
Total volatile fatty acids	15.5 ^{d}	12.8 ^{ef}	20.4 ^{b}	17.3 ^{c}	22.1 ^{a}	10.8 ^{g}	8.1 ^{h}	13.9 ^{de}	12.0 ^{fg}	6.5 ^{h}	0.33	<0.001	<0.001	<0.001
Total fermentation acids [5]	109 ^{bc}	127 ^{a}	117 ^{ab}	104 ^{c}	77 ^{d}	63 ^{e}	77 ^{d}	74 ^{de}	64 ^{e}	26 ^{f}	2.5	<0.001	<0.001	0.204
Total fermentation products [6]	114 ^{bc}	131 ^{a}	121 ^{ab}	105 ^{c}	84 ^{d}	65 ^{e}	79 ^{d}	76 ^{de}	65 ^{e}	26 ^{f}	2.5	<0.001	<0.001	0.029
LA/AA ratio	6.13 ^{b}	9.11 ^{a}	4.85 ^{bc}	5.06 ^{bc}	2.48 ^{e}	4.92 ^{bc}	8.62 ^{a}	4.36 ^{cd}	4.35 ^{cd}	3.02 ^{de}	0.266	0.012	<0.001	0.053
Losses, g/kg initial DM	9.3 ^{cd}	8.7 ^{cd}	9.5 ^{cd}	6.6 ^{d}	12.3 ^{abcd}	16.3 ^{ab}	14.7 ^{abc}	18.3 ^{a}	16.8 ^{a}	9.9 ^{bcd}	1.32	<0.001	0.262	0.001
Microbial counts, cfu [7]/g														
Yeasts	1.5×10^{4}	1.7×10^{4}	4.0×10^{3}	1.0×10^{2}	2.0×10^{2}	4.4×10^{4}	1.0×10^{4}	4.0×10^{2}	9.8×10^{3}	1.6×10^{3}	1.5×10^{4}	0.524	0.320	0.761
Moulds	3.8×10^{3} ^{a}	1.4×10^{3} ^{ab}	1.5×10^{3} ^{ab}	2.0×10^{2} ^{b}	1.7×10^{3} ^{ab}	3.0×10^{2} ^{b}	2.0×10^{2} ^{b}	2.0×10^{2} ^{b}	1.0×10^{2} ^{b}	1.0×10^{2} ^{b}	6.2×10^{2}	<0.001	0.080	0.136

[1] Additives: C, control; HO, homofermentative strains of lactic acid bacteria inoculant; HE, heterofermentative and homofermentative strains of lactic acid bacteria inoculant; SA, salt-based additive; FPA, formic- and propionic-acid-based additive. [2] SEM, standard error of the mean. [3] *p*-value: DM, effect of dry matter content; Add, effect of additive; DM × Add, interaction between dry matter content and additive effects. [4] Corrected for its amount in the FPA-based additive. [5] Total volatile fatty acids + lactic acid. [6] Total fermentation acids + ethanol. [7] cfu, colony-forming unit. Values with the same letter in a row are not significantly different ($p > 0.05$) based on Tukey's test. If there were no differences in Tukey's test, letters were removed.

The additives used in the current experiment present different modes of action, and their established effects [37] were clearly visible: HO boosted lactic acid production, the HE increased acetic acid concentration, and the FPA restricted fermentation; meanwhile, the effects of the SA on the fermentation profile were minor under the conditions of the current experiment (Table 2). The WSC concentration of the grass was relatively high and the application of the HO increased ($p < 0.01$) the conversion of WSCs into lactic acid in both DM contents. On the other hand, fermentation was restricted by the FPA, resulting in a higher residual WSC concentration and a lower lactic acid concentration. This difference was greater in the high DM silage ($p < 0.01$), but no differences were found among the C, HE and SA treatments for these parameters.

Lactic acid is usually the acid found in the greatest concentration in silages and is the one that most contributes to the drop in pH, as it is about 10 to 12 times stronger than acetic and propionic acids [4]. The highest lactic acid production (Table 2) and the greatest drop in pH (Figure 1) occurring in the HO-treated silages produced under a low DM content is in line with that. The residual WSC concentration in the silages treated with the FPA and produced under a high DM content (Table 2) was much higher than in other treatments and even higher than in raw material, which was also found by Franco et al. [34] when applying the FPA in timothy grass silages. According to McDonald et al. [2], this result can be explained by the acid hydrolysis of NDF, which may have released sugars. The concentration of NDF was analysed only in the raw material, but Huhtanen et al. [38] observed that in a dataset of 52 grass silages treated with formic acid, the NDF concentration in the silages was on average 27 g/kg DM lower than in the grass raw material. Although the production of lactic acid in large amounts is beneficial for the silage fermentation process, it can have a negative effect on the voluntary intake of silage DM by dairy cows [39]. Therefore, the restriction of silage fermentation can increase the nutrient supply and the subsequent milk production of cows.

There were interactions between the DM content and additive treatment for most parameters evaluated in this study except for a few, such as lactic acid production (Table 2), which was similarly restricted by the FPA at both silage DM levels. The additive effects were, in general, greater in the low than in the high DM silages, as higher DM contents restrict fermentation.

The interaction effect ($p < 0.01$; Table 2) for acetic acid concentrations in silages was mainly due to the different effects of FPA application on both DM contents. Acetic acid was higher for low DM silages and highest for FPA silages, but the application of the FPA in high DM silages resulted in the most restricted fermentation, with the lowest production of acetic acid among the silages. The same pattern was observed for total VFAs, as it consists mainly of acetic acid. The DM content ($p < 0.01$) and additive application ($p < 0.01$) affected the ratio between lactic and acetic acids, as this ratio was higher in silages produced using low DM raw material. This ratio was the lowest for silages treated with the FPA, and the highest for silages treated with the HO, while the other additives showed intermediate results. Proper silage fermentation patterns result in an approximately 2.5 to 3 times greater production of lactic acid than acetic acid [4]. Considering this index, only silages treated with the FPA at both DM contents approached this ratio.

The evaluation of the yeast and mould presence in silages is important, because they may reduce the aerobic stability of silages. The fermentation during the ensiling process decreased the yeast and mould counts in relation to the raw material for all treatments, as all the values identified in the silages were lower than those found in the grass before ensiling. Probably due to high data dispersion, no significant differences were found regarding yeast counts ($p > 0.05$; Table 2), which are usually considered as starters of aerobic spoilage as they consume WSC and fermentation acids, and raise the silage temperature and pH [36]. Moulds were affected only by the DM content ($p < 0.01$) of the raw material, since the silages produced using a low DM content had higher mould concentrations. In order to guarantee the appropriate aerobic stability of silages when exposed to air, both yeast and mould counts should be below 10^6 [4,40], which was achieved in the current study.

However, care must be taken when interpreting data regarding microbial counts, and some of the reasons for this are as follows: 1. analyses do not discriminate between yeasts that assimilate lactate and those that do not; 2. although yeasts grow in laboratory culture media, this does not necessarily indicate their metabolic capacity in silages; 3. silages that have undergone an extensive deterioration process may have low yeast and mould counts, as these communities have already disappeared due to a lack of substrate; and 4. microbial counts can vary widely from sampling to laboratory analysis [4]. Therefore, silages with high or low yeast concentrations can be both stable or unstable when exposed to air.

Aerobic instability, or silage heating, is a process typically initiated by yeasts and continued by moulds, and as grass is wilted to higher DM contents, this becomes an increasingly relevant problem [29]. Silages treated with homolactic inoculants, as in the case of the HO, have a higher ratio between lactic and acetic acids, because homolactic bacteria exclusively produce lactic acid. These silages might be more prone to aerobic deterioration when exposed to air, as there is not enough acetic acid to inhibit lactate-assimilating yeasts. Such an effect was observed in this study, where the silages treated with the HO at a low DM content showed the highest ratio between lactic and acetic acids (Table 2), and the lowest aerobic stability (Figure 3). On the other hand, silages treated with heterofermentative inoculants tend to produce more acetic acid, which decreases this ratio, as found in this study for the HE (Table 2).

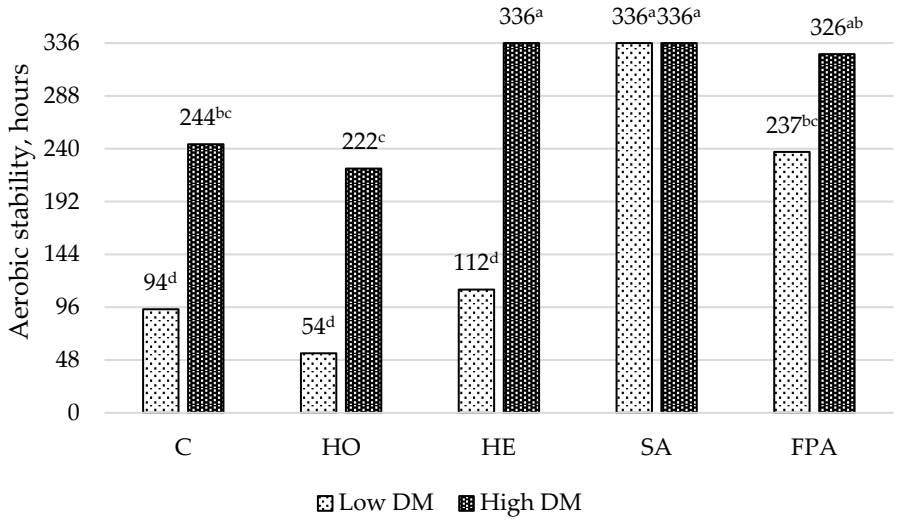

Figure 3. Aerobic stability (hours to reach 2 °C above room temperature) of grass ensiled at two dry matter (DM) contents and treated with additives (Add). Additives: C, control; HO, homofermentative strains of lactic acid bacteria inoculant; HE, heterofermentative and homofermentative strains of lactic acid bacteria inoculant; SA, salt-based additive; FPA, formic- and propionic-acid-based additive. *p*-values: DM, <0.01; Add, <0.01; DM × Add, <0.01. Standard error of the mean: 18.2 h. Values with the same letter are not significantly different ($p > 0.05$) based on Tukey's test. Evaluation conducted for 14 days (336 h), thus silages with 336 h did not heat.

An interaction between the DM content and additive application was found ($p < 0.01$; Figure 3) for the aerobic stability of silages. In low DM silages, the longest aerobic stability was found for the SA followed by the FPA treatment, while the C, HO and HE treatments did not differ from each other. With a high DM content, the HE and SA remained unheated even during the maximum time of aerobic stability evaluation (336 h), and were followed by the FPA, while the C and HO treatments had the lowest aerobic stability. The lowest aerobic stability for the HO-treated silages is in line with McEniry et al. [41] and Wilkinson and Davies [29], who also reported that the application of homofermentative LAB resulted in silages of poor aerobic stability and consequently in the greater aerobic deterioration of the silages during the feed-out phase. This effect is mainly due to the greater production of lactic acid, which is a substrate for aerobic microorganisms, and the lower acetic acid

production in the HO treatment. Undissociated acetic acid has an inhibitory effect on the development of yeasts and moulds in silages, improving silage aerobic stability [42–44].

The extended aerobic stability of the silages treated with the SA and FPA in this study (Figure 3) agrees with previous results reported by Franco et al. [34] and Franco et al. [45] when studying the application of different additives on grass and red clover silages, respectively. The action mode that explains the improvement in aerobic stability may be the antimicrobial actions of the SA on yeasts and moulds. For the FPA, the efficacy may be due to the intentional application of the propionic acid included in the additive, which also has strong antimicrobial activity.

A relatively common problem with highly wilted silages is that they may exhibit poor aerobic stability [27], but this effect was not found in the current study, as the high DM content silages showed longer aerobic stability than the low DM silages (Figure 3). In general, silages prepared using raw material with a high DM content are difficult to compact and more porous, allowing air ingress into the feed, which can cause problems in aerobic stability; however, this effect may not be mimicked in airtight laboratory silosin the same way as in practical large-scale silos.

3.2.2. Experiment 2—Resin Acids Used as Silage Additives

Coniferous resin acids are part of the biological defence mechanism of coniferous trees with various biological activities [10]. They have been suggested as potential feed additives [11], so their inclusion in silage should not be harmful. Although no positive effects on dairy cow digestion or performance could be demonstrated [11], their ability to manipulate silage fermentation has not yet been evaluated.

Table 3 shows the results referring to the application of resin acids on the fermentation profile of silages. The application of resin acids had no quadratic effects, and only minor linear effects were observed. All silages reached a pH below 4.2, which is a threshold established by McDonald et al. [2], indicating that all silages were well fermented.

Protein to ammonia-N conversion (Table 3) was decreased ($p < 0.05$) with the highest dose of FOR when compared to the control, but ROS did not have the same effect ($p > 0.05$). In this experiment, only the FPA and FOR at the highest dose resulted in an ammonia-N concentration below 50 g/kg total N, which is the value established by Wilkinson [35], who stated that these silages are extremely well fermented. The ROS dose had an increasing linear effect on the concentration of ethanol, as well as propionic acid, in the grass silages.

In general, there were no differences ($p > 0.05$; Table 3) between the control treatment and the treatments using FOR and ROS. However, resin acids resulted ($p < 0.05$) in the higher production of ammonia-N, ethanol, and lactic and propionic acids, and a higher ratio of lactic to acetic acid than the FPA-treated silages. The application of resin acids when compared to the FPA also resulted ($p < 0.05$) in a greater amount of residual WSC, a lower production of acetic acid and lower aerobic stability.

The differences between FOR and ROS were minimal regarding the fermentation profile of the grass silages, such as the lower production ($p < 0.05$; Table 3) of ethanol and propionic acid when the grass was treated with FOR than when treated with ROS. Most of the silages, including the control, resulted in an ethanol concentration between 5 and 10 g/kg DM, except for both doses of ROS, which, according to Kung et al. [4], were higher than the values regarded to provide the silages with good fermentation.

Based on the current results, the resin acid products used were not able to affect silage fermentation. The amounts of products used were already very high, so that increasing the level of application would not be a practically feasible approach to increasing the efficacy. Products with higher concentrations of resin acids could potentially be evaluated in future studies.

Table 3. Chemical composition, fermentation quality, aerobic stability and microbial quality of grass treated with resin acids at different doses.

Additives [1]	C	FPA	FOR		ROS		SEM [2]	*p*-Value [3]				
			13 L/t	26 L/t	13 L/t	26 L/t		Lin FOR	Lin ROS	C vs. Res Acids	FPA vs. Res Acids	FOR vs. ROS
Dry matter (DM), g/kg	222 ^{b}	229 ^{b}	228 ^{b}	245 ^{a}	226 ^{b}	227 ^{b}	2.0	<0.001	0.174	0.003	0.349	0.001
pH	3.89	3.92	3.88	3.86	3.89	3.89	0.020	0.257	0.907	0.658	0.098	0.332
Ammonia-N, g/kg N	59 ^{a}	34 ^{c}	58 ^{a}	47 ^{b}	55 ^{ab}	56 ^{ab}	2.2	0.003	0.390	0.064	<0.001	0.205
Chemical composition, g/kg DM												
Water-soluble carbohydrates	32 ^{a}	17 ^{b}	27 ^{a}	27 ^{a}	34 ^{a}	27 ^{a}	1.8	0.141	0.125	0.221	<0.001	0.095
Ethanol	9.3 ^{bc}	5.6 ^{c}	10.4 ^{abc}	8.2 ^{bc}	15.2 ^{ab}	16.9 ^{a}	1.53	0.603	0.006	0.079	0.002	0.001
Acids, g/kg DM												
Lactic (LA)	99.5 ^{a}	65.1 ^{b}	94.9 ^{a}	94.8 ^{a}	95.0 ^{a}	94.7 ^{a}	3.36	0.345	0.340	0.245	<0.001	0.984
Acetic (AA)	19.9 ^{b}	26.3 ^{a}	20.7 ^{b}	18.2 ^{b}	18.0 ^{b}	17.8 ^{b}	0.89	0.198	0.128	0.240	<0.001	0.120
Propionic [4]	0.16 ^{bc}	0 ^{c}	0.17 ^{bc}	0.18 ^{bc}	0.40 ^{ab}	0.57 ^{a}	0.075	0.879	0.003	0.075	0.003	0.002
Butyric	0	0.03	0	0.01	0	0	0.008	0.290	1.000	0.731	0.033	0.448
Total volatile fatty acids	20.1 ^{b}	26.5 ^{a}	20.9 ^{b}	18.4 ^{b}	18.4 ^{b}	18.4 ^{b}	0.95	0.239	0.239	0.341	<0.001	0.221
Total fermentation acids [5]	120 ^{a}	92 ^{b}	116 ^{a}	113 ^{a}	113 ^{a}	113 ^{a}	3.64	0.242	0.239	0.191	<0.001	0.755
Total fermentation products [6]	129 ^{a}	97 ^{b}	126 ^{a}	121 ^{a}	129 ^{a}	130 ^{a}	3.99	0.210	0.843	0.608	<0.001	0.190
LA/AA ratio	5.03 ^{a}	2.47 ^{b}	4.59 ^{a}	5.23 ^{a}	5.33 ^{a}	5.34 ^{a}	0.268	0.626	0.433	0.776	<0.001	0.139
Aerobic Stability (2 °C), hours	40 ^{b}	313 ^{a}	36 ^{b}	58 ^{b}	40 ^{b}	37 ^{b}	15.7	0.434	0.903	0.875	<0.001	0.592
Microbial counts, cfu [7]/g												
Yeasts	1.2×10^5	2.0×10^2	2.0×10^5	2.4×10^5	1.9×10^5	2.0×10^5	6.5×10^4	0.251	0.434	0.269	0.017	0.725
Moulds	5.1×10^2	2.0×10^2	1.0×10^2	1.0×10^2	1.0×10^2	3.0×10^2	1.6×10^2	0.108	0.384	0.078	0.792	0.558

[1] Additives: C, control; FPA, formic- and propionic-acid-based additive; FOR, resin acid oil; ROS, resin acid soluble in water. [2] SEM, standard error of the mean. [3] *p*-value: Lin FOR, linear effect of resin acid oil application; Lin ROS, linear effect of resin acid soluble in water application; C vs. Res acids, control versus resin acids, FPA vs. Res acids, formic- and propionic-acid-based additive versus resin acids; FOR vs. ROS, resin acid oil versus resin acid soluble in water. Applied doses of FOR and ROS had no quadratic effect, and then had their *p*-values excluded. [4] Corrected for its amount in the FPA-based additive. [5] Total volatile fatty acids + lactic acid. [6] Total fermentation acids + ethanol. [7] cfu, colony-forming unit. Values with the same letter in a row are not significantly different ($p > 0.05$) based on Tukey's test. If there were no differences in Tukey's test, letters were removed.

The same low DM grass material and additive treatments, C and FPA, were used in both Experiment 1 and 2. This enables a comparison to be made of two experimental techniques used in silage research: cylindrical silos vs. vacuum plastic bags. The preservation characteristics were similar in both types of silos, as indicated by the high correlation coefficients of lactic acid ($R^2 = 0.92$; $p < 0.01$), acetic acid ($R^2 = 0.94$; $p < 0.01$) and aerobic stability ($R^2 = 0.89$, $p = 0.02$).

4. Conclusions

Silage management factors, such as the dry matter content, combined with the use of additives, greatly affected the fermentation quality of grass silages. The use of additives with different modes of action, including homofermentative and heterofermentative strains of lactic acid bacteria, a salt-based additive and formic- and a propionic-acid-based additive, improved the preservation characteristics of grass silage, but different additives modified the silage quality in different ways. The current grass raw material was relatively easy to ensile, so all silages could be characterized as well preserved. Increasing the dry matter content and the use of the formic- and propionic-acid-based additive effectively restricted silage fermentation, while the salt-based additive was particularly efficient in improving the aerobic stability of the silages. Resin acid products did not affect the fermentation profile and preservation characteristics of the silages, but evaluating higher doses of active compounds and different ensiling matrices remain subjects for further studies.

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