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Evaluating the effects of wilting, mixing with timothy and silage additive application on red clover silage quality

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Red clover is typically produced as a mixed stand with grasses, which may lead to suboptimal fertilization and harvest time practices, resulting in poor survival of red clover. Further, unknown proportions of red clover in mixed swards complicate ration formulation for livestock. Thus, producing red clover as pure stands could offer benefits, but it may be difficult to ensile. The objective of the current study was to evaluate major management possibilities such as wilting, inclusion of timothy grass and different types of additives in optimizing the ensilage of red clover. Low DM silages were poorly preserved with high pH, elevated acetic acid concentrations and proportions of ammonia-N in total N, as well as depleted water soluble carbohydrate levels. Moderate wilting clearly improved the fermentation quality of silages. Further, the use of formic acid-based additives improved the fermentation quality of the silages, the effect being more pronounced in the low than medium dry matter silages. The composition of red clover and timothy were rather similar regarding dry matter content and ensilability. This was probably the reason why clear benefits in silage quality from timothy inclusion could not be demonstrated.

Key words: aerobic stability, fermentation, formic acid, lactic acid bacteria, *Phleum pratense*, *Trifolium pratense*

Introduction

Current ecological and global trade crises have increased the value of legume cultivation in agricultural systems due to their ability to fix atmospheric N_2 by the symbiotic *Rhizobium* bacteria in their root nodules. Red clover (*Trifolium pratense* L.) is a winter-hardy forage legume and offers opportunities to utilize the nitrogen-fixing ability even in the harsh environmental conditions of high latitudes (Järvenranta et al. 2016). Carlsson and Huss-Danell (2003) reported that red clover captured annually up to 373 kg N per ha in the above-ground plant tissues.

There are however challenges in including red clover in silage production systems for ruminants. The proportion of red clover can be highly variable over the years due to winter damage. In general, mixed leys with grasses and red clover become grass dominant over time as red clover winter survival is lower than that of timothy (*Phleum pratense* L.) and meadow fescue (*Festuca pratensis* Huds.), which are commonly used as companion species with red clover in Boreal leys. The proportion of red clover is also typically lower in the primary growth after winter than in the regrowth (Rinne and Nykänen 2000). In addition, the red clover content may be highly variable even within the field (Nykänen et al. 2008). All these factors result in an unpredictable proportion of red clover in the harvested forage, which makes efficient ration formulation for livestock difficult.

Red clover has positive effects as a forage source for dairy cows, such as increasing feed intake as a component in grass silage-based rations (Huhtanen et al. 2007, Vanhatalo and Jaakkola 2016). The higher N content typically found for red clover than grasses may be helpful in providing a N source for rumen microbes. However, if N is provided in excess, it results in poor N use efficiency (Huhtanen et al. 2008, Vanhatalo et al. 2009) and potential environmental burden. Further, if red clover silage is fed to dairy cows during the periparturient period, the high Ca content of it predisposes them to hypocalcemia at calving. All these reasons make it important to be able to control the red clover content in the rations offered to ruminants. One solution to achieve that would be to cultivate red clover as a pure stand and mix it at the stage of total mixed ration preparation to be able to create optimized rations for different animal groups. If red clover is produced as pure stands instead of mixtures with grasses, compromises in N fertilization, plant protection and harvest time are not required, and this may favour the longevity of red clover. Such pure red clover biomasses could also be valuable feedstocks in green biorefineries, where various end products are manufactured from green biomass (Jørgensen et al. 2022).

Herbages with high concentration of red clover are known to be challenging to ensile due to their inherent high moisture content, low water soluble carbohydrate (WSC) concentration and high buffering capacity (McDonald et al. 1991, Krawutschke et al. 2013).There is also experimental evidence showing poor fermentation quality of red

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clover-dominated herbage, although the quality has been improved when efficient additives were used (McEniry et al. 2014, Rinne et al. 2018). Several management factors can influence the preservation quality of difficult-to-ensile materials, and the development of ensiling methods is constantly being developed, including improved additives (Huhtanen et al. 2013, Wilkinson and Rinne 2018).

The objectives of the current experiment were to evaluate major management possibilities such as wilting, inclusion of grass and different types of additives in optimizing the ensilage of red clover. We hypothesized that wilting, grass inclusion and use of additives would improve the preservation characteristics of red clover.

Material and methods

Herbage production

The leys used in the current experiment were grown at Ruukki Research station of Natural Resources Institute Finland in Siikajoki, Finland (64°66'N, 25°09'E). Both red clover and timothy were grown as pure stands. Red clover variety Selma (Boreal Plant Breeding Ltd., Jokioinen, Finland) was used, and the stand was one year old. For timothy, the variety was Nuutti (Boreal Plant Breeding Ltd., Jokioinen, Finland) and the stand was four years old. Both stands were cut from first regrowth of the season at around 9 am on 18 August 2021 using a farm-scale mower conditioner, picked up by a precision chopper, and transported to the laboratory without any additive application. The primary growth for both plant species had been harvested on 23 June 2021 so that the growing time of the aftermath was 56 days. Red clover was blooming at the time of harvest, while timothy was mainly vegetative. After the first harvest, the stands were fertilized with a commercial mineral fertilizer (YaraMila NK2 (N-P-K 22-0-12), Yara Ltd., Oslo, Norway) at 100 and 300 kg per ha for red clover and timothy, respectively.

Pilot scale ensiling procedures and experimental treatments

At the laboratory, both plant materials were divided into two batches. One batch was ensiled immediately (low dry matter [DM]) and the other batch after artificial drying in a forced-air open-circuit dryer using air temperature of 30 °C for approximately 3 hours (medium DM). Samples of red clover and timothy were taken from both DM levels for the evaluation of chemical composition and microbial counts. Within both DM levels, silages of both plant species were prepared separately, and additionally a 1:1 mixture on a fresh matter basis was prepared from them.

The following additive treatments were used:

- Control (CON), tap water
- Heterofermentative lactic acid bacteria (LAB) inoculant (HE; Josilac® Combi, containing *Lactobacillus buchneri* DSM22501 1k20738, *Lactobacillus kefiri* DSM 19455 1k20742, *Lactobacillus plantarum* LSI NCIMB 30083 1k20736 and *Pediococcus pentosaceus* P6 DSM 23688 1k20736; Josera GmbH, Kleinheubach, Germany)
- Formic acid (FA) based additive 1 (FA1; AIV2 Plus Na, containing 76% FA and 5.5% sodium formate; Eastman, Oulu, Finland)
- FA based additive 2 (FA2; AIV VIA, containing 40% FA, 10% sodium formate and 17.5% propionic acid; East man, Oulu, Finland)
- FA based additive 3 (FA3; non-commercial product, containing 47.5% FA, 22.5%, sodium formate, 10% propionic acid and 1.7% sodium benzoate; Eastman, Oulu, Finland)

Commercial application levels were used so that HE was applied at 3×10^5 cfu/g of fresh forage. For the acid-based additives, 6 litres per ton fresh matter were used for low DM red clover, while other plant materials received 5 litres per ton. All additives were mixed in tap water and the amount of liquid applied was 10 litres per ton for all treatments, including CON.

Additive treatments were applied to the six plant materials so that pure red clover at both DM levels received all additive treatments, while timothy and mixture of both plant species received only the CON and FA1 treatments (Fig. 1). Four replicates were prepared for each treatment. For additive application, batches of 3 kg were prepared,

the additive was manually sprayed on the herbage and carefully mixed by hand separately for each replicate. Then the herbage was packed into two plastic bags (BK3550; 300mm × 500mm, 52µm; Sealed Air Food Care, Duchnice, Poland) to form two sub-replicates (approximately 1.5 kg per bag) for each replicate to have enough material for all analyses. The bags were sealed using an industrial scale vacuum machine (GK187R/2R; Supervac, Vienna, Austria) for 25 s with a vacuum of 5 mbar, sealing for 1.8 s and cooling for 2.0 s. After sealing, the bags were weighed and stored in room temperature protected from light until opening. The plastic bags were weighed and opened after a storage time of 3 months, and the two sub-replicates were combined before sampling for analyses.

Plant species	Dry matter level	Additive treatment				
		Control	FA1	FA2	FA3	HE
Red clover	Low	Table 2 & 3	Table 2 & 3	Table 3	Table 3	Table 3
	Medium	Table 2 & 3	Table 2 & 3	Table 3	Table 3	Table 3
1:1 mixture of red clover and timothy	Low	Table 2	Table 2			
	Medium	Table 2	Table 2			
Timothy	Low	Table 2	Table 2			
	Medium	Table 2	Table 2			

Fig. 1. Experimental design including information in which Table the results for each treatment are presented. FA1, FA2 and FA3 are different formic acid-based additives and HE is a lactic acid bacteria inoculant, while control did not receive any additive treatment.

Laboratory analyses and calculations

The samples were kept frozen (–20 °C) before being analysed using the routine methods at the Jokioinen laboratory of Luke, which has a quality system following the SFS-EN ISO/IEC 17025:2005 standards and is accredited by FINAS (Finnish Accreditation Service, Espoo, Finland) with number T024. The herbage samples were analysed for DM, buffering capacity, ash, crude protein (CP), nitrate-N, WSC, neutral detergent fibre (NDF), *in vitro* organic matter digestibility and microbial counts, while silage samples were analysed for DM, pH, ammonia-N, WSC, ethanol, lactic acid, volatile fatty acids (VFA) and microbial counts as previously described by Franco et al. (2022a).

Aerobic stability was estimated as the time in hours required for a 2 °C increase in silage sample temperature relative to the ambient when exposed to air (for details of determination, see Franco et al. 2022a). The length of the follow-up period was 220 h. Not all samples reached the 2 °C threshold during that time, and in that case the end point time was used as the result. Silage CP concentration was not analysed, so that the N concentration of raw materials was used to present ammonia-N as the proportion of total N in the silages. Total VFA included acetic, propionic and butyric acids as well as minor C4-C6 VFA. Total fermentation acids were calculated as: total VFA + lactic acid; and total fermentation products as: total fermentation acids + ethanol. Two of the additives used (FA2 and FA3) contained propionic acid, and the analysed propionic acid concentration was corrected by subtracting 80% of the added amount from the analysed value. The correction rate is based on subjective experience to not overcorrect for the added amount. The corrected propionic acid value was used to calculate the combined fermentation product parameters mentioned above. Buffering capacity was calculated according to Pahlow et al. (2002) and ensiling losses according to Knický and Spörndly (2015). The fermentability coefficient (FC) was calculated as described by DLG (2020) using the following equation:

FC = DM (g/kg) + 8 WSC (g/kg DM) / buffering capacity (g lactic acid/100 g DM),

and values below 35 indicate a herbage difficult to ensile.

Statistical analyses

The data were analysed in two parts (Fig. 1). The first part contained both plant materials and their mixture at two DM levels, but only two additive treatments were included (CON and FA1), so that the design was 3 × 2 × 2 factorial. The second part covered only pure red clover at two DM levels and five additive treatments resulting in a 2 × 5 factorial design. The data were analysed using a MIXED procedure of SAS (SAS Inc. 2002–2012, Release 9.4; SAS Inst. Inc., Cary, NC, USA). The experimental factors were considered as fixed effects, while replicate was a random

effect in the statistical model. The UNIVARIATE procedure was used to test the normal distribution of data utilizing the Shapiro-Wilk test. Least squares means and standard errors of the means were reported. The linear and quadratic effects of red clover proportion as well as combined additive comparisons (non-FA vs. FA containing additive treatments) and interaction comparisons in the first part of data analysis were evaluated using contrasts. There were practically no significant quadratic effects of red clover proportion, so they are not presented. Pairwise comparisons among all treatment means were performed using Tukey's test at a probability level of $p < 0.05$.

Results

The DM concentration of both fresh plant materials was exceptionally low and almost identical (Table 1). The artificial wilt increased the DM content of red clover slightly more than that of timothy, but both increased substantially, which also improved their fermentation coefficient. The ash content was higher and NDF content lower in red clover compared to timothy, but regarding CP and WSC concentrations, the plant species were very similar. Yeast and mould counts were at the same level in both plant materials, but epiphytic LAB and *Clostridia* counts were somewhat higher in red clover than in timothy.

The low herbage DM contents and confining the effluent within the plastic bags resulted in low DM of the subsequent silages, the values being as low as 117 and 129 g kg^{-1} in red clover and timothy, respectively. For the medium DM red clover and timothy, the silage DM contents were 303 and 255 g k^{-1}g, and for the mixture, the values were 121 and 284 g kg^{-1} for low and medium DM, respectively (p-value for DM and species < 0.001). The species effect on silage pH was significant ($p < 0.001$), with the values being 4.39. 4.30 and 4.18 for red clover, mixture and timothy, respectively. The pH of low DM CON silages averaged 4.77, but FA1 application reduced it to 4.07. For medium DM CON silages, the pH was 4.24 and FA1 was still able to reduce it to 4.07. The species effect was significant ($p < 0.001$) also for the proportion of ammonia-N in total N, with the values being 52, 61 and 70 g kg^{-1} total N for red clover, mixture and timothy, respectively.

Table 1. Characterization of fresh (low DM) and wilted (medium DM) red clover and timothy herbages before ensiling

	Red Clover		Timothy	
	Low DM	Medium DM	Low DM	Medium DM
Dry matter (DM), g kg^{-1}	110	303	118	244
Buffering capacity, g lactic acid/100 g DM	2.7	4.2	1.9	1.4
Fermentation coefficient	30	36	32.6	52.0
In DM, g kg^{-1}				
Ash	113	117	78	76
Crude protein	197	188	186	192
Nitrate-N	0.2	0.2	1.1	1.2
Water soluble carbohydrates	64	32	50	50
Neutral detergent fibre	360	377	607	613
In vitro organic matter digestibility	0.713	0.713	0.678	0.679
Microbial counts				
Yeasts, colony forming units (cfu) g^{-1}	8.0×10^4	2.8×10^5	3.2×10^5	1.3×10^5
Moulds, cfu g^{-1}	1.6×10^5	2.1×10^5	2.7×10^5	5.6×10^4
Lactic acid bacteria, cfu g^{-1}	7.9×10^5	1.2×10^6	8.2×10^4	2.1×10^5
Clostridia spores, n g^{-1}	9.2	9.2	3.6	<3.0

Depletion of WSC was observed in all except medium DM FA1-silages (Table 2). FA1 restricted fermentation as acetic acid, total VFA, total fermentation acids and total fermentation products were all reduced by it compared to CON ($p < 0.001$). For lactic acid, there was a numerically marked and statistically significant interaction, as lactic acid was lower in low DM CON silages than in those treated with FA1. This was due to an excessive acetic acid production whose concentration reached 76 g kg^{-1} DM in low DM CON silages on average, while the value for low DM FA1-silages was 22 g kg^{-1} DM. Propionic acid was also elevated (on average 9.8 g kg^{-1} DM) in low DM CON silages compared to an average of 0.3 g kg^{-1} DM in all other silages.

Table 2. Effects of extent of wilting and additive treatment on silage preservation characteristics of red clover, timothy and a mixture of them

Plant Species	Red clover				Mixture				Timothy				SEM	*p*-value [1]				
Dry matter (DM)	Low		Medium		Low		Medium		Low		Medium			Species	DM	S × DM	Add	Add × DM
Additive [2]	CON	FA1	CON	FA1	CON	FA1	CON	FA1	CON	FA1	CON	FA1						
DM, g kg^{-1}	114^{e}	121^{e}	305^{a}	302^{a}	118^{e}	125de	284^{b}	283^{b}	123^{e}	135^{d}	252^{c}	258^{c}	2.6	<0.001	<0.001	<0.001	0.001	0.002
pH	4.78^{a}	4.18^{d}	4.42^{b}	4.17^{d}	4.76^{a}	4.08^{ef}	4.30^{c}	4.05^{fg}	4.77^{a}	3.96^{h}	4.01^{fgh}	3.99^{gh}	0.020	<0.001	<0.001	<0.001	<0.001	<0.001
Ammonia-N, g kg^{-1} N	72^{bc}	30^{f}	62^{cd}	44^{def}	95^{b}	59^{cd}	57^{cde}	34^{ef}	126^{a}	71^{bc}	53^{cdef}	31^{f}	5.5	<0.001	<0.001	<0.001	<0.001	<0.001
In DM, g kg^{-1}																		
WSC [3]	2^{e}	12^{cde}	3^{e}	12^{cde}	2^{e}	8de	3^{e}	24^{bc}	2^{e}	5^{e}	4^{e}	44^{a}	3.1	<0.001	<0.001	<0.001	<0.001	<0.001
Ethanol	11^{bc}	8^{cd}	2^{ef}	3^{def}	15^{ab}	10^{c}	2^{ef}	4^{def}	15^{ab}	20^{a}	3^{def}	7^{cde}	0.9	<0.001	<0.001	<0.001	0.562	0.001
Lactic acid	36^{fgh}	54^{def}	99^{a}	60^{cde}	22^{hi}	72^{bcd}	88^{ab}	47^{efg}	9^{i}	56^{def}	83^{ab}	28^{ghi}	4.4	<0.001	<0.001	0.048	0.135	<0.001
Acetic acid	78^{a}	19^{defg}	40^{b}	23de	75^{a}	26^{cd}	32^{bc}	17^{efg}	75^{a}	21^{def}	20^{def}	10^{g}	2.1	<0.001	<0.001	<0.001	<0.001	<0.001
Propionic acid	8.6^{bc}	0.4^{d}	0.3^{d}	0.1^{d}	10.1^{a}	0.6^{d}	0.4^{d}	0.2^{d}	10.8^{a}	0.5^{d}	0.3^{d}	0.2^{d}	0.17	<0.001	<0.001	<0.001	<0.001	<0.001
Butyric acid	0.1	1.6	0	0	0.2	1.3	0	0	0.1	1.1	0	0	0.40	0.640	0.001	0.690	0.005	0.004
Total volatile fatty acids	87^{a}	22^{def}	41^{b}	23^{cdef}	85^{a}	28^{cde}	33^{bc}	17^{fg}	86^{a}	23^{cdef}	21^{ef}	11^{g}	2.2	<0.001	<0.001	<0.001	<0.001	<0.001
Total fermentation acids	123^{ab}	76^{fgh}	140^{a}	83^{defgh}	107^{bcd}	100^{bcdef}	120^{abc}	64^{h}	95^{cdefg}	79^{efgh}	104^{bcde}	39^{i}	5.7	<0.001	0.086	<0.001	<0.001	<0.001
Total fermentation products	134^{ab}	84^{efg}	142^{a}	86^{def}	122^{abc}	110^{bcde}	123^{abc}	68^{gh}	111^{bcd}	98^{cdef}	107^{cde}	45^{h}	6.0	<0.001	<0.001	<0.001	<0.001	<0.001
Lactic to acetic acids ratio	0.5^{c}	2.7^{b}	2.4^{b}	2.6^{b}	0.3^{c}	2.8^{b}	2.7^{b}	2.8^{b}	0.1^{c}	2.6^{b}	4.1^{a}	2.7^{b}	0.15	<0.001	<0.001	<0.001	<0.001	<0.001
Aerobic stability, hours	61^{b}	202^{a}	220^{a}	204^{a}	63^{b}	188^{a}	220^{a}	213^{a}	55^{b}	216^{a}	194^{a}	158^{a}	13.5	0.097	<0.001	0.022	<0.001	<0.001
Losses, g kg^{-1} initial DM	29^{bcd}	23^{d}	38^{ab}	34^{abc}	30^{abcd}	27^{cd}	40^{a}	35^{abc}	28^{bcd}	33^{abcd}	37^{abc}	36^{abc}	2.4	0.064	<0.001	0.192	0.069	0.361
Microbial counts, colony forming units g^{-1}																		
Yeasts	1.3×10^{2}	5.0×10^{1}	5.0×10^{1}	8.8×10^{1}	5.0×10^{1}	8.8×10^{1}	5.0×10^{1}	5.0×10^{1}	8.8×10^{1}	5.0×10^{1}	5.0×10^{1}	5.0×10^{1}	3.3×10^{2}	0.203	0.921	1.000	0.974	0.921
Moulds	8.8×10^{1}	2.2×10^{3}	5.0×10^{1}	5.0×10^{1}	1.6×10^{2}	8.8×10^{1}	8.8×10^{1}	8.8×10^{1}	1.8×10^{2}	8.8×10^{1}	5.0×10^{1}	3.1×10^{2}	5.6×10^{2}	0.327	0.273	0.157	0.262	0.391

1) Species = Linear effect of gradually decreasing red clover content in the ensiled herbage; DM = Effect of herbage DM; S × DM = Interaction between Species and DM; Add = CON vs. FA1; Add × DM = Interaction between Add and DM; 2) CON = control without additive; F1 = formic acid-based additive; 3) WSC = water soluble carbohydrates
Means within the same row without same superscript differ significantly (p< 0.05, Tukey test).

Table 3. Effects of red clover dry matter (DM) content and additive treatment on silage preservation characteristics

Red clover DM	Low					Medium					SEM	*p*-value [1]		
Additive [2]	CON	HE	FA1	FA2	FA3	CON	HE	FA1	FA2	FA3		DM	Non-FA vs FA	IA
DM kg^{-1}	114^{b}	113^{b}	121^{b}	127^{b}	121^{b}	305^{a}	303^{a}	302^{a}	297^{a}	305^{a}	2.9	<0.001	0.077	0.004
pH	4.78^{b}	4.91^{a}	4.18^{d}	4.03^{e}	4.12^{de}	4.42^{c}	4.43^{c}	4.17^{d}	4.15^{d}	4.13^{d}	0.020	<0.001	<0.001	<0.001
Ammonia-N, g kg^{-1} N	72^{b}	92^{a}	30^{f}	30^{f}	33^{ef}	62^{bc}	63^{bc}	44de	49^{cd}	46de	2.9	0.383	<0.001	<0.001
In DM, g kg^{-1}														
WSC [3]	2^{c}	3^{c}	11^{b}	21^{a}	14^{b}	3^{c}	2^{c}	12^{b}	15^{ab}	14^{b}	1.5	0.206	<0.001	0.343
Ethanol	11^{b}	14^{a}	8^{c}	2de	7^{c}	2de	3de	4^{d}	1^{e}	1de	0.5	<0.001	<0.001	<0.001
Lactic acid	35^{e}	33^{e}	54de	87^{ab}	65^{bcd}	99^{a}	85^{ab}	60^{cd}	78^{abc}	73^{bcd}	4.6	<0.001	0.043	<0.001
Acetic acid	78^{b}	87^{a}	19^{d}	22^{d}	25^{d}	40^{c}	42^{c}	23^{d}	26^{d}	25^{d}	1.7	<0.001	<0.001	<0.001
Propionic acid	8.6^{c}	10.3^{b}	0.4^{f}	14.1^{a}	5.2^{d}	0.3^{f}	0.5^{f}	0.1^{f}	5.0^{d}	1.9^{e}	0.25	<0.001	0.007	<0.001
Propionic acid, corrected [4]	8.6^{b}	10.3^{a}	0.4^{d}	1.7^{c}	0.5^{d}	0.3^{d}	0.5^{d}	0.1^{d}	0.6^{cd}	0.3^{d}	0.20	<0.001	<0.001	<0.001
Butyric acid	0.1^{b}	0.1^{b}	1.6^{a}	0.1^{b}	0.8^{ab}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0.24	0.002	0.032	0.028
Total volatile fatty acids	87^{b}	97^{a}	22^{d}	24^{d}	27^{d}	41^{c}	42^{c}	23^{d}	26^{d}	25^{d}	1.8	<0.001	<0.001	<0.001
Total fermentation acids	123^{abc}	131^{ab}	76^{e}	110^{bcd}	92de	140^{a}	127^{ab}	83de	105^{bcde}	98^{cde}	6.0	0.302	<0.001	0.595
Total fermentation products	134^{abc}	145^{a}	84^{e}	112^{bcde}	99de	142^{ab}	129^{abcd}	86^{ef}	106^{cde}	99^{e}	6.2	0.539	<0.001	0.778
Lactic to acetic acids ratio	0.5^{d}	0.4^{d}	2.7^{bc}	4.0^{a}	2.6^{bc}	2.5^{bc}	2.0^{c}	2.6^{bc}	3.0^{b}	3.0^{b}	0.14	<0.001	<0.001	<0.001
Aerobic stability, hours	61^{b}	59^{b}	202^{a}	213^{a}	215^{a}	220^{a}	220^{a}	204^{a}	218^{a}	220^{a}	7.8	<0.001	<0.001	<0.001
Ensiling losses, g kg^{-1} initial DM	29^{bcd}	25^{cde}	23de	20^{e}	19^{e}	38^{a}	37^{a}	34^{ab}	33^{ab}	31^{abc}	1.5	<0.001	<0.001	0.622
Microbial counts, colony forming units g^{-1}														
Yeasts	1.3×10^{2}	5.0×10^{1}	5.0×10^{1}	5.0×10^{1}	5.0×10^{1}	5.0×10^{1}	5.0×10^{1}	8.8×10^{1}	1.3×10^{3}	5.0×10^{1}	4.0×10^{2}	0.341	0.449	0.369
Moulds	8.8×10^{1}	4.8×10^{2}	2.2×10^{3}	5.0×10^{1}	5.0×10^{1}	5.0×10^{1}	1.4×10^{2}	5.0×10^{1}	5.0×10^{1}	5.0×10^{1}	6.9×10^{2}	0.260	0.631	0.565

1) DM = effect of red clover DM; Non-FA vs FA = CON and HE contrasted against F1, F2 and F3, IA = interaction between DM and Non-FA vs FA; 2) CON = control without additive; HE = heterofermentative lactic acid bacteria inoculant; F1, F2 and F3 = different formic acid-based additive; 3) WSC = water soluble carbohydrates; 4) Analysed propionic acid concentration corrected for the amount added via the additive

Means within the same row without same superscript differ significantly ($p < 0.05$, Tukey test).

The same pattern was observed for aerobic stability, as it was the shortest for low DM CON silages, with the other silages being similar to each other. Weight losses during fermentation were lower at low rather than medium DM ($p< 0.001$). The values for yeast and mould counts were low for all silages, and no pattern based on experimental treatments could be detected.

The effects of the five different additive treatments on low and medium DM red clover are presented in Table 3. The fermentation quality parameters clearly showed that preservation of low DM red clover was poor in CON and HE, which had high pH and high concentrations of ammonia-N, acetic acid and propionic acid. Application of FA-based additives ($p< 0.001$) and wilting improved the fermentation quality ($p< 0.001$). A significant interaction was also found between non-FA and FA-containing additive treatments with the DM level indicating that the differences in the fermentation quality and aerobic stability between these two additive groups were smaller or non-existing at medium rather than at low DM level. The differences between the three FA-containing additives were minor, but FA2 was slightly less effective than FA1 in restricting the fermentation at low DM with e.g., higher lactic acid concentration ($p< 0.05$). The fermentation losses were higher at medium rather than low DM ($p< 0.001$) and lower in FA rather than non-FA treated silages ($p< 0.001$). The different FA additives did not differ from each other in terms of aerobic stability.

Discussion

Raw material characteristics

The benefits of mixed production of grass and forage legumes are partly based on increased yields in case environmental conditions result in loss of leguminous species, but the different plant groups are also considered to complement each other related to the nutritional value, and grass may improve the ensiling characteristics of the mixed material. Fair comparisons between different plant species are difficult because the variation within species is also large. It is however typical, that gramineous and leguminous forages clearly differ from each other in terms of chemical composition and subsequent ensilability. When grown as a mixed stand but analysed separately, red clover had a clearly lower DM content and higher CP content than timothy (Rinne and Nykänen 2000). Such characteristics were also detected by Huhtanen et al. (2006) and Kuoppala et al. (2009) from red clover and grass silages harvested from pure stands. Typically, also the WSC concentration of red clover is lower than that in grasses (Hetta et al. 2003, Rinne et al. 2018, Kuoppala et al. 2009, King et al. 2012). Somewhat unexpected, there was no difference in the current herbage materials regarding WSC concentration, although McEniry et al. (2014) also noted that WSC concentrations were similar in different species although at a higher level than in the current material (on average 114 g kg^{-1} DM for red clover and 106 g kg^{-1} DM for timothy).

Humid weather conditions during harvest contributed to the exceptionally and equally low DM of both red clover and timothy in the present experiment. The extent of wilting for timothy remained somewhat lower than for red clover due to practical difficulties in controlling the drying process. Overall, timothy was very similar to red clover so that the beneficial effect of timothy addition to red clover was not achieved, although the buffering capacity was lower in timothy than in red clover (1.7 vs 3.5 g lactic acid per 100 g DM), in line with McDonald et al. (1991) and McEniry et al. (2014). There were also minor differences in the fermentation coefficients, but both low DM materials were difficult to ensile (fermentation coefficient <35). Medium DM red clover barely reached the intermediate to ensile level (FC 35–45), while medium DM timothy was in the category of easy to ensile (FC>45).

It is much easier to achieve anaerobic conditions in pilot-scale silos than in farm silos. Thus, it may be difficult experimentally to produce challenging enough conditions for the management treatments to be able to show their potential. Under such conditions, even untreated silage can be well preserved and it may be difficult to prove the benefits of experimental treatments. In the current material, the silages ensiled without wilting had extremely low DM content due to unfavourable weather conditions, which resulted in challenging conditions for ensiling and an efficient test for the management factors to affect silage fermentation quality. Concentrations of DM were however clearly below the recommended levels, or the observed values on farms. On Finnish dairy farms the average DM content of about 9000 silage samples analysed in 2022 was 339 g kg^{-1} (personal communication, Laura Vaarnas, Valio Ltd., Helsinki, Finland). It is obvious that in practise, such low DM herbage should not be harvested, but the results can still serve as a demonstration of unfavourable conditions. Risk of extreme weather events may also be increasing due to climate change. Similar low levels of unwilted red clover have also been reported by Hetta et al. (2003), McEniry et al. (2014) and (Rinne et al. 2018). The artificial wilting was quick, but if the materials would have been wilted in field conditions, and particularly if unfavourable weather conditions would have continued, losses of WSC and decrease in hygienic quality would have been probable (McDonald et al. 1991).

Effects from the experimental method used, e.g., using plastic bags as model silos, cannot be excluded as effluent could not be released from the silos. In farm-scale silos, the effluent would have been released, and final silage DM of the low DM herbage would have been clearly higher, but also some soluble substances (minerals, CP, WSC, additives) would have been lost with the effluent. Effluent losses from the medium DM raw materials would probably be insignificant when ensiled at the larger scale (Jones and Jones 1995), so that those results are more directly comparable to farm-scale conditions.

Silage fermentation quality

Achieving good fermentation quality of silage is a sum of several factors. The principal prerequisites are anaerobic conditions in which the epiphytic and/or inoculated LAB ferment WSC into fermentation acids, mainly lactic and acetic acids, which drop the pH to a level where microbial activity is minimized (McDonald et al. 1991). In the current case, WSC were depleted in all except FA treated silages which is a sign of suboptimal silage quality. This was linked with elevated silage pH in low DM CON and HE silages.

The high acetic acid concentration in low DM CON and HE silages indicates excessive secondary fermentation, but in spite of predisposing conditions, clostridial fermentation was not evident as indicated by low butyric acid concentrations, and also ammonia-N proportions in total N were not excessive. This is somewhat surprising as clostridia were detected in the raw material. In case of a very high hygienic quality raw material, clostridia may not be present. To effectively challenge the additive treatments, an experimental technique of adding spoilage microbes into the ensiled material can be used (König et al. 2019, Franco et al. 2022a,b). This may help to show the responses to treatments as in many cases, also the CON silage may be adequately preserved. This should however not have been the case in the current study as evidenced by detection of some butyric acid in the low DM FA1 treated silages. The low DM non-FA treated silages had a high pH so that their fermentation quality could potentially continue to decline over a longer ensiling period, but it is generally accepted that three months is a suitable endpoint for silage experiments (EFSA FEEDAP Panel 2018).

It is well established that LAB inoculants are not suitable for low DM silages, and most commercial brands recommend their use for biomass with a DM content above 250–300 g kg^{-1} DM. Thus, it was expected that HE would not improve the fermentation quality at low DM, but no benefits were observed at medium DM either, which had a DM content above 300 g kg^{-1} DM. The ability of LAB to influence the fermentation quality depends on several factors, particularly on the access to fermentable substrate, and has been found to be limited in several experiments (Krawutschke et al. 2013, McEniry et al. 2014, Franco et al. 2022b). However, in other cases, e.g., in Franco et al. (2022a), LAB significantly boosted lactic acid fermentation in grass silage with a DM concentration of 344 g kg^{-1}.

Direct acidification has long traditions as a silage preservation method and has been proven to be an efficient method for ensiling challenging low DM herbages (Virtanen 1933). The acid-based additives currently in the market consist mainly of FA, and product development has resulted in blends including several components and buffered acids (Huhtanen et al. 2013, Rinne et al. 2016). FA-based additives have improved the fermentation quality of red clover (Seppälä et al. 2013, Rinne et al. 2018, Franco et al. 2018, 2022b) and grass (Jaakkola et al. 2006, Lorenzo and O'Kiely 2008, Seppälä et al. 2016, Rinne et al. 2018, Franco et al. 2018, 2022a) silages, although sometimes responses to highly buffered acids have been rather limited (Rinne et al. 2022).

As a sole component, FA is not particularly effective against clostridia (König et al. 2019), and indeed slightly elevated butyric acid concentrations were observed for low DM FA1 treated silages compared to other silages. The FA-based additives with additional antimicrobial components were, however, able to limit butyric acid production, particularly FA2 with higher amount of added propionic acid in it. The low efficiency of FA against yeasts may result in relatively high ethanol concentration in FA-treated silages (Rinne et al. 2016), but in the current data, ethanol contents were below those of non-FA treated silages.

The proportion of ammonia-N in total N is a sensitive indicator of silage hygienic quality and feeding value, and according to Wilkinson (1990), silages with ammonia-N proportion below 50 g kg^{-1} N are considered very well fermented, those with 50–100 g kg^{-1} N as well fermented, and those above 100 g kg^{-1} N as poorly fermented. According to these guidelines, only low DM CON timothy silage was not considered well fermented even though all low DM CON silages exhibited excessive acetic acid production and somewhat elevated ammonia-N proportions. Use of FA consistently decreased the proportion of ammonia-N in total N in all materials with simultaneous restriction of fermentation, and the efficacy of FA has also been proven earlier (McEniry et al. 2014, Franco et al. 2022a,b). Increased silage ammonia-N concentration and concentrations of fermentation acids decrease the voluntary feed intake of silage resulting in reduced milk production of dairy cows (Huhtanen et al. 2007, 2013) emphasizing the economic importance of good silage quality.

Red clover contains enzyme polyphenol oxidase (PPO), which has been shown to restrict in-silo protein degradation (Sullivan et al. 2006, Lee et al. 2008) and it may have been responsible for the linear effect of plant species on proportion of ammonia-N in total N in low DM silages, as the other fermentation parameters would not support lower N degradation in red clover compared to timothy. The reduction of protein breakdown in-silo is beneficial from nutritional point of view, particularly if the material would be used as a feedstock for protein extraction in a green biorefinery concept (Keto et al. 2021). Red clover inclusion did however not protect the protein in timothy from degrading, as the trends on low DM were linear (Fig. 2). This confirms the observations of Syrjälä-Qvist et al. (1984) and Krawutschke et al. (2013), where no curvilinear trends were found in ammonia-N proportion in response to including grass into red clover in incremental proportions. In medium DM silages, other factors restricting protein degradation seem to have overridden the potential effect of PPO as ammonia-N proportions numerically decreased by decreasing red clover proportion (Fig. 2).

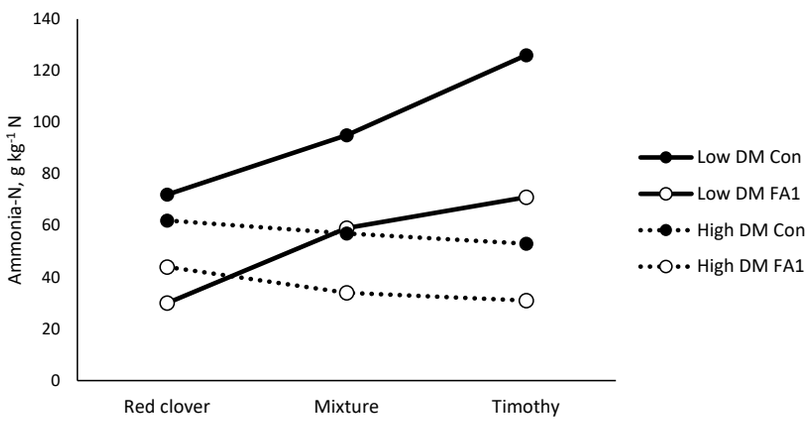

Fig. 2. Interactions between silage raw material species with dry matter (DM; $p < 0.001$), and DM and additive (CON = control; FA1 = formic acid-based additive; $p < 0.001$) on silage ammonia-N proportion

Aerobic stability of silages

Acetic acid has strong antifungal characteristics and has been shown to improve the aerobic stability of silages (Driehuis et al. 2001, Wilkinson and Davies 2013). In the current experiment, extremely high acetic acid concentrations were observed in low DM CON silages and yet their aerobic stability was shorter than in the other silages that had clearly lower acetic acid concentrations (Fig. 3a). The antifungal activity is related to the concentration of undissociated acetic acid, which is pH dependent. Thus, less than 0.5 of the acetic acid was undissociated in the low DM CON silages due to their elevated pH, although for those silages with a pH around 4, the proportion was 0.85. Thus, the differences in the concentration of undissociated acetic acid were smaller than in total acetic acid, but still the values were very high for the low DM CON silages (on average 37 g undissociated acetic acid per kg DM) compared to the other silages ranging from 9 to 28 g kg^{-1} DM. For the antifungal activity, the acetic acid should be expressed per kg fresh matter, which again changes the pattern making the low DM CON silages actually close to the mean of all silages when using this unit (Fig. 3b). They still clearly deviate from the general relationship indicating that some other factors, such as the elevated pH, have contributed to the observed warming. Wilkinson and Davies (2013) suggested a value of 8 g undissociated acetic per kg FM as the threshold for a low risk of silage warming, and indeed, silages above that did not warm at all during the measurement period of 220 h in the current experiment.

The antifungal activity of acetic acid has led to the use of heterolactic LAB inoculants due to their ability to promote acetic acid formation (Muck et al. 2018). Because of that, a product including heterolactic LAB strains was included in the current experiment. At low DM, use of it increased acetic acid production by 12%, but no change in aerobic stability was detected. At medium DM, acetic acid production was not altered, and both CON and HE silages remained unheated over the 220-h follow-up period. The FA application improved aerobic stability of low DM silages concomitantly with an overall improved fermentation quality in line with previous work (Rinne et al. 2016, Franco et al. 2022b). In some cases FA-based additive may even decrease aerobic stability, if the non-additive treated silage has undergone excessive secondary fermentation resulting in high acetic acid concentration (Franco et al. 2022a). In medium DM silages, the stability of CON was good and there was no scope for FA to show further improvements.

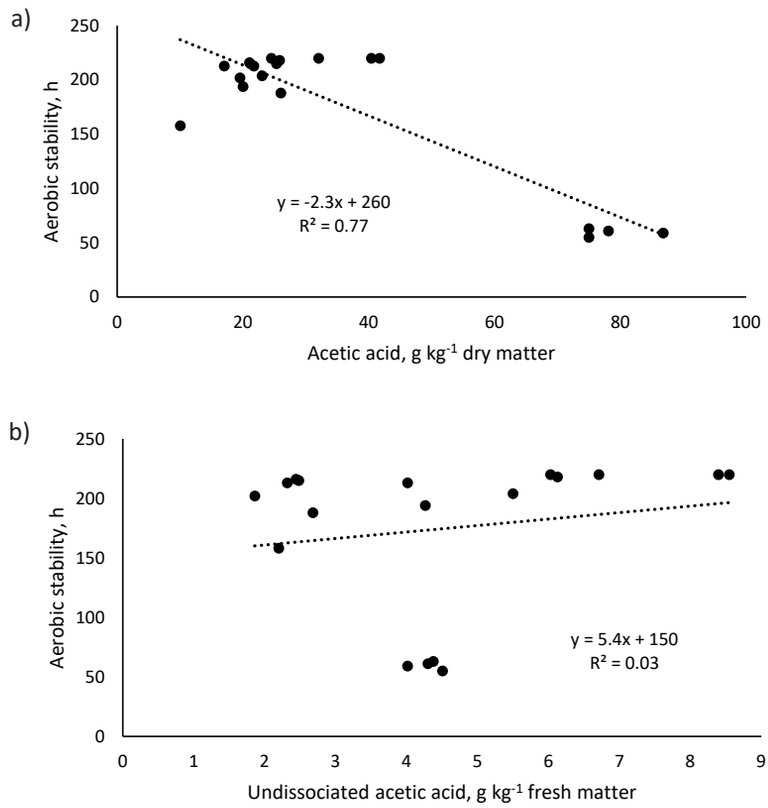

Fig. 3. The relationships between the aerobic stability with the acetic acid concentration in silage dry matter (a) and with the undissociated acetic acid in silage fresh matter (b)

Pahlow et al. (2002) found that silages prepared from forage legumes (red clover, white clover and lucerne) had better aerobic stability than grasses. In our case, the plant species did not significantly affect the aerobic stability, but DM, additive and their interaction were significant. Increasing DM has often led to prolonged aerobic stability in pilot scale experiments (Knický and Lingvall 2004, Seppälä et al. 2016) although in practical farms, heating tends to be a greater problem in high DM silages (Wilkinson and Davies 2013). The reason for this phenomenon may be related to the experimental technique used in the current experiment where airtight plastic bags were used, and the aerobic stability test was initiated immediately after silo opening, while in practice, the silage is exposed to air gradually during the feed-out phase. Under practical conditions, higher DM content results in more porous mass leading to easier air ingress into the feed mass providing opportunities for aerobic spoilage microbes to thrive.

Conclusions

Although production of very low DM silages is not recommended in practice, using them in the current experiment provided an efficient test for the additives used. Both increased DM content and use of the formic acid-based additives improved the fermentation quality of silages supporting the hypotheses, although responses to the lactic acid bacterial inoculant remained minor even in the medium DM red clover. Under the conditions of the current experiment, mixing timothy with red clover did not improve the fermentation quality of the silages, against our hypothesis. This is at least partly linked with the unexpected characteristics of the timothy raw material, i.e., low DM and water soluble carbohydrate concentrations. Under challenging conditions, low DM red clover and timothy silages without additive treatments were very poorly preserved, emphasizing the importance of good silage management practices including the choice of additives used.

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