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ORIGINAL ARTICLE

Forage type and additive effects on fermentation quality and biorefinery performance of silages

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Abstract

The concept of a green biorefinery has recently gained interest and can be defined as a process where green biomass is processed to a variety of products. Most green biorefineries rely on fresh green biomass as the feedstock, but using a stable ensiled biomass could provide benefits. We evaluated the effects of silage additive treatments on silage fermentation quality and performance in a laboratory scale liquid–solid separation to simulate the first step of a green biorefinery. In Experiment 1, red clover and fresh and wilted grass were ensiled without additive or treated with a lactic acid bacteria inoculant or a formic acid based additive. In Experiment 2, grass or red clover were treated with a fibrolytic enzyme, formic acid or a combination of them, and a control without additive was also included. Silage fermentation quality was improved by additive use. Biomass dry matter concentration was negatively related to liquid yield, but effects of additive treatments on the biorefinery performance were minor and inconsistent between different forages. Optimizing agronomic and feedstock conservation management plays an important role for the success and sustainability of the biorefinery process. Good silage management practices with minimal losses during storage should be targeted, but no clear patterns in biorefinery outputs were observed in the current study when different types of additives were used in grass and clover silage production.

KEYWORDS
enzyme, formic acid, green biorefinery, liquid–solid separation, red clover, timothy

1 | INTRODUCTION

The concept of a green biorefinery has recently gained a lot of interest and it can be defined as a process where green biomass is processed to a variety of products. Green biorefineries can provide much-needed alternatives to fossil resources such as materials (biocomposites, insulation, bioplastics, nanofibres), bioenergy (biogas, biofuels, carbon source in single cell production), and novel protein sources (feed and food materials directly extracted or produced by cell cultures) both for livestock and human consumption (Gaffey et al., 2023). Cultivation of perennial plant species to produce green biomass provides ecosystem services such as high nutrient use efficiency, build-up of soil carbon and low pesticide usage (Jørgensen et al., 2022), and additional benefits can be gained if forage legumes are included in the swards (Ditzler et al., 2021). Most green biorefinery approaches rely on using fresh green biomass as the feedstock but using a stable raw material available year-round, i.e., ensiled biomass, could provide benefits depending on the business model (Rinne, 2024).

There is a vast amount of knowledge regarding the agronomic factors as well as harvesting and conservation techniques on biomass

quality for feed use that should be exploited in novel uses of green biomass. The major management factors of forages affecting final silage quality include choices of plant species, harvesting strategy (stage of development of plants at harvest), fertilization, extent of wilting, technology choices in harvesting and storage options, and additive use. In addition, the climate and weather conditions modify both the quantity and quality of biomass. Some of the variations on feedstock quality can be manipulated by management decisions, while others are more difficult to control and may result to suboptimal feedstock quality. In addition, the large variability in the biorefineries themselves regarding technology, processing options and product portfolio emphasizes the need to optimize the feedstock quality case by case.

During silage fermentation, lactic acid bacteria convert water-soluble carbohydrates (WSC) into fermentation end products, which lowers the pH to approximately 4 and ensures the stability of silage under anaerobic conditions (McDonald et al., 1991). At the same time, plant and microbial enzymes and acid hydrolysis modify the chemical constituents of the biomass so that similar processes and products as from fresh grass cannot be obtained from ensiled grass. On the other hand, ensiling may act as a bioprocessing step synthesizing e.g., lactic acid or butyric acid, which can be extracted from the biomass to be used as platform chemicals for further processing in the chemical industry (Haag et al., 2016; Steinbrenner et al., 2022). Ensiling may also act as a pre-treatment to increase the yield of soluble components in liquid–solid separation (Ayanfe et al., 2023; Rinne et al., 2020).

In commercial silage production for ruminant livestock, different types of additives are used to manipulate the type and extent of silage fermentation (McDonald et al., 1991; Muck et al., 2018). Lactic acid bacteria inoculants are used to boost and direct the fermentation, which otherwise depends on the characteristics of the epiphytic flora. Application of organic acids restricts, but not totally prevents the fermentation and inhibits detrimental microbes. Fibrolytic enzymes have been used to liberate more substrates for the lactic acid bacteria, but in case of a biorefinery use, could also positively affect extraction of various components from the biomass (Rinne et al., 2020).

The objective of the current study was to quantify how plant species and different types of additive treatments affect silage fermentation and subsequently the quantity and quality of press juice in a simulated first step, i.e., liquid–solid separation, of a green biorefinery process. We hypothesized that the use of silage additives improves the capture of protein in the liquid fraction irrespective of forage type used.

2 | MATERIALS AND METHODS

2.1 | Conduction of the ensiling experiments

Two pilot scale ensiling experiments were conducted. The fresh forages were produced at the experimental farm of Natural Resources Institute Finland (Luke) in Jokioinen, Finland (60°48′ N, 23°29′ E). All swards were mown and precision chopped using farm scale machinery. The forages were ensiled in 12-l cylindrical plastic laboratory silos with 3 replicates per treatment. Representative samples of the fresh forages were collected at the laboratory before filling the silos.

In Experiment 1 (Exp. 1), a mixed timothy (*Phleum pratense*) and meadow fescue (*Festuca pratensis*) sward was harvested on 7 June 2016 from a field that was used for silage production for dairy cows. The grass was ensiled after a short field wilt (4 h; G4) and after an additional 20-hour wilt indoors (G24). Indoor wilting was chosen due to rainy weather conditions. Red clover (RC1; *Trifolium repens*) was harvested from a pure stand on 20 June 2016 and ensiled on 21 June after a field wilt of 24 h under good wilting conditions. The following additive treatments were used for all three forages:

- Control without additive (CON).
- Lactic acid bacteria inoculant (LAB; Sil-All 4 × 4+; Microbial Developments, Worcestershire, UK at 5 g/t resulting in bacterial inoculation of 10^5 cfu/g fresh forage. The additive contained *Lactiplantibacillus plantarum* ≥1 × 10^{11} cfu/g, *Pediococcus acidilactici* ≥4 × 10^{10} cfu/g, *Pediococcus pentosaceus*, ≥4 × 10^{10} cfu/g, *Propionibacterium acidipropionici* ≥2 × 10^{10} cfu/g, α-amylase from *Bacillus amyloliquefaciens* ≥ 3600 BAU/g, cellulase from *Trichoderma reesei* ≥ 60 CMCU/g, β-glucanase from *Aspergillus niger* ≥ 1000 IU/g and xylanase from *Trichoderma longibrachiatum* ≥ 1500 IU/g).
- Formic acid based additive (FA; AIV 2 Plus, Eastman Chemical Company, Oulu, Finland at 5 L/t. The additive contained 760 g/kg formic acid and 55 g/kg ammonium formate).

In Experiment 2 (Exp. 2), the forages were mown on 24 August 2016 from first regrowth of large experimental plots, where timothy (Tim) and red clover (RC2) were grown as pure stands. The plots had not been fertilized after the first cut. Both swards were immediately harvested without field wilting under humid weather conditions. Additive treatments applied for both forages were:

- CON
- Fibrolytic enzyme (ENZ; Flashzyme Plus containing cellulase and hemicellulase activities, Roal Ltd., Rajamäki, Finland at a rate of 0.5 mL/kg DM)
- Combination of FA and ENZ (FA + E; first FA and then ENZ from separate bottles).
- FA

The experimental procedures were the same in both experiments. The commercial additives were applied at doses recommended by the manufacturers while the dose of ENZ was chosen based on a previous study (Rinne et al., 2020), where different levels of enzyme application were evaluated. Additives were applied separately for each replicate and mixed with tap water so that the actual volume applied was 10 L/ton to ensure even spreading. Bottles with perforated caps were used for additive application, and the forage material (10 kg per replicate) was thoroughly mixed manually. After that, the forage was placed into

the cylindrical silos in ca. 500 g batches and an 8-kg lead plummet was let to drop freely 10 times on the top of the forage to compact it. Densities obtained were 599, 540 and 816 kg/m^3 for G4, G24 and RC1 in Exp. 1, and 760 and 1016 kg/m^3 for Tim and RC2 in Exp. 2. After silos were filled, they were closed with a plastic bag, a lid, an 8-kg lead plummet and finally a plastic bag filled with water.

The silos were stored at room temperature (ca. 20°C) with protection from light and opened after an ensiling period of 111, 110 and 97 days, respectively, for G4, G24 and RC in Exp. 1, and after 92 days for both Tim and RC2 in Exp. 2. The silos were weighed before and after filling, and immediately before opening to measure the weight loss during fermentation. The fermentation weight loss was multiplied by 1.44 according to Knický and Spörndly (2015) to account for the H_2O production during CO_2 formation. Approximately 10 cm of silage from the surface and 5 cm from the bottom of each silo were discarded before sampling for analyses. Representative samples were stored in −20°C until processed and analysed. The fresh forage samples were dried overnight at 105°C for dry matter (DM) determination and at 60°C for 2 days for analyses, and ground using a Wiley mill with a 1 mm sieve.

2.2 | Laboratory measurements and calculations

Fresh forage, silage and juice fractions were analysed for chemical composition and fermentation quality by routine methods as described by Ayanfe et al. (2023). The fermentability coefficient (FC) was calculated according to DLG (2020) as:

$$\mathrm{FC} = (\mathrm{DM}\ (\mathrm{g/kg}) + 8\ \mathrm{WSC}\ (\mathrm{g/kg\ DM})/\text{buffering capacity} \times (\mathrm{g\ lactic\ acid/100g\ DM}))/10,$$

and values below 35 indicate a forage difficult to ensile. The amount of ammonia-N added in FA treatments was reduced from the analysed ammonia-N concentration based on the theoretical addition level.

The liquid–solid separation was conducted from frozen and melted samples using a pneumatic press (PP; in-house built equipment; Luke, Jokioinen, Finland). A sample of 150 g was put into a mesh bag and squeezed between two vertical piston plates for 2 minutes at 6 bars (× 100 kPa) of pressure. The separated liquid was quantitatively collected and weighed. Three analytical replicate pressings were conducted per sample and a mean value of them was used for statistical analyses. For chemical analyses, the juice from the three pressings was combined. In Exp. 2, a double screw press (DS; Angel Juicer Ltd., Busan, South Korea) was also used for liquid–solid separation. A single pressing of 300 g was used per sample, and the measurement of liquid yield was taken when the screw press had reached steady state. The division of the fresh material into liquid and solid fractions is described as liquid yield, and it was calculated as follows:

$$\text{Liquid yield}\ (\mathrm{g/g}) = \text{Fresh matter in liquid}\ (\mathrm{g})/\text{Fresh matter in original biomass}\ (\mathrm{g}).$$

To describe how much of a compound in original biomass was captured in the liquid, the following calculations were conducted for DM, ash and crude protein (CP):

$$\text{Amount of compound captured in liquid}\ (\mathrm{g/g}) = \frac{(\text{Liquid yield}\ (\mathrm{g/g}) \times \text{Liquid DM}\ (\mathrm{g/kg}) \times \text{Nutrient concentration in liquid}\ (\mathrm{g/kg\,DM}))}{\text{DM of original biomass}\ (\mathrm{g/kg}) \times \text{Nutrient concentration in original biomass}\ (\mathrm{g/kg\,DM})}$$

2.3 | Statistical analyses

Data was analysed using a MIXED procedure (SAS Inc. 2002–2012, Release 9.4; SAS Inst. Inc., Cary, NC, USA) of SAS. In Exp. 1, the data was analysed in 3 separate parts. First, the grass treatments were analysed with a model including the effects of additive, wilting, and their interaction. The second analysis included only red clover treatments, and the effect of additive was included in the model. In the third analysis, wilted grass treatments and red clover were included to evaluate the effect of plant species, additive, and their interaction. In Exp. 2, only one analysis was conducted, where the effects of plant species, additive, and their interaction were evaluated. In all cases, replicate was used as the random effect in the model. The pairwise comparisons of the treatment means within each analysis were performed using Tukey's test at a probability level of $p < .05$.

3 | RESULTS

The fresh forage composition for both experiments is presented in Table 1. The DM concentrations were higher in Exp. 1 (on average 291 g/kg) than in Exp. 2 (on average 170 g/kg). The CP concentrations were clearly lower in grass (93 g/kg DM) than red clover (187 g/kg DM) materials. With lower WSC concentrations and higher buffering capacity values, the red clover forages had lower fermentation coefficients than the grasses (48 and 27 for grass and red clover, respectively). The results of the fermentation quality and simulated biorefinery outputs are presented in Tables 2 and 3 for Exp. 1 and 2, respectively. A figure showing fermentation quality of all silages is included as Figure S1.

TABLE 1 Chemical composition of grass and red clover in primary growth (Experiment 1) and first regrowth (Experiment 2) before ensiling.

	Experiment 1			Experiment 2	
	Grass				
	4-h wilt	24-h wilt	Red clover	Grass	Red clover
Dry matter (DM), g/kg fresh matter	290	298	285	210	129
Buffering capacity, g lactic acid/100 g DM	6.4	6.5	10.1	4.4	11.2
Fermentation coefficient	48	43	36	52	17
In DM, g/kg					
Ash	82	88	101	59	89
Crude protein	98	103	197	78	176
Water soluble carbohydrates	153	109	89	170	60
Neutral detergent fibre	537	563	334	592	485
In vitro organic matter digestibility, g/g organic matter	0.762	0.746	0.753	0.628	0.599

The increased ash concentrations in control treatments of G4 and RC2 were probably linked with the greater fermentation losses of them. In Exp. 1, the fermentation quality of CON in G4 was poor as indicated by high pH, ammonia N and butyric acid concentrations, and it resulted in highest weight losses during fermentation ($p < .05$). The quality could be improved both by LAB and FA applications, where LAB increased lactic acid fermentation ($p < .05$) and FA restricted it ($p < .05$). A highly significant wilting × additive interaction was observed for all fermentation parameters ($p < .001$) as differences between the additive treatments practically disappeared after the wilting of G24, although the DM concentration of the fresh forage increased only marginally (8 g/kg). For G24 and RC1, the CP concentrations were higher for FA than LAB ($p < .05$). For RC1, FA restricted lactic acid fermentation ($p < .05$) but resulted in higher ethanol concentration than the other treatments ($p < .05$), and LAB had slightly smaller weight losses during fermentation than the other two additive treatments ($p < .05$). The other differences between the RC1 silages were minor.

In Exp. 2, the fermentation pattern of particularly Tim but also RC2 was characterized by high ethanol concentrations (60 and 21 g/kg DM for Tim and RC2, respectively). There were also clear differences between the plant species as RC2 CON was very poorly preserved with high pH, ammonia-N and ethanol concentrations and virtually no lactic acid, which led to significant species × additive interactions. For Tim, ENZ did not affect fermentation quality, but FA + E and FA restricted lactic acid production ($p< .05$). However, for RC2, ENZ clearly improved the fermentation quality by increasing lactic acid concentration and decreasing pH, ammonia N, ethanol and acetic acid concentrations compared to CON ($p < .05$), although acetic acid concentration remained high (61 g/kg DM). Residual WSC were detected in FA + E and FA, while they were depleted in CON and ENZ ($p < .05$). Acetic acid concentration could be controlled by FA application (65 vs 17 g/kg DM for non-FA vs FA treated RC2; $p < .05$) and also lactic acid concentration was reduced compared to ENZ ($p < .05$). There were no benefits of adding ENZ to FA as the only significant difference between FA + E and FA was a decrease in pH in RC2 (3.95 vs 4.07; $p < .05$). Fresh matter losses during fermentation were not affected by additives in Tim, but for RC2, addition of FA either alone or with ENZ reduced the losses compared to CON and ENZ alone ($p < .05$).

The liquid yield using PP varied between silage batches being 0.192 for G4, 0.123 for G24, 0.232 for RC1, 0.359 for Tim and 0.481 for RC2. Further, in Exp. 2 when DS was also used, the liquid yields were clearly higher (0.628 and 0.694 for Tim and RC2, respectively). However, there were no statistically significant effects of additive treatments on liquid yield ($p > .05$). On the other hand, some treatment effects were noted on the chemical composition of the liquids. The FA application increased liquid DM concentration in G24, RC1 and RC2 compared to CON, but in Tim it decreased ($p < .05$). Liquid ash concentration was higher in FA-treated G24 and RC1 but lower in RC2 compared with CON ($p< .05$). For liquid CP concentration, CON resulted in higher value than LAB in G4. In RC2, the FA-containing treatments resulted in lowest CP concentrations and CON in highest, while ENZ was intermediate ($p < .05$).

The CP captured in the liquid may be considered the most important biorefinery output, and it was 0.153, 0.096, 0.127, 0.100 and 0.078 for G4, G24, RC1, Tim and RC2, respectively, when the less efficient PP was used. The values were greatly increased with DS being 0.301 and 0.239 for Tim and RC2, respectively. The differences in liquid yields and liquid CP concentrations tended to compensate for each other so that the only significant additive effect was observed for RC1, where CON resulted in lower captured proportion of CP than LAB and FA ($p < .05$) using PP. For DS, an additive effect for Tim was found so that the capture of CP was higher in ENZ compared to the FA-containing treatments ($p < .05$).

4 | DISCUSSION

The agronomic factors in green biomass production such as choice of plant species, fertilization and timing of harvest relative to plant developmental stage greatly affect the characteristics of the feedstock, and

TABLE 2 Composition and fermentation quality of timothy and red clover silages treated with additives, and yield and retained compounds of liquid–solid separation (Experiment 1).

	Grass										Red clover						*p*-Value[‡]
	4 h wilting			24 h wilting				*p*-Value[†]			24 h wilting				*p*-Value		
	CON	LAB	FA	CON	LAB	FA	SEM	Wilt	Add	Wilt × add	CON	LAB	FA	SEM	Add	SEM	Species×add
Dry matter (DM), g/kg fresh matter	261^{d}	286^{c}	287^{c}	294^{b}	298^{b}	306^{a}	1.5	<.001	<.001	<.001	285	279	287	2.1	.122	1.8	<.001
pH	4.93^{a}	3.92^{c}	4.06^{bc}	4.21^{b}	4.13^{bc}	4.05^{bc}	0.058	.004	<.001	<.001	4.30^{a}	4.22^{b}	4.27^{ab}	0.014	.029	0.058	<.001
Ammonium-N, g/kg N	98^{a}	47^{b}	62^{b}	65^{b}	82^{b}	62^{b}	11.7	.919	.126	.015	39^{a}	27^{b}	38^{a}	0.9	<.001	7.6	.042
Corrected[‡] ammonium-N, g/kg N	98^{a}	47^{b}	50^{b}	65^{ab}	82^{a}	52^{b}	11.7	.878	.027	.015	39^{a}	27^{c}	32^{b}	0.9	<.001	7.7	.030
Chemical composition, g/kg DM																	
Ash	97^{a}	87^{c}	88^{c}	91^{b}	89^{bc}	88^{c}	0.5	.06	<.001	<.001	106^{a}	103^{b}	105^{ab}	0.7	.068	0.6	<.001
Crude protein (CP)	120^{ab}	115^{b}	117^{b}	119^{ab}	116^{b}	123^{a}	1.3	.095	.007	.039	214^{a}	206^{b}	215^{a}	1.1	.006	1.3	.029
Water soluble carbohydrates	13.2^{bc}	69.5^{a}	18.3^{b}	19.5^{b}	17.4^{b}	8.0^{c}	1.94	<.001	<.001	<.001	12.3^{b}	26.1^{a}	6.7^{b}	1.79	.002	1.70	<.001
Ethanol	19.5^{a}	5.1^{e}	15.9^{b}	9.0^{d}	7.6^{d}	10.9^{c}	0.31	<.001	<.001	<.001	3.5^{b}	2.6^{c}	13.6^{a}	0.15	<.001	0.31	<.001
Formic acid	0.2^{b}	0.2^{b}	15.1^{a}	0.1^{b}	0.3^{b}	14.4^{a}	0.20	.150	<.001	.090	0.2^{b}	0.3^{b}	17.3^{a}	0.18	<.001	0.12	<.001
Lactic acid	32^{e}	120^{a}	69^{cd}	85^{bc}	93^{b}	66^{d}	3.5	.018	<.001	<.001	124^{a}	111^{b}	65^{c}	1.7	<.001	3.7	<.001
Acetic acid	8.7^{d}	9.6^{d}	20.6^{a}	17.1^{bc}	15.5^{c}	20.0^{ab}	0.77	<.001	<.001	<.001	36.3^{a}	17.6^{c}	21.3^{b}	0.96	<.001	0.74	<.001
Propionic acid	1.0^{a}	0.3^{b}	0.4^{b}	0.3^{b}	0.3^{b}	0.3^{b}	0.07	.003	.001	.001	0.4	0.4	0.4	0.01	.257	0.07	.001
Butyric acid	27.9^{a}	0.2^{b}	1.8^{b}	0.7^{b}	0.2^{b}	0.1^{b}	1.62	<.001	<.001	<.001	0.2^{a}	0.2^{a}	0.1^{b}	0.01	.019	1.6	<.001
Fresh matter losses, g/kg	4.1^{a}	1.2^{c}	2.0^{b}	1.8^{c}	1.6^{c}	1.7^{c}	0.8	<.001	<.001	<.001	1.4^{a}	0.9^{b}	1.4^{a}	0.03	<.001	0.05	<.001
Biorefinery performance (pneumatic press)																	
Liquid yield	0.199^{a}	0.199^{a}	0.177^{a}	0.124^{b}	0.127^{b}	0.117^{b}	0.011	<.001	.167	.693	0.235	0.233	0.228	0.0078	.578	0.0090	.422
Liquid DM, g/kg	101^{c}	116^{abc}	118^{abc}	113^{bc}	121^{ab}	132^{a}	4.0	.009	.003	.476	115^{b}	126^{ab}	130^{a}	5.2	.069	5.0	.918
Liquid CP, g/kg DM	261^{a}	220^{b}	237^{ab}	226^{b}	242^{ab}	247^{ab}	8.5	.893	.203	.004	265	269	268	14.2	.940	11.5	.070
Liquid ash, g/kg DM	80^{c}	94^{ab}	96^{ab}	90^{bc}	97^{ab}	107^{a}	3.0	.010	<.001	.357	88^{b}	97^{ab}	101^{a}	3.8	.033	3.81	.577
DM captured in liquid	0.077^{a}	0.081^{a}	0.075^{a}	0.047^{b}	0.051^{b}	0.051^{b}	0.0037	<.001	.473	.605	0.097^{b}	0.106^{a}	0.105^{a}	0.0022	.058	0.0029	.114
Ash captured in liquid	0.061^{bc}	0.083^{a}	0.076^{ab}	0.044^{c}	0.052^{c}	0.057^{bc}	0.0044	<.001	.010	.301	0.082^{b}	0.102^{a}	0.102^{a}	0.0038	.013	0.0037	.670
CP captured in liquid	0.164^{a}	0.151^{a}	0.145^{a}	0.087^{b}	0.104^{b}	0.096^{b}	0.0072	<.001	.659	.115	0.117^{b}	0.137^{a}	0.128^{ab}	0.0067	.077	0.0057	.024

Note: Means within the same row without same superscript (a, b, c, d) differ significantly ($p < .05$, Tukey test) separately for timothy (4 and 24 h wilting) and red clover.

Abbreviations: CON, control without additive; FA, formic acid based additive; LAB, lactic acid bacteria inoculant; SEM, Standard error of the mean.

[†]Add: effect of additive; Wilt: effect of wilting; Wilt×Add: interaction effect of wilting and additive; Species×Add: interaction effect of plant species (wilted grass vs red clover) and additive.

[‡]Corrected for the ammonia-N added via the additive.

TABLE 3 Composition and fermentation quality of timothy and red clover silages treated with additives, and yield and retained compounds of liquid–solid separation (Experiment 2).

	Timothy				Red clover					*p*-Value[†]		
	CON	ENZ	FA + E	FA	CON	ENZ	FA + E	FA	SEM	Species	Add	Species×add
Dry matter (DM), g/kg fresh matter	210^{a}	209^{a}	204^{a}	206^{a}	136^{b}	137^{b}	136^{b}	138^{b}	4.67	<0.001	0.831	0.881
pH	3.77^{e}	3.78^{e}	3.85^{de}	3.93^{d}	5.49^{a}	4.36^{b}	3.95^{d}	4.07^{c}	0.023	<0.001	<0.001	<0.001
Ammonium-N, g/kg N	60^{d}	63^{d}	82^{c}	84^{c}	113^{a}	94^{b}	83^{c}	95^{b}	3.2	<0.001	0.022	<0.001
Corrected[‡] ammonium-N, g/kg N	60^{d}	63^{d}	62^{d}	64^{d}	113^{a}	94^{b}	68^{d}	80^{c}	3.3	<0.001	<0.001	<0.001
Chemical composition, g/kg DM												
Ash	71^{c}	71^{c}	70^{c}	70^{c}	102^{a}	97^{a}	91^{b}	89^{b}	1.2	<0.001	<0.001	<0.001
Crude protein (CP)	86^{b}	92^{b}	95^{b}	95^{b}	194^{a}	194^{a}	185^{a}	184^{a}	2.7	0.005	0.340	0.001
Water soluble carbohydrates	33.3^{a}	30.1^{a}	16.9^{b}	17.6^{b}	3.0^{c}	2.6^{c}	16.4^{b}	14.0^{b}	2.16	<0.001	0.739	<0.001
Ethanol	49.8b^{c}	58.7^{ab}	71.6^{a}	61.4^{ab}	39.8^{c}	12.7^{d}	17.6^{d}	12.0^{d}	2.99	<0.001	0.010	<0.001
Formic acid	0.5^{c}	0.6^{c}	10.5^{b}	12.7^{b}	4.9^{c}	1.6^{c}	32.1^{a}	34.1^{a}	1.24	<0.001	<0.001	<0.001
Lactic acid	105^{a}	100^{a}	60^{b}	53^{b}	2^{c}	56^{b}	21b^{c}	21b^{c}	8.2	<0.001	<0.001	0.002
Acetic acid	19.5^{c}	17.3^{c}	20.4^{c}	20.0^{c}	70.1^{a}	60.8^{b}	17.5^{c}	17.4^{c}	1.15	<0.001	<0.001	<0.001
Propionic acid	0.3^{c}	0.3^{c}	0.8^{c}	0.8^{c}	6.2^{b}	9.0^{a}	0.8^{c}	1.3^{c}	0.230	<0.001	<0.001	<0.001
Butyric acid	0	0	0	0	1.7	0.2	0	1.4	0.406	0.010	0.135	0.135
Fresh matter losses, g/kg	2.0^{c}	2.3^{c}	2.7^{c}	2.9^{c}	18.4^{a}	18.5^{a}	9.8^{b}	9.8^{b}	0.55	<0.001	<0.001	<0.001
Biorefinery performance (pneumatic press)												
Liquid yield, g/g	0.355^{b}	0.362^{b}	0.384^{b}	0.336^{b}	0.479^{a}	0.467^{a}	0.492^{a}	0.488^{a}	0.0133	<0.001	0.229	0.295
Liquid DM, g/kg	57.5^{a}	55.9^{ab}	54.4^{ab}	51.4^{bc}	32.1^{f}	41.4^{de}	47.0^{cd}	40.0^{e}	1.33	<0.001	<0.001	<0.001
Liquid Ash, g/kg DM	147^{c}	150^{bc}	155^{bc}	169^{bc}	282^{a}	254^{a}	221^{ab}	175^{bc}	14.6	<0.001	0.059	0.003
CP, g/kg DM	84^{d}	87^{d}	84^{d}	84^{d}	121^{a}	110^{b}	99^{c}	98^{c}	2.1	<0.001	<0.001	<0.001
DM captured in liquid	0.106^{bc}	0.106^{bc}	0.115^{bc}	0.093^{c}	0.115^{bc}	0.135^{ab}	0.162^{a}	0.137^{ab}	0.0072	<0.001	0.008	0.069
Ash captured in liquid	0.217^{b}	0.220^{b}	0.254^{ab}	0.220^{b}	0.314^{ab}	0.348^{ab}	0.387^{a}	0.271^{ab}	0.0310	<0.001	0.097	0.466
CP captured in liquid	0.107^{a}	0.104^{a}	0.105^{a}	0.084^{ab}	0.073^{b}	0.077^{b}	0.088^{ab}	0.074^{b}	0.0049	<0.001	0.021	0.1001
Biorefinery performance (double screw press)												
Liquid yield	0.623^{c}	0.637^{bc}	0.643^{bc}	0.610^{c}	0.700^{a}	0.671^{ab}	0.707^{a}	0.697^{a}	0.0089	<0.001	0.100	0.048
Liquid DM, g/kg	78.4^{a}	78.5^{a}	71.5^{ab}	70.8^{ab}	51.5^{c}	63.0^{bc}	64.8^{bc}	61.8^{c}	0.75	<0.001	0.025	<0.001
Liquid Ash, g/kg DM	117^{c}	169^{abc}	179^{abc}	148^{bc}	243^{a}	195^{abc}	179^{abc}	227^{ab}	17.5	<0.001	0.963	0.013
Liquid CP, g/kg DM	112^{c}	119^{c}	118^{c}	117^{c}	186^{a}	188^{a}	162^{b}	158^{b}	3.3	<0.001	<0.001	<0.001
DM captured in liquid	0.207^{cd}	0.226^{bc}	0.206^{cd}	0.188^{d}	0.218^{bcd}	0.244^{ab}	0.266^{a}	0.249^{ab}	0.0073	<0.001	0.011	0.004
Ash captured in liquid	0.435^{b}	0.541^{ab}	0.476^{ab}	0.492^{ab}	0.563^{ab}	0.478^{ab}	0.550^{ab}	0.623^{a}	0.0352	0.016	0.386	0.049
CP captured in liquid	0.311^{ab}	0.355^{a}	0.273^{bc}	0.265^{bc}	0.230^{c}	0.274^{bc}	0.232^{c}	0.221^{c}	0.0172	<0.001	0.002	0.405

Note: Means within the same row without same superscript (a, b, c d) differ significantly ($p < .05$, Tukey test).

Abbreviations: CON, control without additive; ENZ, fibrolytic enzyme; FA, formic acid based additive; FA + E, FA and ENZ combined; SEM, Standard error of the mean.

[†]Add: effect of additive; Species: effect of plant species; Species×Add: interaction effect of plant species and additive.

[‡]Corrected for the ammonia-N added via the additive.

subsequently outputs from the biorefinery process (Damborg et al., 2020; Lindorfer et al., 2019; O'Keeffe et al., 2011). The biomasses used in the current experiments were not totally optimal for biorefining, although that actually depends on what is the main product of the process. The large variability of potential approaches (business cases, arrays of products produced, external benefits such as ecosystem services in feedstock production) plays a major role in defining the optimal characteristics of the feedstock. The opportunity to produce local protein and extract it from the fibre matrix so that it is suitable for feeding monogastric animals is often targeted (Keto et al., 2021; Stødkilde et al., 2020, 2023), and in that case the low CP concentration of particularly the grass materials was not optimal. The average CP concentration of the grasses was as low as 93 g/kg DM which is clearly lower than the average value of 145 g/kg DM for commercial grass silages for dairy cows in the same region ($n = 89{,}473$, years 2019–2022; Valio Ltd., Helsinki, Finland, personal communication). Grass CP concentration can be manipulated by N fertilization with subsequent effects on protein yield from the biorefinery (Damborg et al., 2020).

Forage legumes such as red clover are not dependent on the N fertilization due to their ability of biological N fixation by the Rhizobium bacteria in their root nodules and provide a valuable way to produce protein. Typically, the CP concentration is higher in red clover than in grasses, which was the case for the current material as well. Fair comparisons between different plant species are however difficult because the variation within species is also large and depends greatly on the environmental conditions and management decisions taken. When an organic non-N-fertilized ley was sampled and different species manually separated, red clover had a clearly lower DM and higher CP concentration than timothy (Rinne & Nykänen, 2000), and similar trends were reported by McEniry et al. (2014) from pure stands of grasses and red clover. It should however be kept in mind that the choices in plant species are case-specific, and many factors such as ability to increase manure nutrient circulation may affect it (Tampio et al., 2019). For example, a green biorefinery in conjunction of a piggery may benefit from using grasses rather than forage legumes in the swards, as grasses can more efficiently utilize the slurry N applied in the fields.

The focus of the current research was to evaluate how the ensiling process manipulated by additive use affected the biorefinery outputs of the different silage batches. Most of the biorefinery development has concentrated on using fresh grass (Jørgensen et al., 2022), but as a stable year-around available feedstock, ensiled biomass would provide benefits, so that studying also silages is justified (Rinne, 2024). There are also indications that ensiling might benefit e.g., liquid–solid separation (Ayanfe et al., 2023), but direct comparisons of fresh and ensiled materials are scarce.

The current set of forages showed quite distinct characteristics and particularly G4 and RC2 were challenging to ensile as shown by the poor fermentation quality of CON silages. The significant interactions revealed that the effects of additive treatments varied depending on fresh forage characteristics. The FA treatments consistently increased the fermentation quality even with difficult to ensile materials in line with e.g., McEniry et al. (2014) and Rinne et al. (2023). In our previous study, ENZ improved silage fermentation quality and increased liquid yield (Rinne et al., 2020), but here the efficacy was not so obvious although CP captured in liquid increased significantly in Tim and numerically in RC2, and the fermentation quality of RC2 improved.

Although the additive effects on the biorefinery parameters evaluated in the current study were not consistent, silage quality may have important effects on the biorefinery output. In the current study, more detailed analysis of the nitrogenous compounds was not included, but Rinne (2024) reported that poor fermentation quality resulted in greatly reduced proportion of amino-N in total silage N the values being 0.53 in a very poorly preserved control silage and 0.75 in formic acid treated silage. This would significantly affect the value of the product if for example used as a liquid feed for pigs. Winters et al. (2001) evaluated the N quality of non-treated, formic acid treated and inoculated silages, and noted that additive treatments conserved amino-N, although differences were relatively small (proportion of amino-N in total N 0.60, 0.64 and 0.65 for control, formic acid treated and inoculated silages, respectively), which may be explained by the relatively good fermentation quality of even the control silage. In the data sets of Nadeau et al. (2019), use of chemical additives (formic acid or salt based products) increased the proportion of true protein in silages. Formic acid application also decreases the solubility of silage CP (Ayanfe et al., 2023; Jaakkola et al., 2006; Nadeau et al., 2019), which could decrease the CP capture into the liquid (Ayanfe et al., 2023), although that was detected in the current data set only for Tim when the more efficient press was used.

Silage DM concentration was closely linked to liquid yield as previously reported by Franco et al. (2019). In the current data set, liquid yield decreased by 0.02 per 10 g increase in silage DM concentration, when the less efficient PP was used ($R^2 = 0.95$). The fit with the equation presented by Franco et al. (2019) was good between silage DM concentration and liquid yield with R^2 equalling 0.95. When the more efficient DS was used, the effect of silage DM concentration on liquid yield was smaller (0.009 per 10 g increase in silage DM concentration), but still affected by it ($R^2 = 0.87$).

The overall liquid yields and amounts of compounds captured in the liquid were low in the current data when the low efficiency PP extraction method was used. In Exp. 2, when a more efficient press was used, the amounts of CP captured in the liquid tripled. This highlights the importance of technological choices to the performance of a biorefinery (Lindorfer et al., 2019). When low efficiency press is used, the feedstock characteristics may be more important as shown by Franco et al. (2019) and Ayanfe et al. (2023). This was also observed in the current data set referring to the discussion above about the magnitude of the effect of silage DM on liquid yield using different presses.

The wilting period of 24 hours was not effective in increasing the DM concentration of grass in Exp. 1. Due to rainy weather, wilting was conducted indoors, and it resulted in only a minimal (8 g/kg during 20 h) increase in fresh forage DM concentration. However, substantial losses in WSC concentration appeared showing that extensive

respiration had taken place during the wilting period. Even more interestingly, the fermentation quality was greatly improved, which must have been due to changes in the epiphytic microflora during the wilting period.

High moisture content in the biomass increases the logistics costs and compromises silage fermentation quality (McDonald et al., 1991; Rinne et al., 2023), so it should in general be avoided. Technological solutions such as blending or pulping the drier biomass with liquid prior to pressing could be a viable option to be able to benefit from the effects of wilting in biomass conservation for biorefineries. Further, higher liquid yield as such may not be a proper goal, and in the current data set, liquid yield and CP captured in the liquid were even slightly negatively correlated ($R^2 = -0.33$).

The losses during storage can be separated into fermentation and effluent losses. More extensive fermentation and particularly secondary and clostridial fermentation and losses caused by them are linked with low DM silages (McDonald et al., 1991). In current silage production practises for livestock feeding, effluent losses are in general prevented by efficient wilting prior to ensiling as effluent production ceases when biomass DM reaches 250–300 g/kg (Jones & Jones, 1995). For successful mechanical liquid solid separation, relatively low DM feedstock is however required (Franco et al., 2019) so that risks for effluent losses may increase, if low DM biomass is preserved for biorefineries. Further, if formic acid based additives are used, effluent losses are increased (Jones & Jones, 1995; Winters et al., 2001). If spontaneous effluent production takes place, the collection of the effluent needs to be arranged to prevent environmental damages. Issues related to silage losses have been discussed from livestock feeding point of view (Borreani et al., 2018; Wilkinson & Davies, 2013), but aspects related to low DM forages might require further attention specifically concerning biorefineries. Further, losses related to spoiled top and side silage in the silos as well as aerobic spoilage after silo opening may importantly affect the overall efficiency and environmental impact of a biorefinery operation. Thus, silage management practises such as optimized DM concentration and use of effective additives are recommended in silage production for green biorefineries.

5 | CONCLUSIONS

Optimizing the management of agronomic and feedstock conservation techniques will play an important role for the economic and environmental sustainability of the biorefinery process, but actual choices depend on the particular business case regarding feedstock sourcing policy and array of products produced. Good silage management practises with minimal losses during storage should be targeted, but against our hypothesis, no clear patterns in biorefinery outputs were observed in the current study, when different types of additives were used in grass and red clover silage production.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author.

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REFERENCES

Ayanfe, N., Franco, M., Stefański, T., Pap, N., & Rinne, M. (2023). The effects of grass biomass preservation methods, organic acid treatment and press type on the separation efficiency in the green biorefinery. *Bioresource Technology Reports*, *21*, 101356. https://doi.org/10.1016/j.biteb.2023.101356

Borreani, G., Tabacco, E., Schmidt, R. J., Holmes, B. J., & Muck, R. E. (2018). Silage review: Factors affecting dry matter and quality losses in silages. *Journal of Dairy Science*, *101*, 3952–3979. https://doi.org/10.3168/jds.2017-13837

Damborg, V. K., Jensen, S. K., Weisbjerg, M. R., Adamsen, A. P., & Stødkilde, L. (2020). Screw-pressed fractions from green forages as animal feed: Chemical composition and mass balances. *Animal Feed Science and Technology*, *261*, 114401. https://doi.org/10.1016/j.anifeedsci.2020.114401

Ditzler, L., van Apeldoorn, D. F., Pellegrini, F., Antichi, D., Bàrberi, P., & Rossing, W. A. H. (2021). Current research on the ecosystem service potential of legume inclusive cropping systems in Europe. *A Review. Agronomy for Sustainable Development*, *41*(2), 26. https://doi.org/10.1007/s13593-021-00678-z

DLG. (2020). *DLG testing guidelines for the award und use of the DLG quality mark for ensiling agents*. DLG TestServicen GmbH.

Franco, M., Hurme, T., Winquist, E., & Rinne, M. (2019). Grass silage for biorefinery – A meta-analysis of silage factors affecting liquid-solid separation. *Grass and Forage Science*, *74*, 218–230. https://doi.org/10.1111/gfs.12421

Gaffey, J., Rajauria, G., McMahon, H., Ravindran, R., Dominguez, C., Ambye-Jensen, M., Souza, M. F., Meers, E., Macias Aragonés, M., Skunca, D., & Sanders, J. P. M. (2023). Green biorefinery systems for the production of climate-smart sustainable products from grasses, legumes and green crop residues. *Biotechnology Advances*, *66*, 108168. https://doi.org/10.1016/j.biotechadv.2023.108168

Haag, N. L., Grumaz, C., Wiese, F., Kirstahler, P., Merkle, W., Nägele, H.-J., Sohn, K., Jungbluth, T., & Oechsner, H. (2016). Advanced green biorefining. Effects of ensiling treatments on lactic acid production, microbial activity and supplementary methane formation of grass and rye. *Biomass Conversion and Biorefinery*, *6*, 197–208. https://doi.org/10.1007/s13399-015-0178-2

Jaakkola, S., Rinne, M., Heikkilä, T., Toivonen, V., & Huhtanen, P. (2006). Effects of restriction of silage fermentation with formic acid on milk production. *Agricultural and Food Science*, *15*, 200–218. https://doi.org/10.2137/145960606779216290

Jones, D. I. H., & Jones, R. (1995). The effect of crop characteristics and ensiling methodology on grass silage effluent production. *Journal of Agricultural Engineering Research*, *60*, 73–81. https://doi.org/10.1006/jaer.1995.1001

Jørgensen, U., Jensen, S. K., & Ambye-Jensen, M. (2022). Coupling the benefits of grassland crops and green biorefining to produce protein, materials and services for the green transition. *Grass and Forage Science*, *77*, 295–306. https://doi.org/10.1111/gfs.12594

Keto, L., Perttilä, S., Särkijärvi, S., Immonen, N., Kytölä, K., Alakomi, H.-L., Hyytiäinen-Pabst, T., Tsitko, I., Saarela, M., & Rinne, M. (2021). Effect

of silage juice feeding on pig production performance, meat quality and gut microbiome. *Livestock Science*, *254*, 104728. https://doi.org/10.1016/j.livsci.2021.104728

Knický, M., & Spörndly, R. (2015). Short communication: Use of a mixture of sodium nitrite, sodium benzoate, and potassium sorbate in aerobically challenged silages. *Journal of Dairy Science*, *98*, 5729–5734. https://doi.org/10.3168/jds.2015-9332

Lindorfer, J., Lettner, M., Hesser, F., Fazeni, K., Rosenfeld, D., Annevelink, B., & Mandl, M. (2019). *Technical, economic and environmental assessment of biorefinery concepts*. IEA Bioenergy. ISBN: 978-1-910154-64-9 Available at: https://www.ieabioenergy.com/wp-content/uploads/2019/07/TEE_assessment_report_final_20190704-1.pdf

McDonald, P., Henderson, A. R., & Heron, S. J. E. (1991). *The biochemistry of silage* (2nd ed.). Chalcombe Publications.

McEniry, J., King, C., & O'Kiely, P. (2014). Silage fermentation characteristics of three common grassland species in response to advancing stage of maturity and additive application. *Grass and Forage Science*, *69*, 393–404. https://doi.org/10.1111/gfs.12038

Muck, R. E., Nadeau, E. M. G., McAllister, T. A., Contreras-Govea, F. E., Santos, M. C., & Kung, L. (2018). Silage review: Recent advances and future uses of silage additives. *Journal of Dairy Science*, *101*, 3980–4000. https://doi.org/10.3168/jds.2017-13839

Nadeau, E., Sousa, D. O., & Auerbach, H. (2019). Forage protein quality as affected by wilting, ensiling and the use of silage additives. In *Proceedings of the 10th Nordic feed science conference, Uppsala, Sweden. Report 302* (pp. 28–33). Swedish University of Agricultural Sciences, Department of Animal Nutrition and Management. Available at: https://www.slu.se/globalassets/ew/org/inst/huv/konferenser/nfsc/nfsc2019/nfsc-proceedings-2019-06-05-torsten-eriksson.pdf

O'Keeffe, S., Schulte, R. P. O., Lalor, S. T. J., O'Kiely, P., & Struik, P. C. (2011). Green biorefinery (GBR) scenarios for a two-cut silage system: Investigating the impacts of sward botanical composition, N fertilisation rate and biomass availability on GBR profitability and price offered to farmers. *Biomass and Bioenergy*, *35*, 4699–4711. https://doi.org/10.1016/j.biombioe.2011.06.051

Rinne, M. (2024). Novel uses of ensiled biomasses as feedstocks for green biorefineries. *Journal of Animal Science and Biotechnology*, *15*, 36. https://doi.org/10.1186/s40104-024-00992-y

Rinne, M., Franco, M., Manni, K., & Huuskonen, A. (2023). Evaluating the effects of wilting, mixing with timothy and silage additive application on red clover silage quality. *Agricultural and Food Science*, *32*, 207–218. https://doi.org/10.23986/afsci.137136

Rinne, M., & Nykänen, A. (2000). Timing of primary growth harvest affects the yield and nutritive value of timothy-red clover mixtures. *Agricultural and Food Science*, *9*, 121–134. 10.23986/afsci.5654

Rinne, M., Winquist, E., Pihlajaniemi, V., Niemi, P., Seppälä, A., & Siikaaho, M. (2020). Fibrolytic enzyme treatment prior to ensiling increased press-juice and crude protein yield from grass silage. *Bioresource Technology*, *299*, 122572. https://doi.org/10.1016/j.biortech.2019.122572

Steinbrenner, J., Mueller, J., & Oechsner, H. (2022). Combined butyric acid and methane production from grass silage in a novel green biorefinery concept. *Waste and Biomass Valorization*, *13*, 1873–1884. https://doi.org/10.1007/s12649-021-01626-4

Stødkilde, L., Ambye-Jensen, M., & Jensen, S. K. (2020). Biorefined grass-clover protein composition and effect on organic broiler performance and meat fatty acid profile. *Journal of Animal Physiology and Animal Nutrition*, *104*, 1757–1767. https://doi.org/10.1111/jpn.13406

Stødkilde, L., Mogensen, L., Bache, J. K., Ambye-Jensen, M., Vinther, J., & Jensen, S. K. (2023). Local protein sources for growing-finishing pigs and their effects on pig performance, sensory quality and climate impact of the produced pork. *Livestock Science*, *267*, 105128. https://doi.org/10.1016/j.livsci.2022.105128

Tampio, E., Winquist, E., Luostarinen, S., & Rinne, M. (2019). A farm-scale grass biorefinery concept for a combined pig feed and biogas production. *Water Science and Technology*, *80*, 1043–1052. https://doi.org/10.2166/wst.2019.356

Wilkinson, J. M., & Davies, D. R. (2013). The aerobic stability of silage: Key findings and recent developments. *Grass and Forage Science*, *68*, 1–19. https://doi.org/10.1111/j.1365-2494.2012.00891.x

Winters, A. L., Fychan, R., & Jones, R. (2001). Effect of formic acid and bacterial inoculant on the amino acid composition of grass silage and on animal performance. *Grass and Forage Science*, *56*, 181–192. https://doi.org/10.1046/j.1365-2494.2001.00265.x

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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