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Chemical Characterisation and Biorefinery Efficiency of Timothy Grass and Pulp Silages

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ABSTRACT

This study investigated the effects of increased dry matter concentration by screw-pressing or wilting and additive application on fermentation quality of primary growth (PG) and first regrowth (ReG) timothy grass. Additionally, the nutritional quality of the liquid produced during the screw-pressing of pre-ensiled and ensiled biomasses was assessed. Two experiments were conducted: PG grass (Experiment 1) was ensiled fresh (Intact) and following liquid extraction via screw-pressing of fresh biomass (Pulp), whilst ReG included wilted biomass in addition to Intact and Pulp (Experiment 2). Biomasses were ensiled without any additives (Control), with lactic acid bacteria inoculant (LAB), or with a formic acid-based additive (FA). The PG biomasses were ensiled in vacuum bags and ReG in laboratory-scale cylindrical silos for 3 months. The silages were subjected to screw-pressing, and the chemical composition of the liquid was analysed. In both experiments, the Pulp had reduced water-soluble carbohydrates and ash compared to the Intact biomass, but crude protein concentration was not affected. In Experiment 2, pulping and wilting improved ensilability. Silages in both experiments exhibited good fermentation quality, with low pH and ammonia nitrogen concentration. Additionally, FA further reduced protein degradation. Wilting restricted silage fermentation, resulting in slightly elevated pH and reduced lactic acid production, alongside decreased ethanol production. Additives improved fermentation quality in different ways; LAB decreased the pH in wilted silages, and FA initiated fibre hydrolysis, leading to an increase in water-soluble carbohydrate concentration, which surpassed levels present in the raw material. In Experiment 2, ensiling increased liquid yield and protein capture into the liquid fraction compared to fresh biomass. Screw-pressing silage treated with LAB increased crude protein concentration in the liquid whilst FA reduced it. Different processing methods demonstrated possibilities to vary feedstock composition for biorefineries, which can be optimised based on the target end products.

1 | Introduction

Within the circular bioeconomy framework, considerable focus is being directed towards the green biorefinery concept due to its potential to promote sustainability and improve resource efficiency (Gaffey et al. 2023). Grass, a nutritious and widely abundant resource that is inedible for humans, plays a crucial role in maintaining environmental stability (Jørgensen et al. 2022). So far, it has been used primarily for ruminant feeding. However, the green biorefinery concept offers a compelling opportunity to expand grass utilisation beyond ruminant nutrition, including application for monogastric livestock and the production of various value-added products (Gaffey et al. 2023; Rinne 2024).

The biorefinery approach facilitates the cascade processing of green biomass, such as grass, into nutrients, materials, energy

Summary

- Wilting and pulping improved the ensilability of timothy grass biomass and decreased effluent losses.
- Ensiling process improved the recovery of nutrients in the liquid.
- Lactic acid bacteria inoculant increased liquid crude protein concentration, whilst formic acid treatment reduced protein extractability in liquid.

and other side streams, effectively minimising waste. At the core of this processing is the fractionation of green biomass, involving the separation of soluble contents into a liquid fraction, whilst retaining the fibrous part in a solid fraction (Franco et al. 2019). The liquid fraction, valued for its high protein content, holds promising potential for meeting the protein demands of monogastric farm animals (Keto et al. 2021) and even humans (Møller et al. 2021). Conversely, the solid residue (pulp) retains about half of the protein, which is fibre-bound and particularly suitable for ruminant feed. Several studies have explored using pulp as a feed for dairy cows (Hansen et al. 2023; Savonen et al. 2020; Serra et al. 2023). Serra et al. (2023) revealed that pulp silage could enhance nitrogen (N) and phosphorus (P) utilisation compared to grass silage in dairy cows.

For a successful biorefinery, the quality of the feedstock and processing technology plays a crucial role in determining the yield and composition of end products. Factors such as crop type, N fertilisation, harvest timing/plant maturity and weather conditions are known to influence the quality of grass biomass and its conserved forms. Ensiling, the dominating conservation technique for grass, effectively preserves its nutrients and ensures its continuous availability throughout the year (McDonald et al. 1991). Within the green biorefinery context, ensiling ensures product stability, allowing year-round operation, enhances hygiene and extends the shelf life of biorefined products, addressing their inherent susceptibility to rapid degradation. The use of grass silage as a biorefinery feedstock has garnered significant interest (Ayanfe et al. 2023; Resch et al. 2024; Rinne 2024). Compared to fresh biomass, ensiled biomass appears to improve liquid yield (Ayanfe et al. 2023), primarily due to cell wall hydrolysis and degradation of fibre matrix, which enhances the release of cell wall content during mechanical fractionation. Nevertheless, there remains an urgent need to optimise the quality of biomass used in green biorefinery and address logistic challenges.

During ensiling, water-soluble carbohydrates (WSC) serve as substrates under anaerobic conditions for lactic acid bacteria producing fermentation acids that lower pH, thereby stabilising the biomass (McDonald et al. 1991). Simultaneously, protein breakdown takes place, which may have important effects on the biorefinery operation depending on the target end-products (Rinne 2024). Effective silage production hinges on controlling key preservation factors such as anaerobiosis, water activity and epiphytic flora interactions with available substrates. Whilst low dry matter (DM) biomasses yield high liquid output in green biorefineries (Franco et al. 2019), high DM is preferable during silage making due to reduced effluent losses and risks of poor fermentation quality (McDonald et al. 1991; Gebrehanna et al. 2014).

Wilting is an effective method to adjust moisture levels, but weather conditions may impede adequate wilting, and artificial drying can prove energy and cost intensive. Fractionation of grass biomass into pulp and liquid provides another means of managing DM concentration in forage, minimising effluent loss associated with low DM levels during ensiling. In this approach, the nutritious liquid can be extracted prior to ensiling for high-added value uses, and the remaining pulp can be successfully ensiled for later use as, e.g., feed for ruminants, bioenergy or fibre applications.

Another approach to manipulate silage fermentation quality is through the application of silage additives with various modes of action, as reported in Kung et al. (2003). Formic acid is an additive commonly used in Northern Europe to restrict silage fermentation that may increase effluent production by breaking plant cells (Gebrehanna et al. 2014; Kung et al. 2003), which might, on the other hand, improve liquid–solid separation in a biorefinery process. Lactic acid bacteria inoculants are also commonly used as silage additives with the aim to boost fermentation resulting in higher fermentation acid concentrations and degradation of feed protein.

This study had two primary objectives. The first was to compare the fermentation quality of timothy grass when ensiled either intact (fresh grass as such), after moderate wilting, or as pulp after screw-pressing to remove solubles, and to evaluate the effects of different additives on the fermentation quality of these ensiled materials. The second aim was to assess the nutritional quality of the liquid produced during screw-pressing of both pre-ensiled and ensiled grass. We hypothesised that wilting and pulping would increase the DM of the grass, thereby improving fermentation quality, and that additive treatments would enhance the overall fermentation quality of the silages. We also hypothesised that the performance of the biorefinery would be directly linked with the biomass DM concentration and would be improved by silage additive treatment.

2 | Materials and Methods

2.1 | Ensiling Experimental Procedures

The grass biomass for both experiments was obtained from a 4-year-old pure timothy (*Phleum pratense*) stand (variety Hertta BOR, Boreal Plant Breeding, Jokioinen, Finland) in Jokioinen, Finland (60°48′ N, 23°29′ E). The experimental area was cleaned of dead material from the previous season with a chopper on 21 April 2023 and fertilised with YaraMila Y5 (Yara Suomi Ltd., Uusikaupunki, Finland) at the end of April and again with the same fertiliser after harvesting the primary growth (PG). The nutrients provided per hectare were 130 kg N, 30 kg P and 66 kg potassium (K) for the PG and 110 kg N, 26 kg P and 56 kg K for the first regrowth (ReG). The soil type was loam. The PG ley was harvested without wilting on 15 June 2023 whilst the ReG was harvested from the same area as PG on 31 July 2023. The experimental design is presented in Figure 1.

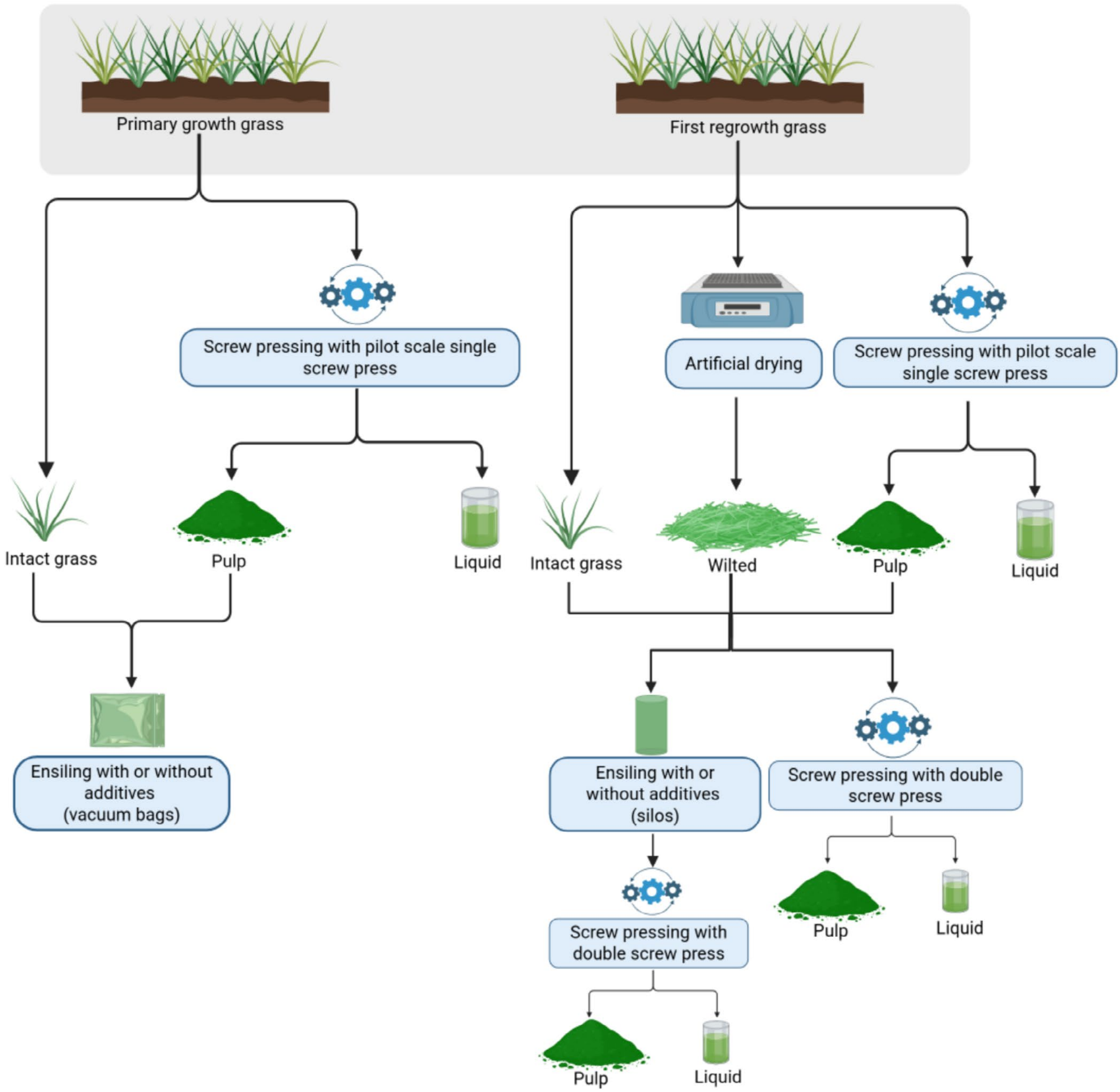

FIGURE 1 | Schematic illustration of the experimental design for primary growth and first regrowth grass.

2.2 | Experiment 1 (Exp. 1)—Primary Growth Timothy

During the growing period for PG, the conditions were dry and cool as the cumulative temperature was 300°-days and rainfall 30 mm since the onset of the growing season on 17 April 2023. The grass was mowed and chopped immediately after cutting with a farm-scale forage precision chopper (JF FCT 1350, JF-Fabriken- J Freudendahl A/S, Sonderborg, Denmark).

The grass biomass was subjected to mechanical separation using a pilot scale single screw press (PSS; Smicon MAS SP300 filter press, Wanroij, The Netherlands) to separate the liquid from the solid fraction, producing pulp. The press, measuring 2417 × 405 × 550 mm, operated at a standard low shaft speed of 24 rpm, with a throughput capacity of 800 kg biomass/h, powered by a 2.2 kW drive and equipped with a filter mesh size of 0.75 mm gap width. The biomass was circulated through the press three to four times to maximise liquid extraction. Due to the high DM content (276 g/kg) of the fresh grass, water was added (0.4 L of water per kg fresh grass) during the first and second pressing cycles to facilitate the extraction of soluble components into the liquid fraction. The liquid extract was collected, weighed and sampled for subsequent laboratory analysis.

The ensiling experiment was arranged as a 2 × 3 factorial design to evaluate the two biomass types and three additive treatments. The two biomass types were the fresh grass (Intact) and the solid fraction (Pulp) obtained from the mechanical separation of the grass. Representative samples of biomasses were collected before ensiling to determine their chemical composition in the laboratory.

Three additive treatments were applied to both biomass types, following the manufacturers' recommended application rates as outlined below:

- Control: distilled water without additive.
- LAB: lactic acid bacteria inoculant [Kofasil Duo, Addcon, Bitterfeld-Wolfen, Germany at 1 g/t, with an inoculation rate of 2.0×10^5 cfu/g of fresh matter. The inoculant contained both homofermentative and heterofermentative lactic acid bacteria strains (*Lactiplantibacillus plantarum* (DSM 3676, DSM 3677) at 1×10^{10} cfu/g and *Lactiplantibacillus buchneri* (DSM 13573) at 1×10^{11} cfu/g)].
- FA1: formic and propionic acid-based additive (AIV Ässä Na, Eastman, Oulu, Finland at 5 L/t. The additive contained 583 g/kg formic acid, 52 g/kg sodium formate, 201 g/kg propionic acid and 25 g/kg potassium sorbate).

The additives were mixed with distilled water to achieve a uniform application rate of 20 mL per 2 kg of fresh biomass (10 L/t), ensuring a consistent liquid volume and even additive application across all treatments. The additives were sprayed on the biomasses manually, and the biomasses were thoroughly mixed to ensure even distribution.

Each treatment was ensiled in four replicates using vacuum-sealed plastic bags (BK3550; 300 × 500 mm, 52 μm; Sealed Air Food Care, Duchnice, Poland). The bags were vacuum sealed (25 s at 5 mbar vacuum, followed by sealing for 1.8 s and cooling for 2.0 s) using an industrial vacuum machine (GK187R/2R; Digimat 5-, Supervac, Vienna, Austria). The sealed bags were stored at room temperature (approximately 20°C) for 88 days, protected from light. The bags were weighed before and after ensiling to determine the weight losses. The ensiling losses (g/kg initial DM) were estimated following the methods described by Knický and Spörndly (2015), by multiplying the weight loss by 1.44 to account for CO_2 production, which also generates equivalent moles of H_2O. Representative samples were taken from each bag post-ensiling for chemical analysis and aerobic stability testing.

2.3 | Experiment 2 (Exp. 2)—First Regrowth Timothy

For ReG grass, the cumulative temperature was 559°-days and rainfall 136 mm during the regrowth period of 46 days. The experimental procedures for harvesting were conducted in the same manner as described for Exp. 1.

The ensiling experiment was conducted according to a 3 × 3 factorial design, evaluating three biomass types [the same as in Exp. 1 (Intact and Pulp), along with an additional wilted biomass (Wilted)], and three additive treatments. Wilted grass was produced through drying of fresh grass using a forced-air open-circuit dryer at 30°C for approximately 5 h to reach a DM content of 300 g/kg. Representative samples from all biomasses were collected before ensiling to evaluate their chemical composition.

Mechanical separation of the three biomass types before ensiling was conducted using a laboratory scale double screw press (DSP; Angel Juicer Ltd., Busan, South Korea) according to Ayanfe et al. (2023). The liquid produced from pre-ensiled materials using PSS from Intact and those produced from Intact, Pulp and Wilted using DSP were weighed and sampled for further analysis of DM, ash, N, WSC and minerals.

Three additive treatments similar to Exp. 1 were used except that FA1 was replaced with another formic acid-based additive (FA) more suitable for lower DM biomass (FA2; AIV 2 Plus Na, Eastman, Oulu, Finland at 5 L/t containing 757 g/kg formic acid and 55 g/kg sodium formate). The additives were applied to each biomass type with three replicates per treatment. The additives were diluted in distilled water to ensure even distribution, resulting in a final liquid volume of 100 mL per 10 kg of fresh biomass (10 L/t). After thorough mixing of the additives, the grass was gradually packed into 12 L cylindrical pilot-scale silos, properly compacted by using an 8-kg lead plummet dropped 10 times freely on the biomass and later weighed. The silos were sealed with a plastic cover, plastic lid, 8 kg lead weight and a water bag and stored at room temperature (approximately 20°C) away from light.

Effluent was periodically collected through tubes attached to the base of the cylindrical silos, weighed and sampled for further chemical composition analysis. After an ensiling period of 88 days, silos were opened and weighed, and the height of silage was measured to calculate silage densities. Ensiling losses were determined as described in Exp. 1. Representative samples were collected from each silo for chemical analysis and aerobic stability testing. Samples used for chemical analyses and further mechanical processing were frozen at −20°C.

The frozen biomasses were later thawed. All silages were pressed using DSP. The liquid from the mechanical fractionation was weighed and sampled. The effluent and liquid samples were analysed for DM, pH, ash, N, WSC, volatile fatty acids (VFA) and minerals.

2.4 | Laboratory Analyses and Calculations

Chemical analyses were conducted at Natural Resources Institute Finland (Luke) Laboratory in Jokioinen, Finland, which operates under a quality management system compliant with the SFS-EN ISO/IEC 17025:2017 standards and holds accreditation from FINAS (the Finnish Accreditation Service) under the code T024. The chemical composition [DM, N, neutral detergent fibre (NDF), ash] of raw materials and all silages, as well as fermentation quality (lactic acid, VFA, ethanol, WSC, ammonia N, pH) of all silages, effluent and liquid samples were analysed according to procedures described by Ayanfe et al. (2023). Buffering capacity of the raw materials was also analysed as described in Ayanfe et al. (2023). Determination of nitrate-N was done according to Walinga et al. (1989) with the use of Skalar San++ autoanalyser (HQ—Skalar Analytical B.V., Breda, Netherlands). Samples for the mineral analysis were digested using the closed wet HNO_3-digestion method in a microwave (CEM MARS 6 Microwave digestor, CEM Corporation, Matthews, Canada) and the extract was analysed by PerkinElmer Optima 8300 ICP-OES Spectrometer (PerkinElmer Inc. Waltham, USA).

The fermentation coefficient (FC), liquid yield from screw-pressing and retained compounds in liquid were calculated according to the equations described in Ayanfe et al. (2023). The total VFA was calculated as the sum of the individual VFA (acetate, propionate, butyrate, isobutyrate, isovalerate, caproate and valerate), whilst total fermentation acids included both total VFA and lactic acid. Total fermentation products were considered to be the sum of the total fermentation acids plus ethanol.

Aerobic stability was evaluated by weighing 700 g of silage and placing it into a polystyrene box with internal dimensions of 13.3 × 13.3 × 10.3 cm, designed to facilitate air ingress. Thermocouple wires were inserted into the centre of the silage samples, which were connected to a data logger for continuous monitoring. Temperature data were automatically recorded at 10-min intervals over a period of 7 days, in accordance with the guidelines set forth by the European Food Safety Authority (EFSA 2024). Aerobic stability was defined as the duration required for the sample temperature to rise by 2°C above the ambient temperature.

2.5 | Statistical Analyses

The data from silages and liquid obtained from screw-pressing ensiled biomasses were analysed using the MIXED procedure in SAS (SAS Institute Inc., 2002–2012, Release 9.4; Cary, NC, USA) separately for Experiments 1 and 2. The model included biomass type, additive and their interaction as fixed effects, whilst replicates were treated as a random effect. To assess the normality of the data, the Shapiro–Wilk test was applied using the UNIVARIATE procedure. Treatment least squares means and their standard errors of the means were reported, and differences between treatment means were considered significant at a 5% probability level. Pairwise comparisons amongst treatment means were conducted using Tukey–Kramer's test. Data pertaining to the chemical composition of liquid from fresh grass and silage effluents were presented descriptively. Although effluent samples were derived from each silo, they were combined for each treatment due to their small volume in order to perform chemical analyses, thus limiting statistical comparisons because of the absence of repetitions. Additionally, statistical analysis was not conducted for the characteristics of original biomasses before ensiling because there were no replicates. Regression analysis (SAS REG procedure) was performed for correlations.

3 | Results

3.1 | Raw Material Characteristics

The composition of raw materials used in both experiments is presented in Table 1. The DM concentration of Intact grass in PG was notably higher than in ReG. Additionally, crude protein (CP) and WSC concentrations were higher in PG, whereas ash, nitrate-N and NDF contents were lower compared to ReG. In PG, pulping reduced DM content due to water addition during screw-pressing, but CP content remained comparable to Intact grass. Conversely, in ReG, pulping increased DM content relative to Intact, with wilting further increasing the DM content whilst maintaining a stable CP concentration. The pulping process led to a substantial reduction in nitrate-N levels, with a decrease of 46% in PG and 43% in ReG. Screw-pressing of the grass also reduced the ash content in both experiments, whilst wilting had no effect on it. Pulping increased the NDF content in PG but had no effect in ReG. The concentration of WSC was clearly higher in PG compared to ReG and slightly reduced by pulping and wilting in ReG. Furthermore, pulping reduced the buffering capacity in both PG and ReG, whilst wilting caused a slight increase, leading to a marginally higher FC of Wilted vs. Intact in ReG.

3.2 | Fermentation Quality of Experimental Silages

The fermentation quality of PG and ReG silages is presented in Tables 2 and 3, respectively. The DM concentration of all silages mirrored the trend observed in the raw material. In Exp. 1, all silages were well-preserved, characterised by low pH and ammonia N concentration, resulting in minimal weight losses during ensiling (average 26.2 g/kg initial DM). The effect of additive treatment on the fermentation pattern of silages varied based on the biomass type (Intact or Pulp), highlighted by the significant interaction observed between biomass type and additive for most parameters evaluated. The effect of LAB and FA1 in reducing ethanol concentration was more pronounced in Pulp than in Intact ($p < 0.05$). An increase in residual WSC was observed in Intact compared to Pulp ($p < 0.05$), and LAB-treated silages had the highest residual WSC followed by FA-treated, which was higher than that of Control silages ($p < 0.05$). Application of LAB increased lactic acid concentration in Intact but restricted it in Pulp, whereas FA1 decreased lactic acid production only in Pulp but not in Intact ($p < 0.05$). Conversely, LAB increased acetic acid concentration more than FA1 in Pulp, whilst acetic acid levels remained consistent in Intact regardless of the additive ($p < 0.05$). The concentration of propionic acid was highest in FA1 treated silages ($p < 0.05$), but this effect was due to the propionic acid added as a component of the additive used. The butyric acid concentration in the Intact Control silage was higher than in all other silages (2.46 vs. 0.05 g/kg DM; $p < 0.05$). Intact silages had longer aerobic stability than Pulp ($p < 0.05$) and both additives prolonged the stability of silages compared to Control ($p < 0.05$).

In Exp. 2, the silages from the different biomass types followed the same differences in DM as the raw material, and no effects of the additive treatments on DM concentration were observed (Table 3). Ash concentration was lower in Pulp than in Intact and Wilted (88 vs. 95 g/kg DM; $p < 0.001$). The effect of additive treatment on the silage fermentation pattern depended on the type of biomass because the fermentation pattern of FA2 and LAB treated silages was similar in some biomasses but differed in other biomasses ($p < 0.05$). Intact and Pulp had relatively low pH values, averaging 3.94 and 3.85, respectively, whilst those of Wilted were above 4 as lower water activity was partly restricting fermentation. However, LAB was effective in reducing the pH in the wilted biomass from 4.43 in Control to 4.11 ($p < 0.05$). All silages exhibited a low proportion of ammonia-N in total N (average 54, 41 and 47 g/kg N for Intact, Pulp and Wilted, respectively) and FA2-treated silages had the lowest ammonia-N proportion compared to LAB and Control ($p < 0.05$).

TABLE 1 | Chemical composition of intact timothy grass and pulp in primary growth (Exp. 1) and intact grass, pulp and wilted timothy grass in first regrowth (Exp. 2) before ensiling.

	Primary growth		First regrowth		
	Intact	**Pulp**	**Intact**	**Pulp**	**Wilted**
Dry matter (DM), g/kg fresh matter	276	249	195	230	301
Buffering capacity, g lactic acid/100 g DM	4.89	3.90	4.73	4.31	5.09
Fermentation coefficient	55	58	33	35	41
In DM, g/kg					
Ash	70	66	90	83	91
Crude protein	146	145	130	130	132
Water soluble carbohydrates	169	160	80	63	69
Nitrate-N	0.011	0.006	0.144	0.078	0.076
Neutral detergent fibre	492	549	596	597	578
In vitro organic matter digestibility, g/g organic matter	0.837	0.834	0.753	0.735	0.747
Macro minerals, g/kg DM					
Calcium	3.4	—	4.3	3.4	4.1
Phosphorus	2.5	—	2.8	2.6	2.7
Potassium	28	—	31	29	31
Magnesium	1.6	—	2.1	1.8	2.0
Sodium	0.04	—	0.04	0.04	0.04
Sulphur	2.0	—	2.1	1.9	2.0
Trace minerals, mg/kg DM					
Iron	277	—	119	158	127
Copper	5.4	—	5.7	6.4	5.8
Manganese	35	—	33	32	34
Zinc	22	—	21	22	21

Ethanol concentration was quite stable in Control and LAB silages regardless of the biomass type, but within biomass types, FA2 resulted in the highest ethanol concentration in Intact ($p < 0.05$) compared to Control and LAB. The effects of additives on WSC concentration were rather typical in Intact, where LAB numerically reduced and FA2 increased WSC concentration. In Pulp, all silages had depleted WSC, as well as in Wilted for Control and LAB treatments, whilst FA2 resulted in very high WSC concentration ($p < 0.05$) even exceeding the one present in the raw material (Table 1). Formic acid treatment restricted fermentation across all biomass types, as evidenced by the lower concentrations of lactic acid, with the most pronounced reduction observed in Wilted silage ($p < 0.05$). However, LAB increased concentrations of acetic acid in all biomasses but more profoundly in Intact, whilst FA2 decreased it only in Wilted ($p < 0.05$). Butyric acid concentration in all silages was generally low, but FA2-treated Intact silage had numerically slightly higher butyric acid than all other silages (1.08 vs. average 0.07 g/kg DM; $p > 0.05$). The total concentration of fermentation acids and fermentation products was lower in FA2-treated silages compared to Control and LAB but more profoundly in Wilted, whilst LAB increased total fermentation products only in Wilted silages. The aerobic stability results were somewhat inconsistent across biomass types and additives, the only significant additive effect within biomass types being the longer aerobic stability of LAB than FA2 in Wilted ($p < 0.05$). The average aerobic stability for Intact, Pulp and Wilted was 132, 199 and 165 h, respectively ($p < 0.05$).

Effluent excretion was observed only in Intact silages, with the FA2-treated silage producing the highest amounts (Table 4). The DM concentration of the effluent ranged between 58 and 72 g/kg, with a pH higher than that of the silages (Table 3). Ammonia-N was undetectable in the effluent from FA2-treated silage, which also had the lowest ash and CP concentrations, but the highest WSC levels compared to Control and LAB.

3.3 | Yield, Nutritional Composition and Fermentation Quality of Liquid From Fresh and Ensiled Biomass

In Exp. 1 and Exp. 2, intact grass was screw-pressed using PSS, while biomass types in Exp. 2 underwent freezing, thawing and

TABLE 2 | Chemical composition and fermentation quality of primary growth timothy ensiled as intact and pulp and treated with additives (Exp. 1).

Biomass type (BT)	Intact			Pulp				P-value		
Additive (Add)[1]	Control	LAB	FA1	Control	LAB	FA1	SEM[2]	BT	Add	BT × Add
Dry matter (DM), g/kg	268^{b}	277^{ab}	279^{a}	244^{c}	242^{c}	249^{c}	2.2	< 0.001	0.006	0.073
pH	4.05^{a}	3.76^{cd}	3.81^{c}	3.74^{cd}	3.92^{b}	3.71^{d}	0.018	< 0.001	< 0.001	< 0.001
Ammonia-N, g/kg total N	48^{a}	34^{b}	28^{cd}	32^{bc}	33^{b}	25^{d}	0.9	< 0.001	< 0.001	< 0.001
Chemical composition, g/kg DM										
Water soluble carbohydrates	50^{ab}	71^{a}	44^{bc}	24^{c}	44^{bc}	38^{bc}	5.8	< 0.001	0.003	0.109
Ethanol	31.8^{b}	14.7^{c}	9.6^{d}	43.5^{a}	13.3^{cd}	8.9^{d}	1.12	0.002	< 0.001	< 0.001
Lactic acid (LA)	66^{b}	105^{a}	63^{b}	97^{a}	65^{b}	60^{b}	2.6	0.069	< 0.001	< 0.001
Acetic acid (AA)	20.6^{bc}	25.3^{b}	26.8^{b}	15.8^{c}	47.1^{a}	27.0^{b}	1.53	< 0.001	< 0.001	< 0.001
Propionic acid[3]	0.06^{b}	0.10^{b}	2.75^{a}	0.04^{b}	0.04^{b}	2.96^{a}	0.048	0.259	< 0.001	0.028
Butyric acid	2.46^{a}	0.04^{b}	0.06^{b}	0.05^{b}	0.04^{b}	0.05^{b}	0.053	< 0.001	< 0.001	< 0.001
Total volatile fatty acids[4]	23.2^{b}	25.4^{b}	29.5^{b}	15.9^{c}	47.2^{a}	30.0^{b}	1.53	0.001	< 0.001	< 0.001
Total fermentation acids[5]	90^{c}	131^{a}	93^{c}	113^{b}	112^{b}	90^{c}	3.5	0.786	< 0.001	< 0.001
Total fermentation products[6]	121^{b}	146^{a}	102^{c}	157^{a}	126^{b}	99^{c}	2.9	0.109	< 0.001	< 0.001
LA to AA ratio	3.42^{b}	4.20^{b}	2.36^{c}	6.16^{a}	1.40^{c}	2.23^{c}	0.211	0.709	< 0.001	< 0.001
Fermentation losses, g/kg initial DM	32	25	23	30	25	22	2.5	0.645	0.008	0.895
Aerobic stability, h[7]	88^{b}	172^{a}	172^{a}	42^{b}	156^{a}	172^{a}	10.8	0.025	< 0.001	0.112

Note: Means within the same row without same superscript (a,b,c,d) differ significantly at the 5% Tukey test.
[1]Control = without additive; LAB = lactic acid bacteria inoculant; FA1 = formic and propionic acid-based additive.
[2]SEM = standard error of the mean. $n = 4$ for each treatment.
[3]Upon correction with the amount of propionic acid present in FA1, Intact and Pulp had 0 g/kg DM.
[4]Acetic acid + propionic acid + butyric acid + other volatile fatty acids.
[5]Total volatile fatty acids + lactic acid.
[6]Total fermentation acids + ethanol.
[7]Length of observation period was 172 h.

DSP processing as pre-ensiled (Table 5) and as silages (Table 6). Liquid yields were lower in fresh than in ensiled biomass: 0.607, 0.570 and 0.393 pre-ensiling compared to 0.699, 0.631 and 0.531 (averaged over all additive treatments) post-ensiling for Intact, Pulp and Wilted, respectively. The DM, ash and CP extractability was greater in the liquid extracted from ensiled biomasses than in fresh. Liquid derived from both fresh and ensiled biomasses had similar levels of macro minerals on a DM basis, with K present in the highest concentration and sodium in the lowest.

The chemical composition, fermentation quality of liquid and nutrient recovery in the liquid from Intact, Pulp and Wilted silages using DSP are presented in Tables 6 and 7 (Exp. 2). The highest ($p < 0.05$) liquid yield was found in the Intact, followed by Pulp and Wilted, with no significant effect of additive. However, liquid DM concentration followed an opposite trend with the highest ($p < 0.05$) DM observed in Wilted, lowest in Intact and intermediate in Pulp (average 82, 89 and 130 g/kg for Intact, Pulp and Wilted, respectively). Additionally, the lowest liquid DM concentration was observed in LAB (average 95 g/kg) compared to Control and FA2 (average 103 and 102 g/kg, respectively). Liquid extracted from Intact and Pulp had a pH of about 4.0 (on average) and the effect of LAB treatment on pH was particularly pronounced in Wilted ($p < 0.05$). The

TABLE 3 | Chemical composition and fermentation quality of first regrowth timothy grass ensiled as intact, pulp or wilted and treated with additives (Exp. 2).

Biomass type (BT)	Intact			Pulp			Wilted				*p*-value		
Additive (Add)[1]	**Control**	**LAB**	**FA2**	**Control**	**LAB**	**FA2**	**Control**	**LAB**	**FA2**	**SEM**[2]	**BT**	**Add**	**BT × Add**
Dry matter (DM), g/kg	193^{c}	192^{c}	199^{c}	227^{b}	230^{b}	233^{b}	301^{a}	294^{a}	299^{a}	3.9	< 0.001	0.283	0.696
pH	3.93^{bc}	3.95^{bc}	3.95^{bc}	3.74^{c}	3.99^{bc}	3.82^{c}	4.43^{a}	4.11^{b}	4.58^{a}	0.050	< 0.001	0.062	< 0.001
Ammonia-N, g/kg total N	62^{ab}	65^{a}	34^{c}	52^{b}	51^{b}	20^{d}	51^{b}	54^{ab}	35^{c}	2.7	< 0.001	< 0.001	0.065
Chemical composition, g/kg DM													
Ash	95^{abc}	99^{a}	94^{bc}	89de	89de	86^{e}	94^{bc}	96^{ab}	92^{cd}	0.8	< 0.001	< 0.001	0.153
Crude protein	134	136	131	136	136	133	131	133	128	2.0	0.043	0.080	0.965
Water soluble carbohydrates	25.4^{bc}	15.6^{bc}	39.1^{b}	6.0^{c}	0.2^{c}	0.4^{c}	1.6^{c}	0.6^{c}	135.7^{a}	5.76	< 0.001	< 0.001	< 0.001
Ethanol	7.6^{bc}	9.1^{abc}	11.2^{a}	8.9^{abc}	9.8^{ab}	7.8^{bc}	7.0^{cd}	6.9^{cd}	4.5^{d}	0.55	< 0.001	0.199	< 0.001
Lactic acid (LA)	89.5^{ab}	70.7^{abc}	36.9^{d}	91.6^{a}	86.3^{ab}	49.1^{cd}	44.9^{d}	68.7^{bc}	8.8^{e}	4.46	< 0.001	< 0.001	0.003
Acetic acid (AA)	23.0^{cd}	40.7^{a}	17.3^{d}	22.8^{d}	32.2^{b}	20.2^{d}	16.9^{d}	29.0^{bc}	7.1^{e}	1.24	< 0.001	< 0.001	0.001
Propionic acid	0.08^{b}	0.05^{b}	0.05^{b}	0.29^{ab}	0.74^{a}	0.06^{b}	0.03^{b}	0.03^{b}	0.08^{b}	0.131	0.013	0.161	0.074
Butyric acid	0.10	0.08	1.08	0.08	0.04	0.11	0.07	0.03	0.05	0.231	0.115	0.129	0.145
Total volatile fatty acids[3]	23.2^{c}	40.9^{a}	18.6^{cd}	23.1^{c}	32.9^{b}	20.4^{cd}	17.0^{d}	29.1^{b}	7.2^{e}	1.02	< 0.001	< 0.001	< 0.001
Total fermentation acids[4]	113^{a}	112^{a}	56^{b}	115^{a}	119^{a}	69^{b}	62^{b}	98^{a}	16^{c}	4.6	< 0.001	< 0.001	0.003
Total fermentation products[5]	120^{ab}	121^{ab}	67^{c}	124^{ab}	129^{a}	77^{c}	69^{c}	105^{b}	20^{d}	4.7	< 0.001	< 0.001	0.005
LA to AA ratio	3.96^{a}	1.74^{bc}	2.13^{bc}	4.02^{a}	2.71^{b}	2.44^{bc}	2.66^{b}	2.37^{bc}	1.24^{c}	0.238	< 0.001	< 0.001	0.010
Fermentation losses, g/kg initial DM	11.3^{b}	13.2^{ab}	12.7^{b}	11.0^{b}	12.9^{ab}	11.0^{b}	12.5^{b}	16.7^{a}	9.7^{b}	0.78	0.137	< 0.001	0.007
Aerobic stability, h[6]	118^{bc}	148^{abc}	136^{abc}	166^{abc}	195^{ab}	236^{ab}	134^{abc}	244^{a}	117^{c}	20.4	0.006	0.022	0.008

Note: Means within the same row without same superscript (a,b,c,d,e) differ significantly at the 5% Tukey test.
[1]Control = without additive; LAB = lactic acid bacteria inoculant; FA2 = formic acid-based additive.
[2]SEM = standard error of the mean. $n = 3$ for each treatment.
[3]Acetic acid + propionic acid + butyric acid + other volatile fatty acids.
[4]Total volatile fatty acids + lactic acid.
[5]Total fermentation acids + ethanol.
[6]Length of observation period was 244 h.

TABLE 4 | Amount and chemical composition of effluents excreted spontaneously from additive[1] treated first regrowth silages (Exp. 2).

Item	Control	LAB	FA2
Effluent yield, g/g fresh silage	0.002	0.003	0.020
Dry matter (DM), g/kg	72	58	62
pH	4.14	4.26	4.16
Ammonia-N, g/kg total N	9.2	10.9	0
Chemical composition, g/kg DM			
Ash	250	273	243
Crude protein	190	199	136
Water soluble carbohydrates	3.0	2.6	22.3
Acids[2], g/kg DM			
Lactic	16.6	14.9	0.7
Acetic	2.70	2.40	1.00
Butyric	0	0.10	0
Macro minerals, g/kg DM			
Calcium	9.7	12.4	11.0
Phosphorus	7.3	8.4	7.2
Potassium	88	114	105
Magnesium	5.4	7.0	6.3
Sodium	0.2	0.3	2.7[3]
Sulphur	3.1	3.7	3.1
Trace minerals, mg/kg DM			
Iron	210	220	157
Copper	3.6	5.1	2.4
Manganese	66	55	87
Zinc	56	69	60

[1]Control = without additive; LAB = lactic acid bacteria inoculant; FA2 = formic acid-based additive.
[2]Values for propionic acid and other VFA were 0.
[3]Sodium present in the form of sodium formate in the additive treatment contributed to this value.

WSC concentrations of the liquid were generally low, but Wilted treated with FA2 resulted in a clearly higher concentration ($p < 0.05$) reflecting the WSC concentrations of the silages. The liquid CP concentration in biomass types was comparable (221, 225 and 220 g/kg DM for Intact, Pulp and Wilted, respectively, $p > 0.05$), but LAB application increased liquid CP concentration compared to Control in all biomass types, and this increase was more pronounced in Intact than Pulp and Wilted. In contrast, FA2 application resulted in lower liquid CP concentration compared to Control and LAB, with the greatest reduction in Pulp. The ash and CP recovery in liquid varied by biomass type and additive treatment ($p < 0.05$). The high liquid yield observed in Intact compared to Pulp and Wilted resulted in a greater proportion of original silage DM, ash and CP being captured in the liquid in the Intact silages. Ash captured in liquid decreased in the LAB treated silages compared to Control and FA2, whilst FA2 lowered CP capture into the liquid.

The relationships between silage and liquid DM concentration, liquid yield and CP capture in liquid and silage DM concentration and CP capture in liquid are presented in Figure 2. Silage and liquid DM concentration were strongly positively correlated, whilst liquid yield and capture of CP in liquid were slightly positively correlated. Conversely, silage DM concentration and CP capture in liquid were negatively correlated.

4 | Discussion

4.1 | Raw Material Quality

Grass composition is significantly influenced by various management decisions, such as plant species, N fertilisation, harvesting time and extent of wilting. These factors have substantial consequences on the use of grass biomass as a feedstock in biorefineries. In this study, we focused on timothy grass, which is one of the most widely cultivated grass species in Northern Europe. Timothy is favoured for its good winter hardiness, high yield potential and high nutritive value (Virkajärvi et al. 2015). Although timothy is commonly grown as mixtures with other grasses and clovers, the current study concentrated on pure timothy stands. The utilisation of pure timothy was intended to eliminate the variability that might arise from the presence of other species within the crop composition. Furthermore, it is essential to evaluate the characteristics of individual species independently if the aim of the biorefinery is to produce ingredients for novel food use (EFSA NDA Panel 2016).

Timothy grass is conventionally harvested two to three times during the growing season. The raw materials from PG and ReG can be considered typical in terms of the CP concentration, which in Finnish farm silages analysed by Valio Ltd. (Helsinki, Finland) in 2023 was on average 148 g/kg DM ($n = 11{,}622$). Also, it is typical for PG to have a higher ash content than ReG (Huhtanen et al. 2006). However, the drought preceding the harvesting of PG resulted in an exceptionally high DM concentration in the standing crop and accumulation of WSC in it compared to ReG. Due to the higher DM and WSC concentrations, the PG-Intact biomass was considered easy to ensile (FC > 45) whilst ReG fell into the difficult ensilability category (FC < 35; Weissbach et al. 1974) due to the relatively low concentrations of WSC and DM. The in vitro organic matter digestibility of PG was marginally higher than that of ReG raw materials, corroborating the findings by Huhtanen et al. (2006) and the Valio dataset (*D*-value of 700 vs. 666 g/kg DM in PG and ReG, respectively) which is related to the higher proportion of indigestible NDF in the cell walls of ReG.

In the green biorefinery, the DM concentration of the material is crucial in determining liquid yield (Franco et al. 2019). Ayanfe et al. (2023) highlighted that high-DM forage can still be effectively processed by introducing water prior to pressing, thereby enhancing the extraction of soluble cell components into the liquid. In the current study, the high DM concentration

TABLE 5 | Yield and composition of liquid from primary growth (Exp. 1) and first regrowth (Exp. 2) timothy grass using pilot scale single screw press (PSS) as well as intact, pulp and wilted grass materials before ensiling (Exp. 2) using a double screw press (DSP).

	Primary growth	First regrowth			
Item	**Intact-PSS**	**Intact-PSS**	**Intact-DSP**	**Pulp-DSP**	**Wilted-DSP**
Liquid yield, g/g fresh sample	0.301	0.244	0.607	0.570	0.393
Dry matter (DM), g/kg	158	47	81	88	136
pH	5.78	5.68	5.60	5.66	5.57
In g/kg DM					
Ash	124	254	168	169	197
Crude protein (CP)	129	119	139	155	121
Water soluble carbohydrates	73.8	12.9	33.7	31.5	30.0
Lactic acid	0	0	0.12	0.11	0.07
Acetic acid	0.90	0.50	0.74	0.80	0.59
Captured in liquid, g/g original material					
DM	0.172	0.058	0.251	0.217	0.177
Ash	0.304	0.164	0.467	0.440	0.383
CP	0.152	0.054	0.268	0.259	0.163
Macro minerals, g/kg DM					
Calcium	5.1	16.5	8.8	7.4	8.9
Phosphorus	3.6	4.0	5.9	6.3	6.5
Potassium	51	90	82	82	89
Magnesium	2.6	6.7	4.7	4.3	4.9
Sodium	0.1	0.3	0.1	0.1	0.1
Sulphur	2.1	3.1	2.9	2.9	2.8
Trace minerals, mg/kg DM					
Iron	200	860	149	171	125
Copper	8.5	28.0	14.9	17.1	15.5
Manganese	34	95	55	53	59
Zinc	31	45	38	39	35

of PG hindered liquid extraction, necessitating the addition of water (0.4 L of water per kg fresh grass) during screw-pressing. Consequently, further wilting of the grass biomass for biorefinery evaluation was deemed unnecessary. Due to water addition during pressing, the residual pulp exhibited a 10% lower DM concentration than the intact grass in Exp. 1. Conversely, in Exp. 2, a high amount of solubles was removed into the liquid during the screw-pressing from low DM intact grass, resulting in an 18% increase in DM concentration of Pulp compared to Intact. Wilting also effectively increased the DM concentration of grass by 54%.

In the current study, a relatively low efficiency PSS screw press was used in the fractionation process at pilot scale, which led to only a marginal difference in the composition between Pulp and Intact. The CP concentrations for Intact and Pulp in both PG and ReG were similar, which could be ascribed to the presence of fibre-bound protein, although more CP was expected to be captured in the liquid extracted during screw-pressing (Mangeon et al. 2010). Similar CP concentrations of Intact and Pulp biomasses have also been previously observed, leading to good prospects of using the pulp as an animal feed component (Damborg et al. 2020; Hansen et al. 2023; Savonen et al. 2020). Damborg et al. (2018) observed higher total amino acid (AA) and essential AA concentrations in the pulp than in the original fresh plant, which shows that more non-protein N than amino acid N had been extracted into the liquid during pressing. Their study further revealed that more CP was recovered in the fibre components (aNDF and acid detergent fibre) of pulp than in the original plant material. Ruminants are able to degrade the fibre efficiently in order to access most of the fibre-bound CP for production.

TABLE 6 | Yield, nutrient composition and fermentation quality of liquid fraction from first regrowth timothy grass ensiled as intact, pulp, or wilted and treated with additives pressed using double screw press (Exp. 2).

Biomass type (BT)	**Intact**			**Pulp**			**Wilted**				***p*-value**		
Additive (Add)[1]	**Control**	**LAB**	**FA2**	**Control**	**LAB**	**FA2**	**Control**	**LAB**	**FA2**	**SEM**[2]	**BT**	**Add**	**BT × Add**
Yield, g/g fresh matter	0.702^{a}	0.689^{ab}	0.706^{a}	0.627^{c}	0.633^{c}	0.634^{bc}	0.553^{d}	0.523^{d}	0.517^{d}	0.0110	< 0.001	0.399	0.252
Dry matter (DM), g/kg	86^{cd}	77^{d}	84^{cd}	89^{c}	85^{cd}	92^{c}	135^{a}	124^{b}	130^{ab}	2.1	< 0.001	< 0.001	0.426
pH	3.89^{bc}	4.01^{b}	3.94^{bc}	3.74^{c}	3.91^{bc}	3.76^{c}	4.41^{a}	4.06^{b}	4.46^{a}	0.042	< 0.001	0.210	< 0.001
Ammonia-N, g/kg total N	3.28^{abc}	3.27^{abc}	3.87^{a}	1.97^{c}	2.89^{abc}	3.73^{ab}	2.11^{bc}	2.08^{bc}	2.44^{abc}	0.335	0.001	0.014	0.279
In g/kg DM													
Ash	173^{c}	183^{abc}	183^{abc}	189^{abc}	195^{ab}	179^{bc}	190^{abc}	201^{a}	197^{ab}	4.4	0.001	0.067	0.201
Crude protein	221^{c}	248^{ab}	193de	237^{b}	256^{a}	182^{e}	220^{c}	243^{ab}	198^{d}	4.1	0.308	< 0.001	0.006
Water soluble carbohydrates	5.7^{bcd}	3.5^{d}	12.2^{bc}	2.2^{d}	3.7^{cd}	6.1^{bcd}	13.2^{b}	6.7^{bcd}	41.4^{a}	1.72	< 0.001	< 0.001	0.001
Lactic acid (LA)	29.4^{ab}	26.0^{b}	13.2^{c}	35.8^{a}	29.8^{ab}	15.7^{c}	14.4^{c}	24.1^{b}	3.1^{d}	1.30	< 0.001	< 0.001	< 0.001
Acetic acid (AA)	5.7^{cd}	11.8^{a}	4.6^{d}	6.8^{bc}	10.2^{a}	5.8^{cd}	4.5^{d}	8.0^{b}	2.0^{e}	0.34	< 0.001	< 0.001	0.001
Propionic acid	0.038^{b}	0^{b}	0^{b}	0.112^{ab}	0.278^{a}	0^{b}	0^{b}	0^{b}	0^{b}	0.0379	0.001	0.026	0.009
Butyric acid	0.038	0	0.286	0	0	0	0	0	0	0.0776	0.173	0.289	0.296
Total volatile fatty acids[3]	5.9^{cd}	11.8^{a}	4.9^{d}	7.0^{bc}	10.5^{a}	5.8^{cd}	4.5^{d}	8.0^{b}	2.0^{e}	0.28	< 0.001	< 0.001	0.001
Total fermentation acids[4]	35.3^{bc}	37.9^{abc}	18.1^{d}	42.8^{a}	40.3^{ab}	21.5^{d}	19.0^{d}	32.0^{c}	5.1^{e}	1.27	< 0.001	< 0.001	< 0.001
LA to AA ratio	5.24^{a}	2.20^{bc}	2.84^{bc}	5.26^{a}	2.97^{b}	2.72^{bc}	3.20^{b}	3.01^{b}	1.53^{c}	0.265	< 0.001	< 0.001	0.001
Macro minerals, g/kg DM													
Calcium	8.8^{bcd}	9.6^{a}	8.6^{cd}	8.3^{de}	8.7^{bcd}	8.0^{e}	8.8^{bc}	10.0^{a}	9.1^{b}	0.12	< 0.001	< 0.001	0.028
Phosphorus	6.71^{d}	7.47^{bc}	6.76^{d}	7.73^{abc}	8.03^{ab}	7.23^{cd}	7.79^{abc}	8.30^{a}	7.93^{ab}	0.122	< 0.001	< 0.001	0.080
Potassium	78^{d}	87^{ab}	79^{cd}	84^{bc}	87^{ab}	82^{bcd}	83^{bcd}	91^{a}	87^{ab}	1.2	< 0.001	< 0.001	0.029
Magnesium	4.71^{d}	5.22^{bc}	4.70^{d}	4.85^{cd}	4.94^{bcd}	4.63^{d}	5.11^{bc}	5.70^{a}	5.32^{ab}	0.085	< 0.001	< 0.001	0.045
Sodium	0.06^{c}	0.06^{c}	1.41^{a}[5]	0.06^{c}	0.06^{c}	1.39^{a}[5]	0.05^{c}	0.09^{c}	1.10^{b}[5]	0.018	< 0.001	< 0.001	< 0.001

(Continues)

TABLE 6 | (Continued)

Biomass type (BT)	Intact			Pulp			Wilted				p-value		
Additive (Add)[1]	Control	LAB	FA2	Control	LAB	FA2	Control	LAB	FA2	SEM[2]	BT	Add	BT × Add
Sulphur	3.24^{b}	3.62^{a}	3.04^{cd}	3.29^{b}	3.55^{a}	3.01^{d}	3.28^{b}	3.61^{a}	3.19^{bc}	0.036	0.048	<0.001	0.080
Trace minerals, mg/kg DM													
Iron	167^{bc}	143^{cd}	129^{d}	213^{a}	201^{ab}	163^{cd}	146^{cd}	162^{cd}	159^{cd}	7.1	<0.001	0.001	0.003
Copper	7.9^{cd}	9.3^{ab}	7.5^{cd}	9.6^{a}	10.2^{a}	8.1^{bc}	7.0^{cd}	7.8^{cd}	6.8^{d}	0.26	<0.001	<0.001	0.084
Manganese	68^{c}	78^{b}	71^{bc}	76^{b}	78^{ab}	72^{bc}	73^{bc}	86^{a}	75^{bc}	1.7	0.002	<0.001	0.027
Zinc	56	66	56	62	67	57	47	54	51	4.2	0.010	0.063	0.842

Note: Means within the same row without same superscript (a,b,c,d,e) differ significantly at the 5% Tukey test.
[1]Control = without additive; LAB = lactic acid bacteria inoculant; FA2 = formic acid-based additive.
[2]SEM = standard error of the mean. $n = 3$ for each treatment.
[3]Acetic acid + propionic acid + butyric acid + other volatile fatty acids.
[4]Total volatile fatty acids + lactic acid.
[5]Sodium present in the form of sodium formate in the additive treatment contributed to this value.

4.2 | Fermentation Characteristics of Experimental Silages

Despite the different ensiling methods used in Exp. 1 (vacuum bags) and Exp. 2 (cylindrical silos), the fermentation patterns of silages were comparable, as previously observed in a direct comparison between these two experimental methods (Franco and Rinne 2023). The pH levels of all silages remained below 4.2, which is the benchmark for good fermentation quality as established by McDonald et al. (1991). From a protein degradation point of view, all silages were of good fermentation quality, as illustrated by ammonia-N concentrations being below 100 g/kg total N (Kaiser et al. 2004). However, the application of FA lowered the ammonia-N levels in silages by causing a rapid pH decline due to increased hydrogen ion concentration. This limited fermentation activity, reducing the populations of fermenting microorganisms that degrade proteins and restricting the activity of plant proteases (McDonald et al. 1991).

The efficiency of lactic acid production during fermentation is affected by the epiphytic natural microflora or inoculated lactic acid bacteria, nutrient availability and physical conditions. Factors such as chopping, laceration or bruising can improve the growth of lactic acid bacteria and the overall fermentation process (McGechan 1990). In the current study, two methods were used to manipulate the grass for ensiling: screw-pressing or wilting. The fermentation quality of Pulp silages was comparable to Intact. The pulping affected the ensilability slightly by reducing the WSC concentration but at the same time moisture and soluble minerals were removed, which seems to result in good prospects for successful ensiling of the pulp (Rinne 2024; Sousa et al. 2022).

Generally, the role of additives in modulating the fermentation process aligned with previous research findings as FA2 restricted fermentation, evidenced by decreased lactic acid production compared to other additives (Franco et al. 2022; Kung et al. 2003; Rinne et al. 2024). Application of FA2 in Wilted exhibited higher residual WSC than other treatments and even greater than in the original raw material, which can be attributed to the acid hydrolysis of NDF generating additional sugars during ensiling (McDonald et al. 1991). Cell wall breakdown during ensiling can occur through epiphytic hemicellulases/plant enzymes, microbial hemicellulases or acid hydrolysis through organic acids generated during fermentation and formic acid if added (Huhtanen et al. 2005). The low water activity in Wilted restricted the conversion of WSC to fermentation acids, and further restriction was achieved through FA addition.

On the other hand, LAB boosted lactic acid production in the PG-Intact and, due to the acidic environment, more residual sugars were released through acid hydrolysis of the cell wall. The applied LAB strain also exhibited heterofermentative properties in all silages, enhancing secondary fermentation by increasing acetic acid concentrations, which contribute to the increased aerobic stability of the silage (Wilkinson and Davies 2013). The ability of LAB to improve silage fermentation quality may be limited in low DM material (Rinne et al. 2023) so they are typically recommended for wilted grass. In the current study, LAB treatment was able to promote lactic acid fermentation in Wilted silages and subsequently lowered

TABLE 7 | Captured compounds in liquid from first regrowth timothy grass ensiled as intact, pulp or wilted and treated with additives pressed using double screw press (Exp. 2).

Biomass type (BT)	Intact			Pulp			Wilted				*p*-value		
Additive (Add)[1]	Control	LAB	FA2	Control	LAB	FA2	Control	LAB	FA2	SEM[2]	BT	Add	BT × Add
Dry matter	0.313^{a}	0.277^{b}	0.298^{ab}	0.246^{cd}	0.233^{cde}	0.252^{c}	0.248^{cd}	0.220^{e}	0.224^{de}	0.0049	< 0.001	< 0.001	0.047
Ash	0.569^{a}	0.514^{ab}	0.578^{a}	0.521^{ab}	0.511^{ab}	0.525^{ab}	0.502^{ab}	0.463^{b}	0.483^{b}	0.0164	< 0.001	0.030	0.471
Crude protein	0.518^{a}	0.507^{a}	0.437^{b}	0.429^{b}	0.440^{b}	0.345^{c}	0.416^{b}	0.403^{b}	0.346^{c}	0.0104	< 0.001	< 0.001	0.459
Ammonia N	0.017^{b}	0.014^{b}	0.038^{a}	0.010^{b}	0.013^{b}	0.047^{a}	0.010^{b}	0.008^{b}	0.016^{b}	0.0036	0.001	< 0.001	0.003
Lactic acid	0.104^{a}	0.102^{a}	0.105^{a}	0.096^{ab}	0.081^{bc}	0.081^{bc}	0.080^{bc}	0.077^{c}	0.080^{bc}	0.0034	< 0.001	0.050	0.081
Acetic acid	0.078^{abc}	0.081^{a}	0.079^{ab}	0.074^{abc}	0.074^{bc}	0.072^{cd}	0.066^{de}	0.060^{e}	0.065^{e}	0.0014	< 0.001	0.706	0.044
Calcium	0.635^{a}	0.619^{a}	0.591^{a}	0.600^{a}	0.595^{a}	0.588^{ab}	0.529^{c}	0.533^{bc}	0.496^{c}	0.0120	< 0.001	0.013	0.553
Phosphorus	0.756^{a}	0.744^{ab}	0.723^{abc}	0.721^{abc}	0.709^{abcd}	0.690^{bcd}	0.709^{abcd}	0.672^{cd}	0.654^{d}	0.0125	< 0.001	0.004	0.791
Potassium	0.784^{a}	0.780^{a}	0.755^{ab}	0.703^{bc}	0.692^{bc}	0.705^{bc}	0.659^{c}	0.647^{c}	0.631^{c}	0.0152	< 0.001	0.349	0.735
Magnesium	0.719^{a}	0.706^{ab}	0.682^{abc}	0.683^{abc}	0.658^{abcd}	0.666^{abcd}	0.630^{bcd}	0.624^{cd}	0.594^{d}	0.0154	< 0.001	0.080	0.774
Sulphur	0.496^{a}	0.489^{a}	0.441^{b}	0.424^{bc}	0.434^{bc}	0.396^{cd}	0.404^{bc}	0.395^{cd}	0.356^{d}	0.0080	< 0.001	< 0.001	0.483
Iron	0.439^{a}	0.333^{b}	0.325^{b}	0.333^{b}	0.297^{b}	0.259^{b}	0.285^{b}	0.280^{b}	0.280^{b}	0.0181	< 0.001	0.001	0.035
Copper	0.439^{ab}	0.453^{a}	0.392^{bc}	0.368^{cd}	0.368^{cd}	0.318^{de}	0.297^{e}	0.294^{e}	0.264^{e}	0.0121	< 0.001	< 0.001	0.717
Manganese	0.641^{ab}	0.652^{a}	0.642^{ab}	0.596^{abc}	0.580^{abc}	0.572^{abcd}	0.535^{cd}	0.562^{bcd}	0.496^{d}	0.0168	< 0.001	0.131	0.368
Zinc	0.829^{a}	0.860^{a}	0.790^{ab}	0.692^{abc}	0.710^{abc}	0.648^{abc}	0.551^{c}	0.562^{bc}	0.535^{c}	0.0473	< 0.001	0.404	0.993

Note: Means within the same row without same superscript (a,b,c,d,e) differ significantly at the 5% Tukey test.
[1]Control = without additive; LAB = lactic acid bacteria inoculant; FA2 = formic acid-based additive.
[2]SEM = standard error of the mean. $n = 3$ for each treatment.

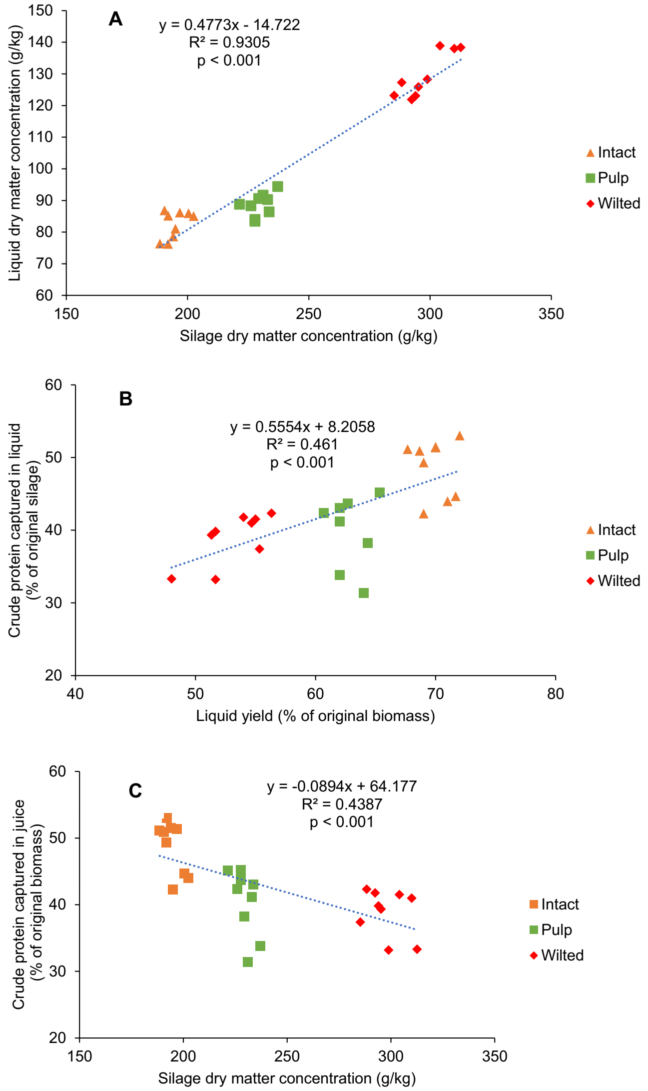

FIGURE 2 | Correlations between (A) silage dry matter concentration and dry matter concentration of the liquid; (B) liquid yield and crude protein extractability rate in the liquid as a proportion of the original silage; (C) silage dry matter concentration and crude protein extractability rate in the liquid as a proportion of the original silage.

the pH. Generally, additive treatments successfully prolonged the aerobic stability of all silages, and the Intact silage was more stable than the Pulp in Exp. 1. However, aerobic stability was similar in silages with or without additives in Exp. 2, except in Wilted, where LAB was more effective in improving aerobic stability compared to FA. This could be due to the high residual WSC left unfermented in the FA-treated silage, which could be utilised by undesirable microbes during the aerobic challenge. The presence of aerobic deterioration inhibitors such as propionic acid and sorbates has been found to improve the stability of silages (Muck et al. 2018). However, the formic acid used in Exp. 2 for the wilted silages lacked these compounds.

Effluent losses were observed only in the low DM Intact grass in Exp. 2, which shows that increasing DM content either through wilting or pulping can effectively decrease nutrient losses in effluent, which is consistent with the findings of Jones and Jones (1995). In alignment with the present study, Larsen et al. (2019) reported no effluent loss in ensiled ryegrass pulp. Furthermore, the time interval between the processing of biomass into pulp and the subsequent ensiling process is crucial for the effectiveness of pulp silage preservation, as a short aerobic phase is essential to minimise ensiling losses (Larsen et al. 2019).

4.3 | Biorefinery Performance of Fresh and Ensiled Biomass

The success of a biorefinery operation depends on its ability to efficiently separate desired components such as protein, WSC, fermentation products, minerals and fibre from the feedstock. The characteristics and chemical composition of green biomass, as well as the efficiency of mechanical processing technologies, are critical in achieving this success. Consequently, to optimise the overall output of a green biorefinery, it is imperative to integrate knowledge regarding these factors. High nutrient recovery in liquid requires optimisation of the mechanical pressing step (Santamaría-Fernández et al. 2017). This was demonstrated in Exp. 2, where the more efficient DSP improved liquid yield and nutrient recovery in liquid per original material compared to the less efficient PSS. The use of DSP increased fivefold the CP captured in the liquid compared to a less efficient PSS, consistent with the findings of Rinne et al. (2018).

Furthermore, the utilisation of frozen-and-thawed biomass within the DSP, as opposed to the fresh biomass processed by PSS, may have contributed to the observed separation efficiency. Ayanfe et al. (2023) highlighted that CP capture in the liquid from frozen-and-thawed biomass was improved when compared with fresh biomass, and the difference was larger with less efficient press than with highly efficient press. The operational mechanism of the freezing and thawing process, which facilitates increased liquid and CP yield, was attributed to the disruption of plant cell walls. This disruption occurs because of the expansion of cell walls due to the formation of large ice crystals during the freezing process, which releases molecules contained within those cells, thereby impacting liquid yield and its composition (Ayanfe et al. 2023). Freezing-and-thawing as a method of preservation may not be practically feasible for commercial green biorefineries. However, freezing of samples prior to processing is often used in research, and this may result in overestimated liquid yields of the feedstock.

In addition to the efficiency of press and type of biomass, incorporating fluids such as water or recycled liquid during the pressing process can increase liquid yield and improve the transfer of constituents into the liquid (Reulein et al. 2007). This was also observed in the current study, where liquid yield and nutrient capture in the liquid were higher in PG-Intact grass compared to the ReG-Intact grass when the same press was used, even though the Intact grass in PG was drier than in ReG. Colas et al. (2013) demonstrated that optimising mechanical pressing techniques and adding water to the biomass enabled the recovery of 58% protein from lucerne biomass. Moreover, the implementation of further screw-pressing of the Pulp can lead to a higher CP concentration in the liquid. This increase is attributable to enhanced fibre degradability, which facilitates the release of proteins interwoven with structural carbohydrates and lignin, particularly when a more efficient press is employed (Santamaria-Fernandez et al. 2019).

The consistent availability of feedstock throughout the year, irrespective of weather conditions, could contribute to the successful operation of a green biorefinery. Ensiling presents a viable logistical solution to ensure the continuous supply of feedstock. Furthermore, the fermentation that occurs during the storage process can also be considered as a pre-treatment for liquid–solid separation, as it significantly enhances this process, as evidenced by our previous research (Ayanfe et al. 2023). In the current trial, ensiling markedly increased liquid yield by 19% and CP captured in liquid by 86% compared to fresh biomass, in a direct comparison using DSP. The CP content of the liquid derived from ensiled biomass ranged from 182 to 256 g/kg DM, aligning closely with earlier findings [176–241 g/kg DM in Ayanfe et al. 2023; 220–261 g/kg DM in Rinne et al. 2024], for ensiled timothy and meadow fescue material. This makes CP extracted from the liquid a potential protein feed source (Stødkilde et al. 2021; Keto et al. 2021).

The extraction efficiency of nutritional constituents from the liquid could be further improved through optimised agronomic practices such as plant species and variety choices, N fertilisation and growth stage of plants at harvest. Rinne et al. (2024) reported a low liquid CP concentration (112–119 g/kg DM) for first ReG timothy, likely due to their experimental plots not being fertilised after harvesting the PG swards. In addition to gramineous forage species, legumes, which are less dependent on synthetic N-fertiliser due to symbiotic nitrogen-fixing capabilities, are a promising feedstock that can promote high protein production in biorefineries (Rinne et al. 2024; Thers et al. 2021).

Despite the elevated liquid CP concentration in ensiled biomass, various factors complicate practical applications of silage use in biorefinery. These challenges include protein degradation during fermentation, changes in the sensory qualities of the products and constraints associated with protein separation (Rinne 2024). Heat coagulation, acid precipitation or ultrafiltration have proven effective in producing protein concentrates from fresh grass juice (Santamaría-Fernández et al. 2017), but they are not readily applicable to ensiled grass juice (Rinne 2024) due to its acidic nature. Ensiling can partly breakdown plant protein into smaller peptides, free amino acids and ammonia (McDonald et al. 1991) which can also reduce protein precipitation (Møller et al. 2021).

The costs associated with the transportation of refinable biomass, particularly during the liquid–solid separation phase, are significantly influenced by the DM content of the biomass. Wilting is a commonly used approach to improve silage quality by increasing the DM concentration of the material. Wilting improves silage quality and simultaneously reduces the weight of feedstock in green biorefinery processes, potentially lowering transportation expenses. The current study found a strong positive correlation between silage DM concentration and liquid DM concentration ($R^2 = 0.93$; Figure 2A), consistent with Franco et al. (2019). Consequently, the highest DM concentration in the liquid was observed in the wilted silage. Despite this, the greatest capture of CP in the liquid was achieved in Intact silage due to the higher liquid yield, as indicated by the slightly positive correlation between liquid yield and CP recovery in liquid ($R^2 = 0.46$; Figure 2B) and slightly negative correlation between silage DM concentration and CP recovery in liquid ($R^2 = 0.44$; Figure 2C). This was contrary to Rinne et al. (2024), who observed a negative correlation between liquid yield and CP recovery in liquid. This discrepancy could be because wilting only increased silage DM concentration by 8% in their study, whereas the effect was much higher in our current study (53%). Nonetheless, since the DM concentration in wilted silage was quite high, adding water or blending the biomass prior to processing might improve liquid yield, thereby increasing CP recovery, as CP concentration in the liquid was comparable in both Intact and Wilted silage.

This study confirmed that the effect of additive application on feedstock is dependent on its characteristics, which subsequently affects biorefinery performance. Additives such as fibrolytic enzymes (Rinne et al. 2020), carbohydrases (Niemi et al. 2013) or proteases (Dotsenko and Lange 2017; Windle et al. 2014) have been found to improve the nutritional characteristics of feedstock and especially protein extraction rates. In this study, the use of a LAB inoculant consistently modified the biomasses and increased CP concentration in the liquid. This effect may be due to a delayed decline in pH at the onset of ensiling, potentially facilitating the activity of plant proteases and other proteolytic microorganisms (Dotsenko and Lange 2017), thereby solubilising the protein. Formic acid, on the other hand, has been shown in several studies to reduce protein solubility, which probably is the reason for the reduced protein extractability of FA-treated silages (Ayanfe et al. 2023; Rinne et al. 2018, 2024). A similar result was observed in this study, where formic acid application decreased CP capture in the liquid by 8%.

The choices made in feedstock manipulation should be considered from the point of view of desired end products from the biorefinery. This study demonstrated that applying formic acid increased the concentration of residual WSC, inadvertently aiding its recovery in the liquid. Consequently, this approach could be an optimal solution for industries targeting soluble sugars as by-products of biorefinery, especially bioethanol. Organic acids present in the silage juice, such as lactic acid and other VFA, also represent major streams that can be used in producing platform chemicals and potentially additives. The silage juice was highly acidic, which contributes to the stability of the liquid product. Feeding such stabilised juice containing organic acids such as lactic and acetic acid or formic acid residues from the additive could be used to enhance the gut function of swine (Khan and Iqbal 2016) in liquid feeding systems. Additionally, monogastric animals are able to metabolise products of protein degradation during ensiling such as free amino acids and peptides (Rinne 2024).

4.4 | Mineral Content of the Liquid and Effluent

Minerals such as K and P which are found in plant cell vacuoles can be readily released into the liquid phase during mechanical processing of biomass (Wachendorf et al. 2009) or through effluent excretion. Understanding the behaviour of these minerals in a biorefinery operation is essential for both livestock production and environmental perspectives. The long-term productivity of grasslands is significantly influenced by their capacity to replenish nutrients extracted during harvest. Although the effluent generated from silage production is generally considered an undesirable waste product, its mineral concentration presents an opportunity for use as fertiliser on grasslands. This application can improve the sustainability and nutrient balance of grassland production. Nevertheless, caution is necessary when handling such effluents as fertiliser, particularly due to their high acidity (Gebrehanna et al. 2014). Additionally, effluent may be used in biogas production and nutrients from the biogas can also be recycled on the field as fertiliser.

The minerals in the liquid derived from grass biomass can be used to fulfil the nutritional requirements of livestock. Compared to other protein feeds used for monogastrics such as soybean meal, the ash content of the liquid is three times greater (Resch et al. 2024), which was also observed in our current study (188 g/kg DM vs. 67 g/kg DM for soybean meal; Luke 2025). The optimal K^+ level in pig feed according to NRC (2012) is 10 g/kg DM, whereas the K concentration in the liquid was about eight times higher. Using diets with high K levels in pig feeding may lead to loose, softened faeces, affecting their hygiene (Keto et al. 2021), so that the high K concentration may limit the levels of grass-derived juice in pig diets.

5 | Conclusions

This study demonstrated the importance of raw material quality and silage management in enhancing the performance of biorefineries using timothy grass biomass. Management practices such as wilting and pulping improved the ensilability of the biomass and minimised effluent-related losses during the ensiling process. All silages were well-preserved, and the application of additive treatments with different modes of action modified the silage fermentation quality differently. Specifically, inoculation with lactic acid bacteria increased lactic and acetic acid production, whilst formic acid treatment restricted fermentation and increased the concentration of residual water-soluble carbohydrates in wilted biomass. The ensiling process improved recovery of nutrients in the liquid, which is an additional benefit of silage as a year-round stable feedstock for green biorefineries. The use of lactic acid bacteria inoculant in ensiling increased liquid crude protein concentration, whereas formic acid treatment reduced it due to decreased solubility of protein in silage. The optimisation of silage management practices to improve the fermentative characteristics of grass silages can pave the way for improved efficiency and sustainability in feedstock utilisation in green biorefineries.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

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

References

Ayanfe, N., M. Franco, T. Stefański, N. Pap, and M. Rinne. 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.

Colas, D., C. Doumeng, P. Y. Pontalier, and L. Rigal. 2013. "Twin-Screw Extrusion Technology, an Original Solution for the Extraction of Proteins From Alfalfa (*Medicago sativa*)." *Food and Bioproducts Processing* 91, no. 2: 175–182. https://doi.org/10.1016/j.fbp.2013.01.002.

Damborg, V. K., S. K. Jensen, M. R. Weisbjerg, A. P. Adamsen, and L. Stødkilde. 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.

Damborg, V. K., L. Stødkilde, S. K. Jensen, and M. R. Weisbjerg. 2018. "Protein Value and Degradation Characteristics of Pulp Fibre Fractions From Screw Pressed Grass, Clover, and Lucerne." *Animal Feed Science and Technology* 244: 93–103. https://doi.org/10.1016/j.anifeedsci.2018.08.004.

Dotsenko, G., and L. Lange. 2017. "Enzyme Enhanced Protein Recovery From Green Biomass Pulp." *Waste and Biomass Valorization* 8, no. 4: 1257–1264. https://doi.org/10.1007/s12649-016-9718-7.

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), V. Bampidis, G. Azimonti, et al. 2024. "Guidance on the Assessment of the Efficacy of Feed Additives." *EFSA Journal* 22, no. 7: e8856. https://doi.org/10.2903/j.efsa.2024.8856.

EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), D. Turck, J. L. Bresson, et al. 2016. "Guidance on the Preparation and Presentation of an Application for Authorisation of a Novel Food in the Context of Regulation (EU) 2015/2283." *EFSA Journal* 14, no. 11: e04594. https://doi.org/10.2903/j.efsa.2016.4594.

Franco, M., T. Hurme, E. Winquist, and M. Rinne. 2019. "Grass Silage for Biorefinery—A meta-Analysis of Silage Factors Affecting Liquid–Solid Separation." *Grass and Forage Science* 74, no. 2: 218–230. https://doi.org/10.1111/gfs.12421.

Franco, M., and M. Rinne. 2023. "Validation of Laboratory-Scale Ensiling Methods to Evaluate Fermentation Quality of Grass Silages Treated With a Formic Acid-Based Additive." In *Proceedings of the XIX International Silage Conference*, edited by F. Yang and X. Guo, 400–401.

Franco, M., I. Tapio, J. Pirttiniemi, et al. 2022. "Fermentation Quality and Bacterial Ecology of Grass Silage Modulated by Additive Treatments, Extent of Compaction and Soil Contamination." *Fermentation* 8, no. 4: 156. https://doi.org/10.3390/fermentation8040156.

Gaffey, J., G. Rajauria, H. McMahon, et al. 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.

Gebrehanna, M. M., R. J. Gordon, A. Madani, A. C. VanderZaag, and J. D. Wood. 2014. "Silage effluent management: A review." *Journal of*

Environmental Management 143: 113–122. https://doi.org/10.1016/j.jenvman.2014.04.012.

Hansen, N. P., S. K. Jensen, M. Johansen, et al. 2023. "Fiber Digestibility and Protein Value of Pulp Silage for Lactating Dairy Cows: Effects of Wet Fractionation by Screw Pressing of Perennial Ryegrass." *Journal of Dairy Science* 106, no. 2: 937–953. https://doi.org/10.3168/jds.2022-21851.

Huhtanen, P., J. Nousiainen, and M. Rinne. 2005. "Prediction of Silage Composition and Organic Matter Digestibility From Herbage Composition and Pepsin-Cellulase Solubility." *Agricultural and Food Science* 14, no. 2: 154–165. https://doi.org/10.2137/145960605774826046.

Huhtanen, P., J. Nousiainen, and M. Rinne. 2006. "Recent Developments in Forage Evaluation With Special Reference to Practical Applications." *Agricultural and Food Science* 15: 293–323.

Jones, D. I. H., and R. Jones. 1995. "The Effect of Crop Characteristics and Ensiling Methodology on Grass Silage Effluent Production." *Journal of Agricultural Engineering Research* 60, no. 2: 73–81.

Jørgensen, U., S. K. Jensen, and M. Ambye-Jensen. 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, no. 4: 295–306. https://doi.org/10.1111/gfs.12594.

Kaiser, A. G., J. W. Piltz, H. M. Burns, and N. W. Grriffiths. 2004. *Successful Silage*. 2nd ed, 419. Department of Primary Industries and Dairy Australia.

Keto, L., I. Tsitko, S. Perttilä, et al. 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.

Khan, S. H., and J. Iqbal. 2016. "Recent Advances in the Role of Organic Acids in Poultry Nutrition." *Journal of Applied Animal Research* 44, no. 1: 359–369. https://doi.org/10.1080/09712119.2015.1079527.

Knický, M., and R. Spörndly. 2015. "Short Communication: Use of a Mixture of Sodium Nitrite, Sodium Benzoate, and Potassium Sorbate in Aerobically Challenged Silages." *Journal of Dairy Science* 98, no. 8: 5729–5734. https://doi.org/10.3168/jds.2015-9332.

Kung, L., M. R. Stokes, and C. J. Lin. 2003. "Silage Additives." In *Silage Science and Technology*, edited by D. R. Buxton, R. E. Muck, and J. H. Harrison, vol. 42, 31–79. American Society of Agronomy.

Larsen, S. U., M. Ambye-Jensen, H. Jørgensen, and U. Jørgensen. 2019. "Ensiling of the Pulp Fraction After Biorefining of Grass Into Pulp and Protein Juice." *Industrial Crops and Products* 139: 111576. https://doi.org/10.1016/j.indcrop.2019.111576.

Luke. 2025. *Finnish Feed Tables and Nutrient Requirements of Farm Animals*. Natural Resources Institute Finland. Accessed January 28, 2025. https://www.luke.fi/en/luonnonvaratieto/science-and-information/finnish-feed-tables-and-nutrient-requirements.

Mangeon, A., R. M. Junqueira, and G. Sachetto-Martins. 2010. "Functional Diversity of the Plant glycine-Rich Proteins Superfamily." *Plant Signaling & Behavior* 5, no. 2: 99–104. https://doi.org/10.4161/psb.5.2.10336.

McDonald, P., A. R. Henderson, and S. J. E. Heron. 1991. *The Biochemistry of Silage*. 2nd ed. Chalcombe Publications.

McGechan, M. B. 1990. "A Review of Losses Arising During Conservation of Grass Forage: Part 2, Storage Losses." *Journal of Agricultural Engineering Research* 45: 1–30.

Møller, A. H., M. Hammershøj, N. H. M. Dos Passos, et al. 2021. "Biorefinery of Green Biomass-How to Extract and Evaluate High Quality Leaf Protein for Food?" *Journal of Agricultural and Food Chemistry* 69, no. 48: 14341–14357. https://doi.org/10.1021/acs.jafc.1c04289.

Muck, R. E., E. M. G. Nadeau, T. A. McAllister, F. E. Contreras-Govea, M. C. Santos, and L. Kung. 2018. "Silage Review: Recent Advances and Future Uses of Silage Additives." *Journal of Dairy Science* 101, no. 5: 3980–4000. https://doi.org/10.3168/jds.2017-13839.

Niemi, P., D. Martins, J. Buchert, and C. B. Faulds. 2013. "Pre-Hydrolysis With Carbohydrases Facilitates the Release of Protein From Brewer's Spent Grain." *Bioresource Technology* 136: 529–534. https://doi.org/10.1016/j.biortech.2013.03.076.

NRC. 2012. *Nutrient Requirements of Swine*. 11th revised ed. National Academies Press.

Resch, R., G. Terler, M. Winter, et al. 2024. "Nutrient Composition, Fermentation Characteristics and Mass Balance of Press Juice and Press Cake Obtained From Biorefining of Grass-Clover and Red Clover Silage." *Grass and Forage Science* 79, no. 3: 449–463. https://doi.org/10.1111/gfs.12682.

Reulein, J., K. Scheffer, R. Stülpnagel, L. Bühle, W. Zerr, and M. Wachendorf. 2007. "Efficient Utilization of Biomass Through Mechanical Dehydration of Silages." In Proceedings of the 15th European Biomass Conference & Exhibition, Germany. 1770–1774.

Rinne, M. 2024. "Novel Uses of Ensiled Biomasses as Feedstocks for Green Biorefineries." *Journal of Animal Science and Biotechnology* 15, no. 1: 36. https://doi.org/10.1186/s40104-024-00992-y.

Rinne, M., M. Franco, K. Manni, and A. Huuskonen. 2023. "Evaluating the Effects of Wilting, Mixing With Timothy and Silage Additive Application on Red Clover Silage Quality." *Agricultural and Food Science* 32, no. 4: 207–218. https://doi.org/10.23986/afsci.137136.

Rinne, M., T. Stefański, M. Franco, T. Jalava, and K. Kuoppala. 2024. "Forage Type and Additive Effects on Fermentation Quality and Biorefinery Performance of Silages." *Grass and Forage Science* 79, no. 2: 170–178. https://doi.org/10.1111/gfs.12671.

Rinne, M., P. Timonen, T. Stefanski, et al. 2018. "Grass Silage for Biorefinery-Effects of Type of Additive and Separation Method." In Proceedings of XVIII International Silage Conference, Bonn, Germany. 182–183.

Rinne, M., E. Winquist, V. Pihlajaniemi, P. Niemi, A. Seppälä, and M. Siika-aho. 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.

Santamaria-Fernandez, M., M. Ambye-Jensen, V. K. Damborg, and M. Lübeck. 2019. "Demonstration-Scale Protein Recovery by Lactic Acid Fermentation From Grass Clover – A Single Case of the Production of Protein Concentrate and Press Cake Silage for Animal Feeding Trials." *Biofuels, Bioproducts and Biorefining* 13, no. 3: 502–513. https://doi.org/10.1002/bbb.1957.

Santamaría-Fernández, M., B. Molinuevo-Salces, P. Kiel, S. Steenfeldt, H. Uellendahl, and M. Lübeck. 2017. "Lactic Acid Fermentation for Refining Proteins From Green Crops and Obtaining a High Quality Feed Product for Monogastric Animals." *Journal of Cleaner Production* 162: 875–881. https://doi.org/10.1016/j.jclepro.2017.06.115.

Savonen, O., M. Franco, T. Stefanski, P. Mäntysaari, K. Kuoppala, and M. Rinne. 2020. "Grass Silage Pulp as a Dietary Component for High-Yielding Dairy Cows." *Animal* 14, no. 7: 1472–1480. https://doi.org/10.1017/S1751731119002970.

Serra, E., M. B. Lynch, J. Gaffey, et al. 2023. "Biorefined Press Cake Silage as Feed Source for Dairy Cows: Effect on Milk Production and Composition, Rumen Fermentation, Nitrogen and Phosphorus Excretion and In Vitro Methane Production." *Livestock Science* 267: 105135. https://doi.org/10.1016/j.livsci.2022.105135.

Sousa, D., M. Larsson, and E. Nadeau. 2022. "Milk Production of Dairy Cows Fed Grass-Clover Silage Pulp." *Agriculture* 12, no. 1: 33. https://doi.org/10.3390/agriculture12010033.

Stødkilde, L., M. Ambye-Jensen, and S. K. Jensen. 2021. "Biorefined Organic Grass-Clover Protein Concentrate for Growing Pigs: Effect on Growth Performance and Meat Fatty Acid Profile." *Animal Feed Science and Technology* 276: 114943. https://doi.org/10.1016/j.anifeedsci.2021.114943.

Thers, H., L. Stødkilde, S. K. Jensen, and J. Eriksen. 2021. "Linking Protein Quality in Biorefinery Output to Forage Crop Crude Protein Input via the Cornell Net Carbohydrate and Protein System." *Applied Biochemistry and Biotechnology* 193, no. 8: 2471–2482. https://doi.org/10.1007/s12010-021-03496-x.

Virkajärvi, P., M. Rinne, J. Mononen, O. Niskanen, K. Järvenranta, and A. Sairanen. 2015. "Dairy production systems in Finland." In *Grassland Science in Europe*, edited by A. van den Pol-van Dasselaar, vol. 20, 51–66. Wageningen Academic Publishers.

Wachendorf, M., F. Richter, T. Fricke, R. Graß, and R. Neff. 2009. "Utilization of Semi-Natural Grassland Through Integrated Generation of Solid Fuel and Biogas From Biomass. I. Effects of Hydrothermal Conditioning and Mechanical Dehydration on Mass Flows of Organic and Mineral Plant Compounds, and Nutrient Balances." *Grass and Forage Science* 64, no. 2: 132–143. https://doi.org/10.1111/j.1365-2494.2009.00677.x.

Walinga, I., W. Van Vark, V. J. G. Houba, and J. J. Van Der Lee. 1989. *Plant Analysis Procedures*. Department of Soil Science and Plant Nutrition, Wageningen Agricultural University. Syllabus Part 7, 197–200.

Weissbach, F., L. Schmidt, and E. Hein. 1974. "Method of Anticipation of the Run of Fermentation in Silage Making Based on the Chemical Composition of Green Fodder." In *Proceedings of the 12th International Grassland Congress*, edited by V. G. Iglovikov and A. P. Movsisyants, 663–673. Russian Academy of Agricultural Sciences.

Wilkinson, J. M., and D. R. Davies. 2013. "The Aerobic Stability of Silage: Key Findings and Recent Developments." *Grass and Forage Science* 68, no. 1: 1–19.

Windle, M. C., N. Walker, and L. Kung. 2014. "Effects of an Exogenous Protease on the Fermentation and Nutritive Value of Corn Silage Harvested at Different Dry Matter Contents and Ensiled for Various Lengths of Time." *Journal of Dairy Science* 97, no. 5: 3053–3060. https://doi.org/10.3168/jds.2013-7586.