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Effect of Wilting Intensity, Dry Matter Content and Sugar Addition on Nitrogen Fractions in Lucerne Silages

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Abstract: Pre-ensiling treatments can significantly influence the composition of lucerne (*Medicago sativa* L.) silages (LS). Besides dry matter (DM) content and availability of water-soluble carbohydrates (WSC), wilting intensity may exert a strong impact on the crude protein (CP; nitrogen [N] × 6.25) fractions. The present study aimed to evaluate the effects of DM level, wilting intensity, and sucrose addition on N compounds and fermentation products in LS. Pure lucerne stand (cultivar Plato) was wilted with either high or low intensity to DM contents of 250 and 350 g kg^{-1}, respectively, and ensiled with or without the addition of sucrose. Non-protein-N (NPN) concentration in LS was affected by all pre-ensiling treatments and with 699 g kg^{-1} CP, NPN was lowest in high-intensity wilted high-DM LS with sucrose addition. No effects were observed on in vitro-estimated concentrations of utilizable CP at the duodenum, a precursor to metabolizable protein. Sucrose addition and higher DM level decreased acetic acid and ammonia-N concentration in the silages. Therefore, the present study demonstrated the beneficial manipulation of CP fractions in LS by high-intensity wilting to higher DM contents and that the provision of WSC may be necessary for sufficient silage fermentation and protein preservation.

Keywords: crude protein; dry matter; lucerne; alfalfa; nitrogen; silage; wilting

1. Introduction

Compared with other forage species, lucerne (*Medicago sativa* L.) has a high crude protein (CP; Nitrogen [N] × 6.25) content and depending on its degradability in the rumen, a considerable part of the ruminant's demand for amino acids (AA) can be supplied by feeding lucerne [1]. Preserved as lucerne silage (LS), this forage is continuously available as a component for dairy and beef cattle diets, independently from vegetative growth periods. However, the vast majority of CP in LS is ruminally readily-degradable non-protein-N (NPN), i.e., from 50 up to 87% of total CP [2–4], which can be ascribed to proteolytic activities of lucerne-derived proteases before ensiling, and microbial enzymes during the ensiling process [5]. Legumes are also characterized by low proportions of water-soluble carbohydrates (WSC) [6], which firstly make them difficult to ensile, and secondly also result in silages with minimal concentrations of rapidly fermentable carbohydrates. Solely feeding LS leads to an inefficient microbial N fixation in the rumen [4,7] and consequently high N excretion causing increased environmental pollution. However, substantial N excretion may still occur in mixed LS-based diets because the provision of rapidly fermentable carbohydrates by concentrate is limited due to the risk of rumen acidosis [8]. Therefore, adequately meeting the microbial energy demand for fixing the N arising from the rapid degradation of high NPN amounts in LS is hardly feasible. Consequently, manipulating the CP fractions in LS should be targeted, and in order to improve this fraction, meaning by increasing true protein (TP) concentrations and decreasing low-molecular-weight CP, high-intensity wilting, i.e., with high solar radiation, may be an effective option that to date has not received much attention. Rapid drying should inactivate plant-derived

proteases, whose functions rely on water, and consequently stabilize TP content of lucerne plants. Likewise, a previous study by Edmunds et al. [9] already showed that high-intensity wilting results in higher TP percentages in grass silages. Thus, we hypothesized that high-intensity wilting alone or in combination with further treatments may influence the CP composition in LS and decrease proteolysis during ensiling. Because lucerne contains limited amounts of WSC [6], the effect of sucrose addition before ensiling on the N fractions in LS was further tested, particularly as there is clear evidence for decreased ammonia-N concentration in glucose- and fructose-added LS [10] and a more stable silage fermentation in general [11]. Therefore, the objective of the present study was to evaluate N fractions in LS wilted with different intensities to DM contents of 250 or 350 g kg^{-1} and with or without the addition of sucrose. The hypothesis was that the highest TP preservation would occur in those LS, which received high-intensity wilting to 350 g kg^{-1} and with sucrose addition.

2. Materials and Methods

2.1. Preparation of Silages

The procedure for the preparation of the LS was adopted from Edmunds et al. [9] and partly modified as described in the following. On the 19th of July 2016, the third cut of a one hectare pure lucerne stand (cultivar Plato) at the early bud stage of maturity was harvested using a disc mower without a mechanical conditioner at 10 cm stubble height at the Educational and Research Centre Frankenforst of the Faculty of Agriculture, University of Bonn (Königswinter, Germany, 7° 12′ 22′′ E; 50° 42′ 49′′ N). The harvested material was immediately collected from the field and equally spread on either black plastic in the sun (high-intensity wilting; HI) or on white plastic in the shade (low-intensity wilting; LI). The lucerne layers on each plastic had a thickness of approximately 10 cm to ensure sufficient and consistent exposure of the entire plant material to the solar radiation. Immediately, a composite sample was taken and stored at −20 °C for later analysis. This composite sample consisted of 20 single samples that were taken from different places of the lucerne layers on the white and the black plastic, respectively. During silage preparation, the sky was clear, and the weather conditions were sunny with a relative humidity of 59%, a maximum temperature of 32 °C and 15 h of sunshine during the day and a minimum temperature of 20 °C during the night. The plant material was wilted to DM levels (DML) of 250 and 350 g kg^{-1}, respectively, and ensiled either without or with sucrose addition (SU) of 125 g kg^{-1} DM. The amount of added sucrose was chosen as it constitutes the difference between the average WSC content of lucerne with 65 g kg^{-1} DM and perennial ryegrass (*Lolium perenne* L.) with 190 g kg^{-1} DM [12], which is good to ensile [13]. The compaction of the lucerne at ensiling was calculated according to the recommendations of the Federal Working Group for Forage Preservation (Bundesarbeitskreis Futterkonservierung; [12]) in Germany with 190.4 (±2.3) kg DM m^{-3} for low-DM LS and 215.8 (±4.6) kg DM m^{-3} for high-DM LS. The lucerne was ensiled in duplicate in 60 l plastic containers and stored for 120 days. Thus, eight different silage treatments were finally prepared, which are referred to as: 250HISU, 250HI, 250LISU, 250LI, 350HISU, 350HI, 350LISU, and 350LI. The required wilting durations were 2.5 h for 250HISU and 250HI, 4.0 h for 250LISU and 250LI, 7 h for 350HISU and 350HI and 22 h for 350LISU and 350LI.

2.2. Basic Analysis

After 120 days, the two plastic containers of each LS were pooled and three composite samples, each comprising 20 single samples from different spots of the silage heap, were taken and checked for the presence of mould or any other signs of spoilage. All composite samples were thoroughly mixed and 800 g fresh matter of each were freeze-dried and ground successively using 3 mm and then 1 mm sieves (SM 100, Retsch, Haan, Germany). These samples were used for the following analyses, except fermentation pattern analysis, which was conducted with two subsamples (50 g) of each LS that were immediately taken after silo opening and stored at −20 °C.

The proximate analyses were conducted in accordance with the Association of German Agricultural Analytic and Research Institutes (VDLUFA; [14]). The DM content was determined by drying the fresh silages overnight at 60 °C and subsequently at 105 °C for at least 3 h (method 3.1). Using the equation from Weissbach and Kuhla [15], DM was corrected for the loss of volatile compounds that occur during drying. Crude protein was determined by the Kjeldahl method (method 4.1.1) using a Vapodest 50s carousel (Gerhardt, Königswinter, Germany) and multiplying N by 6.25. Proportions of neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash (aNDFom), acid detergent fibre expressed exclusive of residual ash (ADFom), and acid detergent lignin (ADL) were determined in accordance with methods 6.5.1, 6.5.2, and 6.5.3, respectively.

2.3. Crude Protein Fractionation and Amino Acid Analysis

The CP fractionation was performed according to the Cornell Net Carbohydrate and Protein System [16], following recommendations and standardizations of Licitra et al. [17]. Briefly, five fractions (all expressed as g kg^{-1} CP; A, B1, B2, B3, and C) were obtained; fraction A represents NPN, fraction B1 represents rapidly ruminally degradable TP, fraction B2 represents moderately ruminally degradable TP, fraction B3 represents slowly ruminally degradable TP and fraction C represents indigestible TP. Fraction A is the difference between total CP and TP, which precipitates in tungstic acid. Fraction B1 is the difference between total TP and borate-phosphate-buffer-insoluble TP. Fraction B2 is borate-phosphate-buffer-insoluble TP minus neutral-detergent-insoluble TP and fraction B3 is the difference between neutral-detergent-insoluble TP and acid detergent-insoluble TP. Fraction C is acid-detergent-insoluble TP. Subsequently, total TP concentrations (g kg^{-1} CP) of samples were calculated by 1000 minus fraction A.

The contents of free AA and total AA (sum of peptide-bound and free AA), including gamma-aminobutyric acid (GABA), were determined by ion-exchange chromatography according to the Commission Regulation (EC) No. 152/2009 of the European Communities [18]. This method is not valid for the determination of tryptophan and cannot differentiate between D and L forms of AA. Briefly, free AA were extracted with diluted hydrochloric acid and co-extracted nitrogenous macromolecules were precipitated with sulfosalicylic acid and removed by filtration before the free AA determination by ninhydrin reaction with spectrophotometric detection at 570 nm. The procedure for total AA determination depended on AA under investigation. Prior to hydrolysis, Cys and Met were oxidized with a performic acid-phenol mixture to cysteic acid and methionine sulphone, respectively, whereas Tyr was determined in unoxidized samples only. All remaining AA were determined in either the oxidized or unoxidized sample. Samples were then hydrolyzed with hydrochloric acid and determined by ninhydrin reaction using spectrophotometric detection at 570 nm or 440 nm for Pro.

2.4. Modified Hohenheim Gas Test

In order to estimate utilizable CP at the duodenum (uCP), the modified Hohenheim gas test [19,20] was conducted as outlined in detail by Edmunds et al. [21]. Briefly, ruminal fluid was collected before morning feeding from two rumen-fistulated sheep receiving a 1:1 grass hay-pelleted compound maintenance ration twice daily. An amount corresponding to 200 mg DM of each sample was incubated in duplicate in each of two runs in 30 mL of ruminal fluid-buffer solution for 8 and 48 h, as recommended for forages [22]. At the end of these incubation periods, syringe contents were analyzed for ammonia-N applying a Vapodest 50s carousel and uCP was calculated using the following equation:

$$\text{uCP (g kg}^{-1}\text{ DM)} = ((\text{ammonia-N}_{\text{blank}} + \text{N}_{\text{sample}} - \text{ammonia-N}_{\text{sample}})/\text{sample weight (mg DM)}) \times 6.25 \times 1000,$$

where ammonia-N is in mg 30 mL^{-1}, blank refers to the ruminal fluid-buffer solution without sample substrate, sample refers to the ruminal fluid-buffer solution with sample substrate, N_{sample} is N added to the syringe through the sample substrate (mg), and sample weight is the amount of sample

substrate (mg DM) weighed into the syringe. When using a live product such as ruminal fluid, small biological fluctuations among runs are inevitable. To correct for this a protein standard provided by the University of Hohenheim was analyzed with every run. The standard was a concentrate mixture of (kg^{-1} DM) 450 g rapeseed meal, 300 g faba beans, and 250 g molasses sugar beet pulp, and had a CP content of 254 g kg^{-1} DM. The correction follows the same method as for gas production [23] whereby the mean uCP value for the standard, provided by the University of Hohenheim for 8 or 48 h, is divided by the recorded value of the standard for that run and all other samples are multiplied by the resulting correction factor. Whole runs were repeated if the correction factor, for either incubation time, lay outside the range of 0.9–1.1. The hay and concentrate standards typically used for correcting gas production were also included in the incubation, not only to correct gas production values, but to ensure the ruminal fluid solution followed typical fermentation. After the correction of obtained uCP, values from the incubation times were plotted against a log ((ln) time) scale and the resulting regression equation was used to calculate the effective uCP at passage rates of 0.02, 0.05, and 0.08 hr^{-1}, which are referred to as uCP2, uCP5, and uCP8, respectively. These passage rates represent the ruminal digesta flow, including the solid and liquid phase, in animals with different production levels [24].

2.5. Fermentation Pattern Analysis

Subsamples (50 g) of all silages were used for fermentation pattern analysis. Procedures, as well as detection limits, are described in detail by Brüning et al. [25]. Briefly, a cold-water extract was prepared from all samples by blending the frozen substrate with 200 mL distilled water and 1 mL toluene and refrigerated overnight at 4 °C. Extracts were then filtered using MN 615 filter paper (Macherey-Nagel, Düren, Germany) and subsequently microfiltered (Minisart RC, 0.45 µm pore size; Sartorius, Göttingen, Germany). Ammonia-N concentration was analyzed colorimetrically based on the Berthelot reaction [26]. The pH of the extracts was determined potentiometrically and lactic acid concentration was analyzed by high-performance liquid chromatography with refractive index detection in accordance with Weiß and Kaiser [27]. Volatile fatty acids, alcohols (methanol, ethanol, propanol, butanol, 2,3-butanediol), ethyl lactate, ethyl acetate, propyl acetate, and acetone were determined by gas chromatography with flame ionization detection [28,29]. The concentrations of WSC were determined using the anthrone method [30].

2.6. Statistical Analysis

Statistical analysis was performed with the GLM procedure of SAS version 9.3 (SAS Institute Inc., Cary, NC, USA) using the following model: $Y = \mu + a_i + b_j + c_k + e_{ejk}$ where μ is the mean, a_i is the effect of the SU, b_j is the effect of the wilting intensity (WI), c_k is the effect of the DML and e_{ijk} is the residual error. The significance level was set at $\alpha = 0.05$. In order to test for interactions, field replicates would have been necessary [31], which were not available in the present study. As a consequence, silos were pooled to avoid an artificially created variation and only the main effects were tested. Particularly due to the limited extent of the present study, we preferred to cautiously draw conditional conclusions from a smaller data set as recommended by Lowry [32].

3. Results

3.1. General Chemical Composition

As shown in Table 1, DM content was affected by SU and was slightly higher in SU LS. Concerning the CP content, effects of all three pre-ensiling treatments could be observed, whereby CP proportions ranged from 188 to 219 g kg^{-1} DM and were higher in LS without SU, LI, and 250DML, respectively. The SU treatment also affected the fibre fractions aNDFom and ADFom, which were lower in SU LS. No treatment factor had an effect on ADL.

Table 1. Effect of sucrose addition (SU), wilting intensity (WI), and dry matter (DM) level (DML) on DM content (g kg^{-1}), crude protein content (g kg^{-1} DM), and fibre fractions (g kg^{-1} DM) in lucerne silages (fresh lucerne values are provided as ease for comparison).

Silage	DM	CP	aNDFom	ADFom	ADL
Fresh lucerne	213.1	213	431	340	91
250HISU	254.8	195	458	322	88
250HI	240.5	215	463	364	88
250LISU	255.0	198	422	325	87
250LI	246.8	219	429	355	86
350HISU	344.5	188	416	325	88
350HI	340.0	211	446	338	90
350LISU	346.8	195	390	312	96
350LI	339.0	213	421	336	95
Results of statistical analyses					
SEM	18	4	9	6	1
SU	**	**	*	*	NS
WI	NS	*	NS	NS	NS
DML	**	*	NS	NS	NS

250HISU = 250 g kg^{-1}, high-intensity wilting and sucrose addition; 250HI = 250 g kg^{-1}, high-intensity wilting and no sucrose addition; 250LISU = 250 g kg^{-1}, low-intensity wilting and sucrose addition; 250LI = 250 g kg^{-1}, low-intensity wilting and no sucrose addition; 350HISU = 350 g kg^{-1}, high-intensity wilting and sucrose addition; 350HI = 350 g kg^{-1}, high-intensity wilting and no sucrose addition; 350LISU = 350 g kg^{-1}, low-intensity wilting and sucrose addition; 350LI = 350 g kg^{-1}, low-intensity wilting and no sucrose addition; DM = Dry matter; CP = Crude protein; aNDFom = Neutral detergent fibre after incineration and amylase treatment; ADFom = Acid detergent fibre after incineration; ADL = Acid detergent lignin; SEM = Standard error of the mean (without consideration of fresh lucerne); NS = not significant; * = $p < 0.05$; ** = $p < 0.01$.

3.2. Crude Protein Fractions and Amino Acids

The CP fractionation revealed various differences between the eight silage treatments (Table 2). Non-protein N was the largest CP fraction in all LS but was more than 110 g kg CP^{-1} higher for 250LI when compared to 350HISU. Likewise, NPN (fraction A) was affected by all three factors, i.e., SU, WI, and DML, with increased proportions in 250DML silages. Both HI and SU decreased the NPN proportion in LS. Moderately ruminally degradable TP (fraction B2) was the second largest fraction and highest in silages with 350DML and SU. As with NPN, the largest difference for moderately ruminally degradable TP was found between 250LI and 350HISU. Rapidly (fraction B1) and slowly ruminally degradable TP (fraction B3), as well as indigestible TP (fraction C), were present in small proportions of total CP and slowly ruminally degradable TP was partly not quantifiable. Thus, the effects of SU and DML on these fractions are negligible. Total TP was calculated by subtracting NPN (fraction A) from total CP. Consequently, 250LI had the lowest TP content and, except for 350LI, was clearly separated from 350DML silages.

Table 2. Effect of sucrose addition (SU), wilting intensity (WI), and dry matter level (DML) on crude protein (CP) fractions (g kg^{-1} CP) and true protein content (g kg^{-1} CP) in lucerne silages (fresh lucerne values are provided as ease for comparison).

	Crude Protein Fraction †					
Silage	A	B1	B2	B3	C	TP
Fresh lucerne	259	289	383	27	42	741
250HISU	772	13	174	0	54	228
250HI	799	6	154	0	53	201
250LISU	782	11	16	0	47	218
250LI	812	11	139	0	58	188
350HISU	699	6	251	2	49	301
350HI	744	6	206	0	49	256
350LISU	718	3	253	2	47	282
350LI	779	7	182	0	46	221
Results of statistical analyses						
SEM	14	1	27	0	1	14
SU	**	NS	**	*	NS	**
WI	*	NS	NS	NS	NS	*
DML	**	NS	**	*	#	**

† According to the Cornell Net Carbohydrate and Protein system [16]; 250HISU = 250 g kg^{-1}, high-intensity wilting and sucrose addition; 250HI = 250 g kg^{-1}, high-intensity wilting and no sucrose addition; 250LISU = 250 g kg^{-1}, low-intensity wilting and sucrose addition; 250LI = 250 g kg^{-1}, low-intensity wilting and no sucrose addition; 350HISU = 350 g kg^{-1}, high-intensity wilting and sucrose addition; 350HI = 350 g kg^{-1}, high-intensity wilting and no sucrose addition; 350LISU = 350 g kg^{-1}, low-intensity wilting and sucrose addition; 350LI = 350 g kg^{-1}, low-intensity wilting and no sucrose addition; TP = True protein; SEM = Standard error of the mean (without consideration of fresh lucerne); NS = not significant; # = $0.05 < p < 0.1$; * = $p < 0.05$; ** = $p < 0.01$.

Both SU and DML affected several AA concentrations determined as peptide-bound and free AA, whereas only a few were influenced by WI (Table 3). Concentrations of Thr, Arg, Ser, Asp, and Glu were increased by SU, whereas it decreased Ile, Leu, Val, and Ala. Besides, a strong tendency ($p = 0.06$) for increased Lys concentrations in SU LS were observed. The HI treatment decreased the concentrations of Ile, and Val. The 350DML treatment increased the concentrations of Cys, Lys, Thr, Arg, His, Ser, Pro, Asp, and Glu. In contrast, concentrations of Ile, Leu, Val, and Ala were decreased in high-DM LS.

The DML treatment affected free AA more than SU or WI (Table 4) and HI tended to decrease Ile concentrations ($p = 0.09$). The SU treatment increased the concentration of free Thr and tended to increase free Glu ($p = 0.07$), whereas it decreased free Ile, Leu, Val, and Ala. The 350DML LS showed higher concentrations of free Lys, Thr, Pro, Asp, Glu as well as free His that was not detectable in 250DML LS. Free Ile, Leu, Val, and Ala were reduced in 350DML LS. Regarding the amount of total free AA, SU decreased total free AA, whereas no influence of other pre-ensiling treatments was observed. Moreover, 350DML and SU reduced the concentrations of free and total GABA (Tables 3 and 4).

Table 3. Effect of sucrose addition (SU), wilting intensity (WI), and dry matter (DM) level (DML) on contents (g kg^{-1} DM) of total amino acids (AA; the sum of peptide-bound and free AA) and gamma-aminobutyric acid (GABA) in lucerne silages.

AA	Ala	Arg	Asp	Cys	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Val	GABA
250HISU	21.1	2.3	11.3	1.2	10.2	9.6	1.9	9.5	14.8	3.2	3.2	9.3	6.2	3.2	3.6	11.8	10.3
250HI	29.4	1.7	4.2	0.7	5	3.5	1.3	10.3	16.5	2.5	2.2	8.5	2.1	2.2	2	13.3	16.7
250LISU	22.3	2.1	10.6	1.3	10.3	9.7	2	9.7	15	3.1	3.2	9.5	6	2.9	3	12	10.7
250LI	28.8	1.7	4.4	0.9	5.5	6	1.6	10.6	16.3	2.6	2.7	8.4	2.3	2.2	2	13.4	16.5
350HISU	14.7	3.1	17.6	0.13	12.5	8.9	3.3	8.9	14.4	7.6	3	9.1	8.7	4.7	6.4	11	8
350HI	19.1	2.3	13	1.4	9.7	10	3.6	10	16	7.2	3.2	9.4	8.4	3	4	12.6	12.5
350LISU	14.4	3.2	19.9	1.4	12.5	9.2	3.5	9.1	14.5	8.6	3.1	9.4	10.3	5.1	6.9	11.7	7.4
350LI	18.1	2.3	14.4	1.4	10.9	10.3	4.4	10.3	16.3	7.8	3.4	10.2	10.9	3.4	4.4	13.2	10.9
SEM	2.02	0.2	1.99	0.16	1.01	0.84	0.4	0.21	0.31	0.95	0.13	0.2	1.18	0.38	0.65	0.31	1.23
Results of statistical analyses																	
SU	**	**	**	NS	**	NS	NS	**	**	#	NS	NS	NS	**	**	**	**
WI	NS	NS	NS	NS	NS	NS	NS	*	NS	NS	NS	NS	NS	NS	NS	*	NS
DML	**	**	**	#	**	NS	**	**	*	**	NS	NS	**	**	**	*	**

250HISU = 250 g kg^{-1}, high-intensity wilting and sucrose addition; 250HI = 250 g kg^{-1}, high-intensity wilting and no sucrose addition; 250LISU = 250 g kg^{-1}, low-intensity wilting and sucrose addition; 250LI = 250 g kg^{-1}, low-intensity wilting and no sucrose addition; 350HISU = 350 g kg^{-1}, high-intensity wilting and sucrose addition; 350HI = 350 g kg^{-1}, high-intensity wilting and no sucrose addition; 350LISU = 350 g kg^{-1}, low-intensity wilting and sucrose addition; 350LI = 350 g kg^{-1}, low-intensity wilting and no sucrose addition; SEM = Standard error of the mean; NS = not significant; # = $0.05 < p < 0.1$; * = $p < 0.05$; ** = $p < 0.01$.

Table 4. Effect of sucrose addition (SU), wilting intensity (WI), and dry matter (DM) level (DML) on contents (g kg^{-1} DM) of free amino acids (AA) and gamma-aminobutyric acid (GABA) in lucerne silages.

AA	Ala	Arg	Asp	GABA	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Thr	Val
250HISU	21.9	0	0.6	9.7	3	6	0	6	10.4	0	2.1	5.9	2.9	1.4	7.8
250HI	32.5	0	0	12.5	0	0.8	0	8	12.8	0	1.2	5.3	0	0.2	10.6
250LISU	23.5	0	0.4	10.1	3.1	6.2	0	6.3	10.7	0	2.2	6.1	2.8	0.9	8.3
250LI	32	0	0.3	12.4	0.5	3.1	0	8.3	12.7	0	1.7	5.3	0.3	0.3	10.6
350HISU	13.4	0	5.4	7.6	3.9	4.7	1	4.8	8.8	3.6	1.5	4.9	4.6	3.7	6.1
350HI	19.8	0	6	9.3	3	6.6	1.7	7.1	11.4	4.1	1.7	5.8	6	2.1	9.1
350LISU	12.7	0	7.4	7.1	4.1	4.6	1.5	4.9	8.7	4.4	1.5	5.1	6.1	4	6.4
350LI	18.4	0	7.5	8.2	4.3	6.5	2.2	7.3	11.5	4.5	1.7	6.5	8.3	2.4	9.4
SEM	2.64	0	1.21	0.72	0.57	0.71	0.32	0.47	0.55	0.79	0.11	0.19	1.03	0.51	0.61
Results of statistical analyses															
SU	**	NS	NS	**	#	NS	NS	**	**	NS	NS	NS	NS	**	**
WI	NS	NS	NS	NS	NS	NS	NS	#	NS	NS	NS	NS	NS	NS	NS
DML	**	NS	**	**	*	NS	**	**	**	**	NS	NS	*	**	**

250HISU = 250 g kg^{-1}, high-intensity wilting and sucrose addition; 250HI = 250 g kg^{-1}, high-intensity wilting and no sucrose addition; 250LISU = 250 g kg^{-1}, low-intensity wilting and sucrose addition; 250LI = 250 g kg^{-1}, low-intensity wilting and no sucrose addition; 350HISU = 350 g kg^{-1}, high-intensity wilting and sucrose addition; 350HI = 350 g kg^{-1}, high-intensity wilting and no sucrose addition; 350LISU = 350 g kg^{-1}, low-intensity wilting and sucrose addition; 350LI = 350 g kg^{-1}, low-intensity wilting and no sucrose addition; SEM = Standard error of the mean; NS = not significant; # = $0.05 < p < 0.1$; * = $p < 0.05$; ** = $p < 0.01$.

3.3. Modified Hohenheim Gas Test

Irrespective of calculated passage rate, pre-ensiling treatments had no effect on effective uCP values of LS (Table 5). Only uCP8 values tended to be higher for 250DML LS ($p = 0.08$).

Table 5. Effect of sucrose addition (SU), wilting intensity (WI), and dry matter (DM) level (DML) on effective utilizable crude protein at the duodenum (g kg^{-1} DM).

Silage	uCP2	uCP5	uCP8
250HISU	72	109	127
250HI	82	114	131
250LISU	74	112	131
250LI	76	110	128
350HISU	74	105	121
350HI	80	107	121
350LISU	75	108	124
350LI	74	103	118
SEM	1.2	1.3	1.7
Results of statistical analyses			
SU	NS	NS	NS
WI	NS	NS	NS
DML	NS	NS	#

250HISU = 250 g kg^{-1}, high-intensity wilting and sucrose addition; 250HI = 250 g kg^{-1}, high-intensity wilting and no sucrose addition; 250LISU = 250 g kg^{-1}, low-intensity wilting and sucrose addition; 250LI = 250 g kg^{-1}, low-intensity wilting and no sucrose addition; 350HISU = 350 g kg^{-1}, high-intensity wilting and sucrose addition; 350HI = 350 g, high-intensity wilting and no sucrose addition; 350LISU = 350 g kg^{-1}, low-intensity wilting and sucrose addition; 350LI = 350 g kg^{-1}, low-intensity wilting and no sucrose addition; uCP2 = effective utilizable crude protein at the duodenum to passage rate of 0.02 hr^{-1}; uCP5 = effective utilizable crude protein at the duodenum to passage rate of 0.05 hr^{-1}; uCP8 = effective utilizable crude protein at the duodenum to passage rate of 0.08 hr^{-1}; SEM = Standard error of the mean; NS = not significant; # = $0.05 < p < 0.1$.

3.4. Fermentation Pattern

Acetone, 2,3-butandiol, i-valeric acid, n-valeric acid and propyl acetate were not detected in any sample during fermentation pattern analysis. The SU treatment decreased silage pH and ammonia-N concentration, but increased lactic acid concentration as well as ethyl acetate and ethyl lactate (Table 6). Besides, LS without SU tended to have higher concentrations of acetic acid ($p = 0.09$), WSC (P = 0.09), and ethanol ($p = 0.06$). In contrast, WI had no effect on response variables. The 350DML reduced acetic acid as well as methanol concentration and tended to decrease ammonia-N ($p = 0.06$) and propanol ($p = 0.09$) in LS compared to 250 DML (Table 6).

Table 6. Effect of sucrose addition (SU), wilting intensity (WI), and DM level (DML) on lactic acid, volatile fatty acids, ester compounds, alcohols, water-soluble carbohydrates (g kg^{-1} DM), and ammonia-nitrogen (N; g kg^{-1} N) in lucerne silages.

Silage	pH	Lactic Acid	Acetic Acid	Propionic Acid	Butyric Acid	Caproic Acid	Ethyl Acetate	Ethyl Lactate	Methanol	Ethanol	Butanol	Propanol	WSC	Ammonia-N
250HISU	4.58	50.6	38.2	0.8	0.7	0.0	0.2	0.1	2.1	7.9	0.1	1.9	10.1	175
250HI	6.12	5.4	42.8	2.1	21.9	0.8	0.1	0.0	2.5	6.2	0.1	0.3	2.3	276
250LISU	4.61	52.4	38.2	1.5	1.5	0.0	0.2	0.1	2.6	8.7	0.1	1.7	10.6	157
250LI	5.85	15.3	48.4	2.0	7.2	0.8	0.1	0.0	3.0	5.4	0.1	0.3	3.1	221
350HISU	4.77	39.7	31.1	1.0	0.6	0.0	0.2	0.1	1.7	6.5	0.0	0.2	17.6	145
350HI	5.81	21.6	34.0	0.3	0.5	0.0	0.1	0.0	2.2	6.3	0.1	0.2	5.0	217
350LISU	4.65	36.2	31.2	0.8	0.3	0.0	0.2	0.1	1.2	5.8	0.0	0.1	46.0	149
350LI	5.73	38.4	31.4	1.3	0.3	0.0	0.1	0.0	1.8	4.3	0.1	0.2	4.8	191
						Results of statistical analyses								
SEM	0.24	5.94	2.22	0.2	2.7	0.13	0.02	0.02	0.20	0.49	0.02	0.26	5.12	15.9
SU	**	*	#	NS	NS	NS	**	**	NS	#	NS	NS	#	**
WI	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
DML	NS	NS	**	NS	NS	NS	NS	NS	*	NS	NS	#	NS	#

WSC = Water-soluble carbohydrates; 250HISU = 250 g kg^{-1}, high-intensity wilting and sucrose addition; 250HI = 250 g kg^{-1}, high-intensity wilting and no sucrose addition; 250LISU = 250 g kg^{-1}, low-intensity wilting and sucrose addition; 250LI = 250 g kg^{-1}, low-intensity wilting and no sucrose addition; 350HISU = 350 g kg^{-1}, high-intensity wilting and sucrose addition; 350HI = 350 g kg^{-1}, high-intensity wilting and no sucrose addition; 350LISU = 350 g kg^{-1}, low-intensity wilting and sucrose addition; 350LI = 350 g kg^{-1}, low-intensity wilting and no sucrose addition; SEM = Standard error of the mean; NS = not significant; # = $0.05 < p < 0.1$; * = $p < 0.05$; ** = $p < 0.01$.

4. Discussion

4.1. General Chemical Composition

Crude protein contents were lower in SU LS, which likely reflects a dilution caused by the SU. The same may apply to aNDFom and ADFom concentrations. Besides a dilution effect, a stronger acidic hydrolysis of hemicelluloses by acids [33] originating from microbial sucrose metabolism may have occurred during ensiling, consequently causing the lower aNDFom concentrations in SU LS. Nonetheless, proportions of aNDFom and ADFom were in a similar range of other LS [34,35]. Likewise, proportions of fibre fractions in fresh lucerne material were similar to previous findings [3,34,35].

The DML also affected CP content of LS, which was higher in 250 DML LS. This slight difference could have been caused by mechanical losses during harvest and consequently a lower leaf proportion in the ensiled plant material. Therefore, LS with higher DM contents seem to be favorable with regards to CP composition, but should not exceed a certain level to preserve the leaf fraction that dries faster than the stem part and thus is more prone to field losses [36]. The WI treatment also effected CP content, but the effect seems negligible as mean CP difference between LS with HI and LI was only 4 g kg^{-1} DM.

4.2. Crude Protein Fractions

Crude protein fractions in fresh lucerne material were in a typical range for this forage legume, although the present proportion of NPN compounds of 259 g kg^{-1} CP was substantially higher than literature data, i.e., 150 g kg^{-1} CP [3], 170-183 g kg^{-1} CP [37] and 180–190 g kg^{-1} CP [35]. It may be noted that the highest discrepancy in NPN proportion, found between the results of the present study and of Guo et al. [3], might partly also derive from different methodologies. In the present study, tungstic acid was used to precipitate TP, which cuts off peptides of an approximate chain length of more than three AA [17]. Guo et al. [3], however, used trichloroacetic acid to precipitate TP, which cuts off at about 10 AA [17].

The pattern of CP fractions in LS with NPN being the largest, and moderate ruminally degradable TP being the second largest proportion of total CP corresponded to the literature [3,35]. It is notable that NPN contents of present LS were similar to those from Broderick [2] and Seale et al. [10], but higher than values reported by others, for instance, 684 g kg CP^{-1} [3] or 599 g kg CP^{-1} [38] in untreated LS. However, these NPN values were determined after only 35 or 30 days of ensiling, respectively, probably underestimating NPN in LS as intrinsic protease and carboxypeptidase were recently shown to remain largely active for more than 30 days after ensiling [38]. Therefore, NPN values of the present LS, which were stored for 120 days, might provide a more realistic insight and should be considered when comparing different results or designing experiments for silage additive evaluation in LS. In this context, it may be noted that the Federal Working Group for Forage Preservation (Bundesarbeitskreis Futterkonservierung; [12]) recommends at least 90 days of ensiling for any silage-related experiment, e.g., when evaluating the effect of silage additives. Besides, the influence of the cut number should also be taken into account as NPN was 10% higher in third-cut LS when compared to NPN proportion of first-cut LS from the same sward [35]. Likewise, present LS was produced from a third cut, which thus may have been a contributing factor and should be investigated in future studies.

The SU reduced NPN along with increasing moderately ruminally degradable TP, which was likely caused by faster and stronger acidification, consequently suppressing proteolytic microorganisms in the silos [11]. These observations were in accordance with Seale et al. [10] who analyzed the effect of glucose and fructose addition with or without microbial inoculants on LS. However, in Italian ryegrass (*Lolium multiflorum* LAM.), Heron et al. [39] found that plant-derived proteases remained active over a wide pH range, which is also true for lucerne with major endopeptidases having optimum activities at pH 4 [40]. Thus, despite SU treatment and probably rapid acidification, the relevant plant-derived proteolytic activity may have taken place, particularly in 250DML LS.

An effect of WI was found for NPN concentration, which was higher in LI LS. Likewise, high-intensity wilted grass silages had approximately 100 g kg^{-1} CP lower NPN proportions compared to low intensively wilted grass silages [9] and, together with the present observations, demonstrate the TP stabilizing effect of HI treatments. The rapid inhibition of plant-derived proteases, which depend on sufficient water availability [5], may be causative. In this context, Owens et al. [41] produced LS with a DM of 350 g kg^{-1} and observed reduced NPN amounts of approximately 50 g kg^{-1} total N in those LS that needed shorter wilting periods to reach the desired DM, which thus can be ascribed to a higher WI. Likewise, when wilting times were different due to varying levels of shade during wilting, they also observed an increase of NPN with shade, thus substantiating the TP stabilizing effect of an HI treatment. However, it must be considered that although there is evidence for a reduction of plant-derived protease activity by HI treatment, it is very arguable whether plant enzymes were completely deactivated as the moisture loss was only until a DM content of maximal 350 g kg^{-1}. Thus, plant-derived proteases may still have contributed to overall proteolytic processes resulting in the still substantial conversion of TP to NPN in high-intensity wilted LS.

The DML treatment also effected NPN proportion, which was lower in 350DML LS. This confirmed previous findings [9,42] and may be explained by a lower water activity in the silos, consequently reducing microbial metabolism [34]. However, this mechanism should be even more pronounced at DM contents above 500 g kg^{-1} [9]. The effect of DML on slowly ruminally degradable TP may be of marginal importance as this fraction could not be determined in six of eight LS. The higher contents of moderately ruminally degradable TP in 350DML LS, however, may be beneficial regarding the quality of CP that is provided to the animal, meaning a decelerated ruminal CP degradation and therefore potentially improved N utilization by rumen microorganisms. As obtained for the TP proportion of 350HISU, the combination of HI, elevated DML and SU should have limited both plant-derived and microbial CP degradation and thus most effectively stabilized the TP content in the present study.

4.3. Amino Acids

A variety of factors influence the AA composition in silages, including wilting rate, acidification, and the microbial activity in the silo, but also plant-associated factors like tannin concentration or activity of plant proteases [5]. To the authors' knowledge, information about the effects of WI, DML, or SU on AA composition of LS is rare [3,43]. However, as the vast majority of AA is degraded in the rumen, knowledge on AA profiles seems to be more important for feedstuffs with high ruminally undegradable CP [9], which does not apply to the present LS. Though, it is worthy of remark that pre-ensiling treatments clearly effected the AA composition of LS. For instance, the higher proportions of free His, Asp, Lys, Thr, Glu, and Pro in 350DML LS should be the result of reduced microbial activity [5]. Likewise, a similar pattern was observed for total AA. The reducing effect of SU on total free AA content supported the TP preserving effect that was also observed for the distribution of CP fractions and should be caused by rapid acidification [5].

Biogenic amines are predominantly formed during proteolysis in silages [44] and Ohshima and McDonald [45] described the decarboxylation of Glu to GABA during lucerne ensiling, which is reflected by the lower Glu concentrations in the present LS without SU. As summarized by Scherer et al. [44], biogenic amines are associated with lower feed intake and potential impairments to animal health. Thereby, GABA is an important biogenic amine and known to act as a neurotransmitter. It is also involved in the sensation of pain and anxiety as well as neurological diseases [46]. Although there is no clear trend for the effect of GABA on feed intake [44], a negative correlation between feed intake and total amine concentration has been observed [47] and the reduced GABA concentration in LS with 350DML or SU may, therefore, be interpreted as beneficial; particularly also because of potential health risks when biogenic amines would be absorbed by host animals, who, however, are more susceptible under acidotic conditions [48].

4.4. Modified Hohenheim Gas Test

None of the pre-ensiling treatments had an effect on effective uCP values at any calculated passage rate. As Edmunds et al. [9] observed higher uCP values for fast wilted grass silages, the absence of any effect was not expected. Although artificially dried lucerne (90 °C for 3 min) showed reduced effective N degradability and degradation rates in the rumen [49], the WI achieved in the present study may not have been high enough to cause a similar impact. Moreover, the generally limited availability of WSC in all present LS may have prevented an effect on uCP as CP was degraded to ammonia-N, but not reused for microbial protein synthesis in the syringes.

Edmunds et al. [9] found that varying CP contents in grass silages from the same sward can confound the detection of possible effects on uCP as uCP values are calculated from the difference between N content in the syringe, which is determined by the CP content of the sample, and ammonia-N in the syringe. Therefore, these authors recalculated the effective uCP values with an average CP concentration. Thus, as the CP concentrations for the present LS also showed a variance, the effective uCP values were recalculated using the average CP content of LS with and without sucrose, respectively. Thereby, no effects of pre-ensiling treatments on uCP were obtained (data not shown). Moreover, a greater standard deviation might further impair the determination of clear effects. However, the inclusion of additional runs did not reduce standard deviation in the present study and thus were not included in the calculation of effective uCP.

4.5. Fermentation Pattern

The pH values were lower for SU LS, which was reflected by higher lactic acid concentration in these silages. Without the SU treatment, the high buffering capacity of lucerne [7] may have hindered rapid and strong acidification and consequently resulted in higher pH. In this context, the higher ammonia-N contents should also be considered, which can limit the pH drop in silages, as well [50]. Besides, low lactic acid concentration may be caused by metabolic activities of lactate-utilizing lactobacilli [51]. Owens et al. [7] stated a pH below 5.0 as a threshold to maintain forage quality and limit protein degradation in the silo, which thus was only met by SU LS. Likewise, the pH of these silages was within the common range for legume silages at this DM content [50]. Water-soluble carbohydrates [52] as well as total non-structural carbohydrates [7] decrease during wilting of lucerne due to plant enzyme activity and respiration, which are both reduced by moisture loss [53]. Therefore, there should have been less WSC degradation in the plant material undergoing HI treatment and consequently, a stronger pH drop along with increased lactic acid concentrations in HI LS was initially expected. However, the lack of a wilting effect may be explained by the overall very low WSC concentration of lucerne [6], which further was cut in the morning when WSC concentrations are again lower compared to the afternoon [7].

An impact of DM content on silage pH was often described in the literature [34,50]. Thereby, silages with DM contents below 300 g kg^{-1} are extremely susceptible to clostridial fermentation [50], which results in elevated pH values as well as high butyric acid concentrations. In the present study, however, DM content had neither an effect on silage pH nor on butyric acid concentration. Possibly the SU treatment superimposed a potential effect of DML, which is indicated by closer examination of butyric acid concentrations, which were numerically but not statistically significantly higher in 250DML LS. Moreover, the influence of DM content on clostridial fermentation and thus silage pH is more pronounced at DM contents of 400 g kg^{-1} or more [11], which is confirmed by the findings of Santos and Kung [34].

The lower ammonia-N concentration in SU LS further strengthens the assumption that addition of rapidly fermentable carbohydrates better inhibited degradation of nitrogenous compounds in these LS compared to LS without SU, and similar trends have been observed previously [10]. Regarding the impact of DML, ammonia-N concentration is generally higher in wet silages, which corresponds to present findings and is often ascribed to clostridial fermentation [50]. Likewise, reduced ammonia-N contents in LS with high DM contents were also observed by Santos and Kung [34]. Thus, a greater

WI seemed to preserve CP from degradation in the silo. However, according to Wyss et al. [35], ammonia-N proportions lower than 100 g kg^{-1} N is preferable for LS. This threshold was not met in the present study, even for 35HISU. In comparison to fresh-cut lucerne [35], plant material of the present study already showed a higher NPN proportion before ensiling, and may explain the high ammonia-N concentration in LS, irrespectively of applied pre-ensiling treatment. However, the increase of NPN from fresh-cut material to silage material were on the same level in the study by Wyss et al. [35] and the present study. Regarding the high ammonia-N concentration in the present LS, the variation of CP composition between different lucerne cultivars [54] should be considered, as well. Moreover, it can be speculated whether chopping of plant material subsequently supporting silo compression would have increased TP proportions as it was described earlier [50,55]. However, as LS was prepared according to recommended guidelines [12], the latter point may be of minor importance. Besides, higher ammonia-N concentration is assumed to be associated with undesirable metabolites like biogenic amines [50], which is in line with the present observations for higher GABA concentration in 250DML LS.

High concentration of acetic acid is associated with high DM and energy losses [50] as well as considerably reduced ad libitum feed intakes [56]. Compared to the literature [34,35], the acetic acid contents of LS in the present study can be classified as slightly high for 250DML LS and thus would negatively impact their nutritive value. Increased activity of Enterobacteriaceae [57], as well as increased deamination [5], could be causative for acetic acid formation, which is further favoured by high moisture contents [50] and in accordance with the higher acetic acid concentration in 250DML LS. However, the presence of acetic acid is not a disadvantage per se. The average 32 g kg^{-1} DM acetic acid in the 350DML LS, however, might be regarded beneficial as such concentrations have a positive effect on aerobic stability of silages [58].

A butyric acid concentration higher than 5 g kg^{-1} DM indicate elevated clostridial activity and due to high losses of energy, this means diminished energy supply to the animal and, consequently, performance may suffer [50]. This threshold was not exceeded for 350DML LS and only applies to 250HI and 250LI. Likewise, these two treatments also had the highest ammonia-N concentration, which further points to clostridial fermentation [50]. Together with the observation that 250HI and 250LI did also not meet the pH threshold for maintaining forage quality in the silo [7], these two LS should, therefore, be classified as poor-quality silages and potentially spoiled material.

Concerning ethanol, SU tended to increase this alcohol in the silages. Though, ethanol concentration was low for all LS and thus does not indicate elevated yeast metabolism [50,59]. Minor amounts of ethanol can also originate from heterofermentative lactic acid fermentation [60], which can never be fully prevented during ensiling. Weiß and Kalzendorf [52] observed higher concentrations of ethanol and ester compounds in LS with low DM contents and further postulated a positive correlation between ethanol and ester concentration in silages, which is both confirmed by the present findings for ethanol and ethyl lactate as well as ethyl acetate. The effect of esters in silages is not fully clear [47,61], but negative correlations to short-term DM intake were observed earlier [61]. Thus, despite lower NPN proportions in SU LS, the effect of SU on ester occurrence could be regarded as critical.

4.6. General Considerations

Up to now, WI has not received much attention in silage preparation and studies investigating the effect of different WI on silage characteristics are rare. It has been reported that wilting per se effects CP composition, for instance by reducing ammonia-N contents in LS [54]. Thus, applying HI treatments may even be more effective for stabilizing TP content in LS, which is underlined by the present findings that confirm our hypothesis of a TP preserving effect by the HI treatment. A variety of silage additives exists that limit proteolysis in LS [38]. However, they cause costs for acquisition, and in case of organic acids, also for maintenance of corroded machinery and concrete [62]. In contrast, HI treatment does not require additional application systems or further technical equipment and in this regard is an easy to apply tool for improving the quality of on-farm produced protein, and consequently

might help to reduce costs for ruminant diets and increase sustainability. Additionally, feed intake of wilted grass silage was increased when compared to non-wilted [63]. In case this also applies to LS, a superior energy and nutrient provision to the animal may be achieved by intensively wilting lucerne to higher DML; provided that mechanical losses during harvest do not exceed the benefits of HI. Regarding the practicality of HI treatment, if possible high solar radiation along with high wind speed should be present during lucerne harvesting. However, this cannot always be guaranteed, which restricts the practicability of HI treatments. To support the effect of rapid dehydration, maceration can be an effective addition to further increase the wilting rate [64] or to compensate weather conditions that may not be as ideal for HI as described in the present study. However, the risk of mechanical losses and thereby associated nutrient changes can be higher when using maceration [64], which needs to be taken into account. Besides, artificial drying treatments are surely a more weather independent option to obtain similar TP preservation [65] as here found for HI in the sun. However, increased production costs due to high energy demands may outweigh the beneficial effects [36] of artificial drying on CP composition.

Concerning the effects of SU on fermentation and CP quality, provision of rapidly fermentable carbohydrates is recommended. Particularly because an inoculation with lactic acid producing bacteria alone may not improve the situation as long as there is not enough easily accessible substrate for lactic acid fermentation [10]. Thus, relating to large scale on-farm conditions, mixing lucerne crop with molasses, crushed cereals, or high WSC forage species before ensiling may constitute a method for equivalently substituting SU treatment in LS. A delayed cutting of lucerne in order to increase non-structural carbohydrates, particularly starch, may not be appropriate as Owens et al. [7] did not find a protein preserving effect in LS differing in WSC content due to different cutting times during the harvest. However, present results revealed concerns about promoted ester and ethanol formation in SU LS that should be kept in mind and require further investigation.

Finally, the pre-ensiling treatment combination of all three factors, i.e., HI treatment to high DML with SU, has the strongest potential to reduce the extent of CP degradation during ensiling, thus improving the protein value and potentially increasing ruminal N retention, particularly when combined with an appropriate carbohydrate source.

5. Conclusions

The effect of WI in silage preparation has not received much attention thus far. However, the present findings underline the importance of HI to limit CP degradation in LS. Therefore, if possible, at harvest, HI should be considered during silage production with lucerne. Regarding the observed effects of SU, providing an additional carbohydrate source to lucerne crop before ensiling is effective to minimize TP degradation and improves silage fermentation quality. However, caution should be paid to volatile organic compounds when operating with SU. Combining an HI treatment to DML of 350 g kg^{-1} with the provision of rapidly fermentable carbohydrates will maintain higher TP proportions along with improving fermentation quality in LS. Otherwise, there is a high chance for poor quality LS that in consequence cannot be fed without the risk of impairing animal performance and health. In order to underpin the present findings and to expand the sparse knowledge on WI, it is necessary to investigate such pre-ensiling treatments over several growth cycles and to further examine if the beneficial effects observed at silage stage can be transferred to rumen fermentation and animals

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