Final report SLU EkoForsk Research Program

Slutrapport Ekoforsk project

Better nutritional value of forage to milk and beef production

Bättre näringsvärde av vallfoder till mjölk- och köttproduktion

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FOREWORD

The present paper is the final report of the EkoForsk project “Better nutritional value of forage to milk and beef production” where the main focus has been to evaluate the effect of prolonged pre-wilting time on the protein and fatty acid fractions in forage crops of grass and clover. The experiments have been performed at Kungsängen Research Station (SLU, Uppsala) and results have been presented at several international conferences and in national popular science magazines. One paper for scientific publication on fatty acid changes and one paper on protein fraction changes are under preparation.

The following publications are published so far.

T. Eriksson, R. Spörndly, and M. Knicky (2009). The effects of wilting time and dry matter on proteolysis and lipolysis in silage. In: Broderick G.A., Adesogan A.T., Bocher L.W., Bolsen K.K., Contreras-Govea F.E., Harrison J.H. and Muck R.E. (eds) Proceedings of XV International Silage Conference, Madison, USA, 2009, pp. 215–216. Madison, WI: U.S. Dairy Forage Research Center, USDA-Agricultural Research Service and University of Wisconsin-Madison, College of Agricultural and Life Sciences.

Spörndly, R. (2010). Effekter av förtorkningstid och ts-halt på ensilage. Svenska Vallbrev nr 3, p 2-3.

Knicky, M., Eriksson, T. and Spörndly, R. 2012. Fatty acid composition of a variety of forages before and after ensiling. In Kuoppala, K. et al (Ed) Proceedings of the XVI International Silage Conference, Hämennlinna, Finland. MTT Agrifood Research, University of Helsinki, Finland, pp 250-251.

Eriksson, T., Knicky, M. and Spörndly, R. 2012. Ammonia-N and α-amino-N in silage determined on either water extracts or solubilized freeze-dried samples . In Kuoppala, K. et al (Ed) Proceedings of the XVI International Silage Conference, Hämennlinna, Finland. MTT Agrifood Research, University of Helsinki, Finland, pp 264-265.

Knicky, M., Eriksson, T. and Spörndly, R. 2012. Effects of different wilting regimes and DM contents on fatty acid profile of fresh forage. In: Udén et al (Ed) Proceedings of the 3rd Nordic Feed Science Conference, Uppsala, Sweden. Report 280. Department of Animal Nutrition and Management. Swedish University of Agricultural Sciences, pp 49-52.

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SAMMANFATTNING

Vallfoder är den gröda som odlas till den största arealen i Sverige. Av jordbruksmarken är 53 % vallfoder (14 % bete och 39 % slåttervall). Det är det största fodermedlet i foderstaten till djur i mjölk-, nötkötts-, får- och getproduktionen samt till hästar och den allra största mängden av slåttervallen konserveras som ensilage. Vid ensilering utsätts näringsämnen som protein- och fettsyrafraktionerna för förändringar som till viss del sätter ned dess kvalitet. Proteinfraktionen utsätts för enzymatisk nedbrytning som resulterar i att den omvandlas till enklare kväveföreningar. Fettsyrafraktionen förändras på ett sätt som återverkar på köttet. Man har visat att kött från djur som betat håller en för människor mer positiv fettsyrasammansättning än djur som utfodrats ensilerat vallfoder på stall.

Inför ensileringen är det praxis att man för-torkar grönmassan för att gynna ensileringsprocessen. Man vill öka torrsubstanshalten från ca 20 % vid slåtter till 30-60 % beroende på vilken silo man ska lägga ensilaget i. Förtorkningen kan ta allt ifrån några timmar till flera dagar beroende på vädret och vilken metod man använt vid slåttern. Olika metoder att behandla den nyslagna grödan kan skynda på förtorkningsförloppet. Föreliggande studie syftade till att på ett systematiskt sätt kartlägga hur protein- och fettsyrafraktionerna förändras under förtorkningen då denna antingen görs på kort tid eller det krävs lång tid för att uppnå en högre ts-halt. Försöket lades upp efter följande matris:

Ts-halt	Tid på slag (förtorkning), tim			
(%)	0	12	24	48
20	x	x	X	x
40	-	x	X	x
60	-	-	X	x
80	-	-	-	x

Vi använde oss i första hand av de två arterna timotej och rödklöver som är dominerande i svensk vallodling i studien men vi undersökte också vitklöver, käringtand, rörsvingel, rajgräs och ängssvingel när det gäller påverkan på fettsyrorna. När det gäller proteinvärdet beräknades också påverkan på det beräknade proteinvärdet AAT_{20} som tillämpas i fodervärderingssystemet NorFor. Förutom tiden kan en stark bearbetning av grödan på skynda torkningen eftersom växtens skyddande vaxskikt påverkas. Det innebär emellertid också en öka syretillförsel som kan ha oxidativ effekt på bland annat fettsyror. Därför testades också en så kallad ”macerator”, en prototyp till en maskin som river sönder grödans struktur vid skörd för att öka förtorknings-hastigheten. Maskinen, en så kallad Comp de Comp” provades i samarbete med Vinnova på arterna timotej och rödklöver. Funktionen av Comp de Comp visade sig emellertid bristfällig så vidare utveckling lades ner av Vinnova.

Resultaten avseende förtorkningstiden och den uppnådda ts-halten för proteinfraktionen kan effekten sammanfattas med att förändringarna under förtorkningen var måttliga men att den uppnådda ts-halten hade stor effekt på det färdiga ensilaget som innebar att en ökad ts-halt gav sänkt andel lösligt kväve, α-aminokväve och ammoniumkväve, men samtidigt en ökad andel NDF-bundet kväve. Sätter man in dessa resultat i fodervärderings-systemet NorFor ger förtorkning ett kraftigt ökat AAT-värde. Det kan emellertid inte från denna studie dras slutsatsen att detta värde realiseras i utfodringen av kon.

Resultaten avseende förtorkningstiden och den uppnådda ts-halten på fettsyrafraktionen kan sammanfattas med att förändringarna som skedde under förtorkningen justerade i stor utsträckning under ensileringsfasen. Sammansättningen av de långa fettsyrorna (LCFA) efter ensileringen var likartad för gräs och rödklöver så att C16:0 och C18:0 ökade vid ökande ts-halt medan C18:3 sjönk. Den ensilerade grödan hade emellertid högre halt fria fettsyror och C18:3 jämfört med före ensileringen. Halten C18:3 skiljde mellan arterna där ängssvingel, rörsvingel och rajgräs hade högre

halter än rödklöver och vitklöver, rödklöver och timotej. En kraftigare fysisk hantering genom macerering med Comp de Comp sänkte halten C18:2 och C18:3 i ensilagen.

Sammanfattningsvis kan till rådgivningen sägas att utdragen förtorkning har mindre effekt på den slutliga ensilagekvaliteten vad beträffa protein- och fettsyrafraktionerna än vad tidigare ansetts. Däremot har den uppnådda ts-halten något större betydelse. Den starka positiva effekt på proteinkvaliteten som NorFor skattar kan dock ifrågasättas.

INTRODUCTION

Organic dairy and beef production in Sweden is based upon legume and grass leys, preserved as silage. From January 2008, ruminants in organic production has to be fed 100% organically cultivated feed (Standards for KRAV certified production, 2007). The limiting factor in dairy cow rations will then in many cases be the protein supply. Protein rich legume seeds such as peas, faba beans and lupins are possible to grow in some areas, but a lot of the organic dairy production is located in areas mostly suitable for forage production from legume-grass leys. Any improvement of protein utilization from these forage crops would have a great influence on the possibilities to maintain dairy production on home-grown feeds. As ensiling is the predominant forage preservation method, optimization of the protein value in silage has high priority. Until today, extensive investigations of chemical fractionation has mostly been done on the green plant material, whereas much less attention has been paid to the silage, actually fed. The studies made on fractionation of the crude protein component in silage suggest large differences between ensiling technologies (Broderick et al., 1999; Guo et al., 2007) and between species (Broderick et al., 2007). The recent development in ration formulation tools greatly improves the possibilities to include a more detailed feed characterization in ration formulation.

Ensiling technologies involving intensive mechanical conditioning at harvest have been proven to enhance the ensiling process by faster drop in pH and reduced NPN formation (Muck et al 1989). Such effects are also seen when these treatments take place just before ensiling at bag ensiling (Muck & Holmes 2003, Sundberg & Pauly, 2006). Older (Broderick et al., 1999) as well as new information (Ouellet et al 2006) show that maceration of forage provide better conditions both for ensiling and for ruminal degradation. The latter has until recently not been possible to consider in the ration formulations due to shortcomings in the feed evaluation systems.

Until the late 1980's, only the apparent digestion of feed N was taken into account when evaluating feeds for ruminants, Although the AAT/PBV system (Madsen, 1985, Madsen et al, 1995) increased the possibilities of a more biologically true description of feeds, the use of constants factors for ruminal degradability and microbial growth resulted in almost identical AAT value for all grass and legume forages (Fodertabeller för idisslare, 2003).

Computerized models, such as CNCPS (Sniffen et al., 1992; Fox et al., 2004) and NorFor Plan (NorFor, 2011), makes it possible to take into account many different feed fractions and also how they interact with rumen microbes to finally result in uptake of amino acids in the small intestine. The fractions are based on commonly available analytical methods, ammonia-N, soluble true protein (also including lower peptides and free amino acids), insoluble but degradable protein and undegradable protein in NorFor Plan. CNCPS also contains a slowly degradable fraction (NDF-N) whereas lower peptides and free amino acids in that system are included in the same fraction as ammonia. However, these models call for improvements of the feed data to be used. An in-depth investigation of silages produced from different crops and by different technologies has the potential to yield accurate data

that can be included in ration formulations. Special attention must be paid to the soluble protein fraction, because this fraction can escape rumen degradation and contribute substantially to the AAT value (Volden et al., 2002; Hedqvist & Udén, 2006; Reynal et al., 2007). Also the most slowly degradable protein fraction, the NDF-bound but available protein, which may constitute about 20% of total N in red clover silage (Broderick et al., 2007) deserves further studies.

Improving protein supply from silage could be accomplished either by restricting protein degradation or by sparing carbohydrates to facilitate higher microbial protein synthesis in the rumen. There is evidence to suggest that rumen undegradable protein (RUP), and in the end milk production, can be increased if silage fermentation is restricted by formic acid (Nagel & Broderick, 1992; Jaakola et al., 2006). At present, formic acid is allowed in organic farming (KRAV, 2016), but it is desired that chemical additives should be avoided. Pre-wilting has also a potential to reduce protein degradation because of a less extensive fermentation (McDonald et al., 1991). In a previous study at our laboratory (SLF project 0330054, 2006), N solubility in silage of ryegrass or red clover pre-wilted to about 45% DM was lowered to the same extent as for treatment of the un-wilted crop with formic acid (8 l/ton). Inhibitory metabolites in forage crops may also affect the extent of proteolysis, with condensed tannins (CT) as the most well-known example (Mueller-Harvey, 2006). Polyphenol oxidase (PPO) in red clover reduces protein degradation compared to lucerne silage (Broderick et al., 2007). Among our grass species, it has recently been discovered that cocksfoot has relatively high PPO activity (Lee et al., 2006). PPO is also assumed to have a role in restricting lipolysis in a way similar to its' action on proteolysis (Lee et al., 2006). Strategies aimed at reducing proteolysis would therefore also spare the long chain n-3 polyunsaturated fatty acids, which have been shown to have beneficial effect on human health (Simopoulos, 2000). Both initial proteolysis (Guo et al., 2007) and lipolysis (Elgersma, 2003) appear to mostly depend on plant enzymes, active during pre-wilting but also in the silo until conditions get unfavourable. Hence, a fast wilting would probably be most effective for reducing degradation in forage crops like timothy, without PPO activity (Lee et al., 2006), but mechanical treatment could perhaps have adverse effect by releasing proteolytic and lipolytic enzymes. In crops like red clover, with high PPO activity (Sullivan & Hatfield, 2006), it is more likely that maceration of the crop would increase the inhibitory effect by allowing contact between PPO and its'substrates.

The main scoop of this project is to investigate the effect of the wilting process on the protein and fatty acid fractions. All ensiled ley crops in Sweden are subjected to pre-wilting before being stored in a silo. The pre-wilting aims to reach a defined DM content of the harvested crop. The time it takes varies in time depending on the weather conditions. We want to separate the effect of wilting time and the effect of achieved DM content.

MATERIAL AND METHODS

Exp 1 – Effect of time and extent of pre-wilting on LCFA and protein fractions

Two experimental crops were each subjected to ten different treatments by combining four DM levels with four wilting times and excluding the impossible combinations of high DM level at short wilting time (Table 1). Because of the large differences in wilting properties between grasses and legumes, the same target DM levels and wilting times could not be used for both crops so wilting levels were classed as "None", "Moderate", "High" and "Extreme" whereas wilting times were classed as "None", "Short"; "One day" and "Two days".

Table 1 Experimental design of the study

DM contents	Time of wilting (h)			
(%)	0	12	24	48
20	x	x	x	x
40		x	x	x
60			x	x
80				x

Crops

Pure stands of red clover (cv Vivi) and timothy grass (cv Grindstad) grown near Uppsala, Sweden (18°E, 60°N) were used for the experiment. The red clover was established the previous year in experimental plots to later on be utilized for tillage experiments. The timothy was from a commercial farm producing grassland seed according to certified organic production regulations (KRAV, Uppsala, Sweden). The ley had been established five years earlier and the timothy stand was at experimental harvest thin with 5-10% dandelions (*Taraxacum spp.*)

Weather during experimental period.

Local weather data was recorded at the Ultuna climate station. The conditions were dry, with totally 15 mm precipitation during the month preceeding the experiment, the last rainfall occurring 12 days before the experimental harvest. During the three-day period of harvest and wilting, temperature was 16°C (5-26°C), relative humidity 57% (35-93%), wind speed 2.7 m/s (0.2-7.9 m/s), average solar radiation 327 W/m^2 and total daily solar radiation 28.2 MJ/m^2.

Development

The crops were cut on June 2, 2008 when the red clover was in an early flowering stage and the timothy in boot stage. Cutting was done without any conditioning of the material. Red clover was cut at 10.30 hrs with a Haldrup plot harvester (J. Haldrup a/s, Løgstør, Denmark) and timothy was cut manually at 14.00 hrs with a scythe. Cutting height was approx. 8 cm.

Triplicate samples in plastic bags, hereafter referred to as "Standing crop", were immediately taken from the cut material and kept in a cooling box until placing in freeze-storage 1 h later. Appropriate amounts for the experiment (approx. 50 kg DM of each crop) were then brought to Kungsängen Research Centre and subjected to the experimental treatments. The transport took about 30 min for the red clover and approx. 1 h 30 min for the timothy. Immediately at arrival, the experimental treatments with 0 h wilting time were processed. Green material for the treatments aiming to maintain harvest DM was put in a swath in the shadow and occasionally turned with a hayfork and dimmed with water. Wilting of green material for the other treatments was done on a lawn exposed to the sun. The lawn had been mown with a grass-collecting mower the day before. For all combinations of a certain DM at the shortest wilting time, the green material was placed in a thin layer, intended to correspond to a wide-spread crop during practical farming conditions. For the intermediate combinations of DM and wilting time, green material was transferred from the swath and wide-spread on the lawn in advance (3-33 h) of the ensiling. Dry matter content in the wilting material was monitored frequently by drying in a micro-wave oven and, if required, drying rate was regulated by turning with a hayfork or raking the material into a low swath.

When targeted DM and wilting time was reached, the wilted material for that treatment was coarsely chopped (5 cm), triplicate samples were frozen for later analysis and ensiling was done without additives in triplicate silos *of black PVC* with 4.5 L capacity. The crop was packet to the maximum possible density and varied depending on DM content of the crop from 122 kg DM m^{-3} for wettest red clover up to 416 kg DM m^{-3} for driest timothy crop. All silos were closed with water-seals and stored in a 21 °C climate chamber for 160-169 days when weight loss was recorded and the silos were opened. The silage was sampled in the same way as the wilted green material and samples were frozen for later analysis.

Sampling, preparation and analysis

Samples of approx. 200 g green material were before freezing weighed into tared bowls for lyophilisation. Samples were also prepared for determination of soluble components by weighing 100 g fresh material into ziplock bags and adding equal amounts of deionized water before freezing. In addition, green material was frozen intact. On silo opening, silage sampling was done in a similar manner as described for the green material. The silo contents were emptied into a separate plastic bag, mixed thoroughly and sampled. Samples were analysed for DM content, pH, ammonia-N (ASN 50-01/92 in FIA-system from FOSS-Tecator, 1992) content, fatty acids (lactic acid, acetic acid and butyric acid), ethanol, and 2.3-butanediol in silages. DM content was analyzed in the same way as with the fresh forage, excepting that a 1.4 % unit as a constant correction for silage volatiles was added to the final calculation. Silage pH was determined using a pH electrode (654 pH-meter Methrom AG, Herisau, Switzerland) in the silage juice. Concentrations of fatty acids, ethanol and 2.3-butanediol were determined from silage juice using HPLC according to (Andersson and Hedlund, 1983).

The freeze-dried samples were weighed immediately when removed from the freeze-drier to get the DM hereafter referred to as "Freeze-DM". After equilibration with air, the samples were weighed again and milled through a 1 mm screen on a Kamas hammer mill. Dry matter was determined by drying at 60°C (Norfor) for 16 h and, after weighing, continued drying at 103°C for 16 h. Ash determination was performed by incineration at 550°C for 3 h. Water soluble carbohydrates (WSC) were analyzed enzymatically (Larsson and Bengtsson, 1983).

Neutral detergent fiber (NDF) and acid detergent fiber (ADF) of the "standing crop" samples were analyzed according to Van Soest et al. (1991). All samples were then analyzed for NDF inclusive of ash (Chai & Udén, 1998) with the use of a heat-stable amylase.

Nitrogen fraction other than amino acid analyses

Total nitrogen in the freeze-dried samples was analyzed by a fully automated Kjeldahl procedure (Kjeltec 2700, Tecator, Höganäs, Sweden). In addition, a sub-set of wet samples stored frozen were analyzed for Kjeldahl N after thawing for comparison with results from the freeze-dried samples. The residues from NDF determination were used for analysis of NDF-bound N by the Kjeldahl method, as previously described. ADF-bound N was determined on a sub-set of 15 samples from each crop after boiling 1 g dry sample with 100 ml AD solution for 1 h in a Kjeldahl heating block. The solution was then filtered through Munktell 00H paper filter, filter papers were dried at 60°C overnight and then analyzed quantitatively for Kjeldahl N as previously described, with deduction of a paper blank.

Silage juice was obtained by hydraulic pressing of the zip-lock bags after thawing and puncturing them. Fermentation products in the juice were determined by HPLC (Ericson and André, 2010) and pH was measured. Soluble Kjeldahl N was determined in the supernatant after centrifugation at 13000

g for 5 min. Non-protein N was determined after precipitation with trichloracetic acid (concentration 50 g/L in the sample) for 30 min in ice water and subsequent centrifugation and Kjeldahl analysis of the supernatant as described for soluble N. Ammonia N and α-amino-N were determined simultaneously on a Technicon AutoAnalyzer (Broderick and Kang, 1980). These results were verified by Kjeldahl distillation on a subset of the silage samples.

For comparison of analysis on silage juice and water extracts from freeze-dried silage samples, 1.2 g freeze-dried sample were mixed with 40 ml deionized water, agitated for 60 min on a tube shaker/rotator with 360° vertical rotation at 21 rpm, centrifuged for 5 min at 1800 × g on a swing-out centrifuge before analysis om the AutoAnalyzer.

Amino acid analyses

Parallel samples of whole silage were freeze-dried in a CD 8 freeze-drier (HETO, Birkerød, Denmark) and milled on a hammer mill to pass a 1 mm screen whereafter 1.2 g sample was weighed into a 50 mL conical plastic centrifuge tube with 40 mL of deionized water. The tubes were agitated for 60 min on a tube shaker/rotator with 360° vertical rotation at 21 rpm, centrifuged for 5 min at 1800 × g on a swing-out centrifuge and the supernatant was decanted to 7 mL tubes, subsequently transferred to Eppendorf tubes and centrifuged for 5 min at 13000 × g. The solubilized samples, as well as centrifuged juice samples from hydraulic pressing, were then diluted with deionized water at ratios 1:4 and 1:19 (v/v), respectively. Sample were analyzed for NH3-N and α-amino-N on a Technicon AutoAnalyzer with phenol-hypoclorite and ninhydrin as main reagents (Broderick and Kang, 1980) and soluble N, peptides, NDF-N and ADF-N with standard methods described by Eriksson et al, (2004) and Hedqvist & Udén (2006). The results were recalculated to a per kg DM basis after DM determination at 60°C on the milled samples.

Fatty acid analyses

Dried and milled samples were used for lipids analyses. Lipids were extracted using the method described by Lourenco et al. (2007). Lipids of dry grass were extracted with chloroform:methanol (C:M) (2:1, vol:vol). Briefly, to 0.5 g of grass was added 3.75 mL of water and 10 mL of C/M. Sample was homogenized for 1 min and rinsed with 5 mL C/M. Samples were extracted overnight. The next morning, 5 mL of 0.8% KCl was added and samples were centrifuged at 4000 rpm for 10 min at 18°C and the C:M layer was recovered. In a second and third extraction step, 10mL of C:M were added and the samples were centrifuged again for every extraction step. The extracts were combined and washed once with distilled water and the C:M layer was recovered. Finally, the extracts were brought to a final volume of 1 mL with C:M (2:1, vol:vol).

The methylation of samples was performed according to the method described by Appelqvist (1968). Approximately 2 mg of lipids in 0.5 mL of hexane were mixed with 2 mL of 0.01 M NaOH in dry methanol. Samples were incubated at 60°C for 10 min and then 3 mL of BF_3 was added and samples were incubated at 60°C for another 10 min. Afterwards 2 mL of 20% NaCl and 2 mL of hexane were added and lipid phase was separated. The methylated fatty acids in this phase were then chromatographically separated on a BPX-70 fused-silica capillary column (50 m x 0.22 mm x 0.25 μm; J&W Scientific, Folsom, CA, USA) in a CP-3800 gas chromatograph (Varia AB, Stockholm, Sweden). Column temperature was programmed to start at 158°C for 5 min, then increase at 2°C/min to 220°C and remain at 220°C for 8 min. Injector and detector temperatures were 220°C and 250°C, resp. Fatty acids were identified by comparison of the respective retention times with the standard mixture GLC-461. Peak areas were integrated using STAR CHROMATOGRAPHY workstation

software version 5.5. Fatty acids were quantified using the internal standard methyl 15-methylheptadecanoate.

Calculations and statistical analysis

The analyzed N fractions and the corresponding calculated fractions according to the CNCPS and NorFor systems are in Table 2. All results are based upon analysis of all replicates (N = 120) with exception for ADF-bound N, where regressions for each sample category on NDF-bound N were developed from the 30 analyzed samples. Regressions were:

Red clover green chop and silage: $Y = 0.85\ X - 20$

Timothy green chop: $Y = 60$

Timothy silage: $Y = 0.13\ X + 30$

Where Y = ADF-bound N (g/kg N) and X = NDF-bound N (g/kg N)

Table 2. Protein (N) fractionation in the experiment and the corresponding fractions according to the current CNCPS and Norfor systems

Analyses in the experiment				CNCPS fraction	Norfor fraction
Total N	Water[1] soluble N	Not precipitable N	NH_3-N	PA1	sCP including NH_3-N
			a-amino-N	PA2	
		Precipitable N			
	Water[1] insoluble N	ND-soluble N		PB1	pdCP
		ND-insoluble N	AD-soluble N	PB2	
			AD-insoluble N	PC	iCP[2]

[1]Borat-phosphate buffer is recommended solvent but the agreement with water solubility is good for silage crops (Huhtanen et al., 2008).

[2]Reference method for Norfor is mobile bag digestibility of ruminal pre-incubated sample

Results were analyzed with procedure GLM in SAS 9.3 with wilting time and ensiling DM as class variables. Each replicate was considered an observation. The model had the main effects Crop (Timothy/red clover), Conservation (Green/ensiled), wilting level ("None", "Moderate", "High" and "Extreme") and wilting time ("None", "Short"; "One day" and "Two days") and all the interactions. As all effects were considered fixed and were tested against the error term, the test is not conservative.

The effects of wilting on NorFor predicted protein value were assessed by selecting a standard red clover silage and a standard timothy silage from the NorFor Feed Table. The analytical results for the extremes of red clover and timothy, namely the directly ensiled crops and the most wilted treatments were then applied to these silages in the NorFor Plan software and the differences in standard AAT_{20} value were taken as the wilting effect. To differentiate between AAT_{20} increase from improved carbohydrate supply after wilting and AAT_{20} increase from reduced ruminal protein degradation, the N fractionation results for the wilted samples were also applied to the directly ensiled crops. The remaining AAT_{20} difference was then ascribed to improved carbohydrate supply.

Exp II – Studies with different crops and maceration and the changes in LCFA

Crops and machines

Forage crops represented by rye-grass, meadow fescue, tall fescue, timothy, red and white clover, and bird's-food trefoil were manually harvested during the period 4-10 of June (first harvest) in the same area as in Exp 1. Forage crops were wilted in a drying cupboard at 25-27 °C to target dry matter (DM) level of approx. 350 g/kg. All crops were mechanically untreated except for red clover and timothy. These two crops were used in the study of effect of mechanical treatments on a crop composition. Accordingly, one half of each crop was wilted mechanically untreated and the second half was macerated with a new developed machine (CompdeComp), developed by Ecolag AB, Uppsala, Sweden with support of Vinnova and Forska & Väx (Dnr 2007-01084) prior to wilting. The same ensiling procedure was followed as in the first experimental year. Black PVC silos without additive treatments were stored in chamber room for 120 days at 21 °C.

Sampling and Analyses

Samples were collected at the harvest time, after the wilting and at the end of ensiling period, and kept frozen at -18 °C. Samples from period before wilting were extracted directly in the field at harvest and transported in cooled box. Wilting time for each forage crop was recorded.
Forages prior to ensiling were analyzed for the dry matter (DM) and ash concentrations. The same procedure of DM and ash determination was applied as in Exp I. The same procedure of sample extraction from silo and the same analyses to assess the silage quality as in Exp I were used.

Fatty acid analyses

The same procedure of lipids extraction (Lourenco et al., 2007) and fatty acid analyses (Appelqvist, 1968) as in Exp I were used.

Statistical analyses

Statistical analyses were performed using the GLM procedure of the SAS computer package (SAS, 1995). Analysis of variance in a completely randomized design was used to evaluate the effect of crop, ensiling stage, and mechanical treatment on forage composition. The mean of three treatment replicates was considered as the experimental unit. When the calculated values of *F* were significant, the t-test was used to interpret any significant differences among the mean values at a 0.05 probability level.

RESULT AND DISCUSSION

Exp I – Results concerning the time and extent of pre-wilting on the LCFA and protein fractions

Fresh crop

Basal composition of standing crop, green chop and silage is in Appendix 1 together with the actual wilting times and obtained DM concentrations. A higher initial DM content and better wilting rate of the timothy crop resulted in higher DM contents during shorter wilting times than planned (Tables 3, 4). The opposite was true for the red clover crop, where a lower initial DM content and less good

wilting ability of the crop resulted in that planned DM contents were not reached within planned wilting times. There was no significant influence of DM content nor wilting time on CP concentration in both timothy and red clover forages. On the other hand, an interaction effect of DM content and wilting time influenced concentration of ash and WSC in timothy forage. It can be seen in Table 3 that prolonged witling reduces WSC concentration in every DM contents. The timothy crop wilted for 45 hours to 75% DM content contained the lowest concentration of ash ($P<0.01$) but the highest concentration of WSC ($P<0.05$) amount the crops wilted for 45 hours. In red clover (Table 4), the concentration of WSC among 51 hours of wilting crops displayed similar trend of being

Table 3. Chemical composition of timothy forage wilted to four DM content levels (%) in four wilting periods (h) prior to ensiling.

DM contents	Wilting time	DM g/kg	Ash	WSC g/kg DM	CP
30	0	287.9	75.8	173.7	137.3
30	5	301.1	75.9	154.4	142.9
30	21	303.1	81.3	146.0	136.7
30	45	356.7	79.0	133.6	141.2
50	5	455.7	77.4	154.1	146.7
50	21	485.2	74.8	160.9	137.6
50	45	515.9	79.0	140.2	146.7
60	21	605.2	74.3	160.7	143.8
60	45	600.5	76.2	150.4	137.8
75	45	751.3	72.2	161.1	147.1
$LSD_{0.05}$			3.39	8.28	9.71
Probability	DM		***	***	NS
	Time		*	***	NS
	Int.		**	*	NS

*, ** and *** at $P<0.05$, $P<0.01$ and $P<0.001$, respectively; NS – not significant; DM – dry matter; WSC – water soluble carbohydrates; CP – crude protein.

Table 4. Chemical composition of red clover forage wilted to four DM content levels (%) in four wilting periods (h) prior to ensiling.

DM contents	Wilting time	DM g/kg	Ash	WSC g/kg DM	CP
16	0	147.8	100.2	96.5	226.3
16	9	144.6	103.4	100.5	223.5
16	29	156.8	104.0	86.7	221.8
16	51	158.1	106.7	81.6	226.0
30	9	265.4	100.0	103.8	221.7
30	29	281.4	104.3	97.8	224.7
30	51	316.8	105.0	93.5	226.8
46	29	440.5	100.3	97.6	220.0
46	51	455.7	105.4	95.7	223.5
62	51	604.9	97.9	106.8	216.3
$LSD_{0.05}$			3.36	16.73	8.64
Probability	DM		***	*	NS
	Time		***	NS	NS
	Int.		NS	NS	NS

*, ** and *** at $P<0.05$, $P<0.01$ and $P<0.001$, respectively; NS – not significant; DM – dry matter; WSC – water soluble carbohydrates; CP – crude protein.

highest in 62% DM content. Wilting of red clover for 29 and 51 hours increased concentration of ash in every DM content, except for 62%. These results can be explained by undesirable activity of enzymes whose activities are seems to be promoted by moist condition. With increasing crop moisture a higher degradation of WSC or other substances (t.ex. hemicelluloses, starch) occur and causing increase in ash concentration.

Fatty acid composition in fresh crop

A faster wilting process was probably the reason for limited changes in LCFA composition in timothy forage (Table 5). The increase in forage DM content from 30% to 50% within five wilting hours was not accompanied by any changes in LCFA profile. However, at a DM content of 62% the content of C16:0 and C18:1 was lower in comparison with initial contents. When timothy had reached 75% DM content, the proportion of C18:0 was reduced ($P<0.05$). On the other hand, the proportion of C18:3 increased as the crop reached higher DM contents ($P<0.001$). This result contrasts previous observations (Dewhurst and King, 1998; Van Ranst et al., 2009), where wilting reduced proportion of C18:3 in forage. On the other hand, Arvidsson et al. (2009) observed no

Table 5. Fatty acid (FA) composition (g/100g FAME (fatty acid methyl esters)), FA content (mg/g DM), and crude fat content (g/kg DM) in timothy forage wilted to four DM content levels (%) in four wilting periods (h).

DM contents	Wilting time	C16:0	C18:0	C18:1	C18:2	C18:3	Total FA	Crude fat
30	0	18.2	1.8	3.5	20.1	51.7	17.0	25.8
30	5	18.3	1.9	3.3	19.6	52.1	14.3	26.0
30	21	18.1	1.8	3.4	19.6	52.0	15.3	29.7
30	45	18.4	1.9	3.4	20.6	50.4	12.7	28.1
50	5	17.8	1.8	3.8	20.8	51.1	15.6	26.4
50	21	18.5	1.8	3.3	20.5	51.0	15.5	27.3
50	45	18.2	1.9	2.8	20.0	51.6	11.3	27.2
60	21	16.6	1.9	2.7	19.1	55.5	18.1	34.7
60	45	17.6	1.8	2.8	21.0	51.9	11.5	26.9
75	45	16.2	1.7	2.6	19.7	55.2	15.1	28.5
$LSD_{0.05}$		0.81	0.12	0.68	1.56	2.76	4.93	2.29
Probability	DM	***	*	*	NS	***	NS	***
	Time	NS	NS	NS	NS	NS	**	***
	Int.	NS	NS	NS	NS	NS	NS	***

*, ** and *** at $P<0.05$, $P<0.01$ and $P<0.001$, respectively; NS – not significant; DM – dry matter.

wilting effect on proportion of C18:3 in timothy forage. A possible explanation can be a higher initial DM content of timothy in this study which limited the activity of lipases. In addition, the crop was not mechanically treated, which could contribute to reduce the activity of lipases (Elgersma et al., 2003). There were no differences in FA content in timothy among wilting times within 30 and within 50% DM content, which indicates limited degradation of LCFA due to longer wilting times. A lower initial DM content and with that an associated higher activity of plant lipases, were probably a reason for large changes in LCFA composition in red clover during wilting compared to timothy (Table 6). Unexpectedly, there was no variation in total FA content in the red clover, but proportions of C18:3 were influenced by an interaction of DM content and wilting time ($P<0.01$). A reduced proportion of C18:3 was observed due to extended wilting (51 h) or increasing DM content up to 46%. The proportion of C18:3 in the red clover crop containing 62 % DM was however similar to the initial proportion. In the crop containing 16 and 30% DM content, the proportion of C18:1 decreased ($P<0.001$) during the entire wilting time. Proportion of C18:1 in the red clover crop containing 46 and 62% DM was lower ($P<0.03$) than the initial proportion. In

contrast to timothy, the trend of increased proportions of C16:0 and C18:0 with increasing DM content was observed in the red clover crop, except for C16:0 at 62% DM content. Moreover, extended wilting time (51 h) increased the proportions of C16:0 ($P<0.05$) and C18:0 ($P<0.001$) compared to initial proportions.

Table 6. Fatty acid (FA) composition (g/100g FAME (fatty acid methyl esters)), FA content (mg/g DM), and crude fat content (g/kg DM) in red clover forage wilted to four DM content levels (%) in four wilting periods (h).

DM contents	Wilting time	C16:0	C18:0	C18:1	C18:2	C18:3	Total FA	Crude fat
16	0	18.3	2.6	2.1	15.4	57.4	17.9	44.1
16	9	18.4	2.5	1.8	15.2	57.7	20.1	40.7
16	29	18.0	2.5	1.5	14.7	58.9	20.7	43.4
16	51	19.1	2.7	1.6	15.2	56.4	15.9	41.0
30	9	19.3	2.8	2.0	16.3	55.0	21.1	39.8
30	29	18.9	2.7	1.6	15.7	56.4	18.6	41.5
30	51	19.4	2.7	1.8	16.5	54.7	18.1	39.5
46	29	19.4	2.8	1.7	16.3	55.2	17.6	41.0
46	51	19.2	2.7	1.7	16.0	55.8	17.6	41.0
62	51	18.7	2.7	1.6	15.5	56.7	17.0	41.5
$LSD_{0.05}$		0.67	0.12	0.16	0.87	1.42	5.20	3.79
Probability	DM	***	***	***	*	***	NS	NS
	Time	NS	NS	**	NS	*	NS	NS
	Int.	*	***	NS	NS	**	NS	NS

*; ** and *** at $P<0.05$. $P<0.01$ and $P<0.001$; respectively; NS – not significant; DM – dry matter.

Fermentation quality of silages

Qualitative parameters of timothy silages are presented in Table 7. There is clear evidence of reduced fermentation intensity with increased DM level of the crop (McDonald et al., 1991). Silages ensiled at 30% DM level were found to have significantly elevated formation of all fermentation products accompanied by significantly lower pH in comparison with the rest of silages ensiled at higher DM levels. On the other hand, concentration of residual WSC in 30 % DM silages was significantly lower than in the rest of silages. Results also showed a high susceptibility of 30% DM silages to undesirable fermentation. High concentration of butyric acid accompanied by a high formation of ammonia-N indicates clostridial activity (Pahlow et al., 2003). Furthermore, high concentration of 2.3 butanediol indicates predominantly undesirable activity of enterobacteria (Pahlow et al., 2003) in these silages. Ethanol is another product of silage fermentation indicating mainly undesirable activity of yeasts (McDonald et al., 1991). Yeasts are known to be resistant to low pH and water availability (McDonald et al., 1991), therefore, it is not surprising that the reduction in ethanol formation at 50% and 60% DM silages was not as remarkable as in other fermentation products. What is however surprising, how wilting time affected the ethanol formation in timothy silages. It is known that wilting cause increases in yeasts count in fresh forage (McDonald et al., 1991) and therefore it would be expected to have a highest yeasts grow problem and consequently highest ethanol content in silages with longest wilting time. The opposite was true in this study. Timothy silages wilted for 45 hours at DM levels 30 and 50 % contained significantly less ethanol than silages of same DM levels wilted for 5 and 21 hours. However, when DM level of forage reached 60%, expected increase ($P<0.001$) of ethanol concentration in 45 hours wilted silages in comparison with 21 hours wilted silages was observed. The effect of wilting was promoted in interaction with effect of DM also on ammonia-N and butyric acid concentrations in silage. Wilting of 30% DM containing timothy forage for 21 and 45 hours gradually reduced butyric acid formation but increased ammonia formation in silages. At 50%

DM level, only 45 hours wilting caused significant increase in ammonia-N silage concentration. In addition, wilting of 30% DM containing timothy forage increased 2.3 butanediol formation.
Ensiling characteristics of red clover silages are illustrated in Table 8. The same as for timothy silages, fermentation intensity was associated with DM content of the crop (McDonald et al., 1991).

Table 7. Chemical composition of timothy silages wilted to four DM content levels (%) in four wilting periods (h).

DM contents	Wilting time	DM	pH	WSC	Lactic acid	Acetic acid	2.3 buta-nediol	Ethanol	Butyric acid	NH_3-N
		g/kg					g/kg DM			g/kg TN
30	0	264.1	4.5	83.3	31.0	3.3	11.6	19.1	17.4	85.7
30	5	273.4	4.6	64.4	26.9	3.5	16.4	20.5	18.8	91.1
30	21	274.9	4.6	27.4	28.7	5.2	15.4	20.2	16.8	96.0
30	45	323.3	4.7	28.1	31.1	5.2	15.3	15.9	10.9	103.6
50	5	427.1	5.4	138.9	5.8	1.9	1.4	17.4	0.1	41.1
50	21	471.5	5.4	125.9	5.1	1.6	0.4	18.9	0.2	43.0
50	45	480.1	5.5	114.4	6.3	1.8	0.4	14.5	0.0	50.2
60	21	556.4	5.4	139.8	3.9	1.2	0.6	10.6	0.0	31.8
60	45	584.3	5.5	110.8	4.7	2.8	0.9	19.9	0.0	31.5
75	45	718.9	5.4	134.5	2.3	0.6	0.2	5.8	0.0	16.2
$LSD_{0.05}$			0.09	6.21	2.92	1.78	2.37	2.48	1.68	4.48
Probability	DM		***	***	***	***	***	***	***	***
	Time		*	***	*	NS	**	NS	***	***
	Int.		NS	***	NS	NS	NS	***	***	*

*, ** and *** at P<0.05. P<0.01 and P<0.001, respectively; NS – not significant; DM – dry matter; WSC – water soluble carbohydrates.

Table 8. Chemical composition of red clover silages wilted to four DM content levels (%) in four wilting periods (h).

DM contents	Wilting time	DM	pH	WSC	Lactic acid	Acetic acid	2.3 buta-nediol	Ethanol	Butyric acid	NH_3-N
		g/kg					g/kg DM			g/kg TN
16	0	134.1	4.5	2.3	99.1	28.7	12.1	15.8	11.2	181.0
16	9	139.7	4.6	0.9	98.0	35.0	12.1	10.3	4.5	195.0
16	29	146.8	4.6	1.1	93.1	31.0	16.3	11.4	8.6	179.8
16	51	154.8	4.9	2.5	81.5	38.3	13.5	10.8	12.9	196.9
30	9	241.9	4.8	4.2	75.7	26.0	33.0	13.5	1.1	182.5
30	29	274.6	4.6	22.9	68.3	13.7	12.8	9.1	0.0	114.0
30	51	315.4	4.9	50.4	44.0	8.9	20.3	8.7	0.0	104.3
46	29	434.0	5.3	108.7	5.4	1.6	0.8	5.3	0.0	27.6
46	51	467.2	5.3	85.5	3.1	1.5	0.4	10.5	0.0	32.7
62	51	596.3	5.3	87.6	0.4	0.7	0.4	6.9	0.0	17.7
$LSD_{0.05}$			0.17	23.50	9.08	5.68	8.81	4.00	4.35	25.02
Probability	DM		***	***	***	***	***	NS	***	***
	Time		***	NS	***	***	NS	**	NS	***
	Int.		*	**	**	***	**	*	*	***

*, ** and *** at P<0.05. P<0.01 and P<0.001, respectively; NS – not significant; DM – dry matter; WSC – water soluble carbohydrates.

The similar DM level where fermentation was reduced as in timothy silages was observed in red clover silages. Silages above 30 % DM level (46 and 62%) were found to have restricted fermentation in comparison with those in 30 and 16% DM. A higher silage pH (P<0.001), higher concentration of residual WSC (P<0.001), but a lower formation of fermentation products such as lactic acid (P<0.001), acetic acid (P<0.001) can serve as evidence of that. Also susceptibility of silages to form undesirable fermentation products such as 2.3 butanediol or ammonia-N was significantly reduced in silages above 30 % DM. These silages were also free of butyric acid, but unlike to timothy silages, butyric acid was not formed either in 30% DM silages. Ethanol as another undesirable product of fermentation revealed a similar pattern as in timothy silages. No wilt at 16% DM level and 9 hours wilting period in 30% DM resulted in silages with a higher ethanol formation than in silages with longer wilting periods in particular DM level. When the DM content of crop reached 46%, longer wilting period resulted in increased formation of ethanol in silages. At 16% DM level, the longest wilting period of 51 hours caused significantly higher silage pH (P<0.03) and acetic acid concentration (P<0.001), but lower lactic acid concentration (P<0.01) in comparison with silages in shorter wilting periods. At 30% DM level, extended wilting from 9 hours to 51 hours resulted in silages with significantly lower lactic acid, acetic acid, 2.3 butanediol, ammonia-N, and a higher concentration of WSC.

Fatty acid composition in silages

The fatty acid composition of timothy silages is presented in Table 9. Fatty acids such as C18:1 and C18:2 with limited variation before ensiling displayed no significant changes in silages as well. On the other hand, the effect of DM was more pronounced in proportion of C18:0 in silages than in crop prior to ensiling and caused significant increase in 60% and 75% DM silages in comparison with 30% DM silages.

Table 9. Fatty acid (FA) composition (g/100g FAME (fatty acid methyl esters)), FA content (mg/g DM), and crude fat content (g/kg DM) in timothy silages wilted to four DM content levels (%) in four wilting periods (h).

DM contents	Wilting time	C16:0	C18:0	C18:1	C18:2	C18:3	Total FA	Crude fat
30	0	16.5	1.8	3.0	20.5	53.9	24.5	34.9
30	5	16.6	1.8	3.0	20.4	53.7	27.5	37.9
30	21	16.6	1.7	3.1	21.0	52.8	24.8	37.0
30	45	16.9	1.8	3.0	20.9	52.2	23.5	37.9
50	5	18.3	1.9	3.4	20.6	51.1	21.4	38.3
50	21	17.9	1.9	3.0	20.1	52.0	20.6	38.1
50	45	18.0	2.0	2.8	20.7	51.3	18.3	37.2
60	21	17.7	1.9	2.9	20.0	52.4	16.7	35.5
60	45	17.2	1.9	2.8	21.1	51.8	16.9	36.8
75	45	17.5	2.0	3.0	20.8	51.5	15.1	31.9
$LSD_{0.05}$		0.50	0.15	0.48	1.05	1.56	2.74	3.41
Probability	DM	***	**	NS	NS	**	***	***
	Time	***	NS	NS	NS	NS	**	NS
	Int.	NS	NS	NS	NS	NS	NS	NS

*, ** and *** at P<0.05, P<0.01 and P<0.001, respectively; NS – not significant; DM – dry matter.

The effect of DM content affected the proportion of C16:0 where significantly highest proportion was found in silages at 50% DM level whereas the lowest C16:0 proportion possessed silages at 30% DM. A lower (P<0.01) proportion of C18:3 in 50% DM silages than in 30% DM silages was observed. Total fatty acid concentration was gradually reduced as DM content of the crop increased being

lowest in 60 and 75% DM contents. Silages in 75% DM content contained the lowest (P<0.001) concentration of crude fat.

Stronger effects of DM content and wilting periods on fatty acid composition in red clover silages than in timothy were observed (see Table 10). Up till 46% DM content, proportions of C16:0 and C18:0 increased whereas C18:3 decreased as DM content increased. In comparison with silages from shorter wilting periods, silages wilted for 51 hours contained significantly a higher proportion of C16:0 in 16% and 46% DM content; a higher proportion of C18:0 in 16% DM silages; and a higher proportion of C18:1 in all DM contents, except for 62 % DM. Any wilting in 16% DM content resulted in silages with significantly lower C18:2 proportion than silage in not-wilted period, 51hours wilting period caused the lowest C18:2 proportion of all silages. From 46% DM content, significant reduction of total FA and crude fat concentrations in silage was observed.

Table 10. Fatty acid (FA) composition (g/100g FAME (fatty acid methyl esters)), FA content (mg/g DM), and crude fat content (g/kg DM) in red clover silages wilted to four DM content levels (%) in four wilting periods (h).

DM contents	Wilting time	C16:0	C18:0	C18:1	C18:2	C18:3	Total FA	Crude fat
16	0	17.2	2.3	1.8	17.2	57.3	28.1	65.0
16	9	17.6	2.3	1.5	15.9	58.5	24.5	62.0
16	29	16.9	2.3	1.2	16.1	59.2	23.2	60.2
16	51	18.6	2.7	1.3	14.6	58.4	21.3	63.0
30	9	19.0	2.8	1.6	15.9	56.0	26.1	66.8
30	29	17.4	2.8	1.6	17.4	56.9	22.4	65.7
30	51	18.3	2.9	2.3	16.4	54.6	22.6	61.5
46	29	18.9	2.9	2.0	17.0	54.1	19.3	49.2
46	51	20.0	3.1	2.6	16.2	52.0	20.6	57.9
62	51	19.4	3.2	1.9	16.8	53.8	18.2	44.1
$LSD_{0.05}$		0.61	0.23	0.39	0.90	1.67	4.09	7.37
Probability	DM	***	***	***	***	***	*	***
	Time	***	***	***	***	**	**	NS
	Int.	**	NS	**	*	NS	NS	NS

*, ** and *** at P<0.05, P<0.01 and P<0.001, respectively; NS – not significant; DM – dry matter.

Statistical comparison of crops prior to ensiling and silages in respect to DM content (Tables 11, 12) revealed than silage fermentation significantly affected fatty acid profile. In comparison with crops before ensiling, silages from both forages contained a higher concentrations of all fatty acids (P<0.001) as well as crude fat (P<0.001). This effect was more obvious in silages of a lower DM contents, particularly in timothy silages up to 50% DM. A possible explanation of this phenomenon could be silage losses during fermentation. Silage losses arise from microbial activities during fermentation and their magnitude is therefore related to the prevailing microbial processes in silage. It has been shown that in silages with domination of undesirable microbes such as clostridia, silage losses can be greater (McDonald et al., 1991). This was the case of low DM silages in present study, those with higher FA concentrations. As fermentation losses account for DM losses, loss one DM constituent (WSC, CP fractions) causes proportional increase another, in this instance crude fat with fatty acids. The increased crude fat content in silages in comparison with fresh forage was found early (Arvidsson et al., 2009, Boufaïed et al., 2003), but increased FA content is a new finding.

Table 11. Comparison of fatty acid (FA) composition (mg/g DM), FA content (mg/g DM), and crude fat content (g/kg DM) between timothy forage prior to ensiling (fresh) and timothy silages wilted to four DM content levels (%).

Forage status	DM contents	C16:0	C18:0	C18:1	C18:2	C18:3	Total FA	Crude fat
Fresh	30	2.7	0.3	0.5	3.0	7.6	14.8	27.4
Fresh	50	2.6	0.3	0.5	2.9	7.2	14.1	27.0
Fresh	60	2.5	0.3	0.4	2.9	8.1	14.8	30.8
Fresh	75	2.4	0.3	0.4	3.0	8.3	15.1	28.5
Silage	30	4.2	0.4	0.8	5.2	13.3	25.0	36.9
Silage	50	3.6	0.4	0.6	4.1	10.4	20.1	37.9
Silage	60	2.9	0.3	0.5	3.4	8.8	16.8	36.2
Silage	75	2.6	0.3	0.5	3.1	7.8	15.1	31.9
$LSD_{0.05}$		0.74	0.08	0.18	0.88	2.56	4.50	3.68
Probability	DM	***	***	***	***	***	***	*
	Status	***	***	***	***	***	***	***
	Int.	**	**	NS	***	***	***	***

*, ** and *** at P<0.05, P<0.01 and P<0.001, respectively; NS – not significant; DM – dry matter.

Table 12. Comparison of fatty acid (FA) composition (mg/g DM), FA content (mg/g DM), and crude fat content (g/kg DM) between red clover forage prior to ensiling (fresh) and red clover silages wilted to four DM content levels (%).

Forage status	DM contents	C16:0	C18:0	C18:1	C18:2	C18:3	Total FA	Crude fat
Fresh	16	3.4	0.5	0.3	2.8	10.8	18.6	42.3
Fresh	30	3.7	0.5	0.3	3.1	10.6	19.3	40.3
Fresh	46	3.4	0.5	0.3	2.8	9.8	17.6	41.0
Fresh	62	3.2	0.5	0.3	2.6	9.7	17.0	41.5
Silage	16	4.2	0.6	0.4	3.9	14.2	24.3	62.5
Silage	30	4.3	0.7	0.4	3.9	13.2	23.7	64.7
Silage	46	3.9	0.6	0.5	3.3	10.6	20.0	53.6
Silage	62	3.5	0.6	0.3	3.1	9.8	18.2	44.1
$LSD_{0.05}$		0.87	0.13	0.13	0.82	2.87	4.88	6.04
Probability	DM	*	NS	NS	*	***	**	***
	Status	***	***	**	***	**	***	***
	Int.	NS	NS	NS	Ns	NS	NS	***

*, ** and *** at P<0.05, P<0.01 and P<0.001, respectively; NS – not significant; DM – dry matter.

Protein fractions in silages

Kjeldahl N concentration analyzed in freeze-dried samples from green material and silage of both crops at lowest DM and shortest wilting time was 0.94 of the level when the corresponding wet samples were analyzed ($P < 0.01$; $n = 12$). This implies that the total N level for the entire experiment may be slightly underestimated and that the proportion may be slightly overestimated for the fractions analyzed in the liquid phase (soluble N, NH_3-N, α-amino-N). However, relative treatment effects should be unbiased.

Both NH_3-N and α-amino-N had positive slopes when results from water extracts of fresh samples were compared to water extracts of freeze-dried samples (Figure A). The largest difference between methods for a treatment mean (n = 3) was 0.55 g kg/DM lower NH_3-N for freeze-dried samples than for pressed juice, and for α-amino-N, the largest difference was 1.69 g/kg DM.The total N from the green crop was quantitatively recovered after ensiling (Figure B), while the ratio N:ash had a slope less than unity (P< 0.001)

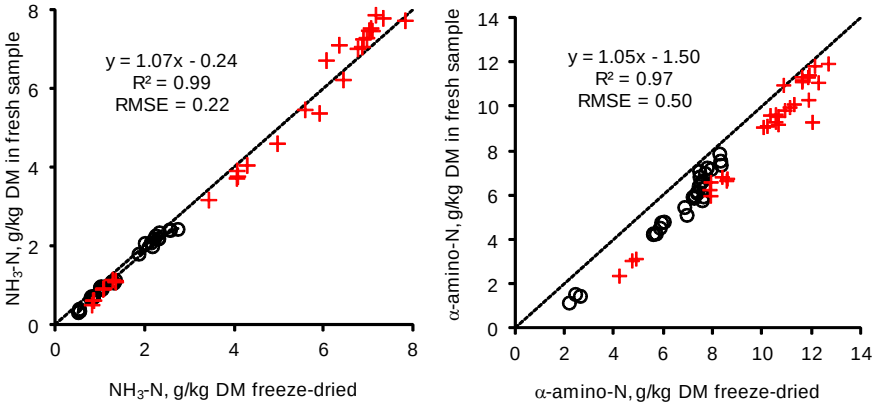

Figure A. Concentrations of silage N fractions determined in water extracts obtained either from fresh samples or from freeze-dried and milled samples of timothy (O) or red clover (+). Dashed line represents y = x. N = 60.

The complete analytical results on N fractionation are in Appendix 2 and the analyzed and calculated CNCPS fractions are in Appendix 3. There were limited effects of wilting on N transformations in the green chop, the most pronounced effect being an increase in soluble N proportion when the two lowest DM levels were maintained in the red clover (Figure C). This effect of wilting was leveled out during ensiling, where the major N transformations occurred as an effect of DM (Figure D, Table B). Water soluble N proportion was reduced from 580 g/kg N to 193 g/kg N in timothy and from 662 g/kg N to 268 g/kg N in red clover by wilting. There was a curvilinear relationship between water soluble N and DM in timothy but a linear relationship in red clover (Figure D).

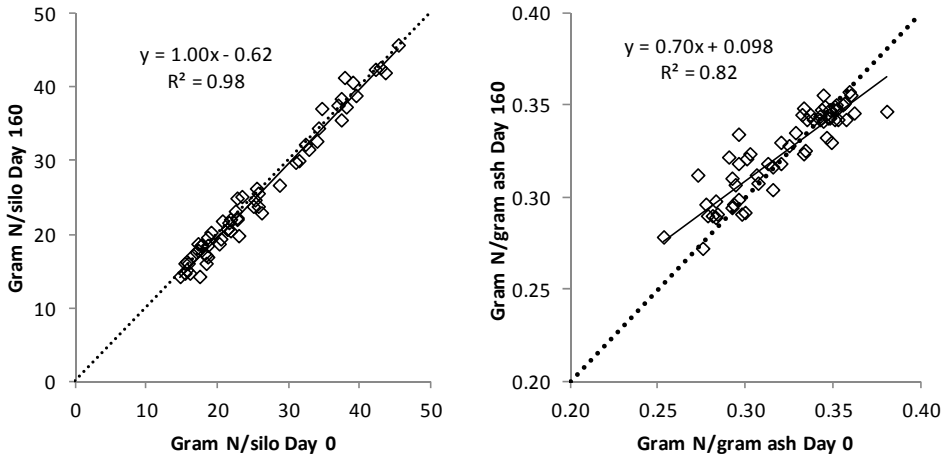

Figure B. Quantitative recovery of N in ensiled crops (left) and corresponding change in the ratio N:ash. Dashed line is y =x.

When water soluble N proportion was regressed against the amounts of analyzed fermentation products in the silage rather than against ensiling DM, the same logarithmic function could be fitted to both red clover and timothy data (Figure E).

The NH_3-N and α-amino-N fractions responded relatively similarly when comparing timothy and red clover (Figure D, Table 13) with declining proportions when DM increased. However, the much lower initial DM concentration in the red clover was coupled to a much larger NH_3-N proportion in the red clover silage with lowest DM than in the timothy silage with the lowest DM. The NDF-bound N fraction increased more in the timothy silage than in the red clover silage (Figure D).

When analytical data was entered into the NorFor model, AAT_{20} for red clover increased from 69 to 98 g/kg DM when comparing directly ensiled silage with the most wilted silage (Figure F). The corresponding increase for timothy silage was from 92 to 106 g AAT_{20}/kg DM. For the red clover silage, the largest contribution was from reduced ruminal protein degradation because of less proportion soluble N. For timothy, reduced ruminal protein degradation and improved carbohydrate supply contributed equally to the increased AAT_{20} value.

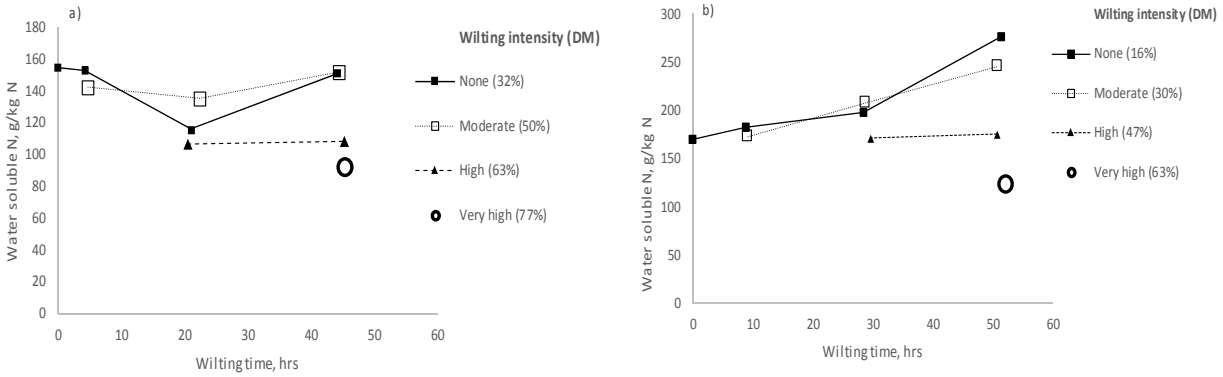

Figure C. Changes in water soluble N fraction of the green chop during wilting of timothy (a) and red clover (b), respectively. Each marker is the mean of three observations from a certain ensiling DM obtained after one of four wilting times from 0 hours to two days.

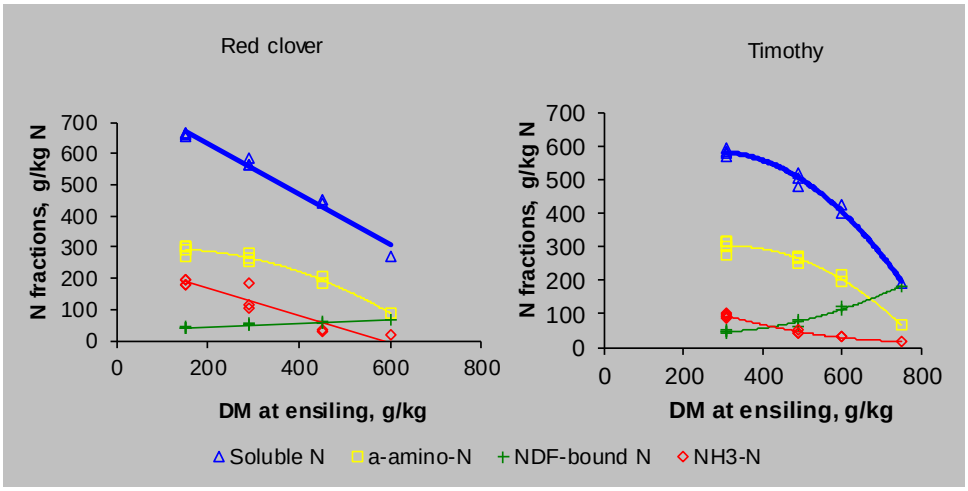

Figure D. Analyzed N fractions in silage of red clover and timothy. Each marker is the mean of three observations from a certain ensiling DM obtained after a certain of four wilting times from 0 hours to two days. Markers within an ensiling DM are in some cases overlapping.

Table 13. Coefficients for regressions of N fractions (g/kg N) against DM (g/kg) in red clover and timothy

	Intercept	Linear	Quadratic	R^2
Red clover				
Water soluble N, g/kg N	794	-0.814		0.976
α-amino N, g/kg N	284	0.184	-0.0008	0.971
NDF-bound N, g/kg N	34	0.059		0.936
NH_3-N, g/kg N	254	-0.439		0.876
Timothy				
Water soluble N, g/kg N	440	0.996	-0.0018	0.987
α-amino N, g/kg N	169	0.819	-0.0013	0.972
NDF-bound N, g/kg N	76	-0.265	0.0005	0.978
NH_3-N, g/kg N	228	-0.539	0.0003	0.973

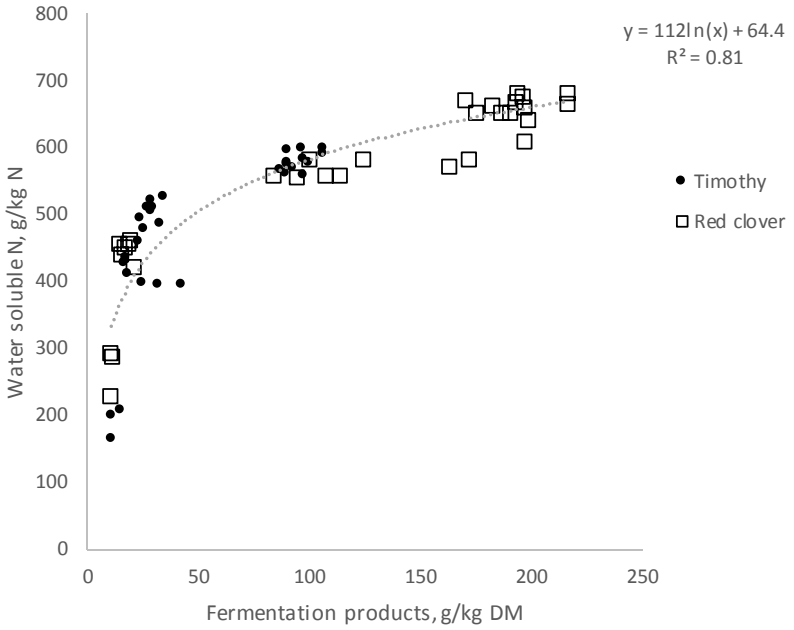

Figure E. Water solubility of N (g/kg N) in timothy and red clover silage, respectively, as a function of fermentation intensity.

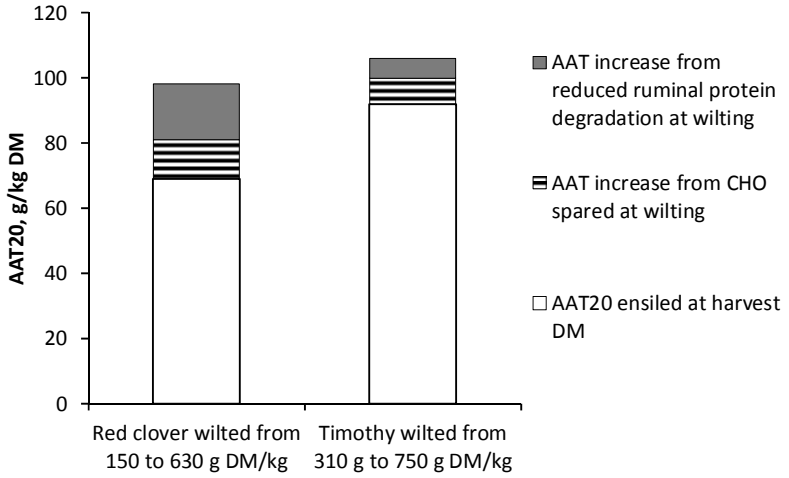

Figure F. Predicted increase in protein value (AAT_{20}) according to the NorFor system by wilting red clover and timothy.

Discussion concerning the time and extent of pre-wilting on the protein fractions

The dry matter concentration at ensiling had far more influence than the wilting time on the distribution of silage protein fractions. Although initial proteolysis may mostly be plant enzyme mediated, during wilting as well as during the first ensiling phase (Guo et al., 2007), the wilting length had small influence on the silage. Considering this, it would be acceptable with some delay of the wilting phase to reach a higher DM level. However, the very high DM levels obtained with red clover in this experiment would be difficult to manage under mechanized harvesting conditions.

The predicted improvements in AAT_{20} are valid if the degradation rate of the potentially degradable N remains the same in spite of its proportion being much larger at higher DM concentration. Huhtanen et al. (2008) concluded in a meta-analysis of 80 dairy cow experiments that the net improvement in protein feeding value from decreased solubility is small. The authors attributed that to a high ruminal degradation rate of the protein protected from proteolysis by wilting or acid application. It is not possible to conclude with certainty to what extent the predicted AAT_{20} improvement should be realized in this case, but a considerable part was from increased carbohydrate supply for microbial protein production and not directly related to reduced protein solubility.

Exp II – Results of studies with different crops and maceration and the changes in LCFA

Fresh crops and fermentation quality

A combination of factors such as unfavorable rainy condition at harvest, a higher resistance to wilt, and coincidental achievement of a higher DM content than planet caused that wilting time of red clover (25 hours), and particularly bird's-food trefoil (39 hours) and white clover (45 hours) were remarkably longer than wilting time of other crops (timothy for 5 hours, meadow fescue for 7 hours, tall fescue for 4 hours).

The fermentation quality of all silages is presented in Table 14. The average pH in all silages was found to be 5.2±0.4, with the lowest value in tall fescue silages 4.5±0.05. The present results confirm suggestion from first experimental year that crop DM content above 30 % eliminates growth of undesirable micro-organisms in silages. Negligible concentration of butyric acid as typical product of clostridia (Pahlow et al., 2003), as well as tiny concentration of 2.3 butanediol indicating enterobacteria (Pahlow et al., 2003) can serve as evidence of that. As consequence of eliminated activity of undesirable micro-organisms during fermentation, silage losses were low.

Table 14. Fermentation quality of silages.

Forage	DM	pH	NH_3-N	Lactic acid	Acetic acid	Butyric acid	2.3 buta-nediol	Ethanol	Losses
	g/kg					g/kg DM			
Tall fescue	343	4.5	1.8	22.7	3.1	0.2	1.8	6.0	43.8
Rye-grass	349	5.3	1.7	10.8	1.6	0.2	0.2	6.2	33.1
Meadow f.	362	5.3	2.2	13.1	2.3	0.2	2.3	3.1	33.8
Bird's-food	521	5.2	1.2	1.2	0.6	0.1	0.1	1.7	16.7
Vitklöver	447	5.3	2.8	6.6	2.4	0.1	0.1	1.4	23.9
Timothy	374	5.0	0.5	3.3	0.7	2.1	0.2	3.9	40.6
Timothy-M*	303	4.0	0.7	20.7	7.5	0.2	0.4	7.3	41.4
Red clover	311	5.1	2.1	12.3	3.0	0.2	3.9	3.0	32.1
Red clover-M	338	4.5	2.4	27.6	7.0	0.2	3.3	2.2	36.6

* Macerated; DM – dry matter.

Fatty acid composition in forages

The LCFA profile and total fatty acids (FA) concentration of all forage crops during the ensiling process are demonstrated in Table 15. Differences between grasses and legumes were detected. Wilting of grasses did not significantly reduce either the total FA content, or proportions of C18:3n-3 and C18:2n-6, which is in contrast to the finding of Van Ranst et al. (2009). Wilted meadow fescue, tall fescue, and timothy displayed even higher proportion of C18:3-n3 than in fresh crops. In legumes, reduced content of the total FA in red and white clover followed by lower proportions of C18:3n-3 in white clover and lower proportion of C18:2n-6 in bird's-food trefoil due to wilting was observed. A possible explanation of the discrepancy between grasses and legumes can be an extended degradation of FA during the transport of grass samples. It is assumed that susceptibility of grasses for FA degradation is much higher than in legumes (Van Ranst et al., 2009). Since time between crop harvesting and receiving sample in the laboratory took occasionally more than two hours, it is a possible that extended degradation of FA in grasses occurred during this time. It is also assumed that extended wilting contributed to the reduction FA of legumes. There were variations in effect of ensiling on FA profile in silages compared with previous study. In line with previous study is the increase ($P<0.001$) in total FA content in silages in comparison with crops prior to ensiling, except for red clover and bird's-food trefoil. In contrary to the previous study is the decrease in C18:3n-3 proportion ($P<0.001$) in silages of present DM contents, except for white clover.

Table 15. Total fatty acid (FA) content and fatty acid composition in tested forages during ensiling process.

Forage	Ensiling stage	DM	C16:0	C18:0	C18:1 cis-9	C18:2 n-6	C18:3 n-3	Total FA
		g/kg	g/100g total FA					mg/g DM
Rye-grass	fresh	191	17.3[a]	1.4[b]	3.4[a]	12.7[b]	62.2[a]	16.0[ab]
Rye-grass	wilted	365	17.6[a]	1.6[a]	2.4[b]	11.9[b]	62.6[a]	14.3[b]
Rye-grass	silage	349	16.4[b]	1.4[b]	2.0[b]	14.1[a]	60.7[b]	22.0[a]
SEM			*0.20*	*0.03*	*0.15*	*0.28*	*0.38*	*7.22*
Meadow f.	fresh	191	17.6[a]	1.2[b]	3.5[a]	12.5[b]	62.9[b]	17.8[b]
Meadow f.	wilted	385	16.5[b]	1.4[a]	2.4[b]	12.3[b]	64.3[a]	18.3[b]
Meadow f.	silage	362	17.5[a]	1.1[b]	2.5[b]	14.3[a]	60.4[c]	24.1[a]
SEM			*0.15*	*0.03*	*0.07*	*0.14*	*0.28*	*2.41*
Tall fescue	fresh	229	17.5[a]	1.5[ab]	5.6[a]	10.9[b]	61.8[c]	15.2[b]
Tall fescue	wilted	374	16.4[b]	1.6[a]	3.2[b]	10.1[b]	65.7[a]	14.6[b]
Tall fescue	silage	343	15.6[b]	1.2[b]	3.0[b]	12.4[a]	63.4[b]	20.7[a]
SEM			*0.23*	*0.08*	*0.27*	*0.23*	*0.32*	*3.40*
Timothy	fresh	261	17.4[a]	1.8	3.8[a]	19.6[b]	53.7[b]	12.5[b]
Timothy	wilted	400	16.0[b]	1.9	2.9[b]	18.8[b]	56.6[a]	13.6[ab]
Timothy	silage	374	16.3[b]	1.7	3.1[b]	21.1[a]	52.0[b]	16.4[a]
SEM			*0.13*	*0.06*	*0.17*	*0.30*	*0.69*	*3.82*
red clover	fresh	169	16.1[b]	2.2[b]	2.0	19.5	57.2[a]	22.0[a]
red clover	wilted	346	17.7[a]	2.7[a]	1.7	18.0	56.2[a]	18.0[b]
red clover	silage	311	17.7[a]	2.7[a]	2.1	19.3	52.8[b]	20.4[ab]
SEM			*0.16*	*0.08*	*0.34*	*0.45*	*0.86*	*3.71*
white clover	fresh	165	16.7[b]	2.2[b]	3.1[a]	16.7	57.3[a]	16.2[b]
white clover	wilted	462	19.7[a]	3.0[a]	2.8[ab]	16.8	52.2[b]	11.1[c]
white clover	silage	447	19.3[a]	2.8[a]	2.8[b]	16.6	51.9[b]	17.8[a]
SEM			*0.17*	*0.05*	*0.09*	*0.34*	*0.46*	*0.79*
Bird's-food	fresh	179	16.7[b]	1.7[b]	1.5	17.7[a]	58.0[a]	17.7
Bird's-food	wilted	535	16.5[b]	1.8[b]	1.3	16.3[b]	59.0[a]	16.4
Bird's-food	silage	521	18.9[a]	1.9[a]	1.4	17.3[a]	53.8[b]	19.4
SEM			*0.16*	*0.06*	*0.11*	*0.11*	*0.30*	*6.73*
Probability	Forage		***	***	***	***	***	***
	Stage		**	***	***	***	***	***
	Int.		***	***	***	***	***	NS

[a,b,c] Significant (P<0.05) differences within forages and between ensiling stage; *, ** and *** at P<0.05, P<0.01 and P<0.001, respectively; NS – not significant; FA – fatty acids; DM – dry matter.

However, expression of LCFA profile as part of DM (Table 16) revealed that concentrations of C18:3n-3 in grass silages were highest during ensiling process or reach the same concentration as in fresh forage in case of legumes. The proportion as well as concentrations of C18:2n-6 in all grass silages was higher (P<0.001) in comparison with fresh and wilted forages. The interaction effect between ensiling stages (wilting + ensiling) on C16:0 was pronounced differently in both forage types. The proportion of C16:0 in grasses was lower (P<0.001) when wilted and ensiled, whereas the opposite trend was observed in legumes. Proportion of C18:0 in legumes followed the same trend (P<0.001) as C16:0. However, when LCFA profile was expressed as part of DM (Table 3), concentration of these FA were often highest in silages. Although significant interaction effect of ensiling stage and wilting on reduction of C18:1cis-9 proportion in grasses was obtained, concentrations in silages was equal to those in fresh crops.

Table 16. Concentrations of fatty acids (FA) and crude fat in tested forages during ensiling process.

Forage	Stage	DM	C16:0	C18:0	C18:1 cis-9	C18:2 n-6	C18:3 n-3	Total FA	Crude fat
		g/kg				mg/g DM			
Rye-grass	fresh	191	2.8	0.2	0.5^{a}	2.0^{b}	10.0^{b}	16.0^{ab}	36.8^{ab}
Rye-grass	wilted	365	2.5	0.2	0.3^{b}	1.7^{b}	9.0^{b}	14.3^{b}	33.7^{b}
Rye-grass	silage	349	3.6	0.3	0.4^{ab}	3.1^{a}	13.4^{a}	22.0^{a}	42.5^{a}
LSD			*1.23*	*0.12*	*0.16*	*0.89*	*4.35*	*7.22*	*6.34*
Meadow f.	fresh	191	3.1^{b}	0.2^{b}	0.6^{a}	2.2^{b}	11.2^{b}	17.8^{b}	39.5
Meadow f.	wilted	385	3.0^{b}	0.2^{ab}	0.4^{b}	2.3^{b}	11.8^{b}	18.3^{b}	42.9
Meadow f.	silage	362	3.6^{a}	0.3^{a}	0.6^{a}	3.4^{a}	14.5^{a}	24.1^{a}	38.0
LSD			*0.51*	*0.04*	*0.11*	*0.32*	*1.40*	*2.41*	*4.41*
Tall fescue	fresh	229	2.6^{b}	0.2	0.8^{a}	1.7^{b}	9.4^{b}	15.2^{b}	30.8^{b}
Tall fescue	wilted	374	2.4^{b}	0.2	0.5^{c}	1.5^{b}	9.6^{b}	14.6^{b}	34.1^{ab}
Tall fescue	silage	343	3.2^{a}	0.3	0.6^{b}	2.6^{a}	13.1^{a}	20.7^{a}	38.4^{a}
LSD			*0.54*	*0.09*	*0.07*	*0.43*	*2.21*	*3.40*	*6.63*
Timothy	fresh	261	2.2	0.2^{b}	0.5^{ab}	2.4^{b}	6.7	12.5^{b}	32.0
Timothy	wilted	400	2.2	0.3^{ab}	0.4^{b}	2.6^{b}	7.7	13.6^{ab}	32.5
Timothy	silage	374	2.7	0.3^{a}	0.5^{a}	3.5^{a}	8.5	16.4^{a}	34.9
LSD			*0.60*	*0.04*	*0.10*	*0.77*	*2.25*	*3.82*	*3.87*
red clover	fresh	169	3.5	0.5	0.4	4.3^{a}	12.6^{a}	22.0^{a}	54.3^{a}
red clover	wilted	346	3.2	0.5	0.3	3.2^{b}	10.1^{b}	18.0^{b}	52.6^{a}
red clover	silage	311	3.6	0.5	0.4	4.0^{ab}	10.8^{ab}	20.4^{ab}	45.8^{b}
LSD			*0.58*	*0.09*	*0.21*	*0.70*	*2.36*	*3.71*	*3.94*
white clover	fresh	165	2.7^{b}	0.4^{b}	0.5^{a}	2.7^{a}	9.3^{a}	16.2^{b}	41.2
white clover	wilted	462	2.2^{c}	0.3^{c}	0.3^{b}	1.9^{b}	5.8^{b}	11.1^{c}	39.4
white clover	silage	447	3.4^{a}	0.5^{a}	0.5^{a}	2.9^{a}	9.2^{a}	17.8^{a}	41.8
LSD			*0.16*	*0.03*	*0.04*	*0.22*	*0.55*	*0.79*	*3.37*
Bird's-food	fresh	179	3.0	0.3	0.3	3.1	10.3	17.7	39.6
Bird's-food	wilted	535	2.7	0.3	0.2	2.7	9.7	16.4	37.1
Bird's-food	silage	521	3.7	0.4	0.3	3.3	10.4	19.4	39.5
LSD			*1.19*	*0.11*	*0.09*	*1.12*	*3.99*	*6.73*	*4.22*
Probability	Forage		***	***	***	***	***	***	***
	Stage		***	***	***	***	***	***	*
	Int.		NS	NS	*	*	*	NS	***

[a,b,c] Significant ($P<0.05$) differences within forages and between ensiling stage; *, ** and *** at $P<0.05$, $P<0.01$ and $P<0.001$, respectively; NS – not significant; FA – fatty acids; DM – dry matter.

The investigation of mechanical treatments on FA composition revealed significantly lower concentrations of C16:0, C18:2n-6, C18:3n-3, and total FA in wilted macerated timothy in comparison with these in wilted mechanically un-treated (see Table 17). Maceration of red clover caused increase in C18:1 concentration but significant decrease in C18:3n-3 concentration in all ensiling stages (Table 18). Total FA concentration was a lower only in fresh macerated red clover crop than in fresh mechanically untreated red clover.

Table 17. The effect of mechanical treatment (M) on concentrations of fatty acids (FA) and crude fat in timothy crop during ensiling process.

Forage	Stage	DM	C16:0	C18:0	C18:1 cis-9	C18:2 n-6	C18:3 n-3	Total FA	Crude fat
		g/kg				mg/g DM			
Timothy	fresh	261	2.2	0.2	0.5	2.4	6.7	12.5	32.0
Timothy	wilted	400	2.2	0.3	0.4	2.6	7.7	13.6	32.5
Timothy	silage	374	2.7	0.3	0.5	3.5	8.5	16.4	34.9
Timothy-M	fresh	277	2.1	0.2	0.5	2.1	5.6	11.0	29.1
Timothy-M	wilted	328	1.4	0.2	0.3	1.4	3.6	7.1	29.9
Timothy-M	silage	303	2.9	0.3	0.7	3.3	7.6	15.6	37.3
LSD			*0.66*	*0.05*	*0.13*	*0.75*	*1.88*	*3.59*	*3.62*
Probability	Forage		NS	NS	NS	*	**	*	NS
	Stage		**	**	**	***	**	**	**
	Int.		NS	*	NS	NS	NS	NS	NS

*, ** and *** at P<0.05, P<0.01 and P<0.001, respectively; NS – not significant; FA – fatty acids; DM – dry matter.

Table 18. The effect of mechanical treatment (M) on concentrations of fatty acids (FA) and crude fat in red clover crop during ensiling process.

Forage	Stage	DM	C16:0	C18:0	C18:1 cis-9	C18:2 n-6	C18:3 n-3	Total FA	Crude fat
		g/kg				mg/g DM			
red clover	fresh	169	3.5	0.5	0.4	4.3	12.6	22.0	54.3
red clover	wilted	346	3.2	0.5	0.3	3.2	10.1	18.0	52.6
red clover	silage	311	3.6	0.5	0.4	4.0	10.8	20.4	45.8
red clover-M	fresh	180	3.3	0.5	0.6	3.7	8.4	17.1	48.0
red clover-M	wilted	343	3.5	0.5	0.5	3.5	8.4	17.2	50.8
red clover-M	silage	338	3.8	0.5	0.5	4.2	9.6	19.8	48.5
LSD			*0.62*	*0.09*	*0.15*	*0.75*	*2.02*	*3.57*	*5.61*
Probability	Forage		NS	NS	*	NS	**	*	NS
	Stage		NS	NS	NS	*	NS	NS	NS
	Int.		NS	NS	NS	NS	NS	NS	NS

*, ** and *** at P<0.05, P<0.01 and P<0.001, respectively; NS – not significant; FA – fatty acids; DM – dry matter.

CONCLUSIONS

Conclusions concerning the time and extent of pre-wiling on LCFA

An expected reduction of WSC in extended wilting time under moist condition was observed similarly in both forages. Effect of wilting time and DM content influenced the LCFA composition differently in timothy and red clover forages. In timothy extended wilting reduced FA content and a higher DM content caused increase in C18:3 proportions. In red clover, extended wilting reduced only the proportion of C18:3 up till 30% DM content.
Silage fermentation was strongly affected by DM content in both forages. A high presence of butyric acid, 2.3butanediol, and ammonia-N indicated undesirable fermentation in low DM silages of both forages. The DM content above 30% was secure to obtain lactic acid fermented silages from both forages.
Unlike to crop prior to ensiling, effect of wilting time and DM content displayed similar trends in the LCFA composition between timothy and red clover silages. A higher DM content caused increase in proportions of C16:0 and C18:0 whereas proportion of C18:3, contents of FA and crude fat were reduced in silages. When comparing silages with crops prior to ensiling, silages contained significantly more of C18:3, total FA and crude fat.

Conclusions concerning the time and extent of pre-wilting on the protein fractions

The changes in the green chop during wilting were generally small while there was a tremendous increase in NorFor predicted protein value of the silage by wilting to a very high DM concentration. However, this prediction is under the assumption that ruminal degradation rates for N fractions are constant even when their size changes. It is therefore not clear that these improvements will be fully realized until they have been shown in animal experiments.

Conclusions on studies with different crops and maceration and the changes in LCFA

The wilting of crop to approx. 35% DM resulted in well fermented silages with low silage losses. Differences in FA profile prior and during ensiling process between grasses and legumes were observed. Unlike in legumes, wilting of grasses did not significantly reduce either the total FA content, or proportions of C18:3n-3 and C18:2n-6 from total FA. Proportions of C18:3n-3 from total FA were decreased due to ensiling in both grasses and legumes, but concentrations of C18:3n-3 in grass silages were highest during ensiling process and in legumes reached the same concentration as in fresh forage. The concentration of total FA and C18:2n-6 in silages followed same trend as C18:3n-3. The effect of forage maceration was pronounced in reduced concentrations of C18:3n-3 and total FA in wilted stage of timothy. Maceration of red clover caused reduction in concentration of C18:3n-3 during the whole ensiling process.

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Appendix 1. Basal composition of green chop and silage of timothy and red clover, respectively

Crop	Wilting			DM, g/kg[1]		Ash, g/kg DM		N, g/kg DM		NDF g/kg DM		WSC, g/kg DM		Silage fermentation	
	Level	Time	Hours	Green	Silage	Green	Silage	Green	Silage	Green	Silage	Green	Silage	Products, g/kg DM[2]	pH
	Standing crop			311		66.5		22.0		472		166.0			
Timothy	None	None	0.0	296	277	73.9	76.4	21.4	23.0	453	443	169.2	75.2	93.7	4.54
Timothy	None	Half-day	4.3	309	286	74.0	77.1	22.3	24.5	455	461	150.6	58.5	97.8	4.57
Timothy	None	One day	21.0	310	285	75.4	82.9	21.4	23.1	459	462	150.0	25.1	98.5	4.58
Timothy	None	Two days	44.3	364	337	79.4	80.8	22.1	23.5	467	471	142.7	25.8	90.3	4.67
Timothy	Moderate	Half-day	4.7	468	449	72.9	71.6	22.8	23.2	479	449	156.9	127.7	30.3	5.40
Timothy	Moderate	One day	22.5	498	495	72.8	75.0	21.5	22.8	473	459	157.5	116.3	30.0	5.43
Timothy	Moderate	Two days	44.5	528	503	77.3	76.3	22.9	22.6	473	466	130.8	105.9	23.8	5.46
Timothy	High	One day	20.5	618	586	77.2	71.8	22.5	22.8	475	457	137.0	129.4	17.4	5.44
Timothy	High	Two days	45.2	619	607	74.0	77.7	21.4	22.9	476	480	145.9	104.0	32.7	5.46
Timothy	Very high	Two days	45.5	771	741	70.4	69.9	22.9	22.8	470	486	157.0	128.0	12.4	5.40
	Standing crop			145		95.0		33.2		225		119.5			
Red clover	None	None	0.0	155	132	95.7	104.4	34.6	36.5	217	242	92.0	2.1	199.7	4.49
Red clover	None	Half-day	8.8	150	137	99.5	110.5	34.4	37.8	226	245	96.7	0.9	194.1	4.57
Red clover	None	One day	28.3	163	145	95.9	110.5	34.2	37.2	233	250	99.6	1.0	191.9	4.56
Red clover	None	Two days	51.3	169	153	100.3	116.0	33.8	38.7	248	264	83.6	2.3	189.3	4.87
Red clover	Moderate	Half-day	9.3	277	253	99.4	105.0	34.0	36.3	241	252	93.2	3.8	178.2	4.81
Red clover	Moderate	One day	28.8	295	285	95.8	102.5	34.3	35.3	241	252	93.2	20.9	115.8	4.57
Red clover	Moderate	Two days	50.8	332	329	99.9	103.7	34.7	35.7	245	254	76.4	46.1	93.5	4.92
Red clover	High	One day	29.6	461	459	100.3	97.2	33.6	33.6	244	242	89.3	99.6	17.4	5.27
Red clover	High	Two days	50.7	477	490	100.8	101.4	34.2	34.7	244	253	91.5	79.1	19.4	5.34
Red clover	Very high	Two days	52.3	632	624	93.8	95.0	33.1	33.6	241	258	102.3	81.7	11.5	5.33

1Defined as remaining proportion after freeze-drying

2(Lactic acid + acetic acid + propionic acid + succinic acid + fumaric acid + butyric acid + ethanol + 2,3 butanediol)

Appendix 2. Analyzed N fractions in green chop and silage of timothy and red clover, respectively (n = 3)

Crop	Wilting level	Wilting time	N, g/kg DM		NDFN, g/kg N		Water soluble N, g/kg N		TCAP N, g/kg N[1]		NH_3-N, g/kg N		α-amino-N, g/kg N	
			Green	Silage	Green	Silage	Green	Silage	Green	Silage	Green	Silage	Green	Silage
Timothy	None	None	21.4	23.0	147	45	155	577	10	10	6	86	47	313
Timothy	None	Half-day	22.3	24.5	123	41	153	594	12	7	5	91	48	314
Timothy	None	One day	21.4	23.1	132	46	116	583	9	10	4	96	36	300
Timothy	None	Two days	22.1	23.5	152	54	151	570	12	8	6	104	46	276
Timothy	Moderate	Half-day	22.8	23.2	188	64	143	502	10	3	5	41	45	268
Timothy	Moderate	One day	21.5	22.8	198	77	135	520	9	4	5	43	42	271
Timothy	Moderate	Two days	22.9	22.6	200	84	152	480	22	2	7	50	46	251
Timothy	High	One day	22.5	22.8	195	110	107	426	6	0	4	32	33	215
Timothy	High	Two days	21.4	22.9	203	121	109	397	6	1	4	31	31	194
Timothy	Very high	Two days	22.9	22.8	172	182	92	193	0	1	3	16	27	65
Red clover	None	None	34.6	36.5	42	40	170	655	19	-3	7	181	25	293
Red clover	None	Half-day	34.4	37.8	40	40	183	657	33	3	7	195	35	301
Red clover	None	One day	34.2	37.2	42	40	198	666	27	12	7	180	41	304
Red clover	None	Two days	33.8	38.7	50	47	276	668	64	15	11	197	58	271
Red clover	Moderate	Half-day	34.0	36.3	54	52	173	587	19	12	6	183	36	253
Red clover	Moderate	One day	34.3	35.3	55	54	207	564	5	-11	6	114	45	279
Red clover	Moderate	Two days	34.7	35.7	52	50	246	564	13	6	9	104	54	265
Red clover	High	One day	33.6	33.6	54	59	171	453	21	6	6	28	35	204
Red clover	High	Two days	34.2	34.7	56	60	175	439	11	8	6	33	37	185
Red clover	Very high	Two days	33.1	33.6	55	69	122	268	9	3	4	18	25	88
[2] Crop			***		***		***		NS		***		†	
Conservation			***		***		***		NS		***		***	
DM			***		***		***		NS		***		***	
Wilting time			*		***		*		NS		*		**	
Crop*Conservation			**		***		**		NS		***		NS	
Crop*Wilting time			†		***		***		NS		**		***	
Crop* Wilting level			***		***		***		NS		***		NS	
Conservation*Wilting time			NS		NS		***		NS		***		***	
Conservation* Wilting level			***		***		***		NS		***		***	
Wilting time* Wilting level			*		*		**		NS		***		***	
Crop*Conservation*Wilting time			*		NS		***		NS		***		NS	
Crop*Wilting time* Wilting level			†		NS		NS		NS		***		NS	
Crop*Conservation* Wilting level			NS		***		†		NS		***		*	
Conservation*Wilting time* Wilting level			NS		NS		NS		NS		***		**	
Crop*Conservation*Wilting time* Wilting level			NS		NS		NS		NS		***		NS	

[1] Trichloroacetic precipitable soluble N determined on the water soluble N fraction

[2] †: P < 0.10; *: P < 0.05; **: P < 0.01; ***: P < 0.001

Appendix 3. N fractions according to the CNCPS system in green chop and silage of timothy and red clover, respectively (n = 3)

Crop	Wilting level	Wilting time	N, g/kg DM		PA1, g/kg N[1]		PA2-N, g/kg N[1]		PB1-N, g/kg N[1]		PB2-N, g/kg N[1]		PC-N, g/kg N[1]	
			Green	Silage	Green	Silage	Green	Silage	Green	Silage	Green	Silage	Green	Silage
Timothy	None	None	21.4	23.0	6	86	149	491	698	378	87	9	60	36
Timothy	None	Half-day	22.3	24.5	5	91	148	502	724	366	63	5	60	35
Timothy	None	One day	21.4	23.1	4	96	111	487	753	371	72	10	60	36
Timothy	None	Two days	22.1	23.5	6	104	145	466	697	376	92	17	60	37
Timothy	Moderate	Half-day	22.8	23.2	5	41	138	461	670	433	128	26	60	38
Timothy	Moderate	One day	21.5	22.8	5	43	130	477	667	402	138	37	60	40
Timothy	Moderate	Two days	22.9	22.6	7	50	145	430	648	436	140	43	60	41
Timothy	High	One day	22.5	22.8	4	32	103	394	698	464	135	65	60	44
Timothy	High	Two days	21.4	22.9	4	31	104	366	688	481	143	76	60	46
Timothy	Very high	Two days	22.9	22.8	3	16	89	176	736	625	112	128	60	54
Red clover	None	None	34.6	36.5	7	181	163	474	788	305	26	26	16	14
Red clover	None	Half-day	34.4	37.8	7	195	176	462	777	302	26	26	14	14
Red clover	None	One day	34.2	37.2	7	180	191	487	760	294	26	26	16	14
Red clover	None	Two days	33.8	38.7	11	197	265	471	674	285	27	27	22	20
Red clover	Moderate	Half-day	34.0	36.3	6	183	167	404	773	361	28	28	26	24
Red clover	Moderate	One day	34.3	35.3	6	114	201	450	738	382	28	28	27	26
Red clover	Moderate	Two days	34.7	35.7	9	104	237	460	702	386	28	27	24	22
Red clover	High	One day	33.6	33.6	6	28	165	425	775	489	28	29	26	30
Red clover	High	Two days	34.2	34.7	6	33	169	406	769	501	28	29	27	31
Red clover	Very high	Two days	33.1	33.6	4	18	118	250	823	664	28	30	27	39
[2]Crop			***		***		***		***		***			
Conservation			***		***		***		***		***			
Wilting level			***		***		***		***		***			
Wilting time			*		*		***		***		***			
Crop*Conservation			**		***		***		***		***			
Crop*Wilting time			†		**		***		***		***			
Crop* Wilting level			***		***		*		***		***			
Conservation*Wilting time			NS		***		***		***		***			
Conservation* Wilting level			***		***		***		***		***			
Wilting time* Wilting level			*		***		***		***		NS			
Crop*Conservation*Wilting time			*		***		+		***		***			
Crop*Wilting time* Wilting level			†		***		***		NS		NS			
Crop*Conservation* Wilting level			NS		***		***		*		***			
Conservation*Wilting time*Wilting level			NS		***		NS		NS		NS			
Crop*Conservation*Wilting time*Wilting level			NS		***		†		NS		NS			

[1] PA1-N = ammonia-N; PA2-N = soluble non-ammonia N; PB1-N = 1000 – (PA1-N + PA2-N + PB2-N + PC-N); PB2-N = NDF-bound N – PC-N; PC-N = ADF-bound N calculated from regression of 30 samples against NDF-bound N

[2] †: P < 0.10; *: P < 0.05; **: P < 0.01; ***: P < 0.001