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Alpha-tocopherol and β-carotene in legume–grass mixtures as influenced by wilting, ensiling and type of silage additive

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Abstract

Effects of wilting, ensiling and type of additive on α-tocopherol and β-carotene contents in legume–grass mixtures were examined. Swards of birdsfoot trefoil + timothy (Bft + Ti), red clover + timothy (Rc + Ti) and red clover + meadow fescue (Rc + Mf) were harvested as a first regrowth in August 2005. Forage was wilted to a dry-matter (DM) content of 273 g kg^{-1} and ensiled without additive or with an inoculant or acid. Wilting decreased α-tocopherol concentration by 30% in the Bft + Ti mixture ($P = 0{\cdot}015$). Untreated Bft + Ti silage had higher α-tocopherol content than red clover silages (56·9 vs. 34·2 mg kg^{-1} DM; $P = 0{\cdot}015$). The α-tocopherol concentration of Bft + Ti forages increased during ensiling from 41·1 mg kg^{-1} DM in wilted herbage to 56·9, 65·2 and 56·8 mg kg^{-1} DM in untreated, inoculated and acid-treated silage respectively ($P = 0{\cdot}015$). The inoculant increased α-tocopherol content in the red clover silages (50·1 vs. 34·2 mg kg^{-1} DM; $P = 0{\cdot}015$) compared with untreated red clover silages. Red clover mixtures had lower β-carotene content than Bft + Ti (32·3 vs. 46·2 mg kg^{-1} DM; $P = 0{\cdot}016$), averaged over treatments. In conclusion, wilting had small effects but the use of bacterial inoculant as an additive and a Bft + Ti mixture increased α-tocopherol concentration in the silage.

Keywords: α-tocopherol, β-carotene, legume, grass, ensiling, additive

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Introduction

Forage is often the largest dietary source of natural fat-soluble vitamins in dairy production (Jensen *et al.*, 1999). Factors affecting vitamin content of the forage, such as species (Brown, 1953; Lynch *et al.*, 2001; Danielsson *et al.*, 2008), harvest time (Lynch *et al.*, 2001), wilting and method of conservation (Brown, 1953; Thafvelin and Oksanen, 1966), can have a large impact on the α-tocopherol and β-carotene contents in plasma of dairy cows, as there is a relationship between the vitamin level of dietary forage and vitamin content in plasma (Havemose *et al.*, 2004; Agabriel *et al.*, 2007; Calderón *et al.*, 2007a).

α-Tocopherol and β-carotene are antioxidants (Granelli *et al.*, 1998; Calderón *et al.*, 2007b) and play important roles in the immune system of ruminants (Burton and Traber, 1990; Hogan *et al.*, 1992; Allison and Laven, 2000). β-Carotene also serves as the most abundant precursor of vitamin A. Most of the α-tocopherol and β-carotene in forage is found in the leaves (Brown, 1953), and plant species with high leaf/stem ratios usually have higher α-tocopherol and β-carotene contents than species with low leaf/stem ratios (Olsson *et al.*, 1955; Thafvelin and Oksanen, 1966; Livingston *et al.*, 1968). Likewise, vitamin contents are highest in immature fresh herbage and normally decrease with advanced forage maturity (Park *et al.*, 1983). β-Carotene is easily oxidized once plants are cut, resulting in significantly lower concentrations of β-carotene in stored forage than in fresh herbage (Bruhn and Oliver, 1978; Kalač and McDonald, 1981; Park *et al.*, 1983). The α-tocopherol content varies between species and is often higher in grasses than in legumes, whereas legumes contain more β-carotene than grasses (Ballet *et al.*, 2000; Danielsson *et al.*, 2008).

As both α-tocopherol and β-carotene are sensitive to oxidation, wilting often reduces their concentrations, especially in sunny weather (Ballet *et al.*, 2000). Carter (1960) reported losses of β-carotene in hay of 80–90%

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and in silage of 40–60% from cutting to feeding. Nozière *et al.* (2006) noted that well-fermented silages usually have β-carotene losses of <20%, whereas Kalač (1983) reported that there was no clear relationship between the quality of silage and its carotene content. Kalač (1983) also found large differences between plant species in losses of β-carotene during ensiling. Use of additives also can affect the vitamin content of forage. There are, however, few ensiling studies that have evaluated the effect of additives on α-tocopherol and β-carotene concentrations in silage. Kalač and Kyzlink (1979) reported larger losses of β-carotene in red clover (*Trifolium pratense* L.) ensiled with acidic additives than in untreated silage, whereas Shingfield *et al.* (2005) found that the use of acids or inoculants as additives resulted in higher contents of α-tocopherol than in untreated silage. A study by Nadeau *et al.* (2004) showed inconsistent results on α-tocopherol concentrations of grass-clover silages when additives were used.

Silages in Sweden are often mixtures of different plant species, in which red clover and timothy (*Phleum pratense* L.) are commonly used. This experiment includes a study of the effects of ensiling on α-tocopherol and β-carotene contents in a mixture of red clover and timothy. The effect of substitution of timothy in the mixture with a leafy grass, such as meadow fescue (*Festuca pratensis* L.), on the content of α-tocopherol and β-carotene was also studied. Birdsfoot trefoil (*Lotus corniculatus* L.) is not common in Swedish forage mixtures, but earlier studies (Loosli *et al.*, 1950) have shown that birdsfoot trefoil has high concentrations of α-tocopherol and it was, therefore, included in the experiment. The aim of this study was to determine the effects of species mixture, wilting, ensiling and additive on the contents of α-tocopherol and β-carotene in legume–grass forages.

Materials and methods

Crops and experimental design

The experiment was performed at Lanna Research Station (58°21′N, 13°08′E) in Skara, Sweden, using three mixtures of forage species. These were birdsfoot trefoil (cv. Oberhaunstaedter) + timothy (cv. Grindstad), (Bft + Ti); tetraploid red clover (cv. Sara) + timothy (cv. Grindstad) (Rc + Ti); and tetraploid red clover (cv. Sara) + meadow fescue (cv. Kasper) (Rc + Mf). These forage mixtures were undersown in a barley crop on 23 April 2004. The experiment had a split-plot design, in which forage mixture was treated as the main plot and forage treatment (unwilted herbage, wilted herbage, control silage, inoculated silage and acid-treated silage) as the subplot. Forage mixture was replicated three times in the field, and the replicates were randomized in a computer programme. Plot size was $1{\cdot}75 \times 12{\cdot}0$ m. Seed rates in kg per hectare of 100% germinative seeds were as follows: 10 + 10, 7 + 20 and 7 + 15 for Bft + Ti, Rc + Mf and Rc + Ti respectively. The soil was fertilized with 40 tonnes slurry ha^{-1} (containing 1% ammonia-N) 1 month before sowing. The aim was to obtain well-balanced mixtures that contained a legume proportion of approximately 50%. However, the mixtures with red clover contained a greater proportion of legumes (>90%) in the primary growth; they were therefore fertilized with another 40 tonnes slurry ha^{-1} on the day after the first harvest.

Harvest and ensiling

The study was conducted on the first regrowth (on 11 August 2005) cut 8 weeks after the primary growth. Forage samples for botanical assessment were cut from five 0·25 m^2 areas randomly allocated in each plot. The botanical composition of the swards was determined by separation of legumes and grasses on a dry-matter (DM) basis. Plant fractions of legumes and grasses were separated into flower ⁄ inflorescence, leaf and stem, and the percentage of each fraction was determined on a DM basis. Stage of maturity of legumes and grasses was determined by sorting into phenology classes according to a scale based on Zadoks *et al.* (1974).

The whole forage plots were harvested and weighed with a Haldrup 1500 forage harvester (Haldrup, Løgstør, Denmark) at a stubble height of 8 cm. Representative samples of 10–15 kg fresh herbage were collected from each plot and transported in coolers to the laboratory, where they were chopped in a machine to 12 mm length and dried outdoors on plastic sheeting. The samples were processed in the order of the layout in the field starting with block 1. Time from cutting until sampling and freezing of the chopped unwilted herbage was approximately 3 h. The target DM was 300 g kg^{-1}, and the achieved DM after wilting for 3 h outdoors was $273 \pm 38{\cdot}1$ g DM kg^{-1}. The average DM content of the mixtures before wilting was $127 \pm 34{\cdot}2$ g kg^{-1}.

Two different silage additives, the acid mixture Proens™ (Perstorps Inc., Perstorp, Sweden) containing two-thirds formic acid and one-third propionic acid, and the inoculant Siloferm® Plus (Medipharm Inc., Kågeröd, Sweden) containing *Lactobacillus plantarum, Pediococcus acidilactici* ($3{\cdot}2 \times 10^{10}$ colony forming units g^{-1}), cellulase and hemicellulase (54 000 HEC g^{-1}) were compared with a control silage without any additive. Proens™ was applied at 5 L $tonne^{-1}$ wilted herbage, and Siloferm® Plus was applied at 4 L $tonne^{-1}$ wilted herbage, resulting in a concentration of 768 000 cfu g^{-1} wilted herbage, as recommended by the manufacturers. Wilted herbage was packed in 1·7-L mini silos, which were glass jars equipped with water seals, to a density of 174 ± 16·4 kg m^{-3}. There were 27 mini silos (three mixtures × three replicates × three silage treatments), and these were stored indoors (20°C) for 100 d before being opened.

Chemical analysis

Samples of unwilted herbage, wilted herbage and silage were analyzed for DM, and additional samples of the same materials were frozen at −20°C for subsequent analysis of vitamins. Samples of wilted herbage and silage were analyzed for crude protein (CP), neutral detergent fibre (NDF), sugar and *in vitro* organic matter digestibility (IVOMD). Silages were sampled for analysis of pH, ammonia-N, organic acids and alcohols.

Analysis of vitamins

Analyses of α-tocopherol and β-carotene were performed at Aarhus University, Faculty of Agricultural Sciences, Research Centre Foulum, Denmark. Both fresh herbages and silages were freeze dried before analysis. α-Tocopherol and β-carotene concentrations were determined by high-pressure liquid chromatography (HPLC) after saponification and extraction into heptane according to Jensen *et al.* (1998) as follows. In the clean-up procedure, 2·00 g of the freeze-dried sample was mixed with 70 mL of 96% v/v ethanol, 30 mL methanol (C 2517; Peter Mark, Valby, Denmark), 30 mL ascorbic acid (5·00074·1000; Merck, Darmstadt, Germany) and 20 mL KOH-water 1:1 (w/v) (5268120; B&B, Herlev, Denmark). The mixture was saponified for 30 min at 80°C in the dark and cooled in cold water. Exactly 2 mL of the saponified mixture was diluted with 1 mL distilled water, after which tocopherols and carotenoids were quantitatively extracted with 2 × 5 mL heptane (C 2514; Peter Mark, Valby, Denmark) and centrifuged at 1500***g*** for 10 min between each extraction.

The HPLC equipment consisted of a Perkin-Elmer LC pump series 410, a Perkin-Elmer Advanced LC Sample Processor ISS 200 and a Perkin-Elmer LC 240 fluorescence detector (Perkin-Elmer GmbH, Überlingen, Germany). Identification and quantification of vitamins were performed by comparisons of retention times as well as peak areas with external standards (Merck). The HPLC column for the determination of tocopherols consisted of a 4·0 × 125 mm Perkin-Elmer HS-5 μm Silica column. For tocopherol analyses, heptane modified with 2-propanol (3·0 mL L^{-1}) and degassed with helium constituted the mobile phase. The flow rate was 3·0 mL min^{-1}. Fluorescence detection was performed with an excitation wavelength of 290 nm and an emission wavelength of 327 nm. Carotenoids were separated on a 4·6 × 250 mm Supelcosil LC-NH_2-5 μm HPLC column (Supelco Inc, Bellfonte, PA, USA). The mobile phase consisted of heptane modified with 2-propanol (50 mL L^{-1}) and trimethylamine (1·0 mL L^{-1}). The eluate was monitored at 450 nm by a Perkin-Elmer LC95 UV/Vis Spectrophotometer Detector.

Analysis of other nutrients

Dry-matter contents of unwilted herbage and wilted herbage before ensiling were determined on a 200 g sample at 105°C for 24 h, and DM content of silage was determined on a 200 g sample at 60°C for 24 h. Chemical analyses were conducted at Eurofins Laboratory, Lidköping, Sweden. Concentrations of NDF, sugar, CP, IVOMD and ash were determined on dried samples that had been ground in a mill (Kamas Kvarnmaskiner AB, Malmö, Sweden) to pass through a 1-mm screen. The concentration of NDF was determined according to Van Soest *et al.* (1991), and sugar was determined according to Ekelund (1966). The CP content as total N concentration and ammonia-N concentration were determined using the Kjeldahl technique in a Tecator Auto Sampler 1035 Analyzer (Tecator Inc, Höganäs, Sweden). The IVOMD was analyzed according to Tilley and Terry (1963) as modified by Lindgren (1979), and the concentration of ash was determined at 550°C for 16 h. Analyses of organic acids, ethanol, 2, 3-butanediol and pH were carried out on a water extract from fresh silage samples. Organic acids and alcohols were analyzed by HPLC (Andersson and Hedlund, 1983), and silage pH was determined with a Metrohm 654 pH meter (Metrohm AG, Herisau, Switzerland). Weight loss of initial DM of the forage during storage was calculated according to Weissbach (2005).

Statistical analysis

Data were analyzed as a split-plot design using the general linear model procedure (PROC GLM) of SAS (2001). In the analysis, forage mixture ($n = 3$) was treated as the main plot, and forage treatment as the subplot. There were five forage treatments (unwilted herbage, wilted herbage, control silage, inoculated silage and acid-treated silage) in the model for α-tocopherol and β-carotene, four forage treatments (wilted herbage, control silage, inoculated silage and acid-treated silage) for the other nutrients and three forage treatments (control silage, inoculated silage and acid-treated silage) for pH and fermentation characteristics. Mixtures were replicated three times in the field. The effect of mixture was tested by using mixture × replicate as the error term. The effects of treatment and its interactions with mixture were tested by using treatment × mixture × replicate as the error term. When a significant F-value was detected, pair-wise comparisons between LSMEANS were analyzed with Tukey's test at $P < 0{\cdot}05$.

Results

Yield and plant characteristics

The Rc + Mf and Rc + Ti mixtures had higher yields than the Bft + Ti mixture (5305 kg DM ha^{-1} and 5182 kg DM ha^{-1} vs. 4102 kg DM ha^{-1}; s.e.m. = 135·1; P = 0·006). The additional slurry applied on the red clover–grass mixture swards after the first harvest reduced the proportion of legumes to 82 and 85% in the Rc + Mf and Rc + Ti respectively. The legume content in Bft + Ti was 65%. Timothy was more mature than meadow fescue: the meadow fescue was only in the sheath elongation stage, whereas 90% of the timothy grown with birdsfoot trefoil was in the stem elongation stage and 90% of the timothy grown with red clover had inflorescences. Birdsfoot trefoil was more mature than red clover; approximately 95% of the birdsfoot trefoil was at least in the flowering stage, whereas only 5–10% of the red clover had started to flower. Meadow fescue had a higher leaf proportion compared with timothy (Table 1).

Vitamin content

There were significant interactions between forage mixture and treatment in terms of α-tocopherol content. The α-tocopherol content decreased during wilting in the Bft + Ti mixture (Table 2). However, the only decrease in α-tocopherol content during ensiling was found in the acid-treated silage of the red clover mixtures (Table 2). In Bft + Ti, there was an increase in the α-tocopherol content during ensiling for both control and treated silages, but there were no differences between silage treatments, whereas inoculated silages of red clover mixtures had higher α-tocopherol contents than both the control and acid-treated red clover silages (Table 2). Control and acid-treated silages of Bft + Ti had higher contents of α-tocopherol than control and acid-treated silages of the red clover mixtures. Also, the Bft + Ti silage treated with the inoculant had more α-tocopherol than inoculated Rc + Ti silage, and acid-treated silage of Rc + Mf had a higher content of α-tocopherol than the acid-treated silage of Rc + Ti (Table 2). There were no differences in vitamin content between mixtures of either the unwilted or wilted herbage (Table 2).

There were no significant interactions between forage mixture and treatment in terms of β-carotene content. There was, however, a significant main effect of mixture on β-carotene content, with the red clover mixtures having lower β-carotene contents than the Bft + Ti mixture, averaged across treatments. There was also a significant main effect of treatment, where acid-treated silage had lower β-carotene content than unwilted herbage, when averaged across mixtures (Table 2).

Table 1 Mean and standard deviation of proportions of leaf, stem and flower/inflorescence for each forage species (n = 3).

	Forage mixture		
	Birdsfoot trefoil + timothy	Red clover + timothy	Red clover + meadow fescue
Legume			
Leaf	0·25 ± 0·020	0·33 ± 0·010	0·37 ± 0·017
Stem	0·60 ± 0·023	0·62 ± 0·026	0·59 ± 0·034
Flower	0·15 ± 0·017	0·06 ± 0·029	0·04 ± 0·020
Grass			
Leaf	0·45 ± 0·044	0·49 ± 0·030	0·87 ± 0·005
Stem	0·55 ± 0·046	0·50 ± 0·035	0·13 ± 0·005
Inflorescence	0 ± 0	0·01 ± 0·008	0 ± 0

Nutritive value and hygienic quality

There were significant interactions between forage mixture and treatment in CP, NDF and sugar concentrations (Table 3). The mixture of Rc + Mf had the highest CP content in the wilted herbage (Table 3). Ensiling decreased NDF concentrations in the Rc + Ti mixture for both control and treated silages, but this effect only occurred in the treated silages of the Rc + Mf mixture (Table 3). There was no difference in NDF concentration between mixtures of the wilted herbage, but all Bft + Ti silages had higher NDF concentration than the red clover silages (Table 3). The sugar content in wilted herbage was highest in the timothy mixtures (Table 3). The sugar content decreased during ensiling and was lower in control silage compared with wilted herbage in all mixtures (Table 3). Inoculated and acid-treated silages generally had higher sugar contents in all mixtures compared with the control (Table 3). Acid-treated silages of Bft + Ti and Rc + Ti had at least as much sugar as in the wilted herbage (Table 3). The DM content and the IVOMD decreased during ensiling, except for acid-treated silage as a mean over forage mixtures (Table 3).

Significant interactions were found between forage mixture and treatment for lactic acid, acetic acid, ethanol, 2,3-butanediol and in weight losses of the initial DM during storage (Table 4). Control and inoculated silages had higher contents of lactic acid than acid-treated silage in all mixtures (Table 4).

Table 2 Alpha-tocopherol and β-carotene concentrations (mg kg^{-1} DM) in unwilted herbage, wilted herbage and silages for each forage mixture (*n* = 3), as a mean over forage mixtures (*n* = 9) and as a mean over forage treatments (*n* = 15).

	Treatment					Statistics of interactions				Statistics of main effects	
Forage mixture	Unwilted herbage	Wilted herbage	Control	Inoculant	Acid	s.e.m.	*P*	$LSD_{0·05}$	Forage mixture mean	s.e.m.	*P*
α-tocopherol											
Bft + Ti	58·8	41·1	56·9	65·2	56·8	4·44	0·015	12·96	$55·8^{x}$	2·34	0·014*
Rc + Ti	51·1	40·1	30·1	46·2	20·7				$37·6^{y}$		
Rc + Mf	59·7	48·6	38·3	54·0	35·2				$47·2^{xy}$		
Treatment mean	$56·5^{a}$	$43·3^{b}$	$41·8^{b}$	$55·1^{a}$	$37·6^{b}$					2·56	<0·0001†
β-carotene											
Bft + Ti	56·2	49·5	41·2	42·7	41·2	3·92	NS		$46·2^{x}$	2·18	0·016
Rc + Ti	39·1	30·3	31·4	32·2	20·5				$30·7^{y}$		
Rc + Mf	35·6	35·9	38·5	31·5	27·9				$33·9^{y}$		
Treatment mean	$43·7^{a}$	$38·6^{ab}$	$37·1^{ab}$	$35·5^{ab}$	$29·9^{b}$					2·26	0·005

Bft + Ti, birdsfoot trefoil + timothy; Rc + Ti, red clover + timothy; Rc + Mf, red clover + meadow fescue.
Different superscripts in the same row (a, b) or column (x, y) differ according to Tukey's test.
**P*-value for the main effect of mixture.
†*P*-value for the main effect of treatment.

Control silages had a higher content of acetic acid than the other treatments except in the Bft + Ti mixture, where there were no differences between control and inoculated silage (Table 4). Inoculated silages had higher acetic acid contents than acid-treated silages of all mixtures (Table 4). Control and inoculated silages of Bft + Ti had lower contents of lactic and acetic acids than the corresponding silages of the red clover mixtures (Table 4). The control silage had the highest content of ethanol whereas acid-treated silages had the lowest ethanol content and this applied to all mixtures (Table 4). The Bft + Ti mixture generally had higher ethanol contents than the red clover mixtures in all silage treatments (Table 4). Control silages had the highest content of 2,3-butanediol of the red clover mixtures and acid-treated silages had the lowest, whereas no differences in 2,3-butanediol were found between silage treatments for the Bft + Ti mixture (Table 4). The control silage of the red clover mixtures had the largest weight losses of the initial DM during storage (Table 4). The weight losses of inoculated silages and acid-treated silages were less than in untreated silages for all mixtures, and acid-treated silages had the lowest weight losses (Table 4). Both inoculated and acid-treated silages had lower ammonia-N concentrations than the control silage, with the lowest ammonia-N concentration in the acid-treated silage when averaged over mixtures. Inoculated silages had the lowest pH, in terms of the mean of all mixtures (Table 4).

Discussion

Vitamin content in forage in relation to botanical characteristics

Meadow fescue had a higher leaf proportion than the other species, which generally generates higher α-tocopherol concentrations (Brown, 1953; Ballet *et al.*, 2000), but the proportion of meadow fescue (18%) in the Rc + Mf mixture in this experiment was probably too low to result in a difference in α-tocopherol concentrations between the red clover mixtures. Stage of maturity is also known to have a large impact on vitamin content of forage (Brown, 1953; Olsson *et al.*, 1955; Hjarde *et al.*, 1963), but it was difficult to distinguish any effect of maturity in this experiment. The maturity stage of timothy varied between forage mixtures probably because of the effects of the different botanical compositions (e.g. more competition in the red clover mixtures than in the birdsfoot trefoil mixtures) and possible differences in N-supply from the legumes. Timothy grown with birdsfoot trefoil was more mature than timothy grown with red clover, and birdsfoot trefoil was more mature than the red clover. Brown (1953) found that the α-tocopherol content in timothy decreased with advancing maturity. However, this decrease is probably related to a decreased leaf proportion as the leaf proportion is the largest factor affecting the α-tocopherol content in forages (Ballet *et al.*, 2000). The greater legume proportion in the red

Table 3 Concentrations of dry matter, crude protein, NDF, sugar and *in vitro* organic matter digestibility (IVOMD) in wilted herbage and silages for each forage mixture ($n = 3$), as a mean over forage mixtures ($n = 9$) and as a mean over forage treatments ($n = 12$).

	Treatment				Statistics of interactions				Statistics of main effects	
Forage mixture*	Wilted herbage	Control	Inoculant	Acid	s.e.m.	*P*	$LSD_{0.05}$	Forage mixture mean	s.e.m.	*P*
Dry matter (g kg^{-1})										
Bft + Ti	310	297	297	293	3·8	NS		299	1·5	NS*
Rc + Ti	256	230	237	253				244		
Rc + Mf	254	237	243	247				245		
Treatment mean	273^{a}	254^{c}	259^{bc}	264^{ab}					2·2	<0·0001†
Crude protein (g kg^{-1} DM)										
Bft + Ti	157	159	148	155	2·3	0·001	6·7	155^{y}	3·6	0·015
Rc + Ti	162	180	176	173				173^{xy}		
Rc + Mf	171	183	187	185				182^{x}		
Treatment mean	163^{b}	174^{a}	170^{a}	171^{a}					1·3	<0·001
NDF (g kg^{-1} DM)										
Bft + Ti	449	450	473	447	6·2	0·002	18·5	455^{x}	4·3	0·005
Rc + Ti	437	417	400	405				415^{y}		
Rc + Mf	437	423	411	396				416^{y}		
Treatment mean	441^{a}	430^{ab}	428^{ab}	416^{b}					3·6	0·002
Sugar (g kg^{-1} DM)										
Bft + Ti	71·3	24·3	40·3	81·0	2·35	0·001	6·97	54·3^{x}	2·44	0·010
Rc + Ti	71·3	7·0	19·3	71·3				42·3^{xy}		
Rc + Mf	62·3	6·0	12·0	54·0				33·6^{y}		
Treatment mean	68·3^{a}	12·4^{c}	23·9^{b}	68·8^{a}					1·36	<0·0001
IVOMD (g kg^{-1})										
Bft + Ti	643	627	587	627	12·2	NS		621	8·3	NS
Rc + Ti	637	600	620	633				623		
Rc + Mf	650	617	620	640				632		
Treatment mean	643^{a}	614^{b}	609^{b}	633^{ab}					7·1	0·009

Bft + Ti, birdsfoot trefoil + timothy; Rc + Ti, red clover + timothy; Rc + Mf, red clover + meadow fescue; NDF, neutral detergent fibre.
Different superscripts in the same row (a, b, c) or column (x, y) indicate a significant difference according to Tukey's test.
**P*-value for the main effect of mixture.
†*P*-value for the main effect of treatment.

clover mixtures than in the birdsfoot trefoil mixture confounded the comparison of the α-tocopherol concentrations in the mixtures, as legumes usually contain less α-tocopherol than grasses (Danielsson *et al.*, 2008). The legumes also had a lower leaf proportion than the grasses. Brown (1953) found four times more α-tocopherol in grass leaves than in stems and three times more α-tocopherol in clover leaves than in stems. Livingston *et al.* (1968) found that plants, which contained a higher proportion of leaves, had higher contents of β-carotene. It is, however, difficult to predict vitamin contents in mixtures based only on the leaf proportion, as climate, season and growth rate also affect vitamin contents in forages (Booth, 1964; Livingston *et al.*, 1968).

Vitamin content in forage in relation to wilting, ensiling and additive

The only losses of vitamins during wilting were found in the Bft + Ti mixture, where the α-tocopherol concentration decreased. The wilting time was short and was the same for all forage mixtures in this experiment (3 h). The short wilting time and the good

Table 4 Fermentation characteristics, pH and weight losses during storage of silages for each forage mixture (n = 3), as a mean over forage mixtures (n = 9) and as a mean over silage treatments (n = 9).

Forage mixture	Treatment				Statistics of interactions		Forage mixture	Statistics of main effects	
	Control	Inoculant	Acid	s.e.m.	*P*	$LSD_{0.05}$	mean	s.e.m.	*P*
Lactic acid (g kg^{-1} DM)									
Bft + Ti	62·1	68·9	32·9	3·41	0·002	10·52	54·6^{y}	4·25	0·026*
Rc + Ti	104·0	95·7	37·3				79·0^{x}		
Rc + Mf	91·5	93·1	48·6				77·7^{x}		
Treatment mean	85·9^{a}	85·9^{a}	39·6^{b}					1·97	<0·0001†
Acetic acid (g kg^{-1} DM)									
Bft + Ti	16·1	12·9	6·6	1·51	0·026	4·67	11·9	2·14	NS
Rc + Ti	27·8	17·9	7·4				17·7		
Rc + Mf	27·2	20·8	8·4				18·8		
Treatment mean	23·7^{a}	17·2^{b}	7·5^{c}					0·87	<0·0001
Butyric acid (g kg^{-1} DM)									
Bft + Ti	0·7	0·7	0·7	0·40	NS		0·7	0·22	NS
Rc + Ti	1·2	0·9	0·8				1·0		
Rc + Mf	2·4	0·8	0·8				1·4		
Treatment mean	1·4	0·8	0·8					0·23	NS
Ethanol (g kg^{-1} DM)									
Bft + Ti	7·8	5·0	1·4	0·23	0·001	0·71	4·7^{x}	0·19	0·006
Rc + Ti	6·6	3·9	0·7				3·7^{xy}		
Rc + Mf	4·4	3·3	0·8				2·8^{y}		
Treatment mean	6·2^{a}	4·0^{b}	0·9^{c}					0·13	<0·0001
2,3-Butanediol (g kg^{-1} DM)									
Bft + Ti	1·0	0·8	0·7	0·18	<0·001	0·56	0·8^{y}	0·13	0·005
Rc + Ti	3·5	1·7	0·8				2·0^{x}		
Rc + Mf	3·0	1·8	0·8				1·9^{x}		
Treatment mean	2·5^{a}	1·4^{b}	0·8^{c}					0·10	<0·0001
Ammonia-N (% of total N)									
Bft + Ti	7·67	7·67	5·67	0·484	NS		7·00	0·607	NS
Rc + Ti	8·00	7·33	4·67				6·67		
Rc + Mf	9·33	6·67	4·33				6·78		
Treatment mean	8·33^{a}	7·22^{b}	4·89^{c}					0·280	<0·0001
pH									
Bft + Ti	4·37	4·20	4·47	0·046	NS		4·34	0·024	NS
Rc + Ti	4·33	4·20	4·37				4·30		
Rc + Mf	4·53	4·30	4·37				4·40		
Treatment mean	4·41^{a}	4·23^{b}	4·40^{a}					0·027	0·001
Weight loss (% of DM)									
Bft + Ti	2·72	2·18	1·30	0·109	<0·001	0·335	2·07^{y}	0·081	0·005
Rc + Ti	3·50	2·40	1·36				2·42^{y}		
Rc + Mf	4·50	2·70	1·54				2·92^{x}		
Treatment mean	3·58^{a}	2·43^{b}	1·40^{c}					0·063	<0·0001

Bft + Ti, birdsfoot trefoil + timothy; Rc + Ti, red clover + timothy; Rc + Mf, red clover + meadow fescue.
Different superscripts in the same row (a, b, c) or column (x, y) indicate a significant difference according to Tukey's test.
**P*-value for the main effect of mixture.
†*P*-value for the main effect of treatment.

weather conditions during wilting, without any precipitation, contributed to only small losses of α-tocopherol and no losses of β-carotene, as losses are dependent on the wilting time and weather conditions, and rain increases the losses (Carter, 1960; Ballet *et al.*, 2000).

In this experiment, an increase in α-tocopherol concentration was found in inoculated red clover silages compared with untreated red clover silages, which was in accordance with results by Shingfield *et al.* (2005), who found more α-tocopherol in silage treated with an inoculant-enzyme preparation and a formic acid-based additive than in untreated silage. The increase in α-tocopherol concentration could not be related to losses of initial DM as inoculated silages had less weight losses than untreated silages. There was also an increase in α-tocopherol concentration of the Bft + Ti mixture during ensiling, especially in inoculated silage. This could not be explained by losses of initial DM as the storage losses were less in the silages of the Bft + Ti mixtures than in the silages of the red clover mixtures, which did not increase in α-tocopherol concentration during ensiling. Shingfield *et al.* (2005) did not present any analyses on weight losses or fermentation characteristics. Based on our results, there is no clear explanation for the increased α-tocopherol concentrations in inoculated red clover silages and in all of the Bft + Ti silages. One explanation might be that α-tocopherol-producing microorganisms may be present on the plants; these microorganisms can enhance their α-tocopherol production when cultured in an optimal medium (Tani and Tsumura, 1989).

Forage conservation usually decreases vitamin content (Brown, 1953; Kalač and Kyzlink, 1980). Ensiling is, however, one of the best ways to preserve vitamins during conservation, at least if the silage quality is good as it was in this experiment as shown by high lactic acid concentrations and low concentrations of acetic acid, butyric acid, ethanol and ammonia-N. The good silage quality probably resulted in no significant losses of α-tocopherol during ensiling of untreated and inoculated forages. However, addition of the acid resulted in losses of α-tocopherol during ensiling of the red clover mixtures, which was in agreement with results by Kalač and Kyzlink (1979). This loss of α-tocopherol during ensiling of the acid-treated Rc + Ti and Rc + Mf cannot be explained by bad fermentation characteristics, as these silages had very low weight losses during storage and low concentrations of volatile compounds. In contrast, there was an increase of the α-tocopherol content of the high-quality acid-treated Bft + Ti during ensiling. These contrasting effects of the acid treatment in terms of effects on α-tocopherol concentrations in the Bft + Ti and the red clover mixtures suggest that there is a forage × acid interaction, and this needs to be investigated further. The numerically lower β-carotene content in the acid-treated silage compared with the control silage averaged over the mixtures is in agreement with Kalač and Kyzlink (1979), who reported greater losses of β-carotene in red clover ensiled with acidic additives than in untreated silage.

Vitamin content in relation to dairy cow requirements

Supplementation of vitamins is expensive, and there is a preference for the natural form RRR-α-tocopherol in cattle (Meglia *et al.*, 2006). β-Carotene is usually not supplemented at all. Instead, vitamin A is provided as a supplement in mineral feed.

In organic farming systems, there is a preference for using only natural sources of feeds for animals, and this can be difficult to achieve in the case of vitamins. The variation in vitamin content of forages is one of the reasons why supplements of synthetic vitamins are permitted for organic dairy cows in the EU. This study shows how the large variation in vitamin content in silage makes it difficult to estimate the amount of natural vitamins from feed that dairy cows consume per day. The vitamin A requirement for adult dairy cattle is based on supplemental vitamin A and is set at 110 IU kg^{-1} body weight (NRC, 2001). The vitamin A activity of β-carotene for cattle is defined as 1 mg of carotene = 400 IU of vitamin A. Thus, a lactating dairy cow weighing 680 kg needs 75 000 IU of vitamin A d^{-1} or 187 mg of β-carotene (NRC, 2001). If this amount were to be provided by one of the silages in our study, the cow would need 9 kg DM d^{-1} of the silage with the lowest β-carotene content and 4 kg DM d^{-1} of the silage with the highest β-carotene content to fulfil its daily vitamin A requirements. NRC (2001) recommends 545 IU of vitamin E d^{-1} for a lactating dairy cow weighing 680 kg. One mg of the natural form of α-tocopherol corresponds to 1·49 IU of vitamin E, which leads to a recommendation of 366 mg of RRR-α-tocopherol d^{-1} (NRC, 2001). This would require 18 kg DM $d^{-1}$of the silage with the lowest α-tocopherol content, which would be difficult for a cow to consume. From the silage with the highest content of α-tocopherol, 6 kg DM d^{-1} would be sufficient to fulfil requirements, which is a low quantity of forage for an organic dairy cow.

Conclusions

Wilting had only a small effect on the vitamin content of the herbage of the forage mixtures, with a decreased α-tocopherol concentration for the Bft + Ti mixture but no decrease in the β-carotene concentrations of the mixtures. Both the untreated and treated silages were of good quality, with low concentrations of acetic and butyric acids, ethanol and ammonia-N, and there were no losses of α-tocopherol or β-carotene during ensiling of the untreated forages. However, use of an acid decreased α-tocopherol concentration of the red clover mixtures during ensiling but increased it in the Bft + Ti mixture, suggesting an interaction between plant species and acidic additives. Inoculated silages had higher α-tocopherol contents than control silage of the red clover mixtures, indicating that the inoculant is an appropriate additive to use when the purpose is to preserve the vitamin content of forage. Ensiling of the Bft + Ti mixture increased the α-tocopherol concentration regardless of silage treatment, and further studies using different inoculants and forage mixtures are needed before firm conclusions can be drawn regarding the positive effects on the α-tocopherol content.

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