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Effects of manipulating the protein content of white clover on silage quality

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Abstract

White clover nitrogen N content is generally high and constant throughout the growing season. It is also difficult to manipulate, as N_2 fixation tends to maintain a high N supply to the plant. White clover is a highly heterozygous, natural out-breeder, but self-fertile lines have recently been developed, including one which fails to develop nodules when grown under appropriate physiological conditions. An advantage of this line is that leaf protein content can be manipulated by altering the N supply to the plant and, in addition, it offers the opportunity to examine the effect of protein content on silage quality. This line was grown under varying levels of nitrate–N, giving rise to plants with a range of protein contents which were subsequently conserved as silage in two separate experiments. In experiment 1, both herbage protein and water-soluble carbohydrate (WSC) content varied, with values ranging from 17 to 31 g/kg dry matter (DM) and 52 to 72 g/kg DM, respectively. Protein content also varied in herbage grown for experiment 2, ranging from 20 to 33 g/kg DM, but variation in WSC content (91–101 g/kg DM) was not statistically significant. Silage quality was dependent on the protein content of the fresh herbage in both experiments. A comparison of herbages with high and low initial protein content, from experiments 1 and 2, respectively, revealed that a high protein content resulted in silages with a higher pH (4.81 versus 3.74 and 4.46 versus 4.23), a lower protein N content when expressed as a proportion of total silage N (536 g/kg versus 752 g/kg and 523 g/kg

Abbreviations: N, nitrogen; WSC, water-soluble carbohydrate; DM, dry matter; TN, total nitrogen; CFU, colony forming units; FM, fresh matter; GABA, γ-amino butyric acid; NPN, non-protein nitrogen; IGER, Institute of Grassland and Environmental Research

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versus 659 g/kg TN), and an increase in free amino acid content when expressed as a proportion of total N (267 g/kg versus 90 g/kg and 323 g/kg versus 200 g/kg TN). A higher initial protein content results in a higher proportion of protein degradation during ensiling.

Keywords: White clover silage; Protein content; Silage quality; Protein degradation

1. Introduction

White clover is the most widely grown clover in the world. It has a high nutritive value and is very palatable to ruminants. Current shifts towards sustainable, low cost, low input agricultural systems in temperate regions of the world, have resulted in increased interest in inclusion of white clover in grazing systems (Frame et al., 1997). White clover can also be conserved as hay or silage, and several studies have demonstrated the high nutritive value of white clover silage (Castle et al., 1983; Weigand et al., 1994; Auldist et al., 1999; Dewhurst et al., 2003). Whilst white clover has many desirable agronomic traits, these can also present problems with preservation and feeding. A high N content, which can benefit animal production response, can also lead to low efficiency of N use by animals and high losses in waste from animals fed grazed (Peyraud, 1993; Cruickshank et al., 1992) and ensiled forage (Bertilsson et al., 2000; Dewhurst et al., 2003). In addition, high buffering capacity, a consequence of a high protein content (Muck et al., 1991) and low water-soluble sugar content, make this crop difficult to ensile (McDonald et al., 1991). Poor fermentation is associated with increased protein degradation during ensilage (McDonald et al., 1991; Winters et al., 2000; Winters et al., 2001) which tends to decrease efficiency of N utilisation by ruminants (Charmley, 2001, 2002).

White clover N content is generally high and only varies to a small extent with stage of maturity. Thomson et al. (1985) reported a mean N content of 44 g/kg DM in white clover over a growing season. In contrast to temperate grasses, it is difficult to manipulate the N content of legumes such as white clover, as N_2 fixation makes it difficult to restrict N supply. As a consequence, there has been limited scope for examining the relationship between protein content and the ensiling characteristics of white clover.

White clover is a natural tetraploid out-breeder (Frame et al., 1997), and it is highly heterozygous. Recently, self-fertile (in-breeding) lines of white clover have been developed which are now revealing previously masked recessive traits. These include a white clover line which fails to develop nodules when grown under certain physiological conditions (e.g., low pH with a supply of N; Abberton et al., 1998; Michaelson-Yeates et al., 1998). A useful trait for silage studies in this line, in contrast with nodulating lines, is that leaf protein content can be manipulated by altering the supply of mineral N to the plant. This model system offers the unique opportunity to study the effect of varying the protein content on the ensiling characteristics of white clover, thereby providing valuable information that can be used to devise appropriate strategies for optimising white clover silage quality.

In the present study, the N content of a non-nodulating line of white clover was manipulated by varying the supply of mineral N, thereby resulting in plants with a range of protein

contents. These were subsequently ensiled and the effect of protein content on silage quality was examined.

2. Materials and methods

2.1. Plant material

Inbred non-nodulating white clover plants with a range of protein contents were grown by varying mineral N application to rhizobia-free plants. These plants were subsequently ensiled in two separate experiments started on 28 July 1999 and 7 June 2000. White clover plants for both experiments were grown in a controlled environment glasshouse at IGER in Aberystwyth (UK). Supplementary lighting was provided by a bank of 18 Phillips Son T Agro 400 W bulbs which were computer-controlled to supply a minimum photosynthetic photon flux density of 200 μmol/s/m^2 for a 16 h photoperiod starting at dawn. Temperature was regulated to 20 and 15 °C during the light and dark periods, respectively.

Seeds of white clover were sown in Fison's Levingtons compost (Levingtons Horticulture Ltd., Ipswich, UK) at a density of 50 seeds to a 15 cm pot and watered daily. After 3 weeks, 20 selected seedlings were transferred to 15 cm pots of Agsorb (8–16 mm diameter baked clay granules, pH 5.2) and watered. The plants were maintained free of rhizobia and, after 4 days, selected pots were provided with an N-containing nutrient solution (10.0 mM NO_3^-), prepared by adding KNO_3 to an N-free nutrient stock solution (Ryle et al., 1978).

In order to produce enough biomass for silage experiments, stolons of mature plants provided with the 10 mM NO_3^- nutrient stock (8–10-week-old) were cut at the third internode back from the tip and cuttings were planted (without rooting hormone) in Levingtons compost (25 cuttings per 15 cm diameter pot) and watered. After 3 weeks, rooted cuttings were transferred to 15 cm diameter pots of Agsorb and watered. After 4 days selected pots were fed one of four N containing nutrient solutions (2.5, 5.0, 7.5 and 10.0 mM NO_3^-) prepared as described above. These concentrations were selected because preliminary studies had confirmed that these treatments gave a range of herbage N contents from low to physiological levels.

Plants were harvested for ensiling at the early flowering stage following 8 wk growth on Agsorb (28 July 1999 for the first experiment (experiment 1) and 7 June 2000 for the second experiment (experiment 2)). Plant material was either chopped by hand with scissors or chopped with a garden shredder (Viking Junior Shredder, Bob Andrews, Ascot, UK) to lengths of approximately 3 cm, and mixed thoroughly. A silage inoculant consisting of a freshly cultured strain of *Lactobacillus plantarum* (*L. plantarum*) (IGER strain no. L54) was applied to chopped herbage at a rate of 6 ml/kg to provide 10^6 colony forming units (CFU)/g fresh matter (FM). After spraying, herbage was thoroughly mixed by vigorous shaking in large polythene bags and subsequently packed in glass test tube silos with 100 g FM capacity (109 g FM mean weight/tube). Three replicate tubes per NO_3^- level were used in experiment 1, and five replicate tubes per treatment were used in experiment 2. Tubes were sealed with airlocks containing liquid paraffin and packed tubes were incubated at a constant temperature (23 °C) for 60 (experiment 1) or 90 days (experiment 2). After opening, silages were thoroughly mixed and sub-samples were stored at −20 °C until subsequent analysis.

2.2. Chemical analyses

Dry matter (DM), pH, lactic acid and ammonia were analysed as described by Winters et al. (1998). Volatile fatty acids were analysed by gas chromatography according to Zhu et al. (1996). Water-soluble carbohydrate (WSC) was determined by the anthrone method of Thomas (1977). Buffering capacity was estimated as described by Playne and McDonald (1966). Total and soluble N were determined by a Kjeldahl method (Bradstreet, 1969). Protein N was determined by subtracting trichloroacetic acid soluble N from total N according to the method of Kuchroo and Fox (1982). Total amino acids were extracted and analysed as described by Winters et al. (2001).

2.3. Microbiological analysis of herbage and silages

For microbiological analysis, replicate samples were pooled to provide 30 g FM of silage, which was mixed with quarter-strength Ringer solution (Oxoid, BR52; Unipath, Basingstoke, UK) in a sterile polythene bag and pummelled for 3 min in a Colworth Stomacher (model 400, A.J. Seward, London, UK). Numbers of lactic acid bacteria, enterobacteria, yeasts and moulds in herbage and silage extracts were determined as described by Merry et al. (1995). No statistical analysis was completed on microbial count results because of lack of replicate data.

2.4. Statistical analysis

Differences in herbages and silages resulting from nitrate treatment were determined using one-way ANOVA (Genstat 5 Committee, 1987).

3. Results

3.1. Herbage

Herbage DM content was similar in both experiments (Tables 1 and 2) ranging from approximately 160–200 g/kg DM. DM content showed a linear decline ($P < 0.05$) with increasing N content with both sets of plants. Herbage used in experiment 1 showed a *linear* increase ($P < 0.001$) in pH with nitrate application, but this did not occur in experiment 2. The WSC content was lower in herbage in experiment 1 versus experiment 2 (52–72 g/kg DM versus 91–101 g/kg DM), and declined linearly ($P < 0.001$) with increasing levels of nitrate application, in experiment 1. Herbage grown for both experiments showed linear increases ($P < 0.001$) in total N and protein N (expressed on a DM basis) content with increasing levels of nitrate application with values ranging from approximately 19–38 and 17–33 g/kg DM, respectively (Fig. 1). Total N also showed a quadratic relationship ($P = 0.016$) with increasing nitrate in experiment 2. Protein N expressed on the basis of total N (TN) decreased linearly ($P = 0.039$) from 925 to 870 g in experiment 1, but was not affected in experiment 2. Free amino acid N levels increased linearly ($P < 0.001$) with rate of nitrate application in experiment 1 both on a DM and TN basis. In herbage from experiment 2, free

Table 1
The effect of nitrate application level on herbage used in experiment 1

	Level of nitrate application (mM)					Significance of effect[a]	
	2.5	5	7.5	10	S.E.D.	L	Q
DM (g/kg FM)	180.8	181.9	157.5	161.5	11.02	0.046	NS
pH	5.83	5.84	6.13	6.13	0.062	<0.001	NS
WSC (g/kg DM)	72.0	70.0	58.3	52.0	4.25	<0.001	NS
Total N (g/kg DM)	18.6	23.8	29.8	35.3	1.78	<0.001	NS
Protein–N (g/kg DM)	17.2	21.9	26.9	30.7	2.06	<0.001	NS
Protein–N (g/kg TN)	925.3	917.6	904.4	869.9	21.46	0.039	NS
Free amino acid N (g/kg DM)	0.07	0.32	0.48	1.13	0.129	<0.001	NS
Free amino acid N (g/kg TN)	3.7	13.45	16.2	31.9	5.41	<0.001	NS
Buffering capacity (meq/kg DM)	328.3	333.7	382.7	381.5	10.35	<0.001	NS

[a] Polynomial contrasts; L, linear; Q, quadratic.

amino acid N levels (kg/DM) showed a quadratic effect ($P < 0.001$) with increasing nitrate application level and, when expressed on a TN basis, both linear and quadratic effects were significant ($P < 0.001$). Buffering capacity increased linearly ($P < 0.003$) with the level of nitrate application in both experiments, but was markedly higher in herbage ensiled in experiment 2.

Differences in numbers of lactic acid bacteria, enterobacteria and yeasts, as well as moulds were observed on herbages grown in both years. Herbage grown in 1999 had higher numbers of enterobacteria and yeasts plus moulds compared with herbage grown in 2000 (approximate values of 5×10^5 CFU/g FW versus 8×10^3 CFU/g FW for enterobacteria, and 3×10^5 CFU/g FW versus 2×10^2 CFU/g FW for yeasts plus moulds) but showed no apparent relationship with level of nitrate application (data not shown). Numbers of lactic acid bacteria on herbage grown in 1999 were not markedly affected by nitrate levels (1.4×10^5 to 7.6×10^5 CFU/g FW) whilst they showed an inverse relationship with level of nitrate application on herbage grown in 2000. Counts ranged from 1.04×10^6 on herbage treated with 2.5 mM nitrate to 2.3×10^4 on herbage treated with 10 mM nitrate.

Table 2
The effect of nitrate application level on herbage used in experiment 2

	Level of nitrate application (mM)					Significance of effect[a]	
	2.5	5	7.5	10	S.E.D.	L	Q
DM (g/kg FM)	195.5	191.9	187.2	172.0	5.45	0.002	NS
pH	6.43	6.31	6.50	6.30	0.027	NS	NS
WSC (g/kg DM)	92.0	98.0	101.0	91.0	6.28	NS	NS
Total N (g/kg DM)	23.1	28.9	36.5	38.0	1.01	<0.001	0.016
Protein–N (g/kg DM)	20.1	25.6	32.7	33.3	0.82	<0.001	NS
Protein–N (g/kg TN)	870	885	895	877	37	NS	NS
Free amino acid N (g/kg DM)	1.18	0.02	0.24	1.04	0.218	NS	<0.001
Free amino acid N (g/kg TN)	51.1	0.1	6.6	28.1	5.27	0.002	<0.001
Buffering capacity (meq/kg DM)	513.7	557.7	593.0	642.0	30.0	0.002	NS

[a] Polynomial contrasts; L, linear; Q, quadratic.

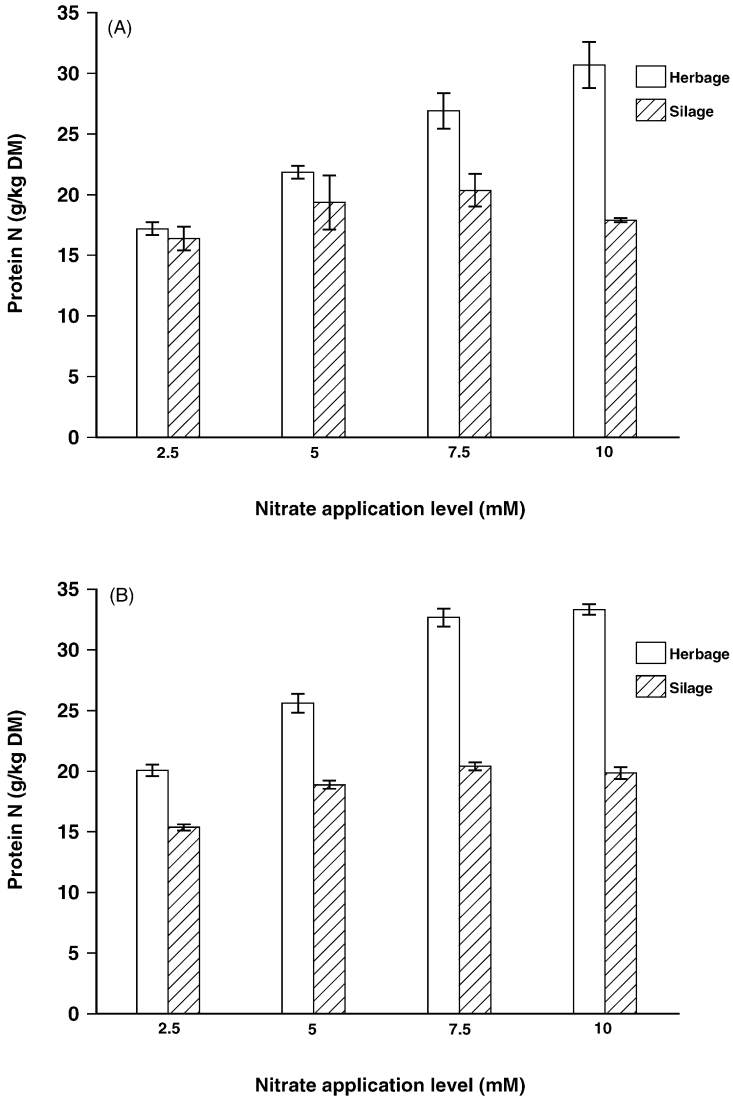

Fig. 1. Effects of nitrate application level on protein N content of herbage and silage in (a) experiment 1 and (b) experiment 2. Vertical bars represent the standard error of the mean (n = 3 or 5).

3.2. Silage

The composition of 60 days silages ensiled in experiment 1 is in Table 3 and the composition of 90 days silages ensiled in experiment 2 is in Table 4. Silage pH values increased linearly with levels of nitrate application in experiments 1 and 2 (P = 0.036 and <0.001, respectively), and a quadratic effect (P < 0.001) also occurred in experiment 2. In general,

Table 3
The effect of nitrate application level on 60 days silage used in experiment 1

	Level of nitrate application (mM)					Significance of effect[a]	
	2.5	5	7.5	10	S.E.D.	L	Q
DM (g/kg FM)	183.9	176.6	148.6	163.1	7.35	0.005	NS
pH	3.74	3.81	4.07	4.81	0.440	0.036	NS
Total lactic acid (g/kg DM)	84.8	82.1	78.3	59.9	18.81	NS	NS
L:D lactic acid ratio	0.16	0.20	0.36	0.38	0.077	0.009	NS
Acetic acid (g/kg DM)	13.7	12.0	18.0	27.7	2.25	<0.001	0.005
WSC (g/kg DM)	9.83	6.80	5.60	5.57	0.81	<0.001	0.031
Total N (g/kg DM)	21.7	27.8	32.4	33.4	0.70	<0.001	<0.001
Protein–N (g/kg DM)	16.4	17.8	20.4	17.9	2.25	NS	NS
Protein–N (g/kg TN)	752.1	636.9	630.0	535.6	72.90	0.017	NS
Free amino acid N (g/kg DM)	1.96	3.68	6.69	8.91	0.587	<0.001	NS
Free amino acid N (g/kg TN)	90.2	133.0	208.0	266.9	20.31	<0.001	NS
Ammonia–N (g/kg TN)	11.1	11.0	15.8	35.5	4.65	<0.001	0.017

[a] Polynomial contrasts; L, linear; Q, quadratic.

lactic acid concentrations were higher in silages in experiment 1 versus experiment 2, but there was no relationship with level of nitrate application in either experiment. It is notable that the L:D lactic acid isomer ratio increased linearly with increasing level of nitrate application in experiments 1 and 2 ($P = 0.009$ and 0.003, respectively). This corresponded with linear ($P < 0.001$) increases in acetic acid concentrations, with a quadratic effect also observed in experiment 2 ($P = 0.005$). Residual WSC levels were low, and showed inverse linear ($P < 0.001$) and quadratic ($P = 0.04$) relationships with level of N application in experiments 1 and 2.

Table 4
The effect of nitrate application level on 90 days silage used in experiment 2

	Level of nitrate application (mM)					Significance of effect[a]	
	2.5	5	7.5	10	S.E.D.	L	Q
DM (g/kg FM)	203.2	195.5	196.8	175.7	1.91	<0.001	<0.001
pH	4.23	4.21	4.37	4.46	0.217	<0.001	0.013
Total lactic acid (g/kg DM)	59.4	68.0	60.4	62.7	4.88	NS	NS
L:D lactic acid ratio	0.22	0.26	0.35	0.44	0.049	0.003	NS
Acetic acid (g/kg DM)	13.2	13.2	16.2	17.0	1.14	<0.001	NS
WSC (g/kg DM)	11.1	8.9	6.9	6.5	0.49	<0.001	0.023
Total N (g/kg DM)	23.3	30.4	36.4	37.9	0.56	<0.001	<0.001
Protein–N (g/kg DM)	15.4	18.9	20.4	19.8	0.51	<0.001	<0.001
Protein–N (g/kg TN)	659.1	620.9	561.4	523.6	11.72	<0.001	NS
Free amino acid N (g/kg DM)	4.70	8.01	10.71	12.29	0.020	<0.001	0.002
Free amino acid N (g/kg TN)	200.4	264.8	291.9	322.8	10.06	<0.001	0.023
Ammonia–N (g/kg TN)	47.4	27.4	20.4	27.5	1.037	0.004	0.012

[a] Polynomial contrasts; L, linear; Q, quadratic.

Table 5
Effects of nitrate application level on amino acid content (mmol/kg DM) of herbage and silage from experiment 2

	Treatment (level of nitrate application (mM))													
	Herbage					Significance of effect[a]		Silage					Significance of effect[a]	
	2.5	5	7.5	10	S.E.D.	L	Q	2.5	5	7.5	10	S.E.D.	L	Q
Cysteine	8.4	10.6	12.8	11.9	2.14	<0.001	<0.001	7.0	9.3	12.1	12.9	1.64	<0.001	<0.001
Aspartic acid[b]	79.4	111.3	147.9	163.5	2.87	<0.001	<0.001	75.6	107.3	144.1	152.8	2.94	<0.001	<0.001
Methionine	7.7	10.9	12.4	11.3	1.08	<0.001	<0.001	8.3	11.4	11.5	12.1	0.58	<0.001	<0.001
Threonine	29.1	37.8	48.5	44.5	1.47	<0.001	<0.001	29.8	37.6	45.5	45.7	0.39	<0.001	<0.001
Serine	46.9	60.3	72.3	73.7	1.34	<0.001	0.003	46.6	63.5	72.2	70.1	0.30	<0.001	<0.001
Glutamic acid[b]	80.7	104.6	130.5	136.1	3.63	<0.001	0.007	77.4	100.9	121.1	122.1	2.37	<0.001	<0.001
Proline	64.6	90.4	124.4	104.1	8.38	<0.001	<0.001	71.2	99.3	124.6	115.3	4.97	<0.001	<0.001
Glycine	71.8	92.7	120.8	115.0	3.25	<0.001	<0.001	77.5	96.2	121.1	122.4	2.36	<0.001	<0.001
Alanine	68.5	89.0	106.1	115.8	3.04	<0.001	0.036	68.2	85.0	111.3	115.2	2.17	<0.001	0.002
Valine	53.9	69.2	84.8	86.8	2.39	<0.001	0.004	56.6	72.8	88.0	90.8	2.79	<0.001	<0.001
Isoleucine	36.0	46.2	57.2	57.4	0.82	<0.001	0.002	38.2	49.6	59.6	62.2	0.90	<0.001	<0.001
Leucine	64.1	82.5	101.8	105.9	2.85	<0.001	0.007	65.1	87.0	106.4	108.0	2.07	<0.001	<0.001
Tyrosine	10.4	16.0	24.0	21.4	2.11	<0.001	<0.001	12.2	18.7	24.3	20.4	1.61	<0.001	<0.001
Phenylalanine	36.0	46.2	56.8	58.2	1.15	<0.001	0.004	36.2	46.6	55.5	57.2	1.37	<0.001	<0.001
Gaba	9.6	10.6	9.6	11.0	0.34	NS	NS	17.4	23.1	37.9	37.8	1.21	<0.001	<0.001
Histidine	15.0	20.1	24.6	26.2	0.33	<0.001	0.008	16.0	20.5	24.2	24.9	0.03	<0.001	<0.001
Ornithine	0.5	0.5	1.2	1.2	0.08	<0.001	NS	2.0	2.3	2.3	2.4	0.07	<0.001	0.012
Lysine	38.8	52.7	66.0	73.9	1.20	<0.001	NS	37.8	50.6	67.5	72.2	0.21	<0.001	0.001
Arginine	22.4	29.9	34.1	37.3	2.85	<0.001	0.016	22.9	28.9	33.9	35.5	2.45	<0.001	0.001
Total	743.7	981.6	1235.8	1255.1	34.00	<0.001	0.002	766.1	1010.6	1263.1	1280.3	24.46	<0.001	<0.001

[a] Polynomial contrasts; L, linear; Q, quadratic.
[b] Glutamine and asparagine are hydrolysed to glutamic acid and aspartic acid.

Total N levels were broadly consistent with that of the initial herbage, and they increased (linear; $P < 0.001$) at a decreasing rate (quadratic; $P < 0.001$) in both experiments. Protein N, expressed on a DM basis, was not affected by nitrate application level in experiment 1, whereas it increased (linear; $P < 0.001$) at a decreasing rate (quadratic; $P < 0.001$) in experiment 2. When expressed as a proportion of total N, protein N decreased linearly with level of nitrate application in both experiments ($P = 0.017$ and <0.001 in experiments 1 and 2, respectively). These declines were matched by an increase in free amino acid N content with increasing nitrate application level. Free amino acid content increased linearly ($P < 0.001$) in both experiments when expressed either on a DM or a TN basis, with a quadratic effect ($P < 0.03$) in experiment 2. As a consequence, increasing herbage protein N (g/kg DM) content did not result in a proportional increase in silage protein yield (Fig. 1). Ammonia levels were generally low and had linear and quadratic effects ($P < 0.02$) in both experiments although the form of the responses differed.

The amino acid composition of herbages and silages from experiment 2 is in Table 5. In general, levels of amino acids in herbages reflect total N concentrations. With the exception of gaba, ornithine and lysine, levels of individual and total amino acids increased linearly ($P < 0.001$) at a decreasing rate (quadratic; $P < 0.04$) with level of nitrate application. There was no effect of treatment on gaba levels whilst ornithine and lysine showed a linear ($P < 0.001$) effect. Gaba and ornithine were the only amino acids which notably increased during ensilage and, in contrast with herbage, gaba levels in silage showed a linear ($P < 0.001$) and quadratic ($P < 0.001$) relationship with treatment. Individual and total amino acids in silage increased linearly ($P < 0.001$) at a decreasing rate (quadratic; $P < 0.02$).

4. Discussion

The most obvious effects of increasing nitrate supply to these white clover plants was the increase in total N content, protein N content and buffering capacity of the herbage and a corresponding drop in DM content. Changes in DM content are possibly associated with increased stress as N content becomes more limiting. This study confirms findings of Michaelson-Yeates et al. (1998), that a nitrate supply of between 2.5 and 10 mM has a direct effect on N and protein content in inbred, non-nodulating white clover lines. The amino acid data indicates little variation in protein profiles with increasing nitrate. However, nitrate supply had an effect on buffering capacity, although it is difficult to interpret the marked differences between the herbages grown in 1999 and 2000. Tissue nitrate content may contribute to buffering capacity, but data for experiment 2 (not presented) showed negligible levels with no relationship to treatment. Playne and McDonald (1966) observed that organic acids made a major contribution to buffering capacity, and their studies showed that glycerate and malate were the main buffers in red clover. Thus, it is possible that the organic acid content varied between the two sets of plants. The lower buffering capacity of herbage ensiled in the first year may, in part, account for the lower pH of the resulting silages.

The pattern of herbage WSC content varied between the two sets of plants. The decrease in WSC content with increasing N content observed in plants grown in 1999 is similar to the pattern observed with temperate grasses, where a limiting N supply results in slower growth and increased accumulation of fixed carbon in the form of WSC (Smith, 1973).

However, this trend was not observed in plants grown in 2000, where the WSC content was more typical of that observed in field grown white clover. Thomson et al. (1985) reported a mean WSC content of 102 g/kg DM in field-grown white clover over a 14-week period. The low herbage WSC contents, and the relationship with nitrate application level, suggest that the rate of carbon fixation was limiting at the time of harvest in experiment 1. The residual levels of WSC were similar in silages from both experiments, despite higher levels of WSC in herbage harvested in 2000, whilst fermentation acid levels were much higher in the 1999 silages. Considering the low initial WSC content of 1999 herbage, this finding suggests that other sources of fermentable substrates were available in the herbage ensiled in experiment 1. Hydrolysis of hemicellulose and starch has previously been observed during ensilage of lucerne and orchardgrass (Jones et al., 1992; Jaurnena and Pichard, 2001; Yahaya et al., 2002), although these were not measured in the present experiments.

In general, the silages were relatively well preserved with low ammonia contents (<50 g/kg TN). This was despite the low DM content of the initial herbage, ranging from 160 to 200 g/kg DM, which are well below the levels generally recommended for ensiling white clover. For example, Wilman and Williams (1993) suggested wilting herbage with a high white clover content to 260 g/kg FM before ensiling in order to ensure good fermentation. However, Cussen et al. (1995) produced low pH silages from mixed herbage, containing 300 g/kg DM ryegrass and 700 g/kg DM white clover, with DM contents as low as 150 g/kg FM.

The present study demonstrated that varying the N content in white clover affected its ensiling characteristics. In both experiments, silage pH, acetic acid concentrations, free amino acid contents and L:D lactic acid ratios were positively correlated with the nitrate application level, while protein N, expressed as a proportion of total N, and lactic acid content in experiment 1, showed an inverse relationship. These findings suggest that increasing the total N content of the herbage had a negative influence on ensilage, especially with respect to protein degradation. It is noteworthy that the results from experiment 2 show that this was independent of the initial WSC content of the crop and the lactic acid content of the silage. A number of factors may account for this increased protein degradation. Higher pH conditions, or a slower decline in pH, may have resulted in more extensive plant proteolytic activity. Previous studies indicate that varying white clover N content affects protease enzyme profiles (Kingston-Smith et al., 2000) and this may have been a contributory factor.

Consistent changes in the L:D ratios of lactic acid observed in both experiments may indicate a shift in the population of lactic acid bacteria. Garvie (1967), in a study on the specificity of lactic acid isomers produced by lactic acid bacteria, concluded that *L. plantarum* produces a fairly constant ratio of L:D isomers during growth and that this ratio is characteristically low with this species. Thus the lower ratios observed in low N silages, produced in these experiments, may reflect the profile of lactic acid produced by the inoculant *L. plantarum* strain. In contrast, the increasing L:D ratios with increasing N in silages may indicate a shift in bacterial populations due to other species successfully competing with the inoculant species. Winters et al. (2000) showed that an inoculant strain of *L. plantarum* made little contribution to protein degradation during ensilage of grass whilst other species of lactic acid bacteria, dominating in non-inoculated silages, made a significant contribution.

The finding that the extent of protein degradation during ensilage is related to the initial N content of white clover herbage has relevance to ruminant nutrition. Charmley (2001)

reviewed recent literature on silage quality and cited data which demonstrated that increased non-protein N (NPN) content was correlated with reduced voluntary intake and bodyweight gain in sheep and steers, leading to the conclusion that soluble NPN content was a major factor in reducing efficiency of silage protein utilization. Decreases in WSC content observed in high N silages may also affect intake. Huhtanen et al. (2002) surveyed 21 studies, and concluded that WSC content was positively correlated with voluntary intake. This illustrates the desirability of developing strategies to minimise the extent of protein degradation during ensilage of white clover.

Increased protein degradation is due to either a direct effect of protein on silage fermentation, or to the protease profile of the crop. If the effect is solely due to the high protein content, then ensiling mixtures of white clover with grass in optimised ratios could benefit the protein quality of the ensiled crop and potentially result in more efficient use by ruminants. In particular, a better balance of available N and carbon in the rumen could be achieved if the mixture included a grass with a high sugar content. Dewhurst et al. (2003) showed some improvement in the efficiency of N incorporation into milk with mixed white clover/grass silage, compared with white clover silage alone. However, at present it is difficult to predict white clover content of a mixed sward during the growing season. Thus, summer dominance by white clover may be an important consideration in situations where mixed grass and white clover swards are cut for silage.

Increased protein degradation with high protein herbage may also be a consequence of changing protease profiles with changing protein content. In this case, there is a need to characterise these enzymes and explore the possibility of breeding for desirable protease levels in high protein, N-fixing, white clover to minimise protein degradation during ensilage.

5. Conclusion

A relationship between N and protein levels in white clover herbage and the ensiling process has been demonstrated. Most notably, the extent of protein degradation during ensilage was influenced by the initial N content of the fresh herbage. Non-nodulating lines of white clover offer further opportunities to examine relationships between white clover N content and the ensilage process, and also to establish whether the extent of protein degradation is determined by microbial fermentation or the plant protease profile.

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