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Original article

Ammonia treatment of lucerne and cocksfoot harvested at two growth stages: Effect on cell wall composition and digestibility

Nathalie Ballet, Jean-Michel Besle*, Camille Demarquilly

Unité de la digestion microbienne, station de recherches sur la nutrition des herbivores, Inra de Clermont-Ferrand/Theix, 63122 Saint-Genès-Champanelle, France

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Abstract – *Medicago sativa* L. var. Europe and *Dactylis glomerata* L. var. Amply were harvested at the middle and the end of first growth. Lucerne and cocksfoot samples were treated with 30 g ammonia per kg DM for 4 days at 80 °C to study the effects on nitrogen content, cell wall composition, and 48 h in situ and pepsin-cellulase digestibilities of dry matter (DM) and cell walls (CW). Lucerne had a very different CW composition than cocksfoot and changed less over maturity. Lucerne had a greater digestibility, but on a cell wall matrix (CWM) basis, it had a higher lignin content, reflecting essentially differences in tissue organization. In spite of a great increase of lignin concentration in the undigested residues, the ratio of CWM lignin concentration in lucerne to that in cocksfoot was very close, on average, in initial cell walls and in in situ undigested residues (1.72), suggesting a prominent effect of lignin partitioning in the initial cell wall on inhibition of digestibility. Ammonia treatment significantly increased the nitrogen content of all four samples both in DM and in CW. It improved DM digestibility slightly for lucerne and more strongly for cocksfoot (about 3.4 and 11.7 points in situ respectively for young stage). Increased digestibility tended to be greater at full flowering only for cocksfoot. In lucerne, the fraction solubilized by ammonia was low, composed mainly of polyuronic acids, which are highly digestible. In cocksfoot, the solubilized fraction was greater and composed of hemicelluloses and phenolic acids. The in situ digestibility of the treated CW fraction was enhanced slightly in lucerne and more strongly in cocksfoot. For both forages, the chemical extractability by ammonia reflected the biological susceptibility of the CW to the rumen microbes. (© Elsevier / Inra)

ammonia treatment / lucerne / cocksfoot / digestibility / cell wall

Résumé – Traitement à l'ammoniac de la luzerne et du dactyle prélevés à deux stades de maturité : Effet sur la composition des parois et la digestibilité. Les effets d'un traitement à l'ammoniac (30 g par kg de MS pendant 4 j à 80 °C) ont été mesurés sur la teneur en azote, la composition des parois et la digestibilité in situ (48 h) et pepsine-cellulase d'une luzerne et d'un dactyle récoltés en

* Correspondence and reprints
Tel.: (33) 04 73 62 40 52; fax: (33) 04 73 62 42 73; e-mail: besle@clermont.inra.fr

milieu et fin du premier cycle de végétation. Les parois de luzerne ont une composition très différente de celle du dactyle et qui évolue moins avec la maturité de la plante. La luzerne est plus digestible bien que la teneur en lignine de la matrice pariétale (polyosides + lignines) soit plus élevée, traduisant ainsi des différences dans l'organisation des tissus. En dépit d'une forte augmentation de la teneur en lignines, dans les résidus non digérés (fermentation in situ), le rapport matrice pariétale/teneur en lignines, de la luzerne et du dactyle reste très proche dans les parois, avant et après fermentation (1,72). Ce résultat suggère l'effet déterminant de la répartition des lignines dans les parois, avant fermentation, sur l'intensité de la dégradation de ces parois. Le traitement à l'ammoniac a augmenté significativement la teneur en azote de la matière sèche (MS) et des parois. La digestibilité in situ de la MS de la luzerne a été légèrement améliorée alors que celle du dactyle l'a été plus fortement, tant au stade jeune (respectivement 3,4 et 11,7 points) qu'au stade mature (3,9 et 12,7 points). Chez la luzerne, la fraction solubilisée par l'ammoniac a été faible et composée principalement de polyuronates, constituants très digestibles. Chez le dactyle, elle a été plus importante et composée d'hémicelluloses et d'acides phénoliques constituants plus ou moins digestibles. La digestibilité in situ des parois a donc été moins augmentée pour la luzerne que pour le dactyle. Les différences de solubilisation chimique suite au traitement à l'ammoniac reflètent celles de la susceptibilité des parois traitées à la dégradation par les microorganismes du rumen. (© Elsevier / Inra)

traitement à l'ammoniac / luzerne / dactyle / digestibilité / parois

1. INTRODUCTION

The improvement of the digestibility of forage plant cell walls obtained by ammonia treatment appears to be linked to disruption of cell wall organization. Ammonia modifies both the physical structure of the cell walls by reducing cellulose crystallinity [12] and their biochemical structure, by cleaving ester bonds linking xylan glucuronic units to lignins, and phenolic acids to hemicelluloses or lignins, thereby disrupting the hemicellulose-lignin matrix [12]. These modifications lead to an increased swelling of the cell walls [4, 12, 23] and consequently, an improved accessibility of cell wall polysaccharides to glycolytic enzymes. Ammonia treatment appreciably increases the digestibility of grasses [25, 31–33] but its effect on lucernes is less marked [3]. It has been suggested that the greater effect of alkali on the digestibility of grasses might be due to cleavage of ester bonds between phenolic compounds and polysaccharides of grass cell walls [14, 15], as this type of bond is not abundant in the hemicellulose-lignin complex of legumes. However, the mechanism of action of alkalis is not yet fully elucidated.

The purpose of this work was to compare the influence of ammonia treatment on cell wall composition, nitrogen content and digestibility of lucerne and cocksfoot harvested at two growth stages, in order to study the mechanisms of action of ammonia and thereby account for the differences in effect observed in grasses and legumes.

2. MATERIALS AND METHODS

2.1. Forages

Lucerne (*Medicago sativa* L. var. Europe) and cocksfoot (*Dactylis glomerata* L. var. Amply) were grown in the field of Inra station of Theix, France. During the first vegetative cycle, whole plants of lucerne were harvested at the early budding stage and at the full flowering stage and whole plants of cocksfoot at early earing and full flowering. The forages were dried for 72 h at 50 °C and then milled through a 1 mm screen.

2.2. Treatment of samples

The milled samples were placed in 850 mL glass jars and treated with 30 g of ammonia per kg DM. The volume of water added with the

ammonia was adjusted to obtain a moisture content of 40%. This level was chosen as it is reported to be optimal for the efficiency of the treatment [26, 33]. The glass jars were then placed in an oven at 80 °C for 4 days. They were weighed at the beginning and at the end of the experiment to detect any ammonia leakage.

After the treatment, the samples were dried for 48 h at 35 °C to eliminate free ammonia.

2.3. Animals

Two castrated male Texel sheep weighing about 60 kg, equipped with permanent rumen cannulas, were used for measurements of in situ digestibility in the rumen. They were fed with about 1200 g DM of lucerne hay in two daily meals (8:00 and 16:00 hours).

2.4. Estimation of digestibility

The dry matter (DM) digestibilities of the lucerne and cocksfoot samples were estimated by two methods: in situ digestibility and cellulase digestibility.

The in situ DM digestibility was measured by the nylon bag method. Nylon bags (Ankom, USA, 5 × 10 cm, pores 50 μm ± 1), containing 3 g of milled sample, were incubated for 48 h in the rumen of fistulated sheep. Four measurements were made per sample (2 sheep × 2 bags). The control and treated samples were placed simultaneously in the rumen of each sheep.

After removal from the rumen, the bags were rapidly washed in water and frozen. After thawing they were washed again in a washing machine (5–6 cycles of 5 min) until rinse water was clear, and then dried at 60 °C for 48 h.

The initial solubility, corresponding to the fraction immediately soluble within the rumen (water solubility), was estimated according to Ballet et al. [3]. Briefly, bags containing 3 g of sample were placed individually in 350-mL glass jars containing 250 mL of water, shaken thoroughly for 4 h at 40 °C, and dried for 48 h at 60 °C.

The cellulase digestibility of the DM was determined by the pepsin-cellulase method (cellulase Onozuka R10) according to Aufrère and Michalet-Doreau [2].

2.5. Chemical analyses

The cell wall residue (CWR) of treated and untreated samples was obtained after washing with water (40 °C) and reflux extraction in a Soxhlet apparatus with ethanol and ethanol/toluene (1/2, v/v). The polysaccharide and lignin contents were determined on the CWRs by the sequential method of Jarrige [19]. After hydrolysis, hemicelluloses and cellulose were analyzed by colorimetry as reducing sugars with xylose and glucose as standards, respectively [5]. Uronic acids were determined on CWRs, after hydrolysis according to Englyst et al. [11], by the colorimetric procedure of Blumenkranzt and Asboe-Hansen [7]. The ester-linked phenolic acids were assayed by the method described by Hartley [13] with slight modifications. In brief, they were released from the CWRs (700 mg for the lucerne and 350 mg for the cocksfoot) by gentle alkaline hydrolysis (35 and 17.5 mL 1M NaOH, respectively) at ambient temperature overnight (16 h) under nitrogen with anisic acid as internal standard. They were then extracted with ethyl acetate and analyzed by HPLC according to Mosoni et al. [29]. Total nitrogen was determined on DM and on CWRs by the Kjeldahl method.

2.6. Statistical analyses

The data were processed by analysis of variance [30]. The Duncan test was used to identify significant differences between means.

3. RESULTS

3.1. Composition of forages and effect of ammonia treatment

The composition of the forages before and after treatment is given in *table I*. With aging, the untreated forages both displayed a significant increase ($P < 0.05$) in their cell wall content and a decrease in nitrogen content.

The cellulose and lignin contents increased ($P < 0.05$) in the CWR with maturity for both forages, whereas that of ester-linked ferulic acid (FA) decreased. The ester-linked *p*-coumaric acid (PCA) content increased with maturity in cocksfoot but decreased in lucerne.

Table I. Chemical composition of young and old[1] lucerne and cocksfoot before and after ammonia treatment.

Samples	TotalN	CWR	H	C	L	UA	FA	PCA	CWR-N
	$g \cdot kg^{-1}$ DM		$g \cdot kg^{-1}$ CWR ($g \cdot kg^{-1}$ DM)						
Lucerne									
Young UT	30^{e}	623^{cd}	111^{c} (69)^{D}	302^{e} (188)^{DE}	132^{bc} (82)^{B}	134^{a} (83)^{A}	0.40^{c} (0.25)^{C}	0.32^{e} (0.20)^{E}	29^{b} (18)^{B}
Young T	36^{b}	585^{e}	105^{c} (61)^{D}	315^{e} (184)^{E}	139^{ab} (81)^{B}	92^{b} (54)^{B}	0.02^{e} (0.01)^{E}	0.10^{g} (0.06)^{G}	33^{a} (19)^{A}
Old UT	26^{d}	699^{b}	110^{c} (77)^{D}	327^{d} (229)^{C}	154^{a} (108)^{A}	115^{a} (80)^{A}	0.19^{d} (0.13)^{D}	0.20^{f} (0.14)^{F}	23^{d} (16)^{C}
Old T	40^{a}	650^{c}	115^{c} (75)^{D}	341^{d} (222)^{CD}	156^{a} (101)^{A}	93^{b} (61)^{B}	0.05^{e} (0.03)^{E}	0.11^{g} (0.07)^{G}	28^{c} (18)^{B}
Cocksfoot									
Young UT	23^{d}	698^{b}	202^{a} (141)^{AB}	383^{c} (267)^{B}	88^{d} (61)^{C}	38^{c} (27)^{C}	10.73^{a} (7.49)^{A}	4.44^{b} (3.10)^{B}	19^{e} (13)^{E}
Young T	34^{b}	603^{d}	172^{b} (104)^{C}	425^{b} (256)^{BC}	78^{d} (47)^{D}	34^{c} (21)^{C}	0.18^{d} (0.11)^{D}	1.33^{d} (0.80)^{D}	23^{d} (14)^{D}
Old UT	11^{e}	780^{a}	205^{a} (160)^{A}	453^{b} (353)^{A}	129^{bc} (100)^{A}	28^{c} (22)^{C}	6.41^{b} (5.0)^{B}	5.26^{a} (4.1)^{A}	8^{g} (6.2)^{G}
Old T	23^{d}	698^{b}	177^{b} (124)^{BC}	513^{a} (358)^{A}	118^{c} (82)^{B}	32^{c} (22)^{C}	0.24^{d} (0.17)^{D}	1.72^{c} (1.20)^{C}	11^{f} (7.7)^{F}
S.E.M.	0	6.4	8 (6)	9 (7)	4 (3)	4 (2)	0.01 (0.00)	0.02 (0.00)	0 (0)

$^{a-g}$ ($g \cdot kg^{-1}$ cell wall) and $^{A-G}$ ($g \cdot kg^{-1}$ DM): for each parameter, values with different letter superscripts are significantly different ($P < 0.05$).

[1] Young stage: early budding for lucerne and early earing for cocksfoot; old stage: full flowering for both forages. UT, untreated; T, treated; H, hemicelluloses; C, cellulose; L, lignins; UA, uronic acids; FA, ferulic acid; PCA, *p*-coumaric acid; totalN, total nitrogen; CWRN, cell wall nitrogen; S.E.M., standard error of the mean.

At each growth stage the CWR, hemicellulose and cellulose contents of the untreated lucerne were significantly lower ($P < 0.05$) than those observed in untreated cocksfoot, whereas the lignin, uronic acid and nitrogen contents were higher. Levels of ester-linked phenolic acids (FA + PCA) in untreated lucerne were 20 to 30 times lower than those measured in untreated cocksfoot.

Ammonia treatment caused a significant decrease ($P < 0.05$) in the CWR content of all four samples. This decrease was about 40 $g \cdot kg^{-1}$ DM (i.e., 6 to 7%) in the lucerne and 90 $g \cdot kg^{-1}$ DM (i.e., 11 to 14%) in the cocksfoot, at both early and late growth stages.

The decrease in the CWR content of the cocksfoot was essentially due to the solubilization of a hemicellulose and some lignin fraction. For lucerne, ammonia solubilized mainly uronic acids with very little other cell wall components being solubilized.

Ammonia treatment also solubilized about 95% of FA and 70% of PCA, for both young and old cocksfoot, and for young lucerne. The release of ester-linked phenolic acids was lower for the old lucerne, which had also a lower initial level.

Ammonia treatment also caused a significant increase in total nitrogen content. This increase was about 12 $g \cdot kg^{-1}$ DM in all the samples except for young lucerne in which the increase was only 6 $g \cdot kg^{-1}$ DM. Nitrogen enrichment was therefore proportionally greater in old forages, which are poorer in nitrogen.

In both forages, very little of the nitrogen supplied by ammonia was bound to the cell walls. On average, the ammonia treatment increased cell wall nitrogen by only 12%.

3.2. Forage digestibilities and effect of ammonia treatment

The initial solubilities and digestibilities of the forages before and after treatment are presented in *table II*. At the same growth stage, water-solubility was significantly higher ($P < 0.01$) in lucerne than in cocksfoot. It decreased with age, but less for lucerne than for cocksfoot. In situ and pepsine-cellulase DM digestibilities were also greater for lucerne than for cocksfoot at a given growth stage, decreasing significantly ($P < 0.01$) with maturity but less in lucerne than in cocksfoot. Similarly, the in situ cell wall digestibility of the two forages decreased with maturity (*table III*). This decrease was more marked for cocksfoot (–26 points) than for the lucerne (–9 points). In cocksfoot the decrease in cellulose digestibility was three times greater than in lucerne, whereas the decrease in hemicellulose digestibility was about twice as great.

After fermentation, lignin concentration increased greatly in the undigested residues (*table III*). The in situ lignin disappearance was moderately high in lucernes and young cocksfoot (42.5%) but was lower in cocksfoot at full flowering stage.

Table II. Influence of ammonia treatment on initial solubility (% DM) and 48 h in situ and cellulase DM digestibilities of young and old[1] lucerne and cocksfoot.

Samples	Water solubility	in situ digestibility	Cellulase digestibility
Lucerne			
Young UT	33.9^{a}	81.1^{c}	70.3^{b}
Young T	34.6^{a}	84.5^{b}	78.3^{a}
Old UT	27.7^{d}	72.7^{e}	61.0^{d}
Old T	29.0^{c}	76.6^{d}	68.5^{b}
Cocksfoot			
Young UT	26.2^{e}	75.9^{d}	65.0^{c}
Young T	31.0^{b}	87.6^{a}	80.4^{a}
Old UT	17.7^{g}	52.4^{g}	36.2^{f}
Old T	23.2^{f}	65.1^{f}	52.7^{e}
S.E.M.	0.3	1.0	0.4

For each parameter, values with different superscripts are significantly different ($P < 0.01$).
[1] Young stage: early budding for lucerne and early earing for cocksfoot; old stage: full flowering for both forages.
UT, untreated; T, treated; S.E.M., standard error of the mean.

Table III. Cell wall composition (g·kg^{-1}undigested CWR) of undigested residues and in situ digestibility (%) of cell walls and cell wall components from untreated and ammonia treated lucerne and cocksfoot.

	Cell wall composition[2]			In situ digestibility[2]			
Samples[1]	H	C	L	DsCWR	DsH	DsC	DsL
Lucerne							
Young UT	149^{c}	302^{g}	250^{c}	70.5^{b}	60.3^{c}	70.5^{b}	44.1^{b}
Young T	103^{f}	347^{c}	290^{a}	74.2^{b}	74.7^{b}	71.6^{b}	46.3^{b}
Old UT	157^{c}	325^{f}	240^{c}	61.7^{f}	45.5^{d}	62.0^{c}	40.6^{b}
Old T	124^{e}	341^{e}	267^{b}	64.9^{ef}	62.2^{c}	65.0^{c}	40.0^{b}
Cocksfoot							
Young UT	218^{a}	376^{d}	150^{e}	66.6^{de}	64.0^{c}	67.3^{bc}	43.1^{b}
Young T	137^{d}	479^{b}	161^{e}	80.2^{a}	84.3^{a}	77.7^{a}	59.2^{a}
Old UT	208^{b}	425^{c}	158^{e}	40.6^{g}	39.8^{e}	44.3^{e}	27.3^{c}
Old T	135^{d}	504^{a}	177^{d}	52.1^{c}	63.5^{c}	53.0^{d}	28.2^{c}
S.E.M.	2.3	2.2	3.5	1.6	2.1	1.8	3.3

For each parameter, values with different superscripts are significantly different ($P < 0.05$).

[1] Young stage: early budding for lucerne and early earing for cocksfoot; old stage: full flowering for both forages; UT, untreated; T, treated.

[2] H, C, L, hemicelluloses, cellulose, lignins; DsCWR, DsH, DsC, DsL, in situ digestibility of cell walls, hemicelluloses, cellulose and lignins, respectively; S.E.M., standard error of the mean.

Ammonia treatment caused a significant increase in water-solubility ($P < 0.01$) except for young lucerne (*table II*). This increase was greater in cocksfoot than in lucerne (about + 5 and 1.3 points, respectively).

The treatment also caused a significant increase in both in situ and pepsin-cellulase DM digestibilities ($P < 0.01$) in the four forage samples. For the young stage the extent of the improvement was greater in cocksfoot (12 and 15 points for in situ and pepsin-cellulase digestibilities) than in the lucerne (3.4 and 8 points for in situ and pepsin-cellulase digestibilities). At the older stage, the increase was roughly similar for lucerne but tended to increase for cocksfoot.

The improvement of DM digestibility was mostly due to an improvement in cell wall digestibility. Ammonia treatment increased the in situ cell wall digestibility by about 13 points in the cocksfoot, but only 3.5 points in the lucerne. This increase was essentially due to improved hemicellulose degradation (+ 14 to 24 points) and, to a lesser extent, to an improvement in cellulose degradation (+ 3 points in mature lucerne and + 9.6 points in cocksfoot). The in situ disappearance of lignin was not improved by the ammonia treatment except in young cocksfoot.

4. DISCUSSION

At the growth stages at which they were compared the chemical compositions of lucerne and cocksfoot had several expected differences [8, 20]. As growth proceeds,

changes in cell wall composition are less marked in lucerne than in cocksfoot. In lucerne, lignins are localized only in the xylem whatever the stage of growth whereas in grasses lignins are observed also in the sclerenchyma and progressively in the parenchyma, both tissues becoming more lignified with maturity [18, 37]. The sheaths and blades of grasses also contain lignified tissues, unlike the leaves of lucerne. This partly explains why the digestibility of lucernes decreases less rapidly with maturity.

The ratio of indigestible cell wall matrix (hemicelluloses + cellulose + lignins) to initial lignin was on average 1.7 and 1.5 for untreated and treated lucerne, in comparison with 3.2 and 2.6 for untreated and treated cocksfoot. The reasons for the differences in these ratios can be attributed primarily to the distribution of lignins which are spread throughout several tissues and organs in grasses and are very localized in legumes. Another contributing factor may be the intra- and inter-polymer cell wall organization: lucerne has more condensed lignins [1] fewer phenolic acid bridges and also probably fewer covalent bonds between cell wall polysaccharides and lignins than grasses [21]. It has been suggested that legumes have a lesser compact cell wall matrix [17]. However, when lignins are expressed on a cell wall matrix basis (CWM), the ratio of CWM lignins in lucerne to that in cocksfoot slightly decreased with maturity but was close, on average, in initial untreated cell walls and undigested residues (1.72) and did not change greatly with the treatment (1.9 and 1.8, respectively). This observation favors lignin localization as the major explanation for the differences in the extent of cell wall degradability observed for lucerne and cocksfoot.

The greater effect of ammoniation on digestibility of grasses than on digestibility of lucerne also was observed by Waiss et al. [36] and Morrison [28]. This effect can be split into a chemical solubilization of cell wall fractions and increased microbial enzymatic hydrolysis of the insoluble cell wall [3]. In lucerne, the fraction solubilized by ammonia is composed mainly of polyuronic acids [3, 28]. These are derived essentially from pectic substances which are highly digestible [34] and their partial solubilization therefore has little effect on cell wall digestibility. In contrast, in cocksfoot the solubilized fraction is composed of hemicelluloses, phenolic acids and some lignins of variable digestibilities. The solubilization of these constituents therefore has a larger effect on cell wall digestibility.

Our results suggest a positive relationship between lignin solubilization caused by ammonia and the improvement of cell wall digestibility in cocksfoot, but a reverse relationship was observed for lucerne. Lignin content is generally considered as a poor predictor of digestibility improvement afforded by ammonia treatment of grass straws [6, 24, 35] and hays [27, 35]. On the other hand, it is has been suggested that the improvement in cell wall digestibility is mainly due to the cleavage of polysaccharide-lignin bonds [9]. Indeed, the variation of optical density in a buffer, which integrates all the modifications undergone by the cell wall aromatic components, is a better predictor of digestibility of alkali-treated straws than lignin content [6, 22].

In lucerne, the limited effect of ammonia on cell wall degradability is partly explained by the small number of covalent bonds, dominated by alkali-resistant bonds [9, 10], between polysaccharides and lignins. The breakdown of some alkali labile linkages may change the swelling or the hydrophobicity and consequently the accessibility to glycolytic enzymes.

5. CONCLUSION

Lucerne and cocksfoot have a very different cell wall composition and cell wall organization. For the same maturity stage,

lucerne cell walls had a greater lignin content but a higher digestibility than cocksfoot. The reason of these differences in digestibility of the cell walls can be attributed primarily to the distribution of lignins in the tissues of the forages. Ammonia treatment was much more effective in improving cell wall digestibility in cocksfoot than in lucerne. The low concentration of alkali labile and slowly degradable compounds in the lucerne cell wall resulted in limited improvement by ammonia treatment. The degree of chemical solubilization of the cell wall by ammonia reflected the respective increases in susceptibility of the residual cell wall to the rumen microbes for both lucerne and cocksfoot.

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