Role of ammonia and biogenic amines in intake of grass silage by ruminants

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M. van Os

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de Landbouwuniversiteit Wageningen,
Dr. C.M. Karssen,
in het openbaar te verdedigen
op woensdag 22 januari 1997
des namiddags te half twee in de aula.

Stellingen

1. Voor het ontstaan van biogene aminen in graskuilen is de aanwezigheid van boterzuurbacteriën niet noodzakelijk. *(Dit proefschrift)*

2. Niet de uiteindelijke pH-waarde, maar de snelheid waarmee deze pH-waarde bereikt wordt, is de belangrijkste factor in de vorming van biogene aminen in graskuilen. *(Dit proefschrift)*

3. De directe relatie tussen de opnamesnelheid per maaltijd en de totale voeropname per dag, zoals verondersteld door Moseley and Manendez, gaat niet op indien de dagelijkse voeropname chemostatisch bepaald is of als een lage opnamesnelheid gecompenseerd wordt door een verhoging van de maaltijdfrequentie. *(Dit proefschrift)*
(Moseley, G. en Manendez, A.A. (1989). In: Proc. of the XVI International Grassland Congress, pp. 789. Nice, France).

4. Ammoniak en biogene aminen als zodanig spelen geen rol in de voeropnamedepressie van graskuilen. *(Dit proefschrift)*

5. Het positieve effect op de voeropname van beperking van fermentatie tijdens het inkuilen houdt meer verband met het positieve effect van behoud van nutriënten dan het negatieve effect van de aanwezige fermentatieproducten in de kuil.

6. Het voeren van laag bemest gras aan rundvee is moeilijk te combineren met verlaging van de methaanproductie.

7. Het toevoegen van probiotica en oligosacheriden aan de menselijke voeding is niet nodig indien het dagelijks diet voldoende vezels en gefermenteerde melkproducten bevat en matig is in vlees.

8. Een overheidsbeleid gericht op stimuleren van eigen huisbezit en mobiliteit in werk draagt niet bij aan vermindering van de automobiliteit.

9. Wachtlijsten voor hartoperaties en het afwijzen van een bypass voor het Groene Hart zijn economisch onverantwoord.

10. Communicatie vereenvoudigd integratie.

11. Een veronderstelling blijft een waarheid als een koe tot het tegendeel bewezen is.

Stellingen behorend bij het proefschrift:
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Monique van Os, Wageningen 22 januari 1997.

Voorwoord / Avant-propos

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Contents

Chapter 1

Introduction

Introduction

In the last two decades ensilage has largely replaced hay making to preserve forage to meet feed requirements during the winter period. While in 1975 in Western and Northern Europe about 69 Mtonne dry matter of hay and 32 Mtonne dry matter of grass silage were made, this shifted towards about 49 Mtonne of hay and 71 Mtonne of grass silage in 1990 (Wilkinson and Stark, 1987; 1992). Underlying factors for this change in forage preservation include; ensiling being less dependent on weather conditions, the mechanisation of silage making and silage feeding, as well as the introduction of new materials, techniques and additives improving silage quality (Zimmer, 1979). Moreover, the increasing livestock production rates needed grass to be preserved at earlier stage of maturity having a better nutritive value and a higher digestibility (Thomas and Thomas, 1985). Hereto, ensiling appears to be a more suitable method than hay making, being less time consuming and fitting better in to-day farm management (Zimmer, 1979). Maximizing nutrient supply from home-grown forage is nowadays of interest with respect to economical and environmental aspects. The advantages of ensilage above hay making made grass silage a major component in feeding rations for dairy and cattle production in Europe.

However, the intake of ensiled grass is often less than the intake of hay or fresh grass of similar digestibility. The degree of difference is highly variable and ranges from 1 to 64% (Demarquilly, 1973). It depends on the ensiling method influencing the changes in chemical composition of the grass between cutting and feeding. Chopping (Castle *et al.* 1979; Deswysen *et al.* 1978; Dulphy *et al.* 1984) or wilting (Marsh, 1979; Zimmer and Wilkins, 1984) the grass before ensiling, and the use of additives including formic acid (Dulphy *et al.* 1984), fermentable nutrients (Thomas, 1978) and inoculants (Rook *et al.* 1988; Sharp *et al.* 1994) improve the nutritive value of the silage through reducing nutrient losses and increasing silage dry matter intake. The adverse role of fermentation products in silage on intake is suggested by the negative correlations found between silage intake and total acid content, concentrations of volatile fatty acids, ammonia as a proportion of the total nitrogen content (Gill *et al.* 1988; Rook and Gill, 1990), or ammonia in combination with amines expressed on dry matter basis (Miettinen *et al.* 1991).

Among the fermentation products in silage, the role of the organic acids in controlling silage intake has excessively been studied (Forbes, 1995). For ammonia and amines, however, a significant role in depressing silage intake has been suggested (Gill *et al.* 1987). Only a few studies have been carried out on the

influence of these end-products from protein fermentation on intake control. Contradictory results have been reported, in part due to differences in levels tested, the way of application (addition to ration or infusion), infusion site in the intestinal tract, or in the case of amines, the type of amine tested. Nevertheless, a direct effect of ammonia and amines on intake control appears to be likely. Both are toxic agents in mammals intermediary metabolism (Visek, 1984; Joosten, 1988) and considering their concentrations is grass silage (Tveit *et al.* 1992) substantial amounts are ingested.

In the present thesis, we investigated the formation of ammonia and biologically active (biogenic) amines in grass silage, and their influence on silage intake control at concentrations generally present in untreated grass silages. In Chapter 2 literature is reviewed concerning voluntary intake of grass silage, the intake control mechanisms involved, and the influence of individual fermentation products and some silage characteristics on voluntary intake. Because in the literature extensive protein degradation and formation of amines are mostly described in poor-quality silages, with only the formation of three or four single types of amines studied per experiment, we investigated the formation of a larger number of biogenic amines in well preserved laboratory silages in relation to the initial fermentation rate (Chapter 3). The most important biogenic amines found in the laboratory scale silages, and ammonia were subsequently tested for their influence on silage intake in sheep (Chapter 4) and dairy cows (Chapter 5). In Chapter 6, we investigated *in vitro,* whether the tested amines are degraded in rumen content of sheep, with additionally considering the influence of the amine concentration in their diet on the extent of rumen degradation of amines. Apparently, the amount of amines tested in the studies of Chapter 4 and Chapter 5 were in the lower part of the range of concentrations found in untreated silages. Therefore, a second experiment was carried out with sheep (Chapter 7) in which the influence of a greater amount of amines supplemented to a good-quality silage on silage intake was studied. In this experiment, we investigated also the possibility that a combination of ammonia and biogenic amines affects silage intake and intake behaviour. It was assumed that amines may depress intake during the first few days of feeding. Their acute effect on intake, and extent of adaptation to biogenic amines were tested in sheep (Chapter 8). In the general discussion (Chapter 9), results of the experiments are discussed and related to the current knowledge on protein degradation and amine formation during ensiling and the intake regulation of grass silage.

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Chapter 2

Voluntary intake and intake control of grass silage by ruminants

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M. van Os and J.P. Dulphy

Abstract

Reviewing the voluntary dry matter intake (DMI) of grass silages in sheep and cattle show that DMI of very good- and good-quality silages is nearly similar to that of the corresponding dry forage prepared from the same sward or the fresh forage. The DMI of more extensively fermented silage is reduced in both sheep and cattle. Especially when the silage contains high amounts of volatile fatty acids (VFA) and ammonia (NH_3). The fermentation products in those poorly preserved silages seem to lower DMI through impaired palatability, and through earlier reaching the state of satiation. This satiation is not the result of a maximum rumen fill. Therefore, the mechanisms controlling silage DMI originate mainly from the oro-pharyngeal level (taste, smell) and metabolic level (metabolites or disturbed homeostasis in rumen or intermediary metabolism). Although significant negative relationships exist between acetic acid, propionic acid, butyric acid and ammonia, these compounds can not be indicated as direct depressors of silage DMI. When added to the diet or infused into the rumen at doses resembling those ingested upon consuming a poorly preserved silage they do not reduce DMI. Possibly, the negative effect of fermentation on DMI of silage is the result of a combined effect of the individual fermentation products and overall fermentation.

Besides lowering silage palatability and inducing satiation, fermentation products in silage can lead to metabolic disregulation in the ruminant depressing DMI. An unbalanced nitrogen and energy supply in the rumen for microbial synthesis may result in an insufficient amino acid supply to the animal.

During protein degradation, which is substantial during silage fermentation and indicated by NH_3 formation, also other nitrogenous compounds are formed including amines. Concentrations of these physiological active agents are not routinely determined in silage. These amines or other possible unknown fermentation products must be considered in the overall negative effect of fermentation in silage on its voluntary DMI.

Introduction

To explain variations in forage intake and to predict the voluntary intake of forages, it is necessary to know which factors and mechanisms are involved in regulating voluntary intake. Ensiled grass is the main forage used in ruminant feeding in northern and western Europe (Wilkinson and Stark, 1992). The voluntary intake of silage shows large variation and is generally lower (1-64 %) than that of the corresponding fresh grass (Demarquilly, 1973) or hay (Chiofalo *et al.* 1992; Thiago *et al.* 1992*a*) prepared from the same sward. Because nutrient supply to the animal depends on the level of voluntary intake, extensive research has been done to explain the lower intake of ensiled forages.

The aim of this paper is to review silage related factors affecting intake and the mechanisms controlling intake.

Grass silage intake

Voluntary daily intake

The fermentation quality of grass silage can be characterized by its levels of fermentation products. Quality classes based on fermentation products have been proposed by several authors (Flieg, 1938; Wieringa and De Haan, 1961; Zimmer, 1966; Dulphy and Demarquilly, 1981; Offer *et al.* 1993). In this paper the system of Dulphy and Demarquilly (1981) is adopted (Table 2.1)

Several comparisons have been made of the voluntary dry matter intake (DMI) of very good-quality grass silages and that of the initial green forage or hay prepared from the same sward. Dulphy and coworkers found with heifers a 2-5% lower DMI of good-quality silages in 32 direct comparisons with the green forage (Dulphy *et al.* 1984; 1987). With dairy cows Dulphy (1987) found in one comparison a 6% higher DMI of silage when compared to hay prepared from the same sward, whereas in 3 other direct comparisons a 5% reduction was found.

In sheep, Dulphy and Michalet-Doreau (1981) report a 17% reduction in DMI of a good-quality silage, when comparing it with the original fresh forage. In this study the intake of fresh forage was measured at long day lengths, whereas silage intake was measured in winter at short day lengths. Feeding the same hay throughout the year, Michalet-Doreau and Gatel (1983) found a 17% reduction in

Table 2.1 *Preservation quality of grass silage (Dulphy and Demarquilly, 1981).*

Class	Level of fermentation products			
	Total VFA (mmol/kg DM)	Acetic acid (g/kg DM)	Butyric acid (g/kg DM)	Ammonia-N (% of N_{total})
Very good	< 330	< 20	0	< 7
Good	330-660	20-40	< 5	7-10
Medium	660-1000	40-55	> 5	10-15
Poor	1000-1330	55-75	> 5	15-20
Very poor	> 1330	> 75	> 5	> 20

VFA, volatile fatty acids; DM, dry matter.

DMI as the effect of day length. So most probably the difference in DMI between silage and grass observed by Dulphy and Michalet-Doreau (1981) has to be attributed to photoperiodicity and not to fermentation products.

It is concluded that DMI of very good- and good-quality grass silages is similar or only slightly reduced as compared to that of hay or the initial green herbage.

Decrease in silage preservation quality, characterized by higher levels of fermentation products (NH_3 and VFA; Table 2.1), declines DMI. Negative correlations exist between concentrations of NH_3, acetic acid and dry matter content of the silage with DMI in sheep (Wilkins *et al.* 1971; Brown and Radcliffe, 1972; Demarquilly, 1973), beef cattle (McCullough, 1966; Waldo *et al.* 1968; Rook and Gill, 1990) and dairy cows (Miettinen *et al.* 1991). Propionic acid correlates also negatively with DMI in sheep (Brown and Radcliffe, 1972), butyric acid with DMI in sheep and cattle (Demarquilly, 1973; Rook and Gill, 1990), and NH_3 plus amines with DMI in dairy cows (Miettinen *et al.* 1991). Dulphy and Michalet-Doreau (1981) linked levels of fermentation products quantitatively to effects on silage DMI. In sheep they calculated intake reductions of; 6.3 g/kg metabolic live weight ($LW^{0.75}$) when pH increases 1 unit; 7.0 g/kg $LW^{0.75}$ per 10% units increase of the NH_3-N content expressed as % of N_{total}; 7.8 g/kg $LW^{0.75}$ when acetic acid concentration increases 100 g/kg DM, and an intake reduction of 5.5 g/kg $LW^{0.75}$ per 1 mol increase of the total VFA content/kg DM.

Because the concentration of these fermentation products are mutually strongly correlated, the calculated effects are not cumulative.

The negative effect of fermentation on silage DMI is also evident when DMI of naturally fermented silages is compared to that of the same herbage ensiled with restricting fermentation by the use of additives (*e.g.* formic acid) or wilting. These treatments generally improve silage DMI (Table 2.2).

Table 2.2 *The positive effect of formic acid addition or wilting on silage dry matter intake (DMI) by ruminants (g/100 g DMI of the untreated silage).*

Animal	Ensiling treatment		Reference
	Formic acid[a]	Wilting[b]	
Heifers	16 (3.0 l/tonne)		Waldo *et al.* 1968
Sheep	15 (4.5 l/tonne)		Dulphy *et al.* 1984
Heifers	23 (4.5 l/tonne)		Dulphy *et al.* 1984
Sheep	18 (4.5 l/tonne)		Chiofalo *et al.* 1992
Heifers	20 (3.0 l/tonne)		Moloney and O'Kiely, 1994
Sheep		44 (25→45% DM)	Marsh, 1979
Cattle		31 (25→45% DM)	Marsh, 1979
Dairy cows		10 (25→45% DM)	Marsh, 1979
Heifers		18 (25→40% DM)	Teller *et al.* 1989

[a] application rate.

[b] increase of dry matter (DM) content.

Intake behaviour and rumination

Voluntary daily DMI is the result of the total number of meals per day and the quantity eaten during the individual meals. Compared to hay, the daily intake pattern of grass silage is characterized by a relatively short first meal after feeding, with lower DMI and a larger number of subsequent small meals in both sheep (Chiofalo *et al.* 1992) and cattle (Thiago *et al.* 1992*b*). The fermentation in silage thus appears to influence intake behaviour by giving earlier signs of satiation

stopping feed consumption.

With good-quality silages, the lower DMI during the principal meal is compensated by a higher DMI during the subsequent meals over the day resulting in similar silage DMI compared with that of hay or fresh herbage. The limited fermentation in good-quality silages thus allows the animal either to increase the number of subsequent small meals or to increase DMI during these meals (Dulphy, 1985).

With medium- to poor-quality silages (Table 2.1), the higher concentrations of fermentation products further reduces the time spent eating and DMI during the principal meal. Additionally, they reduce the number and DMI of subsequent small meals during the day (Dulphy *et al.* 1984; Chiofalo *et al.* 1992). This indicates that with poor-quality silages the satiation induced after the principal meal lasts throughout the day, and the associated intake reduction can not be compensated (Baumont *et al.* 1993).

The average rates of intake during the principal and subsequent meals also characterize forages having different DMI. Both are lower when feeding silage than when feeding hay (Chiofalo *et al.* 1992; Thiago *et al.* 1992*b*) or the fresh herbage (Dulphy *et al.* 1984). Though results of Dulphy *et al.* (1984) indicate that sheep react less to the extent of fermentation than heifers, the negative effect of extensive fermentation on intake rate can not be excluded.

The shorter time spent ruminating in cattle (Thiago *et al.* 1992*b*), dairy cows (Van Bruchem *et al.* 1991) and sheep (Chiofalo *et al.* 1992) fed a good-quality silage when compared to hay, or when feeding silages of inferior quality (Chiofalo *et al.* 1992; Teller *et al.* 1989) is more the consequence of the lower silage DMI than its cause. When feeding silage, the time lag between the end of the principal meal and the beginning of rumination is generally longer than when feeding hay hay. Probably this is caused by the lower rumen fill in silage fed animals (Thiago and Gill, 1986; Chiofalo *et al.* 1992), giving less tactile stimulation in the cardiac region of the rumen which delays regurgitation of the rumen digesta (Dulphy *et al.* 1984).

The lower total chewing efficiency (less g DM chewed per time unit) observed on silage is due to a longer chewing time per g DM during intake, and not due to longer rumination (Chiofalo *et al.* 1992).

In summary, silage gives earlier signs of satiation than hay influencing the silage intake behaviour.

Intake regulating mechanisms

Besides factors controlling intake at long term, such as physiological state, production level and nutrient availability, voluntary DMI during the day is regulated by short term mechanisms. On the short term, intake is controlled by a variety of signals from mechanisms located at oro-pharyngeal, rumen and metabolic level. These act solely or integrated, and determine meal size and meal frequency during the day (Grovum, 1988). Because DMI of good-quality silages is close to that of hay or fresh forage, similar intake regulating mechanisms are assumed. The reduced DMI of medium to poor-quality silages suggests that more fermentation also generates more satiation signals. These satiation signals could be generated at oro-pharyngeal, rumen or metabolic level.

Oro-pharyngeal intake control

Palatability is widely accepted as a factor determining forage intake (Greenhalgh and Reid, 1971; Grovum, 1988). It is generally defined as the sensory response of the animal to its feed, characterized by the willingness to accept the feed and by eating rate. Both, feed related factors (taste, smell and texture) and animal related factors like nutritional state and type and level of production are of influence.

Palatability has a definite impact on the intake of forages with low nutritive value, like straw. Sheep ingest more straw supplemented with sodium glutamate as a taste improver (Colucci and Grovum, 1993), whereas intake of urea treated straw is lower when it contains high concentrations of urea, which is known to be unpalatable (Dulphy *et al.* 1992). Assuming that silage contains fermentation products having strong taste and smell, negative effects of palatability on intake are likely. This was demonstrated by the higher DMI of silage with anosmic sheep (Michalet-Doreau, 1975), and by adding flavouring compounds (Henderson and Anderson, 1986; Corkum *et al.* 1994). Of the fermentation products in silage individually tested on palatability in sham fed sheep (NH_3, amino acids, amines, lactic, acetic and butyric acid), only acetic acid lowers silage DMI (Buchanan-Smith, 1990).

In normally fed animals, as emphasized by Buchanan-Smith (1990), when determining the impact of fermentation products on palatability, it is important to separate oro-pharyngeal intake control from that induced by post-ingestive signals of satiation. The effect of fermentation products on palatability can therefore also be deduced from a lower initial intake rate of the first meal. Here effects of

satiation can most likely be excluded. Just a generally reduced intake rate of the whole meal, as reported for silages when compared to hay and fresh forage by Dulphy *et al.* (1975; 1984), does not totally exclude satiation effects.

Intake control at rumen level

Physical aspects. An important factor determining voluntary intake of roughages is the fill of the reticulo-rumen (Balch and Campling, 1962), monitored by mechanoreceptors in the rumen epithelium (Grovum and Phillips, 1978). Rumen distension highly depends on retention time of the forage in the rumen, being mainly influenced by DM disappearance through digestion and passage of undegraded digesta (Van Vuuren, 1994). Digestibility of silage, when corrected for volatile constituents, does not differ from that of fresh herbage (Beever *et al.* 1971; Michalet-Doreau and Demarquilly, 1981), or hay prepared from the same sward (Beever *et al.* 1971; Thiago *et al.* 1992*a*). Additionally, fermentation quality of the silage has no influence on its digestibility (Sharp *et al.*1994; Cushnahan and Mayne, 1995). The rate of particle size reduction might be lower on silage diets, because of delayed rumination and a high proportion of pseudo rumination (Campling, 1966; Deswysen *et al.* 1978; Dulphy *et al.* 1984). The rumen pool turnover rate, however is similar (Chiofalo *et al.* 1992) or even higher (Thiago *et al.* 1992*a*) with silage than with hay. Moreover, rumen fill is consistently lower in animals fed silage than in animals fed hay. This holds true for sheep (Chiofalo *et al.* 1992), steers (Thiago and Gill, 1986; Thiago *et al.* 1992*b*) and dairy cows (Campling, 1966; Lawlor and O'Shea, 1967). With diminished preservation quality, rumen content is further reduced as demonstrated in sheep by Dulphy *et al.* (1975) and Chiofalo *et al.* (1992). These findings indicate that, unlike feeds with low nutritive value (straw), the voluntary intake of silage is not limited by the rumen capacity. Nevertheless it is possible that, in comparison to hay, a rapid production of fermentation gasses in the rumen upon silage intake can lead to increased rumen distension and depress DMI (Prates *et al.* 1986; Thiago *et al.* 1992*a*). However, comparing silage with fresh grass, this can not be the cause of lower silage intake, regarding the large soluble and easily fermentable fraction in fresh grass (Van Vuuren *et al.* 1991).

Chemical aspects. With silage, the increase in concentrations of fermentation products in the rumen is usually more rapid than with hay (Thiago *et al.* 1992*a*). This rapid increase originates from ingestion of fermentation products present in

silage and the more rapid digestion of the silage than compared with hay. The increase of rumen NH_3 and VFA, and therewith the decrease of rumen pH and increase of rumen osmolality will be detected by the chemoreceptors (Martin and Baile, 1972) respectively osmoreceptors (Gill *et al.* 1987) located in the rumen wall, which then generate signals of satiation (Gill *et al.* 1987). The impact of both types of receptors on intake control has been clearly demonstrated. Increasing rumen fluid osmolality depresses maize silage intake in sheep, whereas DMI totally ceases at values of 550 mosmol/l (Phillip *et al.* 1981). Both, the depressed DMI upon rumen infusions of lactic acid and VFA (Thomas *et al.* 1961*b*), and the depressed DMI in cows upon infusions of acetate or propionate salts, compared to an iso-osmolar control infusion (Anil *et al.* 1993), indicate a role of chemoreceptors in intake control at rumen level. Furthermore, in dairy cows DMI is depressed when NH_3 concentrations of >300 mg/l are present in the rumen for a longer period (Choung *et al.* 1990).

The depressed DMI of the meal after intra-rumen infusion of physiological doses of silage juice (Buchanan-Smith and Phillip, 1986) makes it conceivable that fermentation products act on chemostatic regulation at rumen level. Thiago *et al.* (1992*a*) reported that he meal size is determined by this type of intake control. The grass silage fed steers show a shorter duration of the principal meal with lower DMI, which is accompanied by a faster increase and higher peak concentrations of NH_3 and VFA in the rumen, as well as a more rapid pH drop than when feeding hay. In contrast, Chiofalo *et al.* (1992) found lower pH, higher osmolality and VFA contents in the rumen at the end of a principal meal of hay with a longer duration and higher DMI than with silage. Similar results have been observed when comparing wilted and direct cut grass silage fed to dairy cows (Teller *et al.* 1989). These contradictory results of Thiago *et al.* (1992*a*) and Chiofalo *et al.* (1992) and Teller *et al.* (1989) on the impact of concentrations of fermentation products in the rumen on DMI indicate that the early satiation observed with poorly preserved silages is not only the result of chemostatic regulation at rumen level. Probably, the DMI of good-quality silages is regulated by palatability and chemostatic mechanisms in the rumen and that of poor-quality silages through combined effects of low palatability and signals from intake chemostatic mechanisms in intermediary metabolism.

Almost all studies described in literature lack detailed studies of rumen fermentation patterns while closely monitoring intake behaviour. This type of experimentation might be a method to quantify the impact of single or

combinations of fermentation products in the rumen on chemostatic intake control at this level.

Metabolic intake control

Especially with poor-quality silages, concentrations of fermentation products in the rumen appear not to be the determining factors of meal size and daily DMI (Teller *et al.* 1989; Chiofalo *et al.* 1992). When feeding silage, high amounts of fermentation products are absorbed from the rumen. These fermentation products are present in the silage and are formed upon digestion in the rumen. Concerning the amounts absorbed, it is plausible that control mechanisms at the level of the intermediary metabolism have also an impact on intake control.

Osmo- and chemoreceptors located in the gut, systemic circulation and liver detect changes in levels of nutrients or fermentation products, and induce signals regulating DMI (Baile and McLaughlin, 1987). These feedback signals, are transmitted by hormones, neuropeptides and neurotransmitters via humoral or neural pathways to the hypothalamus, the brain area involved in feed intake control (Morley *et al.* 1984). The gut hormones gastrin, secretin and cholecystokinin, all are secreted upon increasing concentrations of peptides or acids in the proximal duodenum, inhibit DMI in sheep (Grovum, 1981). Whether the impact of these hormones is more important during silage intake than in rations without silage has so far not been studied.

In poor-quality silages, important concentrations of γ-amino butyric acid (GABA), occur (Ohshima *et al.* 1979). This fermentation product of glutamic acid is also an inhibitory neurotransmitter affecting silage intake in sheep (Seoane *et al.* 1984).

Energy supply to tissue cells is another important factor in controlling DMI (Baile and Forbes, 1974). In ruminants the absorbed acetate, by directly being oxidized in the cell, and propionate and butyrate indirectly by being precursors for liver gluconeogenesis, are the main source of energy supply to metabolism (Van Houtert, 1993). Insulin induced glucose uptake in the cell acts as a satiating factor depressing DMI (Bareille and Faverdin, 1996). With poor-quality silages containing low quantities of easily fermentable carbohydrates or lactate, rumen fermentation pattern shifts towards acetic acid instead of propionic acid (Van Houtert, 1993). Intake of this type of silage increases the acetic acid load in intermediary metabolism. The metabolism of acetate in tissue cells yields less energy per mole oxygen used than that of glucose. The consequently lower efficiency of oxygen use might also be a cause of depressed DMI (Ketelaars and Tolkamp, 1992). When

the amount of VFA absorbed exceeds the uptake by liver and tissue cells, a further rise in blood concentrations might depress intake by increased osmolality (Farningham and Whyte, 1993).

Metabolic disregulation. Lower DMI of poorly preserved silages can also be caused by metabolic disregulation (Howie, 1988). Possible disturbances in intermediary metabolism associated with silage intake are acidosis, ketosis, NH_3 intoxication or intoxication from other products of protein fermentation (*e.g.* amines). Further, a shortage of protein or amino acid supply to intermediary metabolism, as well as a lack of energy for metabolic functions are possible. The influence of fermentation products on the incidence of acidosis, ketosis, NH_3 and amine intoxication will be discussed later.

Intake being depressed as the result of a shortage of protein or amino acid supply at metabolic level is deduced from an increase in DMI after duodenal protein infusions in sheep fed a low protein diet (Egan and Moir, 1964). A shortage of protein supply being a cause in lowering silage DMI is acceptable, because in poor-quality silages the flow of rumen undegraded protein to the intestines is low, and the formation of microbial protein in the rumen is reduced due to an asynchronous N and energy supply (Thomas and Gill, 1988). In this situation, increased protein supply to the small intestines through either increasing passage of rumen undegradable protein by supplementing a poor-quality silage with fishmeal (Veira *et al.* 1994), or increasing microbial growth by supplementing the silage with an easily fermentable energy source, improves intake and N retention (Gill and England, 1984; Sanderson *et al.* 1992).

Metabolic disregulation could also be caused by an imbalanced supply of glucogenic (propionate) and ketogenic (acetate and butyrate) fermentation products. This is suggested by Gill *et al.* (1988) who reported increased DMI in sheep upon infusing glucose into the duodenum while also high rates of acetate were infused in the rumen.

In conclusion, the DMI of ensiled forage with normal digestibility is probably controlled by mechanisms at oro-pharyngeal level and by chemostatic control mechanisms at rumen and metabolic level. With poorly preserved silages it is supposed that mainly the low silage palatability and control mechanisms at metabolic level determine its voluntary DMI.

Silage related factors and individual fermentation products

Significant mathematical relationships exist between silage characteristics, including pH, DM content, VFA and NH_3, and silage intake. Extensive work has been carried to determine these silage characteristics or fermentation products responsible for lowering silage DMI, and the different intake control mechanisms involved.

Dry matter content

Silage DMI is positively related to the silage DM content (Dodsworth and Campbell, 1953; Jackson and Forbes, 1970; Wilkins *et al.* 1971; Rook and Gill, 1990). Lowering the DM content of lucerne hay to 250 g DM/kg, however, does not decrease DMI, whereas no increase in DMI is observed when a low DM (250 g/kg) silage prepared from the same lucerne crop is dried. The DMI remains significantly lower than that of the hay compared (Thomas *et al.* 1961*a*; Clancy *et al.* 1977). These observations indicate that silage moisture content *per se* (mainly extra-cellular water) is not a factor limiting intake. Ensiling a forage at higher DM content limits the rate of fermentation and restricts the amount of fermentation products formed. The resulting silage will be of better nutritive value (Wilkins *et al.* 1971), being more likely the factor improving silage DMI.

Organic acids

Silage acidity (pH). Mathematical regressions show that silage intake is reduced when the amount of free acids (acetic plus lactic acid) in silage exceeds 50 g/kg DM, or when its pH value is below 3.65 (Miettinen *et al.* 1991). When correlating silage pH with DMI, two different trends are present; 1, DMI decreases with increased pH value of the silage (Dulphy and Michalet-Doreau, 1981; Demarquilly, 1973), 2, DMI increases with the increased pH value (Wilkins *et al.* 1971; Brown and Radcliffe, 1972; Shaver *et al.* 1985; Erdman, 1988). The observations of Dulphy and Michalet-Doreau (1981) and Demarquilly (1973) indicate that silage acidity is not a cause of the depressed DMI. Here, higher pH values are due to larger amounts of buffering ammonia and the weaker acetic acid in the silage at the expense of lactic acid. Controversially, the increased DMI at high pH values (Wilkins *et al.* 1971; Brown and Radcliffe, 1972; Shaver *et al.* 1985; Erdman, 1988) can be attributed to the inclusion of wilted and formic acid treated silages in the range of silages studied. In these type of silages less extensive fermentation

(less pH decrease) is needed for a proper preservation of the forage.

There is some evidence that a high acidity of the diet lowers DMI through reduced palatability (Grovum and Chapman, 1988). Feeding neutralised silage to dairy cows increases DMI when the control silage pH is below 4 (Fahran and Thomas, 1978; Shaver, *et al.* 1985; Erdman, 1988; Ndwiga *et al.* 1990). These results indicate that with normal silage pH of about 4.0 acidity does lower palatability.

The acids ingested during silage eating are generally neutralized by the saliva produced (Orth and Kaufmann, 1966), which limits the direct effect of silage pH on rumen pH. This agrees with the findings that in sheep and cows the rumen pH values are similar upon intake of low pH silages and intake of the same silages being neutralized with $NaHCO_3$ (Fahran and Thomas, 1978; Ndwiga *et al.* 1990). In addition, comparing sheep consuming low pH silage with sheep consuming neutralized silage, no difference is found in blood and urine variables which indicate acid stress (Phillip and Hidalgo, 1989). These observations exclude metabolic acidosis as a cause for depressed DMI of silage .

So, the mathematical relationships found between silage pH (values from 3.8 to 6.0) and intake are more based on the amounts and type of the different organic acids present in the silage than the silage acidity *per se*.

Volatile fatty acids. Perception of VFA or by chemoreceptors located in the rumen wall depresses DMI (Martin and Baile, 1972). The dose related depression of DMI upon rumen infusions with acetic, propionic or butyric acid (Ulyatt, 1965) is probably induced by a decrease in rumen pH. Infusion of the salts of these acids also decrease DMI (Simkins *et al.* 1965), indicating their direct effect on chemostatic intake control at rumen level. In these studies the effect on intake is only present when high, unphysiological doses are used. The use of more physiological amounts of the individual VFAs appear to have nearly no effect on daily DMI. For acetic acid, neither rumen infusion (Phillip *et al.* 1981; Mbanya *et al.* 1993) nor addition of physiological doses to grass silage (Hutchinson and Wilkins, 1971) depress its DMI in sheep. However, the acetic acid changed the daily intake pattern by shortening the duration of the first meal after feeding. Silage DMI is not lower when physiological doses of propionic acid are infused in the rumen of dairy cows (Thomas *et al.* 1961*b*; Mbanya *et al.* 1993). For butyric acid, rumen infusion of doses higher than those ingested by silage neither affects silage DMI (Huhtanen *et al.* 1993). This study show that the high amounts of butyric acid

in poor-quality silages, does not cause ketosis which is often observed in dairy cows fed butyric acid silage (Howie, 1988). The increase in ketone bodies in the blood, mainly derives from butyrate metabolization by the liver (Van Houtert, 1993), does not depress DMI (Huhtanen *et al.* 1993). Therefore, the incidence of ketosis when feeding low-quality silages results most probably from catabolizing body fat due to the low DMI than being a direct cause (Van Vuuren *et al.* 1995). The metabolic control of DMI by propionate in the blood goes mainly via the increased glucose output from the liver (Farningham and White, 1993) and its subsequent insulin induced uptake in the cell (Sano *et al.* 1995; Bareille and Faverdin, 1996). Increased acetate concentrations in the blood, but also those of propionate when its supply exceeds the gluconeogenic capacity of the liver, will depress DMI via increased osmolality (Farningham and Whyte, 1993).

Lactic acid. Lactic acid is the only fermentation product being positively correlated with DMI (Wilkins *et al.* 1971; Demarquilly, 1973). However, concentrations higher than 100 g lactic acid/kg DM depress DMI probably by reduced palatability due to the increased acidity (Grovum and Chapman, 1988). No direct effect on DMI is observed when lactic acid is added to pelleted grass (120 g/kg DM), whereas rumen infusions of similar amounts depress intake in sheep (Morgan and L'Estrange, 1977). Addition of lactic acid to silage to a content of 120 g/kg DM also reduces DMI in sheep (McLeod *et al.* 1970). The intake depression by lactic acid has been attributed to the decrease of rumen pH, as perceived by the pH sensitive chemoreceptors in the rumen wall (Bueno, 1975). In the studies of Morgan and L'Estrange (1977) the reduced DMI could not be attributed to a decrease in rumen fluid pH. Here the proportion of VFA in the rumen changed by increasing concentrations of propionic acid at the expense of acetic acid. This can be explained by propionic acid being the fermentation product of lactic acid in the rumen (Jaakkola and Huhtanen, 1992). So, the lower intake upon lactate infusions by Morgan and L'Estrange (1977) is probably due to the increased flow of propionate to liver as discussed previously.

In conclusion, the organic acids present in silage have little impact on intake control at oro-pharyngeal level, but effects of acids formed upon silage digestion are more pronounced on chemostatic control mechanisms in the rumen and intermediary metabolism. The post ingestive effects of the fermentation products present in silage can not be separated from those formed upon digestion. Therefore

they must be considered to be cumulative. At physiological concentrations, the impact of the individual organic acids on reducing DMI are low. However, it is acceptable that additive effects of the individual acids with other fermentation products are in cause of depressing silage DMI.

Nitrogenous fermentation products

During ensiling a large part of the plant protein is degraded to peptides and amino acids. A considerable fraction of the amino acids is fermented into N-containing products including NH_3, amines, and N-containing acids, like α-amino butyric acid and γ-amino butyric acid (Ohshima *et al.* 1979). High concentrations of these compounds occur in poor-quality silages (Tveit *et al.* 1992). Their toxicity (NH_3; Visek, 1984) or physiological activity in intermediary metabolism (amines: Joosten, 1988; GABA: Seoane *et al.* 1984) make a negative effect on DMI conceivable.

Ammonia. Negative relationships exist between silage NH_3 and silage DMI when NH_3 is expressed on a N basis (Dulphy and Michalet-Doreau, 1981; Miettinen *et al.* 1991). Weak relationship with DMI is found when silage NH_3 is expressed on a DM basis (Rook and Gill, 1990), suggesting a low direct effect of NH_3 on DMI.

In Sheep, NH_3 does not reduce palatability (Buchanan-Smith, 1990). Whether NH_3 *per se* reduces intake can also be derived from studies in which NH_3 is added to silage or hay. Studies in which ammoniated hay is offered to sheep, no depression of DMI is observed (Benhamed and Dulphy, 1987). Similar results are found in studies in which dairy cows are offered ammonia treated maize silage (Heinrichs and Conrad, 1984). Although total DMI is unaffected, the dairy cows consume fewer meals during the day, but with higher DMI and higher intake rates.

In the rumen, high peak concentrations of NH_3 (>450 mg/l) are tolerated without depressing intake, in sheep consuming ammoniated hay (Benhamed and Dulphy, 1987) and in dairy cows being infused intraruminally with a single doses of urea (Choung *et al.* 1990). A chemostatic regulation at rumen level of NH_3 is therefore not expected. However, lasting rumen NH_3 concentrations of 200 mg/l throughout the day depress intake in cows (Choung *et al.* 1990), indicating regulation or disturbance beyond the rumen. In intermediary metabolism, NH_3 is a toxic agent that is adequately detoxified by the liver into urea (Visek, 1984). In diets containing easily fermentable protein, the continuing absorbtion of high amounts of NH_3 from the rumen may exceed the detoxifying capacity of the liver,

being about 1.84 mmol/min per kg wet weight in dairy cows (Symonds *et al.* 1981). This results in increased plasma NH_3 concentrations (Choung *et al.* 1990) which may depress intake through increased osmolality in the circulation (Carter and Grovum, 1990), or subclinical intoxication disturbing several hormone systems, including that maintaining glucose homeostasis (Visek, 1984).

The NH_3 content in silage is in general used as an indicator for silage quality and as a tool for predicting DMI. However, the direct effects of NH_3 on palatability and intake regulation at rumen level seem to be of no significance.

Amines. These products of protein fermentation are formed during silage fermentation by decarboxylation of amino acids. Small quantities are present in well preserved silages, but amounts increase when preservation quality diminishes (Tveit *et al.* 1992). The predominant amines in grass silage are histamine, tyramine, putrescine, cadaverine, tryptamine and phenyl-ethylamine (Ohshima *et al.* 1979; Tveit *et al.* 1992). Because of their physiological activity (Joosten, 1988), it has been assumed for several decades that amines negatively affect DMI of grass silage (Barry *et al.* 1970; Hole, 1985; Beever and Reynolds, 1994). Moreover, a negative relationship exists between NH_3 plus amine content in silage and its DMI in dairy cows (Miettinen, *et al.* 1991). The effect of amines on DMI through palatability and influences on intake control mechanisms at rumen or metabolic level have not been studied in any detail.

A combination of putrescine, cadaverine and GABA added to silage at the rate of 3.5 g/kg DM does not lower DMI by reducing palatability (Buchanan-Smith, 1990). Neither does adding 1 g of histamine to the daily ration of sheep (McDonald *et al.* 1963), nor adding 3 g to the daily silage ration of heifers (Okamoto *et al.* 1964) reduce DMI. Additionally, no adverse effect on silage DMI is found when histamine, tyramine or tryptamine are infused into the rumen in a single dose of 1 g each, or in combination in one single doses of 3 g in total (Neumark *et al.* 1964). More recent studies show that neither addition to the diet, nor rumen infusions of a combination of putrescine, cadaverine and GABA (2 g/kg DM each) lower DMI by silage fed steers (Dawson and Mayne, 1995). In contrast, intraruminal infusions of large amounts of amines depress DMI significantly. This is found for putrescine in dairy cows upon infusing a single dose of 100 g (Lingaas and Tveit, 1992), and for GABA in sheep upon infusing a single dose of 40 g (Buchanan-Smith, 1982). A study on the fate of histamine in the intestinal tract of sheep (Kay and Sjaastad, 1974) shows that histamine is degraded by the rumen microbes and that

absorption takes place in the lower intestines. Supposing the fate of other amines is similar to that of histamine, it is possible that upon infusing large amounts of amines into the rumen, a considerable part escapes from degradation and passes the rumen to the lower intestines. Whether concentrations of amines comparable to those appearing in the rumen after consuming a poorly preserved silage act on rumen chemostatic intake control is unknown.

The impact of amines alone or combined with other absorbed agents on DMI via metabolic control are evident. Unlike intra-ruminal infusions, infusing formic acid (40 ml 0.15 M) containing 121 mg histamine into the omasum of sheep depresses DMI (Neumark, 1967; Neumark and Tadmore, 1968). The increased respiration rate (Neumark, 1967) and depressed rumen motility (Neumark and Tadmore, 1968) show the physiological effects in intermediary metabolism. Other indications of individual amines affecting intermediary metabolism are a reduced intestinal motility in sheep upon intravenous infusion of GABA (Brikas, 1993) and the release of stress hormones (cortisol and norepinephrine) in sheep induced by tyramine and phenyl-ethylamine (Forbes *et al.* 1994).

In conclusion, the effect of amines on palatability can not be excluded, because with silage intake, a wider range of different amines is ingested than those studied by Buchanan-Smith (1990). Although DMI is not depressed by individual amines added to the diet or infused into the rumen at amounts simulating amine ingestion during silage intake, their influence on metabolic regulation remains possible. Probably there are additive effects between the different amines present in silage, or with other fermentation products as suggested by Buchanan-Smith and Phillip (1986).

Conclusions

The voluntary daily DMI of extensively fermented grass silage seems to be depressed trough impaired palatability, a rapid induction of satiation and most probably also through metabolic disregulation. Negative effects of the individual fermentation on DMI are mainly found when using high, unphysiological doses. When their effect is tested at physiological levels, no fermentation product or group of fermentation products can be indicated as being responsible for the gap observed between DMI of silage and that of the corresponding fresh or dried forage. Among the wide range of different fermentation products in the silage

exists probably additive effects acting on the different intake control mechanisms, which on their turn are complex and not yet fully understood.

Additionally, referring to the composition of the NPN fraction in silage, it contains several compounds (*e.g.* biogenic amines) that might affect DMI about which literature lack information.

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Chapter 3

Formation of biogenic amines in well-fermented grass silages

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Abstract

Biogenic amine formation was studied in silages made from perennial ryegrass. Two batches of grass from the same sward were wilted to either 250 or 450 g dry matter (DM)/kg, and were ensiled in eight 1-litre laboratory silos for each treatment (Expt A). To induce differences in fermentation pattern, the grass was ensiled without additive (CON) or treated with formic acid (5 ml/kg; FA), cell wall degrading enzymes (2.1 ml/kg; ENZ), molasses (50 g/kg; MOL), or inoculated with Lactobacillus plantarum *(10^7 colony forming units (CFU)/g; LP), a combination of* Lactobacillus plantarum *and* Streptococcus faecium *(10^5 CFU/g; LPSF), or* Enterobacter sakazakii *(6×10^6 CFU/g; EB). One silo for each treatment was opened after 1, 2, 4 and 7 d for pH determination and duplicate silos were opened after 10 and 90 d for pH determination and analysis of fermentation products. Two similar experiments (B and C) were performed using the CON, FA and LP treatments. Total amine content of the grass was low (0.1 - 0.2 g/kg DM). The well-preserved silages in each experiment contained considerable amounts of amines, ranging from 0.1 g/kg DM in the wilted LP and FA silages to 7.4 g/kg DM in a low DM CON silage. Tyramine, cadaverine, putrescine and histamine were, in descending order, the principal biogenic amines formed, representing together 90 (SE 9) % of the total amine content measured in the silages. Formation of amines occurred mainly during the first 10 d of fermentation, and was highest in silages with a slow acidification rate. Ensiling at high DM content, with formic acid or inoculation with large numbers of lactic acid bacteria reduced ($P < 0.01$) the amount of amines in the silage. Total and individual amine content of the silages were significantly correlated to concentrations of ammonia and acetic acid. It was concluded that the formation of biogenic amines in grass silage is related to protein degradation, and that amine formation can be reduced by restricting fermentation in the silage, or by achieving a rapid acidification during the first phase of ensiling.*

Introduction

During ensiling, much of the plant protein is degraded to non-protein nitrogen (NPN). The plant proteolytic enzymes are mainly responsible for hydrolysis of the plant protein to peptides and free amino acids, while during fermentation microbial deaminative and decarboxylative enzymes degrade the free amino acids yielding ammonia (NH_3) or amines, respectively (Ohshima and McDonald, 1978; Heron *et al.* 1986). The amines present in silage can be volatile amines (Jackson, 1964) or biogenic amines (Voss, 1966). The biogenic amines are toxic agents, which may cause health problems when they are ingested in large amounts (Joosten, 1988). In the present paper, when amines are mentioned they refer to the category of biogenic amines.

High concentrations of NH_3 and amines are found in clostridial silages (Hughes, 1971; Tveit *et al.* 1992). Proteolytic clostridia deaminate free amino acids to volatile fatty acids (VFA) and NH_3, or decarboxylate them to amines and CO_2 (Ohshima and McDonald, 1978). Amines and NH_3, however, are also found in substantial amounts in non-clostridial silages with low pH and without butyric acid (MacPherson and Violante, 1966*a*). This indicates that during fermentation microorganisms other than clostridia deaminate and decarboxylate the free amino acids.

Based on negative correlations between NH_3 and amine concentration in silage and intake by ruminants (Miettinen *et al.* 1991), adverse effects of NH_3 and amines on the nutritive value of silage have been suggested. Buchanan-Smith and Phillip (1986) reported lower voluntary food intake after infusing a mixture of amines into the sheep rumen. The intake reduction by amines can be caused by impaired palatability or by induction of stress in the intermediary metabolism (Baile and Della-Fera, 1988). Protein degradation in silage is routinely monitored by measuring NH_3 concentration. Because amines are not routinely analysed in silage, it is of interest to know how amines are related to NH_3 in silage.

The aim of the present study was to determine the amount and type of amines formed in non-clostridial silages, and the influence of acidification rate in the initial phase of ensiling on the amounts formed. Differences in rate of acidification were induced by using common silage additives that either stimulate or restrict fermentation (Spoelstra, 1991) and additionally the rate of fermentation was restricted by wilting. The extent to which amines in silages are related to other fermentation products was also examined.

Materials and methods

Silage making

In 1991, grass silages (Expt A) were made from a third cut of the ID-DLO Institute's permanent pastures predominantly of perennial ryegrass (*Lolium perenne*), fertilized with 450 kg N/ha per year. The grass was mown at 14.00 hours with a disc mower with a yield of approximately 3 tonnes dry matter (DM)/ha. About 250 kg of the fresh material was spread out on a black nylon sheet for wilting. Half the material was wilted to 250 g DM/kg in 22 h. The other half underwent prolonged wilting (48 h) to 450 g DM/kg. During the wilting period, the grass was manually turned. When the grass had reached the required DM content, it was chopped to a theoretical length of 2.0 cm with a precision chop harvester.

The chopped grass was divided into seven portions for each DM level, which were ensiled with and without additives. Additives applied were 5 ml of 85% formic acid/kg (FA); 50 g molasses/kg (MOL); 2.1 ml/kg of a cell wall degrading enzyme (CLAMPZYME, Finnfeeds Ltd, Redhill, UK; ENZ), 10^7 colony forming units (CFU)/g of a commercial inoculant containing *Lactobacillus plantarum* (ECOSYL, ZENECA Bio Products, Billingham, UK; LP); 10^5 CFU/g of a commercial inoculant containing a combination of *Lactobacillus plantarum* and *Streptococcus faecium* (Inoculant 1188, Pioneer Hi-bred Ltd, Cheshire, UK; LPSF). Additionally one portion was inoculated with 6×10^6 CFU/g of a strain of *Enterobacter sakazakii* (EB). This strain was isolated from grass silage and identified by the API 20E system (API system, Vercieu, France). The quantity of additives and microorganisms needed for 5 kg fresh material were dissolved or suspended in 125 ml deionized water. These additives were sprayed on to the chopped grass while mixing in a 150 litre concrete mixer. In addition, a control silage (CON) was made, treated with 25 ml deionized water per kg chopped material. After mixing for 5 min, the grass was ensiled in eight 1-litre glass laboratory silos for each combination of treatment and DM level. The silos were stored in the dark at room temperature.

Two similar experiments (Expts B and C) were performed from a second cut of perennial ryegrass, wilted for 48 h (Expt B) or 65 h (Expt C) using the treatments CON, FA and LP.

Sampling and analysis

Immediately before ensiling, grass samples were taken and analysed for pH, DM content, crude fibre (CF), ethanol (40 ml/l) soluble carbohydrates (SC), total

nitrogen (N), NH_3-N, amines and numbers of lactic acid bacteria and enterobacteria. Of the eight silos filled per treatment, one was opened at 1, 2, 4 and 7 d after ensiling for pH determination. After 10 and 90 d of storage, duplicate silos were opened and analysed for pH, volatile fatty acids (VFA), alcohols, NH_3-N, lactic acid and amines. Additionally, in the silos opened after 10 d, numbers of lactic acid bacteria, enterobacteria and clostridial spores were counted.

Grass samples were dried at 70°, and ground through a 1-mm screen for analysis of DM, ash, N, CF and SC, according to the methods of Van Vuuren *et al.* (1993). Biogenic amines were extracted from grass or silage, by macerating 20 g of the material in 100 ml (100 g/l) trichloroacetic acid. After centrifugation (20 min, 12,000 g), amines were determined in the supernatant by ion-exchange chromatography using an automatized amino acid analyser (Biotronik LC 6001, Munich, Germany). The orthophtaldehyde derivatives of the amines were separated on a column (length 7 cm, internal diameter 4 mm) packed with BTC 271 cation exchange resin (Biotronik, Munich, Germany). Composition and preparation of the eluting buffers used, as well as the analysis conditions are described by Villanueva and Adlakha (1978).

Ammonia, pH, VFA, alcohols and lactic acid concentrations in the silage were determined in a water extract, prepared by treating 30 g of the sample, to which 270 g of deionized water had been added for 5 min in a stomacher (Seward Laboratory, London, UK). In the extract, pH was determined immediately. For combined VFA and alcohol analysis, 4 ml of the extract was preserved with 0.8 ml orthophosphoric acid (50 ml/l) and stored frozen (-20°) until analysis by gas chromatography using a capillary CP-Sil-5CB column (Chrompack, Middelburg, NL). For NH_3-N and lactate determination, 4 ml extract were preserved with 4 ml 0.5 M sulfuric acid and 4 ml $CuSO_4$ (50 g/l), respectively. Lactate was measured by the method of Jones-Owen and Lechocki (1974) and NH_3-N using the Berthelot method (Searle, 1984). Additionally, the aqueous extract was used to make dilution series for bacterial counts on numbers of lactic acid bacteria and enterobacteria (Spoelstra and Hindle, 1989) and for counts on clostridia spores according to the method of Spoelstra (1990).

Statistical analysis

Within experiments, data were subjected to analysis of variance using Genstat 5, Release 2 (1987). The influence of wilting (1 df), additive treatment (Expt A, 6 df; Expts B and C, 2 df) and the interactions between the main effects on the

content of the fermentation products were tested. Concerning the amine content, additionally the effect of storage time (1 df) was included in the model, as well as the interactions between storage time and the effects of treatment and wilting. The Bartlett's test (Steel and Torrie, 1980) was used to test homogeneity of the variances. For cadaverine, putrescine and tyramine, variances were not homogeneous. Analysis of variance for these variables was conducted after ln-transformation of the data, using the same model testing effects of treatment, storage time, wilting and their mutual interactions. However, untransformed data of cadaverine, putrescine and tyramine are given in Table 3.4 and 3.5.

Correlations between amine content and other fermentation products included data from all three experiments and were calculated by linear regression. Significance of the correlations was tested using *t*-tests.

Results

Grass and silage composition

The chemical composition of the grass before ensiling is given in Table 3.1. The addition of molasses increased SC content of the grass in Expt A from 160 to 192 g/kg DM. Total concentrations of amines in the three grasses used averaged 166 (SE 37) mg/kg DM. The predominant amines in the grass were putrescine, spermidine, agmatine and tyramine.

Epiphytic lactic acid bacteria of the grass ensiled amounted to 6×10^6, 8×10^7, 6×10^5 and 6×10^6 CFU/g for Expt A with 250 g DM, Expt A with 450 g DM, Expt B and Expt C respectively. Numbers of epiphytic enterobacteria amounted to 6×10^7, 3×10^7, 2×10^6 and 2×10^7 CFU/g for Expt A with 250 g DM, Expt A with 450 g DM, Expt B and Expt C respectively. In the Expts B and C, numbers of lactic acid bacteria and enterobacteria were not changed by prolonged wilting.

The content of fermentation products in the silages of Expt A are given in Table 3.2 and those of Expt B and C are given in Table 3.3. With exception of the low DM CON silage in Expt B, all silages were well preserved as indicated by the low concentrations of acetic acid and NH_3-N (Dulphy and Demarquilly, 1981). All silages were free from butyric acid and contained only traces (< 1 g/kg DM) of propionic acid. Ensiling at high DM content, addition of formic acid or inoculation with LP and LPSF reduced the concentrations of acetic acid and NH_3-N.

Table 3.1 *Chemical composition and biogenic amine content of the grass before ensiling in experiments A, B and C.*

Experiment	A	B	C
	---------- g/kg wet weight ----------		
Dry matter (short wilt)	258	211	325
Dry matter (prolonged wilt)	453	378	541
	---------- g/kg dry matter[a] ----------		
Ash	111	98	141
Crude fibre	200	239	212
Soluble carbohydrates	160	138	92
Crude protein (N x 6.25)	182	177	249
	---------- mg/kg dry matter[a] ----------		
Biogenic amines	198	175	125
Agmatine	19	15	17
Cadaverine	5	3	3
Histamine	2	1	1
Phenyl-ethylamine	19	11	14
Putrescine	77	44	34
Spermine	16	3	3
Spermidine	33	51	32
Tyramine	27	47	21

[a] Means of short and prolonged wilted grass.

In all silages, numbers of lactic acid bacteria remained high (1.6×10^8 CFU/g) after 10 d of fermentation. At the same time, numbers of enterobacteria were low (1×10^2 - 1×10^3 CFU/g) in the silages of Expts A and C, with the exception of both FA silages in Expt A, containing 2×10^6 CFU/g. In the CON and FA treatments of both DM contents in Expt B, numbers of enterobacteria also remained high (5×10^6 CFU/g). In all silages of the present study numbers of clostridial spores were below the detection level of 1×10^2 CFU/g.

Table 3.2 *Fermentation characteristics (g/kg dry matter) of grass silages in Experiment A, 90 days after ensiling.*

Treatment[a]	CON		FA		EB		LP		LPSF		ENZ		MOL		SEM
Wilting period (h)	24	48	24	48	24	48	24	48	24	48	24	48	24	48	(14 df)
Dry matter (g/kg)	248	443	259	457	250	445	250	449	255	448	252	449	270	460	3.09
Lactic acid	110	46	52	15	112	42	130	64	119	57	107	40	105	37	2.89
Acetic acid	17.5	16.4	6.8	2.6	20.9	15.8	10.2	6.3	14.9	14.1	19.7	20.8	19.5	16.4	0.70
Ethanol	4.7	2.7	3.3	2.8	4.7	2.0	19.7	12.7	5.0	2.4	7.0	2.8	6.1	1.8	1.00
NH_3-N (g/kg N)	78	69	55	50	78	71	50	48	62	63	76	71	64	63	2.28
pH	4.0	4.4	4.1	4.5	4.0	4.4	3.9	4.1	3.9	4.1	4.0	4.4	4.0	4.4	0.10

[a] Treatments: ensiled without additive, CON; with formic acid, FA; inoculated with enterobacteria, EB; inoculated with *L. plantarum*, LP; inoculated with *L. plantarum* and *S. faecium*, LPSF; with cell wall degrading enzyme, ENZ; with molasses, MOL.

Table 3.3 *Fermentation characteristics (g/kg dry matter) of grass silages in Experiments B and C, 90 days after ensiling, and pH value 24 h and 10 days after ensiling.*

Treatment[a]	Experiment B							Experiment C						
	CON		FA		LP		SEM	CON		FA		LP		SEM
Wilting period (h)	24	48	24	48	24	48	(6 df)	24	65	24	65	24	65	(6 df)
Dry matter (g/kg)	195	374	206	375	203	370	2.38	317	556	324	527	320	553	1.58
Lactic acid	78	28	13	6	96	57	0.96	88	52	44	10	86	71	2.37
Acetic acid	43.2	4.8	1.7	1.4	5.4	5.3	0.24	22.0	5.4	6.5	1.9	19.6	5.0	0.60
Ethanol	34.1	10.3	5.4	5.9	9.7	6.1	0.96	5.0	1.8	4.5	1.3	2.6	1.1	0.87
NH_3-N (g/kg N)	165	60	50	35	50	37	3.37	90	57	45	48	69	40	2.12
pH	5.1	4.9	4.6	4.8	3.8	4.0	0.21	4.3	4.8	4.3	4.8	4.2	4.3	0.05
pH after 24 h	6.7	6.7	4.2	4.7	4.4	5.4	nd	5.2	6.7	4.6	5.2	4.6	5.8	nd
pH after 10 days	5.8	6.4	4.2	4.8	3.9	4.1	0.11	4.2	5.6	4.3	5.2	4.0	4.3	0.08

[a] For treatment details, see Table 3.2.

nd, not determined.

All silages in Expt A (Fig. 3.1 and Table 3.2), Expts B and C (Table 3.3) maintained a low and stable pH during storage. The initial rate of acidification varied, depending upon the grass ensiled and the additive applied, with all control silages (CON) having the slowest rate of acidification (Fig. 3.1 and Table 3.3).

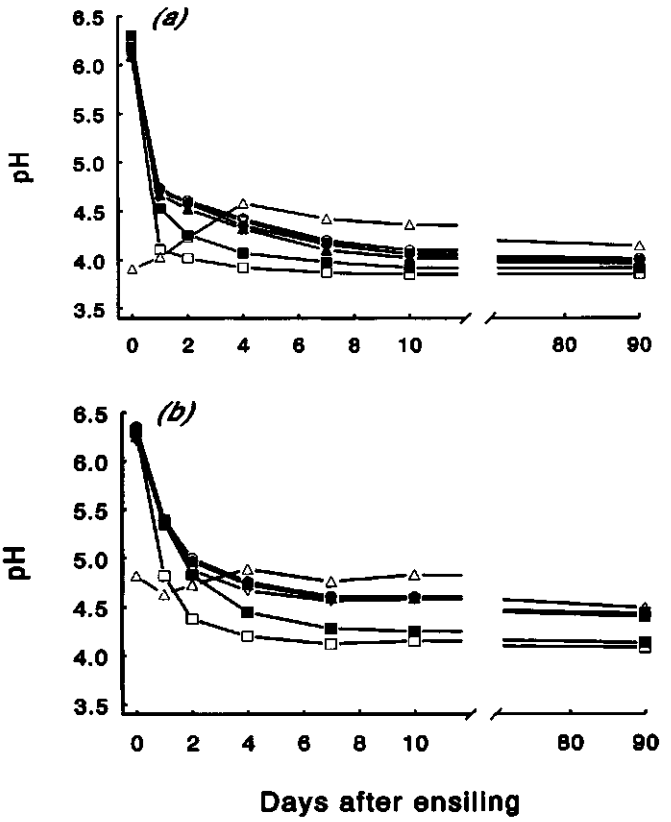

Figure 3.1 *The pH fall in silages of Expt A containing (a) 250 and (b) 450 g dry matter/kg. Ensiling treatments: without additive (○, CON); inoculated with enterobacteria (●, EB); inoculated with* L. plantarum *(□, LP); inoculated with* L. plantarum *and* S. faecium *(■, LPSF); with formic acid (△, FA); with cell wall degrading enzyme (▲, ENZ); with molasses (▽, MOL).*

Biogenic amines

Figure 3.2 shows that ensiling at a high DM or the addition of formic acid or high numbers of *L. plantarum* (10^7 CFU/g), significantly reduced ($P < 0.01$) the total amine content in silage. In the low DM silages in Expt A, a moderate reduction was brought about by the other additives used. This with the exception of inoculation with enterobacteria, which did not affect amine concentrations compared to the silage CON. In almost all silages, except the low DM silages CON and LP in Expt C, amines were formed mainly during the first 10 d after ensiling.

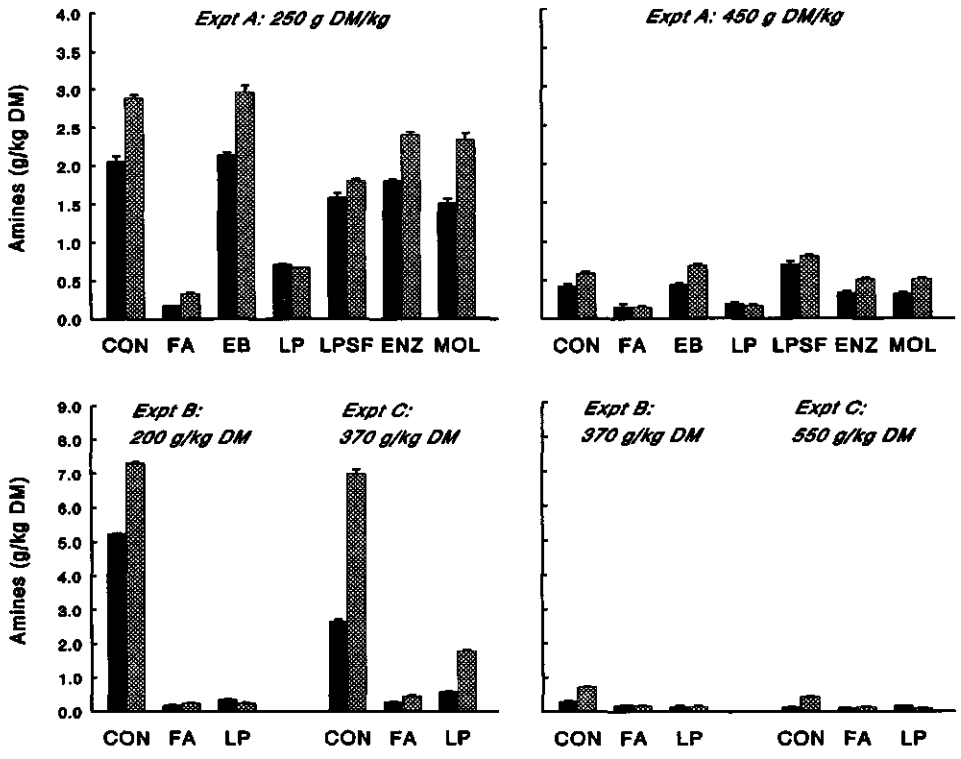

Figure 3.2 *Total content of biogenic amines (+SE) in grass silages in Expts A, B, and C with low and high dry matter (DM) content, 10 days (■) or 90 days (▒) after ensiling. For ensiling treatments, see Fig. 3.1. N.B. note different Y-axis scaling for experiment A and the combination of B and C.*

Table 3.4 *Individual biogenic amine content (mg/kg dry matter; DM) in grass and grass silages of Experiment A containing 250 or 450 g DM/kg, 10 and 90 days after ensiling.*

Treatment[a]	Grass	CON		FA		EB		LP		LPSF		ENZ		MOL		SEM[b]
Days		10	90	10	90	10	90	10	90	10	90	10	90	10	90	(28 df)
DM (g/kg)	250															
Agmatine	21	57	35	19	18	56	28	108	96	67	54	46	34	50	30	
Cadaverine	6	656	663	11	22	635	621	318	306	550	575	471	516	484	465	
Histamine	2	35	62	1	26	32	44	17	16	30	31	24	47	26	36	
Phenyl-ethylamine	17	14	41	4	19	12	22	5	5	33	10	14	15	8	17	
Putrescine	80	138	157	85	97	139	162	104	107	108	125	112	139	118	113	
Spermine	15	42	4	7	3	45	4	34	11	27	4	20	4	27	3	
Spermidine	42	44	3	17	4	47	2	40	17	34	3	26	2	38	1	
Tyramine	31	1065	1930	35	142	1181	2086	95	116	746	1007	1091	1649	767	1674	
DM g/kg:	450															
Agmatine	17	24	23	18	15	23	21	22	21	22	23	22	20	21	17	1.42
Cadaverine	4	44	40	6	5	42	39	20	22	34	36	34	32	25	23	0.06
Histamine	1	3	17	1	3	4	34	1	2	2	2	1	13	2	31	1.34
Phenyl-ethylamine	20	10	14	13	11	7	8	9	4	7	11	6	6	9	6	2.86
Putrescine	73	78	77	77	70	82	81	79	78	78	82	83	82	73	69	0.04
Spermine	16	10	2	5	2	6	2	10	2	8	2	4	2	3	2	2.65
Spermidine	24	15	2	5	2	10	1	13	2	12	1	8	1	6	1	2.46
Tyramine	23	244	409	29	42	267	498	41	44	536	649	175	350	186	360	0.07

[a] For treatment details, see Table 3.2.

[b] Includes means of silages with both DM content, and represents for cadaverine, putrescine and tyramine SEM after ln-transformation.

Table 3.4 and 3.5 give the composition of the individual amines in the silages in all the experiments. When compared to fresh grass, minor changes were found for agmatine, phenyl-ethylamine and spermine. In low DM silages, with the exception of the formic-acid treated silages, small quantities of agmatine and spermine were formed during the first 10 d of acidification, followed by degradation during further storage. In high DM silages, agmatine and spermine content remained similar to those in the fresh grass. Spermidine, which was found in relatively high concentrations in the fresh grass, decreased during ensiling.

Tyramine, cadaverine, putrescine and histamine were, in quantitatively descending order, the most important amines formed. Irrespective of the type of silage, together they represented 90 (SE 9) % of the total amount of amines measured. The extent of formation of these amines depended highly on the rate of acidification. In silages having a slow to moderate acidification rate, about half of the quantity of tyramine and histamine was formed during the initial stage of fermentation. In the low DM silages with high acidification rate (LP and LPSF), with the exception of the low DM LP silage in Expt C, a greater proportion of histamine and tyramine was formed during the first 10 d after ensiling, while in the formic-acid treated silages, most of the tyramine and histamine were formed later in fermentation. In the silages of Expt A, except FA, the formation of cadaverine occurred mainly during the first 10 d of fermentation, but when pH fell more slowly (silage CON in Expts B and C), formation continued during the later stages of fermentation. Significant concentrations of putrescine were only present in the low DM silages showing a slow acidification rate. Here patterns of formation were similar to that of cadaverine, mainly during the first stage of fermentation.

Relationships with other fermentation variables

Data from all 26 silages were used to calculate correlations between concentrations of amines and other fermentation variables. Large positive correlations were found between total and individual amines, and between the amines and NH_3-N and acetic acid content (Table 3.6).

From the data of this study, two regression equations were generated which can calculate total amine content in the silage (Y) from the silage NH_3-N or acetic acid content. For NH_3-N this equation was Y = 0.067 (SE 0.008) X - 2.85 (SE 1.027)

Table 3.5 *Individual biogenic amine content (mg/kg dry matter; DM) in grass and grass silages of Experiments B and C with low and high dry matter (DM) content, 10 and 90 days after ensiling.*

Treatments[a]	Experiment B								Experiment C							
	Grass	CON		FA		LP		SEM[a]	Grass	CON		FA		LP		SEM[a]
Days		10	90	10	90	10	90	(6 df)		10	90	10	90	10	90	(6 df)
DM (g/kg)	200								320							
Agmatine	12	115	82	13	12	35	35		19	28	8	22	19	151	142	
Cadaverine	1	1701	2559	7	95	14	35		3	866	2043	116	168	193	590	
Histamine	1	180	313	3	1	1	1		1	53	139	6	36	6	76	
Phenyl-ethylamine	7	18	7	20	2	7	2		17	34	7	6	2	9	2	
Putrescine	46	1337	2138	57	97	86	99		36	706	1598	62	79	77	328	
Spermine	4	5	1	4	1	4	1		3	3	2	3	13	3	11	
Spermidine	63	56	0	38	3	121	0		30	3	7	19	14	31	15	
Tyramine	55	1829	2215	36	29	85	77		20	966	3197	55	125	100	622	
DM (g/kg)	370								550							
Agmatine	17	21	45	22	25	28	32	4.84	15	11	14	15	12	17	18	2.76
Cadaverine	5	64	124	4	7	6	8	0.26	3	26	220	11	66	12	15	0.06
Histamine	1	17	94	1	3	2	2	2.93	1	1	3	1	0	2	0	3.13
Phenyl-ethylamine	14	7	17	11	11	5	12	1.63	10	7	12	4	1	7	6	2.07
Putrescine	41	65	94	48	39	51	51	0.06	31	42	83	36	30	36	33	0.08
Spermine	2	25	4	51	36	31	3	2.17	2	1	1	2	1	2	1	1.32
Spermidine	39	3	3	2	1	2	15	2.12	33	14	1	14	1	25	0	2.06
Tyramine	39	93	342	53	48	35	29	0.05	22	21	99	16	30	16	21	0.29

[a] For treatment details, see Table 3.2. For SEM description, see Table 3.4.

with r = 0.85, in which Y is the calculated amine content and X the NH_3-N content in the silage in g/kg N. For acetic acid this equation was Y = 0.166 (SE 0.024) X - 0.70 (SE 1.16) with r = 0.81, in which Y is the calculated amine content and X the acetic acid content in the silage in g/kg DM. No significant correlations were found between total or individual amines and DM, silage pH, propionic acid or ethanol content. Silage lactic acid content was only slightly correlated with tyramine (Table 3.6).

The correlations between amines and the different fermentation products that were significant in Table 3.6, were also significant when only the low DM silages of Expt A, B and C were considered (12 df). Here, in addition, significant (P < 0.01) correlations were found between pH value 24 h after ensiling and total amine content (r = 0.86), contents of tyramine (r = 0.65; P < 0.05), cadaverine (r = 0.91) and putrescine (r = 0.88). Correlations with pH after 90 d were only significant for cadaverine (r = 0.63; P < 0.05) and putrescine (r = 0.74).

Table 3.6 *Correlations (r) significantly different from zero (P < 0.01), between total amine content, the amines predominantly present in silage (g/kg DM) and some fermentation products (n = 26).*

	Total amines	Tyr-amine	Hist-amine	Cada-verine	Putre-scine	NH_3-N	Lactic acid	Acetic acid
Total amines	—							
Tyramine	0.92	—						
Histamine	0.89	0.66	—					
Cadaverine	0.95	0.79	0.89	—				
Putrescine	0.91	0.68	0.92	0.94	—			
NH_3-N (g/kg N)	0.86	0.72	0.91	0.83	0.84	—		
Lactic acid	NS	0.56	NS	NS	NS	NS	—	
Acetic acid	0.81	0.76	0.78	0.73	0.71	0.92	NS	—

NS, not significant.

Discussion

Biogenic amines in grass silages

The type and concentrations of amines found in the untreated silages of the present study generally agreed with those reported in the literature (Voss, 1966; Rauramaa *et al.* 1987*a*; Křižek, 1993). Higher concentrations of amines, but with similar proportions of the individual amines, were found in clostridial silages by Tveit *et al.* (1992). Tryptamine was not measured in our study, but others found only traces of this amine in silage (Voss, 1966; Rauramaa *et al.* 1987*a*; Tveit *et al.* 1992). No data are available in the literature to affirm the low concentrations of agmatine.

The formation of large amounts of tyramine, cadaverine, putrescine and histamine in silage, is partly the result of their precursor amino acids, respectively tyrosine, lysine, arginine and histidine being available (Joosten, 1987). These amino acids are liberated by plant proteolytic enzymes during the first stage of fermentation (Heron *et al.* 1986) and must subsequently be decarboxylated to form amines. The contribution of plant decarboxylases in the formation of these amines is unlikely (Ohshima and McDonald, 1978; Heron *et al.* 1986). The bacteria that proliferate during the initial phase of ensiling (Pahlow, 1991), are held responsible for the subsequent degradation of the free amino acids (Heron *et al.* 1986). Enterobacteria (Joosten, 1987), heterofermentative lactic acid bacteria (Joosten, 1987; Choudhury *et al.* 1990), and proteolytic clostridia (Ohshima and McDonald, 1978) possess the specific amino acid decarboxylases to degrade these amino acids. In the present study, amine formation is attributed to the activity of entero- and lactic acid bacteria, because in none of the silages clostridial activity was observed. No data are available in the literature about yeasts forming amines. It was assumed that their role in amine production in silage is limited. This was supported by the low amine content of the low DM silages treated with formic acid, which generally contain high numbers of yeasts (Lindgren *et al.* 1985).

Most amines were formed during the first few days after ensiling. In the low DM silages in Expt A, putrescine and cadaverine were formed mainly during the first 10 d, whereas tyramine and histamine formation continued during subsequent storage, at a low pH level. This was also found by Rauramaa *et al.* (1987*a*; *b*) and suggests that putrescine and cadaverine are formed mainly by enterobacteria which are eliminated early in fermentation, and that histamine and tyramine are formed by lactic acid bacteria which persist during fermentation. This argument is

consistent with the high numbers of lactic acid bacteria and low numbers of enterobacteria observed after 10 d of fermentation in Expt A, and the positive correlation between tyramine and lactic acid content. Edwards *et al.* (1987) and Joosten (1987) have also showed that strains of enterobacteria that are generally present among the fermenting microbial population produce mainly putrescine and cadaverine, while the lactic acid bacteria produce tyramine and histamine (Joosten, 1987; Choudhury *et al.* 1990).

In general, concentrations of amines in silages with a low and stable pH do not change during further storage (Hughes 1970; M. van Os, unpublished). However, after the initial fermentation during prolonged storage, several species of lactic acid bacteria can remain active, either in fermenting sugars or fermenting lactic acid to acetic acid and other products (Rooke *et al.* 1990). In both cases a moderate rise in pH occurs, which possibly explains the relatively high amounts of amines formed during prolonged storage of the low DM CON and LP silages in Expt C.

Influence of fermentation rate

In the silages of all experiments, differences in initial pH drop were observed (Fig. 3.1, Table 3.3). Within the experiments these differences in acidification rate were induced by the use of additives or wilting, whereas between experiments differences in chemical and microbiological composition of the grass ensiled are thought to be the cause. The results show that rate of acidification largely determines the amount of amines formed in the silage. Slow acidification leads to prolonged activity of amine forming enterobacteria and lactobacilli (Pahlow, 1991), and simultaneously to prolonged exposure of the silages to the optimum pH range (pH 5-7) of the bacterial amino acid decarboxylases (Meister, 1965). MacPherson and Violante (1966*b*) also observed reduced amounts of amines in silages with fast pH decline.

Of the additives applied, molasses and enzymes did not affect the acidification rate, but showed some reduction in amines. This agrees with the findings of Rauramaa *et al.* (1987*a*), and could be explained by the preference of bacteria for fermenting free sugars rather than amino acids (Brady, 1966). Inoculation with lactic acid bacteria resulted in faster acidification and a corresponding drop in amine formation. The effect of LPSF, however, was less pronounced than that of LP. This was probably attributed to the lower inoculation rate in LPSF. Inoculation with *E. sakazakii* did not influence any silage variable, although this bacterium is described as a putrescine former (Richard, 1984). This was possibly due to the

rapid elimination of enterobacteria in this experiment.

Restriction of fermentation by formic acid or by ensiling at a high DM content was most effective in lowering silage amine content. The low initial pH in the formic acid-treated silages probably inhibited decarboxylase activity, because high numbers of enterobacteria were still present in these silages 10 d after ensiling. The low amine content of formic acid-preserved silages was also reported by Rauramaa *et al.* (1987*a*) and Křižek (1993). A lower amine content in wilted silages was found by Voss (1967) and Křižek (1993). The factor limiting amines in high DM silages is probably the lack of substrate, because wilting leads to low water activity restricting proteolysis by plant enzymes (Kemble and MacPherson, 1954). Moreover, the low water activity generally inhibits microbial proliferation and the activity of their enzymes (McDonald *et al.* 1991), including amino acid decarboxylases. The influence of acidification rate on the amount of amines formed was of minor importance in the high DM silages. The significant correlations between the individual amines indicated that manipulation of fermentation affects the amount of all amines formed and not one type in particular.

In conclusion, this study shows that significant amounts of amines are formed in non-clostridial silages, and that formation can be limited by increasing the acidification rate in the initial phase of ensiling, or by restriction of fermentation through wilting or formic acid. The high correlation between amines and NH_3 indicates that deamination and decarboxylation are two concomitant processes of protein degradation during the ensiling process. This implies that the observed negative correlation of NH_3 with silage intake could also in part be attributed to amines in the silage.

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Chapter 4

The effect of protein degradation products in grass silages on feed intake and intake behaviour in sheep

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The effect of protein degradation products in grass silages on feed intake and intake behaviour in sheep

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Abstract

The effects of NH_3 and amines on grass silage intake, intake behaviour and rumen characteristics were studied in sheep. From a single sward, two direct-cut grass silages were prepared, either untreated (WAS) or with 4.5 l formic acid/tonne (FAS). Four experimental diets: WAS, FAS, FAS with addition of 2.9 g NH_3/kg dry matter (DM; FAS+N) and FAS with 2.8 g amines/kg DM (FAS+A), were offered ad lib. once daily to four rumen-cannulated wethers in a 4x4 Latin square design. Daily DM intake (DMI) tended to be influenced by dietary treatment ($P=0.09$). Compared with FAS, DMI was lower for WAS. Addition of NH_3 did not alter DMI, whereas amine addition slightly lowered daily DMI. Reduced DMI resulted from lower intake rates during both the principal meal and the subsequent small meals. Lower initial intake rate during the principal meal suggested reduced palatability of WAS and FAS+A. Amines and NH_3, however, did not influence chewing efficiency. No treatment effects were observed on total rumen pool size, DM and neutral-detergent fibre content. Furthermore, NH_3 and amines did not alter rumen pH, NH_3 and volatile fatty acid concentrations to the extent that they could act on chemostatic intake regulation. Amine addition, however, lowered osmolality of the rumen liquid. No treatment effects on rumen motility were observed. In conclusion, daily DMI was not reduced by the addition of NH_3, suggesting that NH_3 per se is not the causal factor in the negative correlations between silage NH_3 content and intake observed by other authors. Amines, however, tended to reduce DMI only by their effect at the oro-pharyngeal level of intake control.

Introduction

The generally lower voluntary intake of grass silages compared with hay prepared from the same fresh forage or with the fresh forage itself is mainly attributed to fermentation endproducts in the silage. This reduction in intake varies widely (ranging from 0 to 64%) and is related to the quantities of the fermentation

products in the silage, which vary with the mode of preservation (Demarquilly, 1973; Donaldson and Edwards, 1976; Thiago *et al.* 1992*a*). These fermentation products (organic acids, NH_3, amines) may regulate intake by their oro-pharyngeal properties (taste, smell) or metabolically (metabolites in rumen and or blood; Gill *et al.* 1987). Rumen fill appears to be of minor importance in controlling silage intake, because rumen dry matter (DM) content remains lower when the animals are fed on silages compared with hays prepared from the same original herbage (Thiago and Gill, 1986; Chiofalo *et al.* 1992). Relationships between silage constituents and intake suggest that NH_3 is one of the factors responsible for reduction in intake of poor-quality silages (Wilkins *et al.* 1971; Dulphy and Michalet-Doreau, 1981). However, other products of protein degradation (*e.g.* amines) may also influence silage intake (Buchanan-Smith and Phillip, 1986; Baile and Della-Fera, 1988). During the fermentation process, cadaverine, histamine, putrescine and tyramine can be formed in considerable quantities (Hole, 1985; McDonald *et al.* 1991). Because of their physiological action when absorbed into the bloodstream (Joosten, 1988), their influence in controlling silage intake is a possibility.

The aim of the present experiment was to investigate the effect of NH_3 and amines on voluntary intake of grass silage, and their possible mode of action in sheep.

Materials and methods

Dietary treatments and feeding

In June 1991 two grass silages were prepared from the first cut of a single sward of cocksfoot (*Dactylis glomerata*) meadow. The grass was harvested directly with a precision-chop forage harvester. One part was ensiled without additive, whereas the other part was ensiled while adding formic acid (4.5 l/tonne fresh material). Four dietary treatments were prepared: the silage without additive (WAS), the silage with formic acid (FAS), FAS with the addition of 2.9 g NH_3/kg DM (FAS + N) and FAS with 2.8 g amines/kg DM (FAS + A). The quantity of NH_3 and amines added, was based on the difference in these fermentation products between WAS and FAS in preliminary samples. The amine addition consisted of a mixture of the four biogenic amines cadaverine (0.6 g), histamine (0.5 g), putrescine (0.7 g) and tyramine (1.0 g). The ratio between amines corresponded

to that found in WAS, and values in the literature (Hole, 1985; McDonald *et al.* 1991). The amines, in hydrochloride form and NH_3 solution (Normapur^{TM}; 200 ml/l) were purchased from SIGMA Chimie, St. Quentin Fallavier, France and Prolabo, Paris, France respectively. All quantities of amines provided in the present study reflect the real amines and not their hydrochlorides. Amines were dissolved in water before being added to the silage, whereas NH_3 was added as purchased (200 ml/l solution). After removal of FAS from the silo, FAS + N and FAS + A were prepared by sprinkling these solutions over the silage, and then firmly mixing. Dietary treatments were prepared every 2 d and stored at +4° in portions of 50 kg (fresh material).

After collection of the refusals of the previous day, feed was offered to the sheep *ad lib.* (10% refusals) once daily (09.00 hours).

Animals and management

Four 5-year old Texel wethers (average live weight 66 kg) fitted with a permanent polyamide rumen cannula (75 mm in diameter) were used. In addition, a group of four non-fistulated 3-year old Texel wethers (average live weight 61 kg) was used to extend data of daily dry matter intake (DMI). Throughout the experiment, non-fistulated sheep were kept in individual indoor pens with sawdust for bedding. The fistulated animals were housed in individual pens in an experimental unit where lighting was provided for 11 h daily. During experimentation the fistulated animals were kept in metabolism cages in which they were placed 5 d before. All animals had free access to water and salt blocks.

Experimental design

The animals were randomly assigned to the four dietary treatments in two concurrent 4x4 Latin square designs, one with fistulated and one with non-fistulated sheep. Each period lasted 4 weeks and consisted of a 2-week adaptation period followed by 1 week of measuring intake behaviour and rumen characteristics, and 1 week of measuring rumen fill. The animals were weighed at the beginning of each period.

Measurements and sampling

Silage. During periods of measurements, samples of the offered silage were taken each day and analysed for DM content. A pooled sample of fresh material was stored at -20° until analysed.

Daily intake and intake behaviour. The daily DMI was calculated throughout the experiment as the difference between amount offered and that refused. The intake behaviour of the fistulated sheep was monitored during five consecutive days. The pattern of intake was registered by feed dispensers placed on sensors fitted with strain gauges. Weight variations of the feed dispensers were continuously recorded by a microcomputer after digitalization of the signal (Baumont *et al.* 1990). Kinetics of intake during the principal meal (first meal after distribution) were determined by fitting the model: $I_{(t)} = a\ (1 - e^{-bt})$ to the data, where I is the feed intake (g DM); t is the time after feeding (min); a is the asymptotic intake (g DM), and b is the rate constant of decrease (/h). Initial and final intake rates were calculated as the values of the first derivative of $I_{(t)}$ at $t = 0$ (ab) and $t = T$ (abe^{-bT}; where T is the end of the principal meal) respectively. Jaw movements were recorded simultaneously using a polyurethane-foam-filled balloon placed submandibularly and connected to a microcomputer via a pressure transducer. Eating and ruminating activities were analysed as described by Brun *et al.* (1984).

Rumen motility. Simultaneously with monitoring intake behaviour, rumen motility was recorded over a 48 h period. Contractions were recorded as air-pressure signals from a small foam-filled balloon (length 10 cm, diameter 3 cm) inserted in the dorsal region of the rumen. The signals were converted by a pressure transducer (LCD 86-110; Sélectronic, Paris, France) and registered on paper by a potentiometric recorder (BS 273; Gould Electronics LTD, London, UK).

Rumen fermentation. While recording intake behaviour, rumen fluid samples were withdrawn from the ventral region during two consecutive days, using a peristaltic pump (ISMATEC SA; Laboratoriumstechnik, Bern, Switzerland). From 08.45 to 11.45 hours sampling was carried out continuously (60 ml/h) and samples (15 ml) were collected every 15 min in order to select for analysis the sample corresponding to the end of the principal meal. Two additional samples were withdrawn at 13.30 and 15.30 hours. The pH was measured immediately and samples to be analysed for volatile fatty acids (VFA) and NH_3-N were stored frozen, preserved with orthophosphoric acid (50 ml/l) and NaCl solution (125 g/l) respectively.

Rumen fill. Total rumen contents were determined by manually emptying the rumen before (08.30 hours) and after the principal meal (10.30 hours). Rumen evacuations were carried out with an interval of at least 72 h to ensure normal digestion (Aitchison, 1985). The four animals were emptied simultaneously. After emptying, rumen contents were weighed, homogenized and sampled for DM

determination, amine extraction and rumen fluid (30 ml). Osmotic pressure was measured in the fluid within 12 h after sampling. Dried and ground (0.8 mm) rumen contents were preserved for analysis for neutral-detergent fibre (NDF).

Chemical analysis

The DM content of silage and rumen contents were determined by drying samples at 80° for 48 h. The DM content of the silage was corrected for volatile components lost by oven-drying, as recommended by Dulphy *et al.* (1975).

Fermentation characteristics of the silages (pH, VFA, alcohols, lactic acid, NH_3 and soluble N (N_{sol})) were determined in the juice pressed from silage. The pH was measured immediately. Total N and N_{sol} were determined by the Kjeldahl method, NH_3 by the gas diffusion method of Conway (1957) and lactic acid by the enzymic method described by Noll (1974). VFA and alcohols were analysed by gas-chromatography (Jouany, 1981).

In rumen fluid, NH_3-N was determined by the method of Berthelot as modified by Van Eenaeme *et al.* (1969), whereas VFAs were analysed as described previously. Osmotic pressure was measured by freezing-point depression (Astor 4000; Humeau, La Chapelle sur Erdre, France). Rumen NDF was determined in the dried material according to Goering and Van Soest (1970).

Amines were extracted from the silages and rumen contents by macerating 20 g of the fresh material in trichloroacetic acid (100 g/l; TCA). After centrifugation (12,000 g, 10 min), amines were determined in the supernatant fraction by reversed-phase HPLC as orthophthaldehyde derivatives by the method described by Gomez *et al.* (1991). Before injection, sample preparation was carried out automatically by an auto-sampler (AS 3000; Spectra physics, San José, USA). For adequate separation of the amine peaks the linear gradient of the solvent mixture was sustained at 84-20% for the acetate buffer, 14-73% for methanol and 2-7% for the tetrahydrofuran. Analysis time was prolonged to 28 min.

Statistical analysis

Data of daily DMI for all sheep, fistulated and non fistulated ($n = 32$), were subjected to analysis of variance, using the general linear model procedure of the Statistical Analysis Systems (1987). In the model, effects of silage (3 df), animal (7 df), fistulation (1 df) and period (3 df) were tested. Data concerning measurements on only fistulated animals ($n = 16$) were subjected to analysis of variance using the model, in which effects of period (3 df), silage (3 df) and animal

(3 df) were tested. Differences between treatments were compared by the Student's *t*-test. The 2-week adaptation periods were sufficiently long to prevent carry-over effects between periods of measurements.

Results

Silage composition

Composition and fermentation characteristics of both prepared silages and the principal changes as a result of NH_3 and amine addition to FAS are given in Table 4.1. The quality of FAS could be considered as good (Dulphy and Demarquilly,

Table 4.1 *Chemical composition and fermentation characteristics (g/kg dry matter) of grass silages preserved without additive (WAS) and with formic acid (FAS) and FAS after addition of ammonia (FAS+N) and amines (FAS+A).*

Dietary treatment	WAS	FAS	FAS+N	FAS+A
Dry matter (g/kg wet weight)	199	209	214	213
pH	4.1	4.0	4.3	4.1
Crude protein (N x 6.25)	144	153	167	159
NH_3-N (g/kg N_{total})	120	60	120	60
N_{sol} (g/kg N_{total})	680	400	430	410
Lactic acid	63	21	19	22
VFA	62	19	23	21
Acetic acid	56.6	15.6	19.0	15.8
Propionic acid	4.8	0.6	0.6	0.7
Butyric acid	0.8	2.6	3.4	3.9
Alcohols	19	5	6	6
Amines	6.5	1.1	1.3	3.6
Histamine	0.9	0.1	0.2	0.6
Tyramine	2.0	0.4	0.4	1.3
Putrescine	1.5	0.3	0.3	0.8
Cadaverine	2.1	0.3	0.4	0.8

VFA, volatile fatty acids.

1981), as indicated by low pH and low NH_3, VFA, alcohol and amine contents. The silage WAS underwent a more extensive fermentation, resulting in higher concentrations of organic acids, NH_3, N_{sol} and amines. Despite the increase of fermentation products, WAS could be classified as a medium-quality silage. As expected, NH_3 addition increased NH_3-N concentration in FAS + N, resulting in slightly higher N_{sol} and crude protein (N x 6.25) contents. Amine addition to FAS increased its amine content.

Intake and intake behaviour

The effect of fistulation on daily DMI was not significant. Pooled data of DMI for fistulated and non-fistulated sheep (Table 4.2) showed that DMI was significantly reduced for WAS compared with FAS. The addition of NH_3 to FAS had no effect on daily DMI, whereas amine addition caused a slight depression. The pattern of effects of dietary silage was reflected in the limited findings for DMI from the 5 d of recording intake behaviour. Dietary treatments did not affect period of time spent eating each day, which averaged 5.8 (SE 0.22) h. Thus, the reduction in intake of WAS and FAS + A was the result of a lower intake rate ($P < 0.05$). For the principal meal, intake of the four silages showed the same pattern as the daily DMI, with a reduced intake ($P < 0.05$) for WAS, a slight reduction for FAS + A and no difference between FAS and FAS + N. Sheep consumed WAS for a shorter period of time (not significant) with a reduced intake rate, while the duration of the principal meal for FAS, FAS + N and FAS + A was similar. A slight reduction in intake rate was recorded for FAS + A. Reduction in the overall intake rate of the principal meal for WAS and FAS + A compared with FAS, was mainly attributed to the lower intake rate at the beginning of the meal, because final intake rates were the same for the four diets.

Both WAS and FAS + N were eaten in a higher ($P < 0.05$) number of smaller meals. In FAS + N, this was the only variable of intake behaviour that differed from FAS. Average intake rate during the small meals was lower ($P < 0.05$) for FAS + A and tended to be lower for WAS. The period of time spent ruminating on a daily basis was least in sheep fed on WAS, whereas no treatment effects on the lag before the start of rumination were observed. Daily duration of mastication (eating and ruminating) was the lowest ($P < 0.05$) for WAS, and was related to daily DMI. Consequently, the efficiency of mastication did not differ among the four diets.

Table 4.2 *Dry matter intake (DMI; g), intake rates (g DM/min) and duration (min) of feed intake activities of sheep offered* ad lib. *grass silage preserved without additive (WAS), with formic acid (FAS) and FAS after addition of ammonia (FAS + N) or amines (FAS + A).*

Dietary treatment	WAS	FAS	FAS + N	FAS + A	Significance of the diet effect[a]	Pooled SED
Daily DMI[b]	1371	1477	1486	1437	0.09	40
Daily DMI[c]	1249	1475	1475	1308	0.09	87
Intake rate	3.7	4.3	4.3	3.6	**	0.14
Principal meal:						
DMI	274	465	452	407	0.06	59
Duration	53	71	67	75	NS	11
Intake rate	5.2	6.7	6.8	5.4	0.07	0.57
Initial rate	10.2	13.9	13.9	11.4	*	1.20
Final rate	3.4	3.4	3.7	3.4	NS	0.64
Small meals:						
No.	12.0	9.2	11.3	10.3	*	0.66
Intake rate	3.4	3.6	3.8	3.1	0.06	0.21
Rumination:						
Duration	352	471	473	407	*	35
Lag time[d]	179	147	163	179	NS	35
Mastication:						
Daily duration	687	817	816	774	*	40
Efficiency (g DM/min)	1.84	1.82	1.82	1.69	NS	0.09

SED, standard error of difference (6 df), except for daily DMI for fistulated and non-fistulated sheep (17 df); NS, not significant ($P > 0.10$); * $P < 0.05$; ** $P < 0.01$.

[a] For tendencies ($0.05 < P < 0.10$) probabilities are given.

[b] Average daily intake of fistulated and non-fistulated sheep during the 2-week period of measurements (days on which rumen emptying was carried out and following day were excluded).

[c] Average daily intake of the fistulated sheep during 5 d of recording intake behaviour.

[d] Start of first rumination after silage distribution (min).

Rumen fill and characteristics of the rumen contents

Rumen fill (Table 4.3) was not significantly influenced by dietary treatment and averaged 9.2 kg wet weight before the principal meal (at 08.30 hours) and 10.7

kg at the end of the principal meal (10.30 hours). Rumen pool size, however, was greatly influenced by animal effects ($P < 0.01$) both before and after the principal meal. Rumen DM content averaged 99.7 g/kg wet weight before the meal and increased slightly with all diets to 116.4 g/kg wet weight after the principal meal. No effects of dietary treatment were observed.

At 08.30 hours, osmolality of the rumen fluid, which was significantly affected by diet, was lowest (249 mosmol/l) for FAS + A and highest for FAS + N (267 mosmol/l). At the end of the principal meal the average increase of osmolality of the rumen fluid was 13% and did not result in different values across treatments. Likewise, no differences were observed in NDF content at both sampling times. Only traces of amines were recovered in the rumen contents after the principal meal, even on treatments WAS and FAS + A.

Table 4.3 *Characteristics of the reticulo-rumen content before (08.30 hours) and after (10.30 hours) the principal meal, in sheep offered* ad lib. *grass silage preserved without additive (WAS), with formic acid (FAS) and FAS after addition of ammonia (FAS + N) or amines (FAS + A).*

Dietary treatment	WAS	FAS	FAS + N	FAS + A	Significance of the diet effect	Pooled SED
08.30 hours:						
Wet weight (kg)	8.9	8.9	9.7	9.5	NS	0.89
Dry matter (DM; g/kg)	97	103	104	96	NS	4.9
Fluid volume (l)	8.0	8.0	8.7	8.6	NS	0.79
Osmolality (mosmol/l)	255	257	267	249	*	4.2
NDF (g/kg DM)	658	684	668	666	NS	7.2
10.30 hours:						
Wet weight (kg)	10.2	10.7	10.9	11.3	NS	0.54
Dry matter (DM; g/kg)	112	118	119	111	NS	4.2
Fluid volume (l)	9.0	9.4	9.6	10.0	NS	0.50
Osmolality (mosmol/l)	295	285	312	291	NS	13.4
NDF (g/kg DM)	650	657	660	648	NS	4.9

SED, standard error of difference (6 df); NS, not significant ($P > 0.10$); * $P < 0.05$.

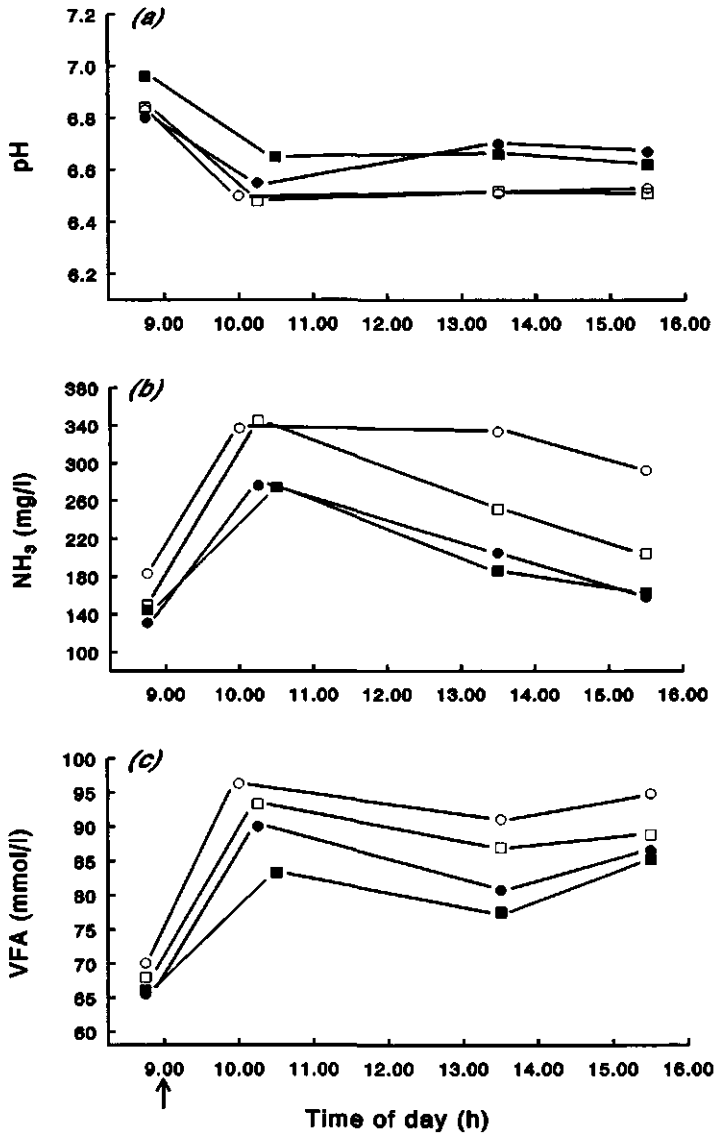

Figure 4.1 *Diurnal changes in (a) pH, (b) ammonia and (c) volatile fatty acid (VFA) concentrations in rumen fluid from sheep offered* ad lib. *grass silage preserved without additive (WAS; ○), with formic acid (FAS; ●) and FAS after addition of ammonia (FAS + N; □) or amines (FAS + A; ■). ↑; Silage distribution.*

The time-course of changes in fermentation products in the rumen fluid is presented in Fig. 4.1. Generally, rumen pH (Fig. 4.1*a*) decreased on average 0.3 (SE 0.05) pH units during the principal meal and remained relatively constant at approximately 6.6 (SE 0.08) during the day. Before silage distribution, rumen fluid pH was significantly higher in sheep fed on FAS + A compared with the other three silages. Although not significant, the pH remained elevated for FAS and FAS + A at subsequent sampling times.

Rumen NH_3 increased after silage distribution (Fig. 4.1*b*) and showed the highest increase for NH_3-rich silages (WAS and FAS + N). Due to considerable variation in data, average NH_3 concentrations in the rumen fluid of 342 (SE 51) mg/l for WAS and FAS + N and 273 (SE 38) mg/l for FAS and FAS + A at the end of the principal meal were not significantly different. After the principal meal, NH_3 concentrations in the rumen decreased for all silages, except for WAS. Here, the high concentration persisted until 13.30 hours and decreased afterwards but remained higher ($P < 0.05$) than those of the other three diets. At 15.30 hours, rumen NH_3 nearly approached the average initial concentration of 138 (SE 13) mg/l for FAS and FAS + A.

Patterns of total rumen VFA concentrations were similar for the four treatments, but differed in level (Fig. 4.1*c*). After silage was offered there was an increase in VFA from the average initial concentration of 67 (SE 2.0) mmol/l. After the principal meal, VFA concentrations decreased slightly until 13.30 hours but increased again thereafter. The highest increase during the principal meal was observed with WAS and the lowest with FAS + A. After the principal meal, rumen VFA concentrations tended to be influenced by dietary treatment ($P = 0.08$). At other sampling times, no further effects of dietary treatment on rumen VFA concentrations were observed. Before the meal, molar proportions of acetic acid, propionic acid and valeric plus caproic acid were 72.5 (SE 0.6), 17.8 (SE 1.2) and 1.9 (SE 0.03) mol/100 mol total VFA respectively and did not differ across treatments. The molar proportion of butyric acid was higher in FAS + A compared with FAS (10.1 (SE 0.7) *v.* 8.6 (SE 0.5) mol/100 mol). An important shift in VFA composition was observed at the end of the principal meal. The proportion of acetic acid decreased to 66.0 (SE 1.02) mol/100 mol for FAS, FAS + N and FAS + A, whereas for WAS it remained significantly higher throughout the day (71.1 (SE 0.5) mol/100 mol). With FAS + A the proportion of propionic acid remained lower ($P < 0.05$) and that of butyric acid higher ($P < 0.01$) compared with the other treatments. Average proportions during the rest of the day (13.30 and 15.30 hours), were 17.6 (SE

0.7), 20.2 (SE 2.0), 18.4 (SE 3.1), 15.1 (SE 2.1) for propionic acid and 7.0 (SE 0.7), 9.9 (SE 1.2), 10.4 (SE 1.0) and 13.0 (SE 1.0) for butyric acid, for WAS, FAS, FAS + N and FAS + A respectively.

Rumen motility

The number of primary contractions and total number of contractions (primary and secondary) of the reticulo-rumen per minute are given in Table 4.4. These results emphasize the difference in contraction frequencies during the different feed intake activities, rumination and rest. Highest frequencies of primary and total contractions were observed during the principal meal and decreased during the consecutive small meals. A further reduction occurred during rumination and rest.

Considering the effect of treatment on rumen motility, only an increase of primary contraction frequency was observed during rumination in sheep offered FAS + A. Moreover, treatments influenced neither the frequency of total contractions nor total number of contractions per d, calculated as the sum of the

Table 4.4 *Rumen contractions (/min) during different activities in sheep offered* ad lib. *grass silage preserved without additive (WAS), with formic acid (FAS) and FAS after addition of ammonia (FAS + N) or amines (FAS + A).*

Dietary treatment	WAS	FAS	FAS + N	FAS + A	Significance of the diet effect	Pooled SED
Primary contractions:						
Principal meal	2.0	2.0	1.9	1.9	NS	0.10
Small meals	1.7	1.6	1.7	1.7	NS	0.10
Rumination	1.3	1.2	1.2	1.4	*	0.04
Rest	1.1	1.0	1.0	1.0	NS	0.04
Primary and secondary contractions:						
Principal meal	3.6	3.4	3.4	3.5	NS	0.13
Small meals	3.0	2.8	2.9	2.9	NS	0.18
Rumination	1.9	1.9	2.0	2.1	NS	0.06
Rest	1.8	1.7	1.7	1.7	NS	0.06

SED, standard error of difference (6 df); NS, not significant ($P > 0.10$); * $P < 0.05$.

duration of the different activities multiplied by their corresponding contraction frequencies. For the silages WAS, FAS, FAS + N and FAS + A, total number of contractions per d were 3061, 2945, 3035 and 3097 respectively.

Discussion

In studies that relate daily intake of grass silages to their quality, NH_3 has been shown to be a protein fermentation product negatively correlated with intake. Amines, endproducts of amino acid decarboxylation, have also been suggested as being responsible for the reduction in intake of poor-quality silages. In the present study the effect of both protein degradation products on silage intake was tested by adding them separately to a grass silage of good quality, with high potential intake.

Silages

The two selected silages ideally suited the purpose of the present experiment. Silages were similar in DM content, pH and crude protein content. Concentrations of fermentation products (NH_3, VFA, alcohols and amines) in the silage conserved with formic acid (FAS) were sufficiently low to ensure a proper intake. This was confirmed by a daily DMI of 65.3 g per kg metabolic weight ($W^{0.75}$) for FAS, which is high compared with other experiments in which sheep consumed grass silages preserved with formic acid (Wilkins *et al.* 1971; Dulphy *et al.* 1984; Chiofalo *et al.* 1992). The lower quality of the silage WAS was indicated by a considerable quantity of fermentation products (organic acids, NH_3 and amines). According to equations formulated by Dulphy and Michalet-Doreau (1981), predicting silage intake in relation to its composition, the increase in NH_3-N of 6% in WAS compared with FAS could result in an intake reduction of 4.2 g/kg $W^{0.75}$, i.e. 6.4% *v.* 7.2% found in the present study.

The quantities of NH_3 and amines added to FAS were based on their concentrations in WAS in preliminary samples. The NH_3 content of FAS + N was equal to that in WAS, but the amine content in FAS + A was only 55% of that found in WAS samples taken throughout the experiment. The difference in amine concentration in the preliminary and ultimate samples of both FAS and WAS is probably due to unequal distribution of amines in the silos (Tveit *et al.* 1992). Nevertheless, amine concentration in FAS + A reflected the lower part of the range

of amine concentrations found in grass silages of medium quality. Likewise, proportions of the different amines used were comparable to those reported in the literature, with in the highest quantity being tyramine, followed by putrescine and cadaverine and the lowest histamine (Hole, 1985; Tveit *et al.* 1992). Addition of NH_3 and amines to the diet, rather than infusion, was chosen in order to follow both pre- and post-ingestive effects of these products on daily intake pattern.

Intake and intake behaviour

Values for daily DMI confirmed the potential for decreased intake of the poor-quality silage (WAS) compared with a silage of superior quality (FAS), on the basis of NH_3 content, as would be expected from the equation of Dulphy and Michalet-Doreau (1981) relating silage intake to NH_3 content. A similar decrease in DMI of 4.6 g/kg $W^{0.75}$ was not achieved on the addition of NH_3. In contrast, DMI for FAS+N was equal to that of FAS. The identical intakes of FAS and FAS+N indicate that NH_3 *per se* is not an intake depressant. This is supported by increased intake of hay treated with high levels of NH_3 in sheep (Benahmed and Dulphy, 1987) and the fact that NH_3 addition to silage did not depress intake in dairy cows (Lingaas and Tveit, 1992). The apparent decrease in daily DMI by the limited quantity of amines in FAS+A, however, explained 40% of the difference in daily intake of WAS and FAS. Data in the literature on the influence of amines on silage intake by ruminants are scarce. Intrarumen administration of 0.5 g histamine or 1 g added to the daily silage ration of sheep did not affect daily intake (McDonald *et al.* 1963). Neumark *et al.* (1964) did not find a response in feed intake after infusion of individual amines or a combination of histamine, tyramine and tryptamine (1 g each). Also, in heifers no reduction in intake could be detected either when 3 g histamine was added to the daily ration (Okamoto *et al.* 1964) or when tyramine was infused intraruminally (Thomas *et al*, 1963). These findings may be explained by the use of relatively low doses or the use of individual amines. For instance, Lingaas and Tveit (1992) detected reduction in intake in silage-fed dairy cows following the introduction of 100 g putrescine into the rumen, whereas intake was reduced in sheep following rumen infusion of a combination of the four amines used in the present study plus γ-aminobutyric acid (Buchanan-Smith and Phillip, 1986).

Silage intake appears to be controlled mainly by oro-pharyngeal factors and chemostatic regulation (Gill *et al.* 1987). In the present study, lower daily DMI for WAS and the trend to lower DMI for FAS+A was related to a decrease in intake

rate and not by the shorter period of time spent eating. The decrease of intake rate could be related to both types of intake control. Generally, grass silages are characterized by a considerable soluble fraction and rather high digestibility (McDonald *et al.* 1991; Tamminga *et al.* 1991). Both properties will lead to a rapid rise in metabolites in the rumen, achieving a state of satiation rapidly (Thiago *et al.* 1992*b*). Moreover, fermentation products do not generally have highly attractive odours and tastes. Silage intake during the day corresponded to intake behaviour reported for silage-fed sheep, by Dulphy (1985) and Chiofalo *et al.* (1992) and silage-fed steers (Thiago *et al.* 1992*b*), *i.e.* one short principal meal after feed was offered, followed by a relative high number of discrete meals throughout the day.

From profiles of the principal meals in the present study it seems likely that for WAS both chemostatic regulation and lower palatability were responsible for reduction in DMI. The lower percentage intake during the principal meal (22 % of the daily DMI) and the shorter period of time spent eating suggested faster achievement of satiation. The influence of oro-pharyngeal factors was suggested by the lower initial intake rate. This variable can be considered as an indicator of the perception of the forage, because at the very beginning of a meal the influence of chemostatic regulation can be excluded. The tendency of amines to decrease DMI can only be explained by a lower palatability of FAS + A, because intake during the principal meal, as a percentage of daily DMI, was equal to that of FAS (31% of the daily DMI). The animals ate for the same period of time, but initial intake rate tended to be lower. The addition of NH_3, however, did not result in a change in intake behaviour during the principal meal. Experiments of Buchanan-Smith (1990), with sham-fed sheep, confirm that NH_3 had no negative effect on silage palatability. The addition of a mixture of the amines putrescine, cadaverine and γ-aminobutyric acid to silage (3.5 g/kg DM), however, showed a positive effect on palatability.

Intake-regulating mechanisms seemed to have less effect over a daily period than over the short period of the principal meal (Dulphy, 1985), because the animals partly compensated the smaller principal meal of WAS by an increased number of small meals during the day. With FAS + A the percentage of DMI during the small meals was equal to that of FAS. The lower average intake rate of FAS + A had to be compensated for by a slight increase in the number of small meals. The increase in number of small meals with FAS + N is hard to explain in this context. More meals with a shorter duration would suggest a role of NH_3 in regulating intake. This is not supported by the similarity of the other intake-

behaviour variables of FAS + N with those of FAS.

The longer duration of rumination (total and per kg/DM) with FAS and FAS + N can be considered to be related to particle reduction to compensate for the shorter chewing time per unit DM during eating due to the higher intake rate (Ulyatt *et al.* 1986). The similarities in total chewing time per kg DM indicate that, compared with a good-quality silage, extra fermentation products do not influence chewing efficiency. This finding agrees with the observations of Thiago *et al.* (1992*b*) and Chiofalo *et al.* (1992).

Rumen fill, characteristics and motility

Among the four dietary treatments there were no significant differences in rumen load (on wet weight basis) or DM and NDF contents before or after the principal meal. The similarity in rumen load after termination of the principal meal indicates the minor importance of rumen fill as a regulating factor of DMI during this meal. Furthermore, Chiofalo *et al.* (1992) did not detect significant differences in rumen fill after termination of the principal meal in low- and high-quality silages. Nevertheless, they found a further increase in the rumen load during the day, which means that termination of the principal meal was not a consequence of limitation of the rumen capacity.

The slightly lower osmolality of rumen fluid observed before the principal meal for FAS + A suggested that amines increased the rumen fluid volume. Although not clearly indicated by calculated values, this diluting effect was also supported by the significantly higher pH for FAS + A before the principal meal and the elevated level during the subsequent period. Tyramine might be responsible for the increased rumen fluid volume, mediated through increased salivation (Joosten, 1988; Okina *et al.* 1993), or decreased water absorption, caused by deleterious effects on rumen epithelial cells (Kutas *et al.* 1986). An influx of water into the rumen is not likely, because osmolality of rumen fluid was far below the level that would induce the influx of water from the blood into the rumen (Carter and Grovum, 1990). Amines that enter the rumen apparently disappeared rapidly because only traces were found, even in rumen contents after the principal meal of WAS. Kay and Sjaastad (1974) found that histamine was metabolized primarily by rumen microbes and there was little evidence for absorption through the rumen wall. Other amines probably undergo the same fate. The absence of amines in the rumen contents supports the hypothesis that they probably do not act as chemostatic regulators of silage intake.

Values for rumen pH and concentrations of VFA and NH_3 were within the range of those found in silage-fed sheep (Chiofalo *et al.* 1992) and cattle (Thiago *et al.* 1992*a*). In terms of chemostatic regulation of DMI of the principal meal, only the influence of VFA was likely. Similar concentrations for rumen NH_3 for WAS and FAS + N at the end of the principal meal, together with the higher DMI for FAS + N, indicate the minor role for NH_3 in chemostatic regulation. This is supported by the fact that postprandial concentrations of 550 mg/l, (cf. 340 mg/l in the present study) were tolerated by sheep without negative effect on intake (Benahmed and Dulphy, 1987). The faster decrease in rumen NH_3 after the principal meal for FAS + N compared with WAS might be explained by a higher rate of NH_3 incorporation in microorganisms. Generally, silages preserved with formic acid contain higher concentrations of soluble carbohydrates (McDonald *et al.* 1991) and addition of NH_3 to this type of silage probably results in a better equilibrium between energy and N, favourable to microbial growth in the rumen (Russell and Hespell, 1981). However, with all treatments, rumen NH_3 concentrations were well above the minimum value of 50 mg/l for microbial growth (Satter and Slyter, 1974).

Differences in rumen contraction frequencies during the various eating activities and during rest are similar to findings of Ruckebush (1988). Differences in fermentation products in the silages did not affect rumen motility, on a per activity or total contractions per day basis. However, the higher number of primary (mixing) contractions during rumination with FAS + A was probably due to slightly higher rumen distention. The absence of an effect of fermentation products in medium- and good-quality silages on rumen motility patterns was supported by the findings of Thiago *et al.* (1992*b*).

In conclusion, the lower voluntary intake of a medium-quality grass silage (WAS) compared with that of good-quality grass silage (FAS), could only be explained partly by the fermentation products of proteins (NH_3 and amines). The addition of NH_3 to FAS did not reduce intake, in contrast to the findings of Wilkins *et al.* (1971) and Dulphy and Michalet-Doreau (1981) who suggested that intake was reduced by NH_3, since there was a correlation between silage NH_3 content and silage intake. Amine concentrations in WAS differed from those of FAS + A and there was a tendency towards reduced intake of FAS + A. The present study suggests oro-pharyngeal regulation of intake by amines. The low levels of amines did not permit conclusions to be made relating to the action of amines on chemostatic intake regulation. Therefore, higher quantities of amines need to be

tested, as well as the fate of amines in the rumen.

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Chapter 5

The influence of ammonia and amines on grass silage intake and intake behaviour in dairy cows

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The influence of ammonia and amines on grass silage intake and intake behaviour in dairy cows

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Abstract

The influence of ammonia (NH_3) and amines on silage dry matter (DM) intake (DMI) and milk yield was studied in four rumen cannulated dairy cows. Four dietary treatments were studied: a direct-cut grass silage without additive (WAS), a formic acid (4.5 l/tonne) preserved silage from the same sward (FAS), and FAS with addition, just before feeding, of either 2.9 g NH_3/kg DM (FAS+N) or 2.8 g amines/kg DM (FAS+A). In three successive cross-over design experiments, the diets WAS, FAS+N and FAS+A were compared to the control diet FAS. Daily DMI was lower ($P < 0.10$) for WAS, and the milk yield also tended to be lower. The DMI reduction was mainly due to reduced DMI during the first meal following silage distribution ($P < 0.10$). Lower intake rates during this principal meal ($P < 0.05$) suggested lower palatability of WAS. Addition of NH_3 and amines to FAS, did not alter milk yield, daily DMI or other variables of intake behaviour, compared to the control FAS. In the rumen, a diluting effect by amines was suggested. In this study, a direct role of NH_3 and amines on feed intake regulation was not established.

Introduction

In temperate regions, grass silage has become an important forage in rations for dairy cattle. Feed intake and animal performance on silage-based diets, however, depend highly on preservation quality of the silage (Waldo, 1978; Jarrige *et al.* 1982), which is determined by the content of fermentation end-products (Dulphy and Demarquilly, 1981). Correlations between fermentation products in silage and intake indicate acetic acid and ammonia (NH_3) as products involved in intake reduction of poor-quality silages in cattle (Rook and Gill, 1990), as well as in sheep (Demarquilly, 1973; Dulphy and Michalet-Doreau, 1981). It is also suggested that other products of protein fermentation (*e.g.* amines) are involved in intake reduction (Hole, 1985*a*; Baile and Della-Fera, 1988), by negatively affecting taste

and odour, or influencing chemostatic regulation of feed intake. The objective of the present experiment was to quantify the effect of NH_3 and biogenic amines in the currently observed intake reduction of poor-quality silages in dairy cows, by adding these products to a good-quality silage, with low concentrations of fermentation products.

Materials and methods

Dietary treatments

Two direct-cut grass silages were prepared in June 1991 from the first cut of a single sward of cocksfoot (*Dactylis glomerata*) meadow. The grass was separated into two batches and ensiled either without additive (WAS) or with addition of (4.5 l/tonne) formic acid (FAS). Four dietary treatments were tested: the silages WAS and FAS without any addition, FAS with addition of 2.9 g NH_3 /kg dry matter (DM) (FAS+N) and FAS with addition of 2.8 g amines/kg DM (FAS+A). The amine addition consisted of a mixture of the biogenic amines: cadaverine (0.6 g), histamine (0.5 g), putrescine (0.7 g) and tyramine (1.0 g). The amines, in hydrochloride form, and the NH_3 solution (Normapur^{TM}; 200 ml/l) were purchased from SIGMA Chimie, St. Quentin Fallavier, France and Prolabo, Paris, France respectively.

The composition and fermentation characteristics of the four studied silage diets are given in Table 5.1. Although FAS was analyzed in each individual cross-over experiment, only the average values are given here, because of similarity of the results. The silage FAS could be qualified as well preserved (Dulphy and Demarquilly, 1981) with low pH, low NH_3-N content and only small quantities of volatile fatty acids (VFA) and amines. However, some butyric acid (in average 3 g/kg DM) was found. In contrast to FAS, WAS could be considered as a medium to poorly preserved silage, indicated by higher NH_3-N and soluble N and higher concentrations of VFAs and amines, but with low level of butyric acid. The increase of NH_3-N in FAS+N and amine concentrations in FAS+A were due to NH_3 and amine addition to FAS respectively. The feeding values of the two basic silages, derived from INRA feed tables (Andrieu *et al.* 1989), were for energy, 0.89 UFL/kg DM for WAS and 0.88 UFL/kg DM for FAS, and for absorbable protein, 63 PDIE/kg DM for WAS and 74 PDIE/kg DM for FAS. It was assumed that NH_3 and amine addition did not significantly alter UFL and PDIE values.

Prior to feeding, daily required quantities of WAS and FAS were removed from the silo, followed by preparation of FAS + N or FAS + A. Detailed procedures of diet preparation and product addition have been published elsewhere (Van Os *et al.* 1995).

Table 5.1 *Chemical composition and fermentation characteristics (g/kg dry matter) of grass silages preserved with formic acid (FAS), without additive (WAS) and FAS after addition of ammonia (FAS + N) and amines (FAS + A).*

Dietary treatment	FAS[a]	WAS	FAS + N	FAS + A
Dry matter (g/kg wet weight)	214	189	210	209
pH	4.0	4.3	4.2	4.1
Crude protein (N x 6.25)	153	144	154	153
NH_3-N (g/kg N_{total})	60	130	120	60
N_{sol} (g/kg N_{total})	410	690	490	430
Lactic acid	26	49	19	24
VFA	19.8	65.8	18.1	20.2
Acetic acid	16.1	60.3	15.1	15.8
Propionic acid	0.7	4.2	0.4	0.7
Butyric acid	3.0	1.3	2.6	3.7
Alcohols	6.4	18.4	6.4	7.4
Amines	1.2	7.2	1.5	4.3
Histamine	0.2	0.9	0.2	0.6
Tyramine	0.5	2.1	0.4	1.4
Putrescine	0.2	1.8	0.3	1.2
Cadaverine	0.3	2.4	0.6	1.1

VFA, volatile fatty acids.

[a] Average value of individual results of FAS in experiments I, II and III.

Animals, feeding and experimental design

Four Holstein dairy cows (average live weight 610 ± 42 kg) were used. At the beginning of the experiment they were in the 7th week of lactation. The animals were fitted with permanent rumen cannulalae. Throughout the experiment, the

animals were kept in an experimental unit where lightning was provided for 11 h daily. Silages were offered to the cows from January through April 1992. The animals were fed *ad lib.* (10% refusals) with a single distribution of feed per day (11.00 h). To meet nutritional requirements, 5.5 kg DM of a commercial concentrate (net energy, 1.05 UFL/kg DM and absorbable protein, 115 PDIE/kg DM) was offered daily plus 190 g of a mineral mixture (14% P, 16% Ca, 3% Mg). Both were given separately from the silage. The daily schedule of concentrate and silage distribution and milking is described in Table 5.2. Animals had free access to water and salt blocks throughout the entire experiment.

Table 5.2 *Daily feeding and milking schedule.*

Time of day (h)	Activity
07.00	Milking
07.30	Refusal collection of previous day; Concentrate (2.25 kg DM) and mineral distribution
11.00	Silage distribution
16.00	Milking; Concentrate (2.25 kg DM) and mineral distribution

The study was divided into 3 sequential 2x2x4 cross-over designs in which the dietary treatments WAS, FAS + N and FAS + A were compared with the control diet FAS (Experiment (Expt) I, comparing WAS with FAS; Expt II, comparing FAS + N with FAS and Expt III, comparing FAS + A with FAS). Expt I consisted of 2 experimental periods of 3 weeks each. In the first 2 weeks, animals were adapted to the silage, and in the third week measurements were made. The Expts II and III consisted of 2 periods of 2 weeks each, with the first week for diet adaptation and the second for measurements.

Measurements and sampling

During periods of measurements, samples of the distributed silages were taken daily, and analysed for DM content. From this daily sampling, a pooled sample of fresh material was stored frozen until analysis for total nitrogen (N), amine content and fermentation products.

Daily dry matter intake (DMI) was calculated throughout the experiment as the difference between distributed amount and refusal. Intake behaviour was monitored during five consecutive days using continuously weighed feed dispensers, while signals of jaw movements were simultaneously recorded using a foam filled balloon placed submandibularly. Weight variations of the dispensers and air pressure signals from the balloon were recorded continuously by a microcomputer. Calculation of initial and final intake rates of the principal meal (first meal after silage distribution) was described by Baumont *et al.* (1990).

On two consecutive days, while recording intake behaviour, rumen fluid was withdrawn continuously (100 ml/h) from the ventral region of the reticulo-rumen, using a peristaltic pump (ISMATEC SA, Laboratoriumstechnik, Switzerland). Rumen fluid samples (50 ml) were collected every half-hour from 10.30 until 13.30 hours. All samples were stored and the sample of this series that corresponded with the time of termination of the principal meal (determined from data of intake behaviour recording), was analysed for pH, NH_3, VFA and biogenic amines. Two additional samples were taken at 14.30 and 16.00 hours. In the samples of rumen fluid, pH was measured immediately, whereas samples for NH_3, VFA and amine determination were preserved and stored at -20° until analysis.

Milk production was measured daily and samples were taken for analysis of fat, protein and lactose content twice a week.

Chemical analysis

Silage DM content was determined by oven-drying (48 h at 80°) and was corrected for volatile components (Dulphy *et al.* 1975). Fermentation characteristics (pH, organic acids, alcohols, NH_3 and soluble nitrogen (N_{sol})) were determined in liquid pressed from the silage, whereas amines were extracted from the fresh material by maceration in trichloroacetic acid (100 g/l). Total N and N_{sol} were determined by the Kjeldahl method, NH_3 by gas diffusion (Conway, 1957) and lactic acid by the enzymic method described by Noll (1974). Alcohols and VFA were analysed by gas-chromatography (Jouany, 1981) and rumen fluid NH_3-N was determined according the method of Berthelot adapted by Van Eenaeme *et al.*

(1969). Amines were analysed by HPLC (Van Os *et al.* 1995).

Statistical analysis

Within each cross-over design, data were subjected to analysis of variance by the general linear model procedure of the SAS Institute Inc. (1987). Effects of dietary treatment (1 df), period (1 df) and animals (3 df) were included in the model.

Results

Daily intake, intake behaviour and rumination

Average silage DMI during days of recording feed intake behaviour (n = 5), as well as variables of intake behaviour and rumination are given in Table 5.3. Compared to FAS, a significant reduction in DMI of 0.8 kg was observed for WAS, whereas DMI of FAS + N and FAS + A did not differ from FAS. Dietary treatments did not affect daily time spent eating, which averaged 336 min (eating time for concentrate being excluded). During the principal meal, DMI of FAS, FAS + N and FAS + A was similar and averaged 3.2 kg (26% of daily silage DMI). For WAS, DMI was 16% of daily silage DMI and was lower ($P < 0.10$) than for FAS, as a result of the combination of a shorter duration of the principal meal ($P < 0.10$) and the lower intake rate ($P < 0.05$). The latter seemed to be reduced during the whole meal, because no significant differences were observed in intake rates during the initial and final phases of the principal meal. For FAS + N, compared with FAS, only duration of the principal meal was prolonged ($P < 0.05$), whereas other variables of intake behaviour were similar. In Expt III, no difference in intake behaviour was observed between FAS and FAS + A. The average number of small meals was 9.0 for diets FAS, FAS + N and FAS + A, and it was slightly higher (+ 1.5) for WAS.

The treatments did not affect the period of time spent ruminating, which averaged 525 min per day. Compared with FAS, rumination cycles were significantly longer for WAS and for FAS + A, but shorter for FAS + N. The rumination lag time, *i.e.* duration of the interval between the end of the principal meal and initiation of rumination, was similar for the four dietary treatments. Daily duration of mastication was similar for the four silages (860 min), but resulted in combination with the lower intake of WAS in a significant lower mastication efficiency (g DM/min mastication) for WAS compared to FAS.

Table 5.3 *Dry matter intake (DMI; kg), intake rates (g DM/min) and duration (min) of feed intake activities of dairy cows offered* ad lib. *grass silage preserved with formic acid (FAS), without additive (WAS), and FAS after addition of ammonia (FAS + N) or amines (FAS + A).*

	Expt I			Expt II			Expt III		
Dietary treatment	FAS	WAS	SEM	FAS	FAS + N	SEM	FAS	FAS + A	SEM
Daily DMI	12.2	11.4*	0.2	12.0	12.6	0.6	12.4	12.5	0.7
Duration	335	349	12	332	339	17	342	327	20
Intake rate	37	34	1	38	38	2	36	39	3
Principal meal:									
DMI	3.3	1.8*	0.3	3.1	3.3	0.2	3.2	3.1	0.2
Duration	62	48*	3	64	71**	2	70	64	9
Intake rate	54	38**	2	50	47	2	46	49	6
Initial rate	79	68	4	78	77	4	68	74	4
Final rate	36	29	2	33	32	2	37	35	9
Small meals:									
No.	9.2	10.5	0.5	9.0	8.9	0.2	8.9	9.2	1.6
Intake rate	32	31	1	35	35	2	34	36	3
Rumination:									
Duration	526	533	11	513	528	21	512	535	24
Duration cycle	61	65**	1	62	60**	1	59	61*	1
Lag time[a]	22	25	5	26	21	3	19	22	3
Mastication:									
Daily duration	861	882	6	835	867	30	854	862	41
Efficiency[b]	14.3	13.1	0.3	14.6	14.6	0.1	14.6	14.5	2.3

SEM, standard error of means (4 df). Significance of the difference with the corresponding control FAS, * $P < 0.10$; ** $P < 0.05$.

[a] Start of first rumination after silage distribution (min). Duration cycle (s).

[b] Efficiency (g DM/min of mastication).

Intake and animal performance

Silage DMI during the total period of measurements (average of 7 days), milk production and the resulting average energy and protein balances are given in Table 5.4. The silage DMI of WAS, was 11.7 kg per day and like on days of intake and

rumination behaviour recordings, significantly lower ($P < 0.10$) than FAS. Daily DMI of FAS+N and FAS+A was similar to that of FAS. Besides the silage, cows consumed 5.5 kg DM of concentrate per day.

Table 5.4 *Dry matter intake (DMI), milk production and average energy and protein balances in dairy cows offered* ad lib. *grass silage preserved with formic acid (FAS), without additive (WAS), and FAS after addition of ammonia (FAS+N) or amines (FAS+A).*

	Expt I			Expt II			Expt III		
Dietary treatment	FAS	WAS	SEM	FAS	FAS+N	SEM	FAS	FAS+A	SEM
DMI (kg):									
Silage	12.5	11.7*	0.2	12.5	12.7	0.3	12.4	12.5	0.4
Concentrate	5.5	5.5	-	5.5	5.5	-	5.5	5.5	-
Milk production:									
kg	23.8	21.8	1.4	20.2	20.6	0.3	19.9	20.6	1.1
kg FCM	23.9	21.7	1.4	19.6	20.6	0.6	19.9	21.1	1.3
Energy ingested[a]	16.8	16.2		16.8	16.9		16.7	16.8	
Energy balance	+1.2	+1.6		+3.1	+2.7		+3.0	+2.4	
Protein ingested[b]	1558	1370		1558	1559		1557	1558	
Protein balance	+11	-72		+218	+184		+203	+145	

SEM, standard error of means (4 df). Significance of the difference with the corresponding control FAS, * $P < 0.10$.

[a] Ingested net energy in UFL, including 5.8 UFL from concentrate (UFL, feed unit for lactation, with 1 UFL is equivalent to 7.1×10^6 Joule (Vermorel, 1989).

[b] Ingested absorbable protein in g PDIE, including 633 PDIE from concentrate (PDIE, rumen undegraded protein plus microbial protein from rumen degraded organic matter).

The average milk production during the three cross-over experiments was 21.1 kg FCM (Table 5.4). In Expt I, a non-significant decrease of 2.2 kg FCM was observed for WAS compared to FAS, whereas no differences were observed in

Expts II and III. Average energy and protein balances per dietary treatment were calculated as the difference between energy and protein ingested from silage plus concentrate and requirements for maintenance and production. These balances indicated that with all dietary treatments the cows covered their energy requirements. Absorbable protein supply was largely sufficient in Expts II and III, but was marginal for FAS and insufficient for WAS in Expt I.

Rumen fluid characteristics

The course of rumen pH until 5 h after silage distribution is given in Fig. 5.1. After the end of the principal meal a comparable pH drop of about 0.5 unit was observed for FAS, FAS+N and FAS+A. The drop was less for WAS (0.2 unit),

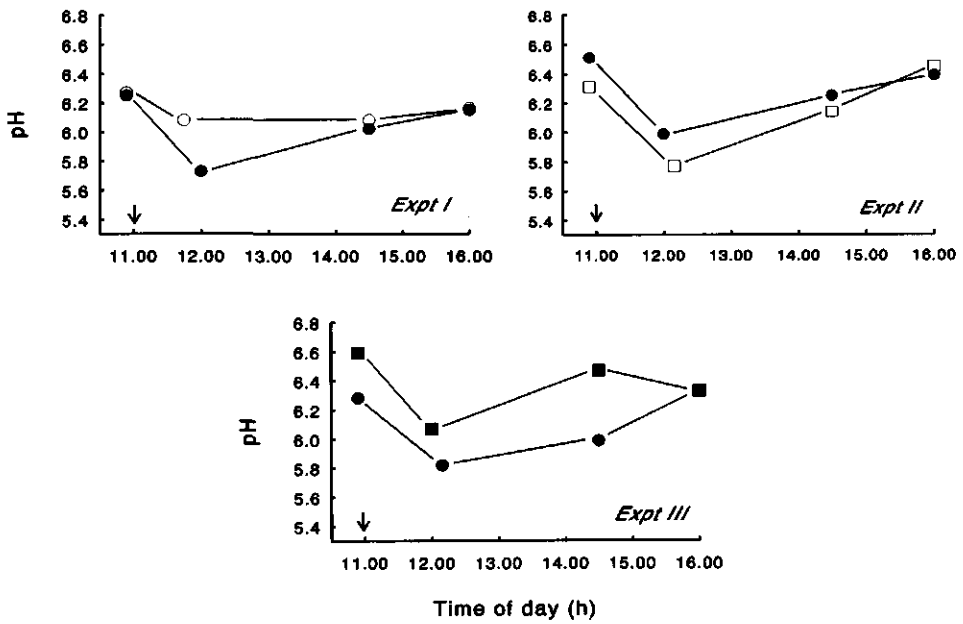

Figure 5.1 *Time course of rumen fluid pH in Expts I, II and III. The cows were offered* ad lib. *grass silage preserved without additive (WAS; ○), with formic acid (FAS; ●) and FAS after addition of ammonia (FAS+N; □) or amines (FAS+A; ■). ↓; Silage distribution.*

resulting in a significant difference in rumen pH between WAS and FAS at the end of the principal meal. During the observation period, rumen pH remained relatively constant for WAS, whereas for the other 3 treatments the pH drop after the principal meal was followed by a gradual increase. In Expt II, at all sampling times, rumen pH was not significantly different for FAS and FAS + N, while in Expt III it was higher ($P < 0.10$) for FAS + A, before silage distribution and at 14.30 hours.

During the principal meal, rumen fluid NH_3 concentrations increased for all dietary treatments (Fig. 5.2). At the end of the meal, it was higher ($P < 0.05$) for FAS + N, whereas at 14.30 and 16.00 hours concentrations were similar to those of FAS. In contrast to the general course of rumen NH_3 concentrations for FAS, FAS + N and FAS + A, a continuing increase over 5 h was observed for WAS. With

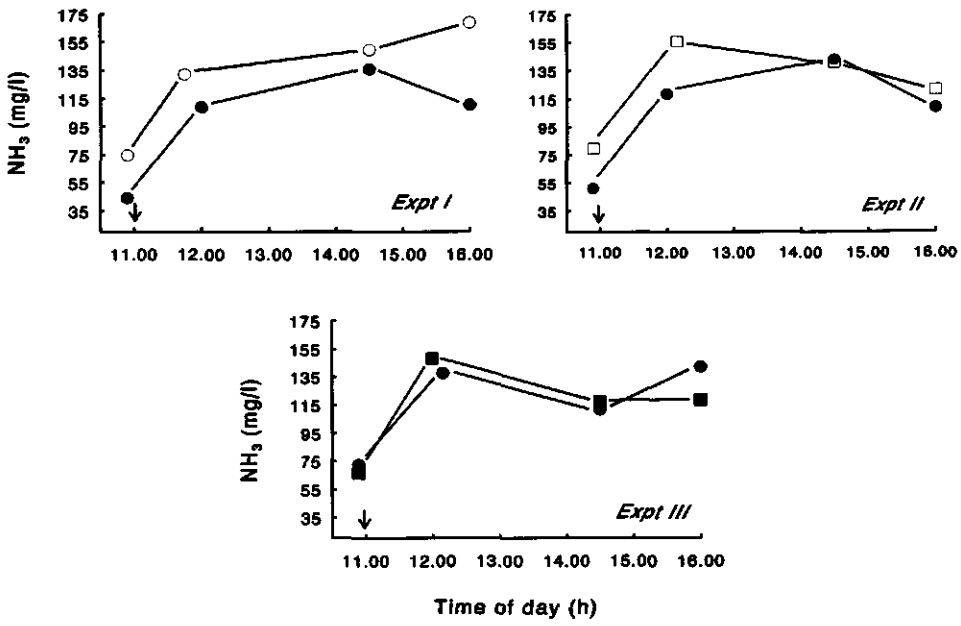

Figure 5.2 *Time course of rumen NH_3 concentrations in Expts I, II and III. The cows were offered* ad lib. *grass silage preserved without additive (WAS; ○), with formic acid (FAS; ●) and FAS after addition of ammonia (FAS + N; □) or amines (FAS + A; ■). ↓; Silage distribution.*

this diet, NH_3 concentrations were generally higher (+32 mg/l on average over the 4 sampling times) and significantly different from FAS at 16.00 hours ($P < 0.01$). Rumen NH_3 concentrations with FAS+A did not differ from those observed for FAS.

The increase of total rumen fluid VFA concentrations over the principal meal was relatively low and ranged from 7 mmol/l for WAS to 27 for FAS+N (Fig. 5.3). Significant differences were never observed between the diets WAS, FAS+N, FAS+A and their corresponding control FAS at the end of the principal meal or at the other sampling times. However, the greatest difference in rumen VFA concentration was found in Expt II, being on average 10 mmol/l higher for FAS+N, during the 4 h following silage distribution. The proportional VFA composition was

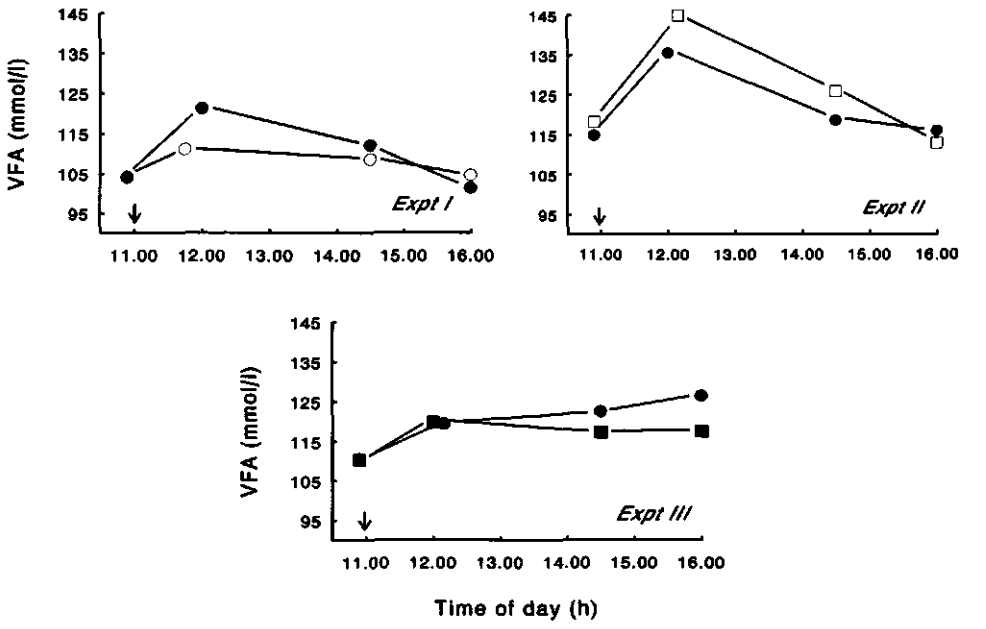

Figure 5.3 *Time course of rumen volatile fatty acid concentrations in Expts I, II and III. The cows were offered* ad lib. *grass silage preserved without additive (WAS; ○), with formic acid (FAS; ●) and FAS after addition of ammonia (FAS+N; □) or amines (FAS+A; ■). ↓; Silage distribution.*

similar for the treatments FAS, FAS+N and FAS+A, without large shifts during the sampling period and averaged 67.4 mol/100 mol for acetic acid, 17.8 mol/100 mol for propionic acid, 11.8 mol/100 mol for butyric acid and 2.7 mol/100 mol for valeric acid. For WAS however, the proportion of propionic acid (18.9 mol/100 mol) was higher ($P < 0.10$) and that of butyric acid (9.8 mol/100 mol) was lower ($P < 0.05$), compared to the proportions observed in FAS.

For all treatments, the rumen liquid after the principal meal contained no histamine and only traces (<20 mg/l) of tyramine, putrescine and cadaverine.

Discussion

Silage quality

The prepared silages WAS and FAS were similar in DM content, pH and crude protein. The low concentrations of fermentation products (NH_3, VFA, alcohols and amines) in FAS indicated that it was well preserved (Dulphy and Demarquilly, 1981). Concentrations of NH_3 and acetic acid in FAS corresponded with those in the silages used for intake studies by Waldo (1978) and Dulphy and Michalet-Doreau (1981). In contrast, relatively high concentrations of NH_3-N, acetic acid and amines indicated a more extensive fermentation in WAS, resulting in a lower silage quality. Its NH_3-N content was intermediate to the low and high quality silages used by the authors previously mentioned, whereas acetic acid content was higher. Generally, the chemical composition of WAS was similar to untreated silages cited by Dulphy and Lienard (1981) (characteristics: pH, 4.26; NH_3-N, 101 g/kg N_{total}; acetic acid, 51 g/kg DM and butyric acid, 4.4 g/kg DM). Total and individual amine concentrations of WAS were within the range of values found by Tveit *et al.* (1992). Addition of NH_3 to FAS increased NH_3-N concentration in FAS+N up to the level as found in WAS, whereas the quantity of biogenic amines finally added to FAS was insufficient to reach concentrations found in WAS (Van Os *et al.* 1995). Nevertheless, amine concentrations in FAS+A were in the lower part of the range of those found in grass silages preserved with little or no formic acid (Hole, 1985*b*; Tveit *et al.* 1992).

Intake, rumination behaviour and performance

Compared to FAS, higher concentrations of fermentation products in WAS led to lower daily DMI. This DMI reduction (0.8 kg) was close to the 1.0 kg observed in

data compiled by Dulphy and Lienard (1981). Milk yield reduction, however, was larger in the present experiment (2.2 kg FCM/d) than in literature (0.9 kg FCM/d; Dulphy and Lienard, 1981). The slightly higher negative response in milk yield was possibly due to the shorter measuring period (7 d in the present study). Consequently, less or no time was left for recovery from a possible decrease in milk yield due to the diet change-over, by increased intake after habituation to the new diet or by mobilisation of body reserves. The overall reaction of cows (lower intake and milk production) to a lower quality silage was thus confirmed, although differences in milk production in our study were not significant, due to the limited number of animals.

The addition of NH_3 or amines had no negative effects on DMI and milk yield. Moreover, in sheep, addition of NH_3 to a well preserved silage had no effect, but DMI tended to be lower when amines were added (Van Os *et al.* 1995). The latter was not confirmed in the present experiment. These results imply that the correlation between reduced intake and increased silage NH_3 content (Demarquilly, 1973; Dulphy and Michalet-Doreau, 1981; Rook and Gill, 1990) is not due to NH_3 *per se*. The results also agree with the absence of negative effects on intake and animal performance when cattle was offered NH_3 treated hay, with considerable residual NH_3 content (Drennan, 1990). Data on direct effects of amines on silage intake are scarce and deal mainly with short term effects. Intraruminal infusions of tyramine did not decrease intake in dairy cows (Thomas *et al.* 1963), whereas infusion of a single dose of 100 g putrescine in the rumen reduced subsequent silage intake and milk yield (Lingaas and Tveit, 1992). It is likely that the influence of amines on silage intake depends on type and quantity used. Buchanan-Smith and Phillip (1986), for instance, found lower intake in sheep during the meal following ruminal infusion of a combination of histamine, putrescine, cadaverine, tyramine and γ-aminobutyric acid.

The principal mechanisms controlling the intake of precision chopped silages are oro-pharyngeal reactions of the animals (taste, smell) and chemostatic regulation (satiation) (Gill *et al.* 1986), whereas rumen fill seems to be of minor importance (Deswysen and Ehrlein, 1981; Chiofalo *et al.* 1992; Thiago *et al.* 1992*a*). The action of chemostatic regulation and oro-pharyngeal factors mainly revealed during the principal meal. The spontaneous end of the principal meal will be initiated by the achievement of a certain state of satiation, caused by the rise of metabolite concentrations in the rumen or blood. Involvement of oro-pharyngeal factors can be established by alterations in average intake rate of the meal and especially from

alterations in the initial intake rate (Van Os *et al.* 1995).

Nearly identical eating behaviour of cows offered FAS + N and FAS + A compared with FAS demonstrated once again that the added NH_3 and amines had no effect on intake regulation. No differences were found in daily intake rate, DMI and intake rate during the principal meal, variables which are normally highly affected by silage quality (Dulphy *et al.* 1984; Chiofalo *et al.* 1992). For WAS, however, a rapid achievement of satiation was observed during the principal meal, expressed by the significantly lower intake and the shorter time spent eating. Lower intake rates for WAS during the whole meal and in the beginning and end phase, indicate its lower palatability. Compared to FAS, the DMI of WAS during the principal meal was only 55% in our experiment *versus* 70% by heifers in the experiments of Dulphy *et al.* (1984). This extra reduction of DMI may be attributed to additional effects on satiation of the preceding concentrate gift. The lower intake of WAS during the principal meal was partly compensated by a small increase in number of small meals during the day. So the above-mentioned intake regulation mechanisms may have less effect on DMI during a larger time span.

Unequal amine content in FAS + A and WAS did not allow us to state precisely their role in intake regulation. However, an identical quantity of biogenic amines added to the same silage FAS (2.8 g/kg DM) resulted in a slightly lower intake in sheep, presumably caused by oro-pharyngal influences (Van Os *et al.* 1995). Although cattle seem to be more sensible to silage quality than sheep (Dulphy *et al.* 1984), the effect of silage quality may be overruled by a high intake motivation of the lactating cows to meet their nutritional requirements, indicated by the positive net energy balances for all dietary treatments, even with WAS. The deficit of absorbable protein with this silage, however, is more probably a consequence of the lower intake than a direct cause. The higher metabolite turnover rates in producing animals may therefore have reduced the treatment effects on intake.

The effects of fermentation products on rumination behaviour were negligible and physiological importance of the differences in duration of the rumination cycles may be questioned.

Rumen fluid characteristics

Rumen fluid pH, NH_3 and VFA concentrations corresponded to ranges of the fermentation characteristics given by Bosch *et al.* (1992). At the end of the principal meal, lowest pH values were found for the silages with highest intake. The higher pH with little fluctuations for WAS may indicate that rumen acidity

could not be considered as a factor limiting intake in this study. However, the slightly higher rumen pH during a great part of the measuring period for FAS + A was remarkable. This was also observed in sheep (Van Os *et al.* 1995) and attributed to a possible diluting effect in the rumen. This dilution, however, could not be established from NH_3 and VFA concentrations, perhaps because concentrate distribution altered rumen VFA and NH_3 content and their relationships to one another markedly, compared to those in sheep offered silage only (Van Os et al, 1995).

The role of rumen NH_3 concentration in rapid achievement of satiation with WAS was also unlikely, because equal DMI was observed during the principal meal and throughout the day for FAS + N and FAS, despite higher NH_3 content in the rumen with FAS + N. The influence of the higher rumen NH_3 content 5 h after WAS distribution may be expected, but generally then concentrations will decrease (Thiago *et al.* 1992*b*; Van Os *et al.* 1995) and even higher intakes per kg metabolic weight were found in dairy heifers with rumen NH_3 concentrations above 200 mg/l (Teller *et al.* 1989). The earlier decrease of rumen NH_3 with FAS + N compared with WAS can be attributed to a higher NH_3 utilization rate for microbial growth, because a better equilibrium between energy and N is generally present in formic acid preserved silages (Chamberlain and Choung, 1993).

Enhanced VFA concentrations and the slightly lower pH during a great part of the measuring period for FAS + N compared with FAS may indicate the increased microbial activity as a result of the extra N. Inversion of average proportions of propionic and butyric acid in the rumen VFA pool for WAS can be explained by its difference in fermentation characteristics compared with the FAS-based diets. The higher lactic acid content in WAS will increase rumen propionate content, whereas less butyric acid will be formed caused by its lower residual sugar content (Chamberlain and Choung, 1993).

The presence of only traces of biogenic amines in the rumen, even of cows offered WAS containing considerable quantities of amines, indicates a fast disappearance of amines. This is possibly due to a considerable dilution that occurs when silage amines enter the rumen and to degradation of amines in the rumen. Degradation of histamine by rumen microorganisms was observed in sheep by Kay and Sjaastad (1974) and it is likely that degradation of other types of amines also occurs.

In conclusion, the hypothesis of possible negative effects of NH_3 and biogenic amines on silage DMI were not confirmed here. Evidently, NH_3 *per se*, is not a

cause for intake reduction of poor-quality silages like WAS. It is probably caused by the combination of high NH_3 content and other fermentation products (organic acids and alcohols) and the low availability of residual sugars that characterise poor-quality silages. The influence of amines on intake control must be verified by higher additions to the animal diets.

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Chapter 6

In vitro degradation of amines by rumen microorganisms

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In vitro degradation of amines by rumen microorganisms

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Abstract

Degradation of biogenic amines was studied in rumen contents obtained from wether sheep adapted to diets with different levels of biogenic amines: high (H), low (L) and without (W), containing 7.4, 2.4 and 0 g amines/kg dry matter (DM) respectively. To 200 g of the rumen contents (RC), 2 ml of a solution containing a mixture of the biogenic amines cadaverine (73.5 mmol/l), histamine (45.0 mmol/l), putrescine (83.0 mmol/l) and tyramine (123.5 mmol/l) were added, followed by a 5 h incubation in vitro. *The fermentation pattern in RC derived from diets H and L differed with that in RC derived from W. This difference was attributed to differences in fermentative properties of silage and hay based diets in the rumen. The addition of amines to RC increased ammonia production, which was the highest in RC from sheep adapted to silage with the highest amine content (diet H). Amines had no influence on gas production. Amine degradation occurred in all types of RC, but the extent depended on adaptation of the rumen microflora, such that 70.9, 54.2 and 25.3% of the added quantity in RC from H, L and W respectively, was degraded. Generally, the breakdown of the individual amines was highest for histamine, followed by tyramine, putrescine and cadaverine. Tyramine breakdown was particularly slow in RC from diet W. These results imply that in animals adapted to grass silage with high concentrations of biogenic amines, the accumulation of amines in the rumen will be prevented by an increase of the amine-degrading capacity of the rumen microbes.*

Introduction

During silage fermentation, extensive protein degradation occurs due to plant and microbial enzyme activity. One type of protein degradation product is biogenic amines, formed by amino acid decarboxylation (Ohshima and McDonald 1978). In grass silages of medium to poor quality, amines can occur in concentrations of up to 8 g/kg dry matter (DM) (Tveit *et al.* 1992; Křižek, 1993), and they have been

suggested to be partly responsible for the observed lower intake of ensiled forages by ruminants compared to the fresh forage or hay (Neumark *et al.* 1964; Hole, 1985; Gill *et al.* 1987). The mode of action of biogenic amines on intake reduction may be oro-pharyngeal (smell and taste) or metabolic (satiation) (Baile and Della-Fera, 1988). The latter requires substantial quantities of amines to be in the reticulo-rumen, and detection by chemoreceptors in the rumen epithelium or absorption in the rumen or in the lower intestines. Once in the blood, biogenic amines can cause undesirable physiological effects, like changes in blood pressure, decreased gut motility and effects on the nervous system (Joosten, 1988), all likely to limit feed intake.

Van Os *et al.* (1995) found only traces of biogenic amines in rumen contents of sheep adapted to grass silage containing 7 g biogenic amines/kg DM, suggesting rapid disappearance of amines from the rumen. Kay and Sjaastad (1974) observed rapid disappearance of histamine from the rumen and concluded from their experiments that histamine was lost via degradation by the rumen microbial mass rather than by absorption.

The aim of this work was to investigate to what extent tyramine, putrescine, cadaverine and histamine, the quantitatively most important biogenic amines in grass silage, are degraded by rumen microorganisms and whether adaptation to diets varying in amine content changes their amine-degrading capacity.

Materials and methods

Animals and diets

The fermentation medium in which degradation of biogenic amines was studied consisted of a mixture of the rumen contents (RC) obtained from two fistulated Texel wether sheep. The animals were housed in individual pens with sawdust for bedding, and they had free access to water and salt blocks.

The RC donating sheep were successively adapted to diets differing in amine content, which were respectively a grass silage with high (H) and low (L) concentrations of biogenic amines and hay without amines (W). For diet L, a formic acid (4.5 l/tonne) preserved grass silage of good quality was used, with low concentrations (2.4 g/kg DM) of naturally formed biogenic amines (Table 6.1). Diet H consisted of the same silage as used for diet L, but was supplemented with a

mixture of the biogenic amines cadaverine (1.1 g/kg DM), histamine (0.8 g/kg DM), putrescine (1.2 g/kg DM) and tyramine (1.8 g/kg DM), in order to obtain total and individual biogenic amine concentrations comparable to those found in grass silages of medium to poor quality (Tveit *et al.* 1992; Van Os *et al.* 1995). The amines, dissolved in water, were added to the silage just before feeding. Diet W consisted of a good quality meadow hay without amines. Chemical composition of the diets and fermentation characteristics and amine concentrations in both silage diets are given in Table 6.1. For the change in diets, 2 weeks were allowed for adaptation of the rumen microorganisms to the silages (diets H and L), and 3 weeks for the hay (diet W). The daily *ad lib.* ration was offered once daily at 09.00 hours.

Two series of incubations were carried out per diet, with an interval of 3 d between sampling, in order to allow recovery of the rumen contents of the donor sheep.

Table 6.1 *Composition and fermentation characteristics (g/kg dry matter) of the diets with high (H) and low (L) biogenic amine content, and without biogenic amines (W), fed to the rumen contents donating sheep.*

Dietary treatment	Grass silage		Grass hay
	H	L	W
Dry matter (g/kg wet weight)	227	230	869
Crude protein (N x 6.25)	169	165	125
Neutral-detergent fibre	596	591	644
pH	4.0	4.0	
Ammonia-N (g/kg N_{total})	80	81	
Lactic acid	75.1	72.6	
Volatile fatty acids	19.8	22.3	
Acetic acid	17.6	19.4	
Biogenic amines	7.4	2.4	
Histamine	1.1	0.4	
Tyramine	3.3	1.2	
Putrescine	1.5	0.4	
Cadaverine	1.5	0.4	

Preparation of RC and amine addition

Amine degradation was determined by *in vitro* fermentation carried out in 250-ml Erlenmeyer flasks, according to the system described by Jouany and Thivend (1986). The fermenting medium (RC) was prepared according the following procedure. About 1.5 kg of rumen contents were withdrawn from each sheep 4 h after feeding, to fill a 1.5 litre airtight container which was then stored in a waterbath (39°) during the time of transport to the laboratory. After pooling the rumen contents of both sheep, the sample was rapidly mixed manually and 1.5 kg was filtered through a 1-mm mesh. This filtered rumen fluid was stored in a closed thermos flask. The fermenters, equilibrated at 39°, were then filled with 100 g of the non-filtered rumen contents (liquid and solid phase) and 100 g of the filtered rumen fluid.

Before closing the fermenters, which indicated the beginning of incubation, biogenic amines were added to the RC in four of the eight fermenters (+A). Two ml of a freshly prepared amine solution, containing 0.147 mmol cadaverine, 0.090 mmol histamine, 0.166 mmol putrescine and 0.247 mmol tyramine (dissolved in 2.00 ml distilled water) was added to each fermenter. The amines, in hydrochloride (HCl) form, were purchased from SIGMA Chimie, St. Quentin Fallavier, France. All quantities and concentrations of amines given in this paper refer to amines and not the HCl form. Since amines were added in the HCl form, an equal quantity of HCl (1.052 mmol) dissolved in 2.00 ml distilled water was added to each of the 4 control fermenters (C).

The fermenters were placed in a shaking water bath (100 rpm) at 39°, and fermentation was followed for 5 h. No additional substrate or buffers were introduced into the fermenters. The entire procedure from collection of rumen contents to the start of incubation did not exceed 20 min. Additionally, freshly prepared RC was sampled, without the addition of amines or control HCl solution, for direct analysis.

Sampling and chemical analysis

Chemical components and fermentation characteristics of the experimental diets were determined according to the methods described by Chiofalo *et al.* (1992). Amine concentrations were analysed, according to the method described below, in the supernatant obtained after centrifugation of 20 g silage macerated in 100 ml trichloroacetic acid (100 g/l; TCA).

Gas production in the fermenters was measured by liquid displacement in graduated cylinders (Jouany and Thivend, 1986). With a syringe, 3 ml liquid samples were taken from the fermenters 15 min ($T_{0.25}$), 1 h (T_1) and 3 h (T_3) after the start of incubation. In these samples, pH was determined immediately, and subsequently 1.5 ml was preserved with 6 ml NaCl solution (125 g/l) and stored frozen for ammonia nitrogen (NH_3-N) analysis (Van Eenaeme *et al.* 1969).

From the freshly prepared RC before incubation and amine addition (T_0) and at the end of the incubation period (T_5), a 13 ml sample was taken for pH, NH_3-N and VFA determination, using the methods described by Jouany (1982). After opening the fermenters at T_5, 50 g of the RC was taken and immediately acidified with 30 ml TCA (200 g/l). The acidified RC was macerated and, after centrifugation at 12,000 g for 20 min, amine concentrations were determined in the supernatant by reversed-phase HPLC according to the method of Gomez *et al.* (1991). An auto-sampler (Spectra Physics AS 3000, USA) was used to prepare the reaction between the amines present in the sample and orthophtaldehyde (OPA) exactly 2 min before the sample was injected. The amines as OPA-derivatives were separated in a C18 column (22 cm in length) connected to a 3 cm precolumn. Linear gradients of the solvent mixture were used for combined analysis of histamine, putrescine and cadaverine at 84-20% for the acetate buffer (pH 6), 14-73% for methanol and 2-7% for the tetra-hydrofuran. For tyramine, separation from an interfering peak necessitated setting the linear gradients at 84-15%, 14-75% and 2-10%, respectively. The solvent flow rate was maintained at 1.0 ml/min throughout the analysis time of 28 min. Amine concentrations were determined in each of the prepared amine solutions added to the fermenters, as well as in samples removed from the fermenters.

Amine degradation was calculated as the difference between the total amine content at T_0 and residual amine content at T_5.

Statistical analysis

Influences of type of RC (2 df), amine addition (1 df) and interaction effects (2 df) were determined by analysis of variance, using the general linear models procedure (SAS, 1987). Differences between the three types of RC for amine degradation were tested using *t*-tests.

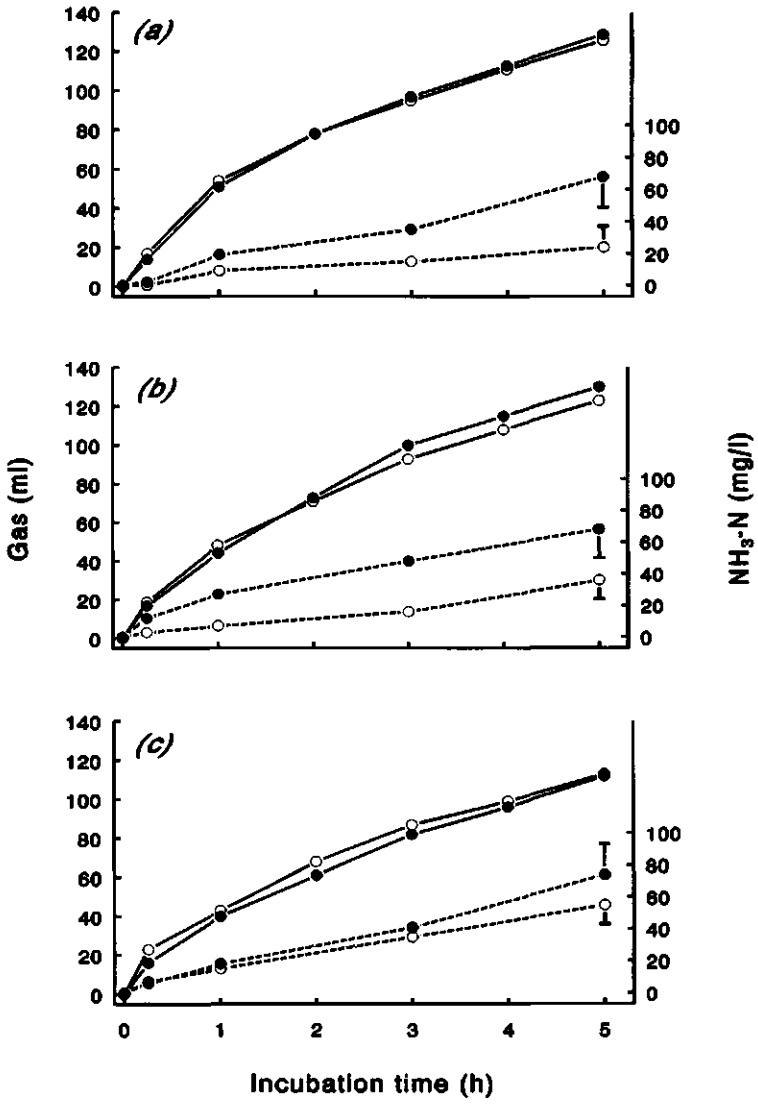

Figure 6.1 *Time course of gas production (—) per fermenter and NH_3-N production (---) in the rumen contents (RC), composed of 100 g filtered rumen fluid plus 100 g total rumen contents (liquid and solid phase), obtained from sheep adapted to grass silage with (a) high and (b) low amine content, and (c) to hay without amines. Control fermenters, ○; Fermenters with amine addition, ●.*

Results

Effect of amines on fermentation.

At the end of the incubation (T_5), average gas production per fermenter amounted to 129 ml (SE 21), 127 ml (SE 7) and 112 ml (SE 7) in RC obtained from H, L and W, respectively (Fig. 6.1). Gas release was significantly higher ($P < 0.01$) in RC from the silage based diets H and L, compared with RC from hay (diet W). Amine addition did not influence total gas production, with the exception of a decrease ($P < 0.05$) in RC obtained from W at $T_{0.25}$. No interactions between type of RC and amine addition were observed.

Initial NH_3-N concentrations in the fermenters at T_0 were 313, 304 and 127 mg/l for H, L and W respectively. During fermentation, NH_3-N concentrations increased in all fermenters. The NH_3-N production curves (Tx, corrected for T_0, x = 0.25, 1, 3 and 5 h) are shown in Fig. 6.1. Production of NH_3-N was significantly higher ($P < 0.01$) when amines were added. The type of RC affected NH_3-N production only at the end of the incubation period ($P < 0.05$), with higher NH_3-N production in RC obtained from W. Interaction effects ($P < 0.05$) between type of RC and amine addition on NH_3-N production were present at sampling times T_3 and T_5. The net NH_3-N production from added amines at T_5 amounted to 45 mg NH_3-N/l RC fluid for H, 31 mg/l for L and 18 mg/l for W.

At T_0, VFA concentrations in the RC fluid obtained from H and L were 118.3 and 116.2 mmol/l respectively. Initial VFA concentration in fluid of the hay based RC (W) was somewhat higher (124 mmol/l). Concentrations of individual VFAs at T_0, in RC fluid from H and L, were 80.1 mmol/l acetic acid (HAc), 23.7 mmol/l propionic acid (HPr) and 10.0 mmol/l butyric acid (HBu). For RC from W these were 93.0, 21.9 and 8.8 mmol/l respectively. Formation of VFA and that of the individual VFAs after 5 h of incubation are given in Table 6.2. Total VFA production (HAc, HPr and HBu) was the highest ($P < 0.01$) in RC of the silage based diets, H and L. No significant effect of amine addition was observed, but a significant interaction effect ($P < 0.05$) between amine addition and type of RC resulted in higher VFA production in fermenters with RC from L and W. This interaction effect and the influence of type of RC appeared also for the production of the individual VFAs, HAc and HPr. HBu production was influenced by both type of RC ($P < 0.01$) and amine addition ($P < 0.05$), resulting in higher values in RC from L and W to which amines were added.

The initial pH value of the unbuffered RC averaged 6.2 (Table 6.2). Addition of the 0.5 M HCl control solution caused an initial decrease of 0.27 pH units in the C fermenters, *versus* the decrease of 0.02 units caused by addition of the amine solution. This initial pH difference at $T_{0.25}$ diminished during the incubation period.

Table 6.2 *Volatile fatty acid (VFA) production (mmol) per litre of rumen contents (RC) incubated without (C) or with amine addition (+A) for 5 h (T_5-T_0), initial pH in the fermenters and changes after 15 min and 5 h of incubation.*

Type of RC[a]	H		L		W		SE (42 df)
	C	+A	C	+A	C	+A	
Total VFA	13.2	9.1	7.5	20.9	3.9	10.5	0.85
Acetic acid	9.6	6.8	3.7	16.2	1.6	6.9	0.63
Propionic acid	2.1	0.9	2.0	2.4	0.8	1.6	0.17
Butyric acid	1.5	1.4	1.8	2.3	1.5	2.0	0.08
pH (T_0)	6.30		6.37		6.13		
$T_{0.25}$-T_0	- 0.33	- 0.07	- 0.21	0	- 0.27	+ 0.01	0.01
T_5-T_0	- 0.60	- 0.51	- 0.68	- 0.60	- 0.60	- 0.54	0.02

[a] Rumen contents composed of 100 g filtered rumen fluid plus 100 g total rumen contents (liquid and solid phase), obtained from sheep adapted to grass silage with high (H) and low (L) amine content and to hay, without amines (W).

Amine degradation

No amines were present in the freshly prepared RC. Amine degradation was determined as the difference between the amount initially added and quantity recovered at the end of the incubation period, expressed as a percentage of the added dose. Amine degradation was highly influenced by type of RC ($P < 0.001$). The highest overall degradation occurred in RC from H (70.9 SE 2.9 %), followed by that of L (54.2 SE 7.6 %) and W (25.3 SE 4.4 %). A similar influence of type of RC was also observed on the degradation of the individual amines (Fig. 6.2). The percentages of histamine, tyramine and cadaverine degraded differed

significantly among the three types of RC, except for the degradation of putrescine, which showed to be significantly higher in H, but similar in the RC from L and W. Generally, the highest degradation rate was observed for histamine, followed by that for tyramine, putrescine and cadaverine. An exception in this sequence was the considerable lower degradation rate of tyramine that was observed in RC from W.

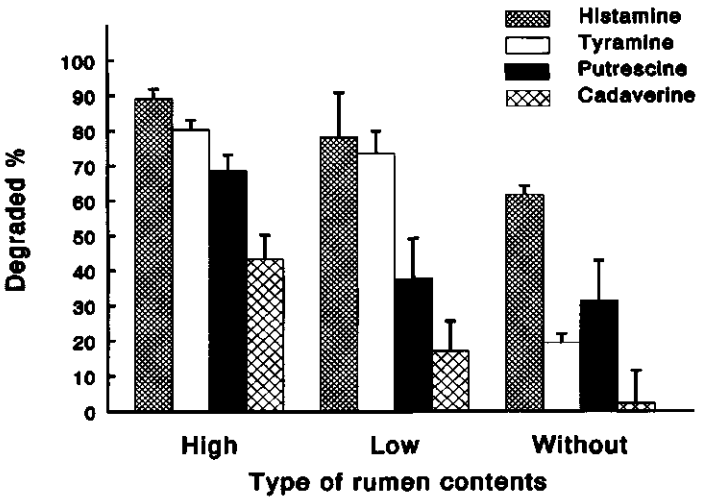

Figure 6.2 *Degradation percentage (+ SE) of the individual biogenic amines added to fermenters containing 100 g filtered rumen fluid plus 100 g total rumen contents (liquid and solid phase), obtained from sheep adapted to grass silage with high and low amine content and, to hay without amines, after 5 h of incubation.*

Discussion

Methodological aspects

The aim of this study was to investigate the capacity of rumen microorganisms to degrade biogenic amines. The rumen content, withdrawn 4 h after feeding, was assumed to contain sufficient nutrients to continue microbial fermentation, which

was confirmed by the ongoing gas production during the incubation period. In our experiment, the decrease of *in vitro* pH was comparable to that observed *in vivo* in the sheep rumen after silage feeding (Chiofalo *et al.* 1992).

The quantity of amines added per fermenter was calculated to approximate 1.4 times the quantity of amines that can be recovered in 200 g rumen contents of sheep after ingestion of an average meal after feeding (350 g DM) of the experimental diet H. It was assumed that the ingested amines were instantaneously and thoroughly mixed in the rumen, and that no degradation occurred. The 40% increment was chosen to ensure detectable amine concentrations at the end of the incubation period, without adding excessive quantities.

The impact on fermentation of the initial pH drop, caused by the added control HCl solution, was expected to be low, because during the incubation period the pH in all fermenters remained above the lower limit of the pH range for optimal growth of the principal rumen bacteria (Therion *et al.* 1982; Wallace and Cotta, 1988).

Fermentation and influence of amines

The influence of type of RC on the courses of fermentation must be attributed to the different fermentative properties of the experimental diets offered. Silage, compared to hay, is characterized by a large, soluble and highly degradable crude protein fraction (Tamminga *et al.* 1991). For the diets H and L, the main part of this fraction will already be degraded within the 4 h before collection of rumen contents, explaining the higher initial NH_3-N content in the RC from both silage based diets and the lower endogenous NH_3-N production (NH_3-N production in C fermenters) during incubation.

The absence of negative influences of amines on gas production suggests that microbial fermentation was not affected, irrespective of degree of adaptation. This in contrast with Tveit *et al.* (1992), who suggested, from *in vivo* measurements in silage fed cows, a negative effect of amines on rumen fermentation. That amines do not have negative impact on microbial activity can also be concluded from the higher quantities of VFAs produced in RC from diets L and W to which amines were added. Although VFA concentrations in the +A fermenters with RC from diet H were lower (not significant), this need not signify a depression of microbial activity, either by the high concentration of dietary amines or by amine addition. This assertion is supported by the higher endogenous VFA and NH_3-N production in RC from H, as compared with RC from L, and similar gas production in the C and +A fermenters. The observed differences in VFA production among

the three types of RC with added amines may possibly be the result of differences in end-products of amine degradation. It may be that in RC from L and W diets these are mainly HAc, HPr and HBu, and that adapted microbes in the sheep fed the H diet possibly convert them to other, not measured end-products, or use the intermediates of amine degradation in anabolic pathways (Prins, 1977). This, however, can only be speculated, because data from the literature dealing with the fate of biogenic amines in the rumen are very scarce. Data that are available are discussed below.

Amine degradation

This study confirms the suggestion of Prins (1977) that amines are degraded by rumen microorganisms with the formation of NH_3. Deamination of amines was also demonstrated by the agreement of the net NH_3-N production (total NH_3-N production corrected for endogenous NH_3-N production) measured in the fermenters (9.4, 6.2 and 3.6 mg N/200 ml RC fluid obtained from H, L and W respectively), and the quantity of N that could theoretically be liberated from the degraded amines (9.5, 6.0 and 2.7 mg N/fermenter for H, L and W respectively). Considerably higher rates of amine degradation occurred in rumen contents adapted to amines. This agrees with the observation of Tveit *et al.* (1992), who found accumulation of biogenic amines in the cow rumen 2 d after a diet change-over from hay to an amine-rich grass silage. They did not study possible adaptation. The results of our study indicate that amines are broken down at pH values below neutrality, in the range of 6.4 to 5.5. At lower pH values, however, amine degradation could be inhibited, as observed by Dain *et al.* (1955), with histamine accumulation in the sheep rumen at pH values below 5.3. This accumulation may be associated with dysfunctions of the rumen microbial fermentation, which occur in this pH range (Mould *et al.* 1983).

Of the individual amines, it appears that histamine and tyramine, which are the most toxic (Joosten, 1988), had the highest degradation rates in adapted rumen contents. This result makes it unlikely that significant amounts of these amines pass the rumen and will be absorbed in the intestines in sheep that are adapted to poor-quality silages containing considerable concentrations of these biogenic amines. Only shortly after a change-over of diets would amines be expected to accumulate in the rumen.

In the rumen, several deaminating pathways are present, which may yield NH_3 from amines (Schlegel, 1986), but also various other organic acids, depending on

the nature of the amines (Scheline, 1978). For example, tyramine would be expected to yield NH_3 and hydroxy-phenylacetic acid (Scheline, 1978). The latter can after ring opening, be fermented to acetic acid (Shlomi *et al.* 1978). Rupture of the imidazole ring of histamine was suggested by Kay and Sjaastad (1974), which was deduced from the recovery of ^{14}C-labelled CO_2 in rumen gases after infusions of ^{14}C ring-labelled histamine. The carbon chains of putrescine and cadaverine would be expected to be degraded to VFAs, but it is also possible that ring formation occurs (Prins, 1977). The absence of net VFA production from amines in the RC from H, however, could not confirm that VFAs are the additional end-products of amine degradation in the rumen, at least not in rumen contents adapted to dietary biogenic amines. Experiments are required to clarify this observation.

It can thus be concluded that biogenic amines are degraded by rumen microbes, and that their degrading capacity increases when they are adapted to diets with high amine content. This mechanism will largely prevent amine transfer to the lower intestines. So, direct effects of dietary biogenic amines on metabolic level of intake regulation are doubtful.

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Chapter 7

The influence of ammonia, biogenic amines and γ-aminobutyric acid on grass silage intake in sheep

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The influence of ammonia, biogenic amines and γ-aminobutyric acid on grass silage intake in sheep

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Abstract

In the present study was investigated whether biogenic amines alone, or a combination of ammonia (NH_3), amines and γ-aminobutyric acid (GABA) influenced grass silage intake, intake behaviour and rumen liquid content in sheep. Three diets were studied: a grass silage preserved with formic acid (4 l/tonne) (FAS), FAS with 4.9 g amines/kg dry matter (DM) added (FAS+A), and FAS supplemented with a combination of N-components at the concentration of 2.7 g amines, 3.0 g NH_3 and 5.0 g GABA/kg DM (FAS+C). The diets were offered ad lib., *once a day to six rumen cannulated Texel wethers in a 3x3 Latin square design. Daily dry matter intake (DMI) tended to be influenced by diet ($P = 0.08$). Compared to FAS, the DMI of FAS+A was similar, whereas that of FAS+C tended to be higher ($P = 0.06$). The mean rate of ingestion over all feeding bouts tended to be the lowest for FAS+A ($P = 0.07$). No differences were found among the diets concerning intake behaviour during the principal meal. Average intake rate of the small meals also tended to be the lowest for FAS+A ($P = 0.06$) Although rumen NH_3 concentration was higher ($P < 0.05$) after the principal meal, rumen pH, osmolality, rumen pool size and liquid content were not significantly altered by adding amines or the mixture of N-components to FAS. We conclude that biogenic amines or N-containing products of protein fermentation in concentrations normally found in poor-quality silages do not reduce the intake of a formic acid treated silage. A direct effect on chemostatic regulation of intake was not observed, but a slight negative effect on silage palatability can not be excluded.*

Introduction

Ensiling grass may alter the nutritional value of the original herbage considerably. Soluble carbohydrates are converted to the preserving organic acids (McDonald *et al.* 1991), and a significant part of the plant protein is degraded into non-protein nitrogen (NPN) comprising peptides, amino acids (Heron *et al.* 1986), amines and

ammonia (NH_3; Voss, 1967). The generally observed lower intake of grass silage when compared with that of hay prepared from the same forage, or the lower intake of silages of inferior quality, may be attributed to the fermentation products in them (Chiofalo *et al.* 1992; Thiago *et al.* 1992). Presumably, they reduce intake by lowering silage palatability or by evoking signals of satiation from intake regulating mechanisms located in the rumen or intermediary metabolism (Gill *et al.* 1987; Baile and Della-Fera, 1988). Among the fermentation products, those of protein degradation, especially biogenic amines are suggested to lower silage intake (Buchanan-Smith and Phillip, 1986).

In a previous experiment we observed that addition of 3 g/kg DM of a mixture of biogenic amines, commonly present in a poorly-preserved silage, to a well-preserved grass silage tended to depress intake by lowering palatability (Van Os *et al.* 1995*a*). Moreover, the rumen fluid volume tended to be higher upon addition of this relatively low quantity of amines to the experimental diet. However, the results did not allow us to judge whether biogenic amines acted on chemostatic intake regulation at ruminal or metabolic level.

The aim of the present experiment was to confirm the negative influence of biogenic amines on silage intake and to establish their interference on chemostatic intake regulation by adding 5 g biogenic amines/kg DM to a good-quality silage. Additionally, the effect of a combination of NPN components (NH_3, biogenic amines and GABA) on silage intake was studied.

Materials and methods

Dietary treatments and feeding

The basal diet consisted of a formic acid-treated (4 l/tonne fresh material) grass silage prepared from the first cut of a cocksfoot (*Dactylis glomerata*) meadow. After a 24 h wilt, the grass was harvested with a precision-chop forage harvester and ensiled in a clamp silo. In the experiment, three diets were studied: the formic acid silage alone (FAS), FAS supplemented with 4.9 g biogenic amines/kg DM (FAS + A) and FAS supplemented with a combination of NPN components consisting of a total of 2.7 g biogenic amines/kg DM, NH_3 and GABA (FAS + C). Amounts of individual amines added was fixed for FAS + A to achieve an amine content of this silage that was higher than that tested in a previous similar study by Van Os *et al.* (1995*a*). For FAS + C the same amounts of amines and NH_3 were

added as by Van Os *et al.* (1995*a*) while the amount of GABA was determined from concentrations commonly found in poorly preserved silages (Ohshima *et al.* 1979). Amounts of each NPN component added to FAS to obtain the diets FAS + A and FAS + C are given in Table 7.1. The amines, in hydrochloric form, and GABA were purchased from SIGMA Chimie, St. Quentin Fallavier. The NH_3 solution (Normapur^{TM}; 200 ml/l) was purchased from Prolabo, Paris. All quantities of amines given in this study represent the free bases and not the hydrochlorides. The amines and GABA necessary to supplement 50 kg fresh material of FAS to obtain either FAS + A or FAS + C were dissolved in 1.5 l water before being mixed through FAS. The NH_3 solution was added as-is. Additionally, the same volume of water without NPN components was mixed through FAS, so that texture and DM content of the control FAS would not differ from the other dietary treatments. Every two days, portions of 50 kg (fresh material) of the three diets were prepared and stored at +4°. After the refusals of the previous day were collected, the diets were offered *ad lib.* to the sheep once daily at 09.00 hours, in amounts sufficient to ensure a refusal of at least 10%.

Table 7.1 *Amounts of individual non-protein nitrogen components added (g/kg dry matter) to the formic acid preserved grass silage (FAS) to obtain the dietary treatments FAS + A and FAS + C.*

Dietary treatment	FAS + A	FAS + C
Cadaverine	1.1	0.6
Histamine	0.8	0.5
Putrescine	1.2	0.7
Tyramine	1.8	1.0
γ-aminobutyric acid	-	5.0
Ammonia	-	3.0

Animals and experimental design

Six 6-year old Texel wethers (average live weight 69 kg) were used, each fitted

with a permanent rumen cannula (75 mm in diameter). Throughout the experiment, the animals were kept in an experimental room, which was lit for 11 h a day. During adaptation periods they were housed in individual pens with sawdust for bedding. During experimentation they were kept in metabolism cages, in which they were placed five days before. During the experiment the animals had free access to water and salt blocks.

The sheep were randomly allocated two by two to each sequence of the three dietary treatments in the 3x3 Latin square design with 3 periods. Each period lasted for four weeks, and consisted of a 2-week period for adaptation to the diet and a further 2-week period in which intake behaviour was recorded and measurements on rumen fill and its characteristics were carried out.

Measurements and sampling

During periods of measurement, samples of the diets were collected daily and analysed for DM content. During each period, pooled samples of each diet were stored at -20° until they were analysed for fermentation products.

The DM intake (DMI) was determined daily throughout the experiment, and was calculated as the difference between the amount of DM offered and that refused. Intake behaviour was monitored during five consecutive days by continuously weighed mangers. Jaw movements of the sheep were recorded simultaneously, by a foam-filled balloon placed submandibularly. Procedures used for recording intake behaviour, fitting the intake curve of the principal meal, rumen fluid sampling and rumen emptying are described by Van Os *et al.* (1995*a*).

During two consecutive days, simultaneously with intake behaviour recordings, rumen fluid was withdrawn continuously by a peristaltic pump. From 08.30 to 12.00 hours sampling was carried out continuously (60 ml/h): samples (30 ml) were collected every 30 min and additionally at 14.00, 15.00 and 16.00 hours.

Chromium ethylene diamino-tetra-acetate (Cr-EDTA) was used as a marker of the liquid phase to determine rumen fluid volume and the turnover rate (Binnerts *et al.* 1968). On the first day of rumen fluid sampling a single dose of 300 ml was introduced into the rumen through the rumen cannula at 07.00 hours. Previously, a fluid sample (100 ml) was taken to be analysed for background Cr levels. All samples were analysed for Cr concentration. The samples collected at 09.00 hours just before feeding and at 09.30, 10.00, 10.30, 14.00 and 16.00 hours were additionally analysed for NH_3-N concentration, pH and osmolality.

Total rumen fill before (08.30 hours) and after (10.30 hours) the principal meal

was determined by manually emptying the rumen at given times at 3-d intervals. The complete collection of digesta was weighed, manually mixed and sampled for DM analysis and amine extraction. Rumen fluid samples were also prepared to determine pH, NH_3-N concentration and osmolality. Additional measurements made of factors being related to the rumen liquid content were: water intake which was measured during the 5 d of intake behaviour recordings (including rumen fluid sampling days) and salivation during mastication. Hereto a series of ingestive mastication boli (n = 10) was collected when expelled from the cardia into the reticulum of the emptied rumen (08.30 hours). These boli were weighed and analysed for DM content. Under the assumption that DM content of the bolus reflected the silage DM, the saliva content was calculated as the difference between total weight and DM content plus the moisture content of the silage. However, it must be noted that salivation during intake differs from that during rumination or rest (Church, 1976). Therefore, the salivation rate measured in this study can not be extrapolated beyond the time point it was measured.

To determine a possible overall effect of amines in the diet on body temperature, this was measured rectally with a clinical thermometer at 08.30 and 17.00 hours on the days that intake behaviour was recorded.

Chemical analysis

The DM content of silage, refusals, rumen content and mastication boli was determined by oven drying at 80° for 48 h. Silage DM content was corrected for the loss of volatile components occurring during oven drying (Dulphy *et al.* 1975). Fermentation characteristics in the silages (pH, VFA, alcohols, lactic acid, NH_3-N, and soluble N) were determined in the juice pressed from the silages, while total N and amine concentrations were determined in the fresh material. Full details of methods used for analyzing silage composition, rumen fluid NH_3-N and osmolality are described by Van Os *et al.* (1995*a*). The Cr concentration in the rumen fluid samples was determined in the supernatant fraction, after centrifugation (20 min, 40,000 g), by atomic absorption spectrometry (Perking-Elmer 23800) at the wave length of 358 nm. α-Aminobutyric acid (AABA) and GABA were extracted from the silage using the same extraction method as used for the biogenic amines (Van Os *et al.* 1995*a*). Concentrations were determined in the supernatant fraction by ion-exchange chromatography (Pharmacia LKB Biochrom ltd., Amino acid Analyzer alpha plus 4151).

Calculations

Liquid passage. The volume of the rumen liquid and its turnover rate were determined by fitting the model $C_{(t)} = C_0 \times e^{-kt}$ to the data of the Cr-concentration curve in the rumen fluid over the two sampling days. In this model, C_t and C_0 are the marker concentrations at time t and time 0, respectively; k is the fractional turnover rate constant (% /h) and t is the time (h) after marker introduction. The parameter k and the rumen liquid volume at t_0 were estimated using the Marquardt method of the non-linear (NLIN) procedure of the SAS Institute Inc. (1987).

Statistical analysis. Data were subjected to analysis of variance using the procedure GLM of the SAS Institute Inc. (1987). Because no carry-over effects were observed between periods, only animals (5 df) and period (2 df) were included in the model to test for dietary treatment effects (2 df). Differences between diets were compared using the Student's t-test.

Results

Silage composition

Fermentation quality of the basic silage (FAS) was considered good according to the classification of Dulphy and Demarquilly (1981), as indicated by low pH, low acetic and butyric acid contents and the low proportions of total N content present as N_{sol} and NH_3-N (Table 7.2). Addition of the different NPN-components to FAS, resulted in the desired increase of biogenic amine content in the FAS + A diet, and the concentrations of different NPN components (NH_3-N, biogenic amines and GABA) in the FAS + C diet. Adding amines did not significantly alter pH and crude protein (CP) content in FAS + A when compared with FAS. The mixture of NPN-components added, however, increased slightly CP content and the proportion of N_{sol} of FAS + C, as well as its pH value as a result of the buffering properties of the added N-components.

Intake and intake behaviour

Daily DMI and means of the most important variables of intake behaviour are given in Table 7.3. Daily DMI was similar for FAS and FAS + A, but tended to be higher for FAS + C. The average rate of intake of the daily ration also tended to be affected by dietary treatment, and was the lowest for FAS + A.

Neither addition of the biogenic amines alone nor addition of the mixture of NPN

components significantly altered the intake behaviour variables at the principal meal. The amount eaten averaged 316 (SE 13) g DM. So, tendencies of dietary treatment affecting intake rate of the daily ration, originated mainly from influences on the average intake rate during the subsequent small meals. For these small meals intake rate tended to be the lowest for FAS + A and the highest for FAS + C. The number of small meals however, was not affected by type of diet. Furthermore, neither biogenic amines nor the combination of NPN components appeared to influence rumination behaviour or mastication efficiency, *i.e.* the amount of DM chewed per min of total mastication.

Table 7.2 *Chemical composition and fermentation characteristics (g/kg dry matter) of the grass silage preserved with formic acid (FAS), FAS after amine addition (FAS + A), or a combination of non-protein nitrogen components (FAS + C).*

Dietary treatment	FAS	FAS + A	FAS + C
Dry matter (g/kg wet weight)	231	227	229
pH	4.0	4.0	4.2
Crude protein (N x 6.25)	165	169	184
NH_3-N (g/kg N_{total})	81	80	154
N_{sol} (g/kg N_{total})	504	522	571
Lactic acid	83	75	73
Volatile fatty acids	22.3	19.8	22.6
Acetic acid	19.4	17.6	19.8
Propionic acid	12	0.8	1.2
Butyric acid	1.7	1.4	1.6
Alcohols	3.7	3.6	3.3
Amines	2.4	7.4	5.1
Cadaverine	0.4	1.5	1.1
Histamine	0.4	1.1	0.7
Putrescine	0.4	1.5	1.1
Tyramine	1.2	3.3	2.2
α-aminobutyric acid	0.1	0.2	0.1
γ-aminobutyric acid	2.0	2.1	6.4

Table 7.3 *Dry matter intake (DMI; g), intake rates (g DM/min) and duration (min) of feed intake activities of sheep offered* ad lib. *grass silage preserved with formic acid (FAS) and FAS after addition of amines (FAS + A) or a combination of non-protein nitrogen components (FAS + C).*

Dietary treatment	FAS	FAS + A	FAS + C	Significance of the diet effect[a]	Pooled SED
Daily DMI	1187	1293	1327	0.08	62
Intake rate	3.8	3.6	4.2	0.06	0.22
Principal meal:					
DMI	304	316	330	NS	25
Duration	48	53	48	NS	4
Intake rate	6.5	5.9	6.9	NS	0.56
Initial rate	12.0	11.0	11.9	NS	1.10
Final rate	3.6	3.2	4.0	NS	0.56
Small meals:					
No.	11.3	10.2	11.2	NS	0.63
Intake rate	3.4	3.1	3.8	0.07	0.17
Rumination:					
Duration (/kg DMI)	310	336	320	NS	21
Lag time[b]	168	145	152	NS	20
Mastication:					
Daily duration (/kg DMI)	578	620	565	NS	27
Efficiency (g DM/min)	1.74	1.64	1.78	NS	0.08

SED, standard error of difference (8 df); NS, not significant ($P > 0.10$).

[a] For tendencies ($0.05 < P < 0.10$) probabilities are given.

[b] Start of first rumination after silage distribution (min).

Rumen fermentation

The average NH_3 concentration in the rumen fluid before feeding was 146 (SE 11) mg/l and did not differ significantly among the dietary treatments (Fig. 7.1*a*). After the sheep were fed, rumen NH_3 increased for all diets. This increase was the largest for FAS + C, attaining the concentration of 478 mg NH_3/l at 90 min after feeding. This NH_3 concentration for FAS + C was significantly higher ($P < 0.05$) than those measured for FAS and FAS + A at 90 min after feeding. At further

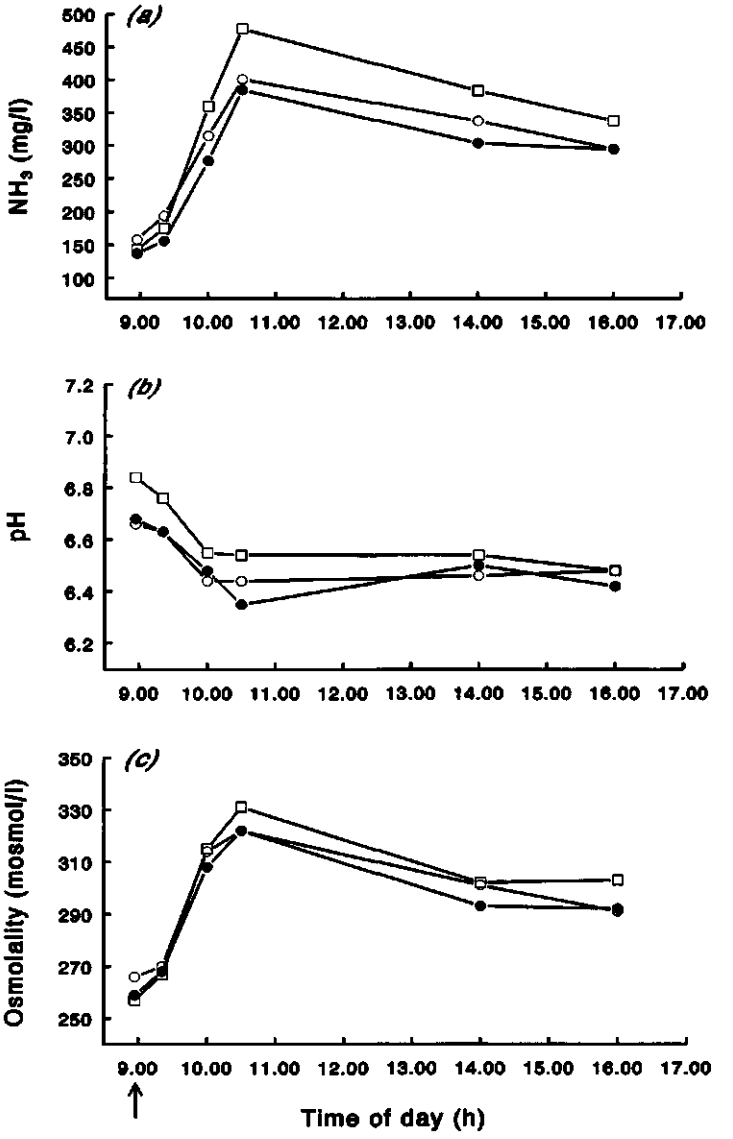

Figure 7.1 *Diurnal changes in (a) ammonia concentration, (b) pH and (c) osmolality in rumen fluid from sheep offered* ad lib. *grass silage preserved with formic acid (FAS; ○) and FAS after addition of amines (FAS + A; ●) or a combination of non-protein nitrogen components (FAS + C; □). ↑; Silage distribution.*

sampling times, rumen NH_3 decreased slowly for all diets, but with FAS + C, the silage with the greatest NPN fraction, rumen NH_3 always remained slightly higher than with FAS and FAS + A.

Rumen fluid pH (Fig. 7.1*b*) before feeding was significantly higher with FAS + C. After feeding, rumen pH decreased for all diets and remained relatively constant with a mean value of 6.46 (SE 0.06) for the remainder of the sampling period without significant differences among diets.

Time courses of rumen fluid osmolality (Fig. 7.1*c*) were similar for the three dietary treatments. Before feeding, osmolality was low and averaged 261 (SE 5) mosmol/l. Subsequently to feeding rumen osmolality increased to an average of 325 (SE 6) mosmol/l for all the diets, and then decreased during the sampling period.

Rumen fill and characteristics of rumen contents

Details regarding the contents of the reticulo-rumen obtained after manual emptyings before (08.30 hours) and after the principal meal (10.30 hours) are presented in Table 7.4. At both times the diets did not apparently influence the total rumen pool size, nor its DM or liquid content.

The rumen fluid was also analysed for pH, NH_3 content and osmolality at both times that the rumen was emptied. Results (not shown) agreed with those measured for determining the patterns of pH, NH_3 and osmolality in the rumen fluid. With all dietary treatments, no biogenic amines were found in the rumen content sampled at 08.30 hours, and only traces were found at 10.30 hours (< 35 mg/kg DM for putrescine and < 19 mg/kg DM for each of the other biogenic amines).

No diet influences were found on rumen liquid volume and turnover rate, estimated by the Cr-EDTA marker (Table 7.5). The rumen volume before feeding, estimated with the Cr-EDTA marker, however, was little higher than that calculated from the characteristics of the rumen content determined by manually emptying the rumen at 08.30 hours (Table 7.4). A higher silage DMI for FAS + C ($P < 0.05$) during days when rumen fluid was sampled, resulted in a significantly higher liquid turnover rate with FAS + C. Including DMI as a covariate in the model for analysis of variance on data of liquid turnover rate eliminated the diet influence related to DMI (Table 7.5).

With these low DM silages, water intake was very low (150 (SE 25) ml/day), without being affected by dietary treatment. Furthermore, type of diet did not

Table 7.4 *Contents of the reticulo-rumen before (08.30 hours) and after (10.30 hours) the principal meal, in sheep offered* ad lib. *grass silage preserved with formic acid (FAS) and FAS after addition of amines (FAS + A) or a combination of non-protein nitrogen components (FAS + C).*

Dietary treatment	FAS	FAS + A	FAS + C	Significance of the diet effect	Pooled SED
08.30 hours:					
Wet weight (kg)	9.0	9.6	8.8	NS	0.41
Dry matter (g/kg)	98	101	91	NS	3.9
Fluid (l)	8.1	8.6	8.0	NS	0.35
10.30 hours:					
Wet weight (kg)	10.5	10.7	10.6	NS	0.41
Dry matter (g/kg)	112	109	110	NS	4.6
Fluid (l)	9.3	9.5	9.4	NS	0.34

SED, standard error of difference (8 df); NS, not significant ($P > 0.10$).

Table 7.5 *Rumen volume (l) estimated by Cr-EDTA marker and rumen fluid turnover rate (%/h), in sheep offered* ad lib. *grass silage preserved with formic acid (FAS) and FAS after addition of amines (FAS + A) or a combination of non-protein nitrogen components (FAS + C).*

Dietary treatment	FAS	FAS + A	FAS + C	Significance of the diet effect[a]	Pooled SED
Rumen volume	9.7	9.8	10.1	NS	0.07
Turnover rate of the liquid phase	6.33	7.26	7.60	NS	0.39

SED, standard error of difference (8 df); NS, not significant ($P > 0.10$).

[a] Inclusion of daily dry matter intake as covariate in the model for analysis of variance.

seem to influence saliva production during ingestive mastication at the beginning of the principal meal, which averaged for all diets 85.0 (SE 0.14) g/100 g fresh silage ingested.

Finally, adding biogenic amines or a combination of NPN-components to FAS did not significantly affect the normal body temperature of the sheep, which averaged 38.8 (SE 0.14)° for both morning and evenings measurements on all diets.

Discussion

Silages

The quantities of tyramine, histamine, putrescine and cadaverine in FAS + A were comparable to quantities detected in medium to poorly preserved silages that led to significantly lower intakes than a good-quality silage (Tveit *et al.* 1992; Van Os *et al.* 1995*a*). The concentration of the NPN fraction in FAS + C was similar to that seen in low fermentation-quality silages, namely with high NH_3 content and high concentrations of biogenic amines (Tveit *et al.* 1992; Van Os *et al.* 1995*a*) and GABA (Ohshima *et al.* 1979). The use of GABA as an additional N component to increase the NPN fraction of FAS + C, was because of its presence in significant amounts in naturally fermented silages (Ohshima *et al.* 1979), the fact that GABA is found to be a neurotransmitter in the central nervous system acting on feed intake control (Seoane *et al.* 1984) and the fact that GABA depressed intake in sheep when infused into the rumen or intravenously (Buchanan-Smith, 1982).

Intake and intake behaviour

The tendency for biogenic amines to reduce silage DMI observed by Van Os *et al.* (1995*a*) was not observed in the present study, even though higher amounts of amines were added than in the previous experiment. Regression analysis of the relationship between levels of fermentation products in silage and silage intake has shown that silage intake may be negatively correlated with the combination of NH_3 and amines in the silage when the total concentration of both exceeds 1.6 g/kg DM (Miettinen *et al.* 1991). However, Van Os *et al.* (1995*a*) concluded that NH_3 *per se* was not an intake depressor, and therefore, interactive effects between NH_3 and other N-containing fermentation products may be responsible for the observed lower intake of poorly preserved silages. The results of the present study did not support this hypothesis. On the contrary, DMI of FAS + C tended to be even higher

than that of FAS.

It must be noted that DMI of FAS in the present study was lower than that of the control silage preserved with formic acid in study of Van Os *et al.* (1995*a*) (50 *vs* 65 g/kg metabolic weight ($W^{0.75}$)). This could be attributed possibly to a lower digestion rate or the higher overall content of fermentation products, especially that of lactic acid (Morgan and L'Estrange, 1976), which was 21 g/kg DM in the study of Van Os *et al.* (1995*a*) *vs* 83 g/kg DM in the present study. Possibly, the slight negative effects of the biogenic amines on DMI as found by Van Os, *et al.* (1995*a*) were hidden by this low intake. On the other hand, DMI of FAS in the present study was not lower than that of the same type of silages used by Dulphy *et al.* (1984), and it was within the range of values predicted using equations based on silage pH and NH_3 content or pH and lactic acid (Wilkins *et al.* 1971). Moreover, daily DMI of FAS covered largely the daily energy and protein requirements of the sheep for maintenance (Andrieu *et al.* 1989).

Silage intake appears to be controlled mainly by oro-pharyngeal factors (taste, smell) and chemostatic regulation (satiation) and not by rumen fill (Gill *et al.* 1987). The similarity between the dietary treatments in the amount eaten and the period of time spent eating during the principal meal indicated that with FAS + A and FAS + C the state of satiation was reached at the same time as that for the control diet FAS. Similar intake rates at the very beginning of the principal meal, when feed intake cannot be inhibited by satiation, as well as during the principal meal, suggested that neither biogenic amines in FAS + A nor the combination of NPN-components in FAS + C lowered palatability of the silage. This did not agree with the results of Van Os *et al.* (1995*a*) which showed a slight negative influence of lower quantities of biogenic amines on palatability during both the principal meal and the small meals. This difference in extent of amines affecting palatability is possibly due to the higher overall content of fermentation products of FAS in the present study, or to the sheep getting accustomed to the taste of amines. The latter could be possible, because four of the six sheep that were also used in the previous study of Van Os *et al.* (1995*a*) showed, in the present study, slightly higher DMI and intake rates during the principal meal than the newly used animals. Nevertheless, the tendency towards lower intake rates during the small meals of FAS + A in the present study does not totally exclude negative effects of biogenic amines on palatability.

In agreement with the findings of Van Os *et al.* (1995*a*), no further influences of the added NPN-components were observed on either rumination behaviour or

chewing efficiency.

Rumen fill and characteristics

Of the rumen fluid variables measured only the concentration of NH_3 was affected by diet, with the highest concentration observed with FAS + C during the first hour after the principal meal. In other studies using sheep (Benahmed and Dulphy, 1987) and dairy cows (Choung *et al.* 1990) no intake depressions have been observed with NH_3 concentrations in the rumen reaching peak values of 450 mg/l. The decrease after peaking was observed to be slightly larger with FAS + C. This might indicate an increased rate of microbial synthesis as a result from the better ratio of N to digestible organic matter in FAS + C (Russell and Hespell, 1981). With this dietary treatment a higher flow of microbial or dietary protein to the lower intestines can be expected which may enhance DMI of FAS + C (Charmley and Veira, 1990). On the other hand, the NH_3 concentration in the rumen on FAS appeared not to be limited for microbial synthesis (Russell and Hespell, 1981). Therefore the faster decrease of rumen NH_3 concentration with FAS + C could also be the result of increased rates of absorption across the rumen wall (Morris and Payne, 1970).

Rumen pH and osmolality followed the patterns which are normally observed after silage feeding (Van Os *et al.* 1995*a*). The post-feeding increase of fermentation metabolites in the rumen increased osmolality and decreased pH, both inducing signals of satiation at the ruminal level (Phillip *et al.* 1981). Similarity of the patterns of both variables for all diets may indicate that biogenic amines, either alone or in combination with NH_3 and GABA, did not alter concentrations of metabolites in the rumen to such an extent that they could depress DMI.

The suggested higher rumen liquid volume as a consequence of amine addition (Van Os *et al.* 1995*a*) was not observed in the present study. Neither amines nor the combination of NPN-components affected total rumen pool size, the liquid content or the factors measured that might have influenced the rumen liquid balance. Biogenic amines did not affect the rate of salivation at intake, which was found to be stimulated by tyramine in rats (Okina *et al.* 1993). Rumen fluid turnover rate seemed to be higher for FAS + C, but this was merely the result of a higher DMI (Warner and Stacy, 1968) rather than the effect of the supplemented N-components. The rumen fluid turnover thus remained unaffected by dietary treatment itself, when fluid turnover rate was expressed relative to DMI.

The traces of biogenic amines in the rumen are not expected to interfere directly

in chemostatic regulation of intake. Physiological effects of amines on intake are possible when significant concentrations are present in the circulation (Joosten, 1988). The highly physiologically active histamine appears not to be absorbed from the rumen (Kay and Sjaastad, 1974). So, assuming no absorption from the rumen of other types of amines, the ingested amines can only enter the circulation after passing the rumen and being absorbed in the lower intestines. A higher amine passage could be expected with the higher liquid passage with FAS + C, but the higher DMI of this silage indicated that the amount of amines that eventually passed the rumen was too low to have negative influences on intake via this route. The rapid degradation in the rumen of the ingested biogenic amines (Van Os *et al.* 1995*b*), however, will largely prevent this amine passage. Additionally, Van Os *et al.* (1995*b*) found that the degradation rate increased when the animals were adapted to diets with high biogenic amine content. This possibly explains the controversial findings of Buchanan-Smith and Phillip (1986) of intake reduction upon biogenic amine infusions into the rumen. In their study, the sheep were fed a high DM lucerne silage, in which biogenic amine concentration was probably low (Voss, 1967). The rumen microflora in those sheep may not have been able to degrade the infused amines rapidly, resulting in an increase of the amount that passed the rumen.

However, it must be considered that the high NPN fraction in poorly preserved silages could be an indirect cause of the lower intake. With this type of silages, when rumen NH_3 content is high and is combined with a relatively low energy supply to the rumen microorganisms or to metabolic processes, subclinical NH_3 intoxication may be a cause of lower intake (Chamberlain and Choung, 1993).

In conclusion, it can be stated that neither biogenic amines nor the combination of the major NPN components present in poor-quality silages play a significant direct role in the lower intake of these silages. In the sheep, adapted to the experimental diets, no direct effects of NH_3, biogenic amines and GABA were found on variables that indicate interference of chemostatic intake control. However, a minor effect of biogenic amines on palatability can not be excluded.

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Chapter 8

Mechanisms of adaptation in sheep to overcome silage intake depression induced by biogenic amines

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Abstract

Effects of biogenic amines on silage intake and rumen fermentation during diet changes were studied in sheep. Two grass silages were prepared from a single grass sward, one untreated (WAS) and one treated with 4.0 l formic acid per tonne (FAS). Diets of FAS, and FAS supplemented with 7.2 g biogenic amines/kg dry matter (DM; FAS + A), were offered ad lib. once daily to four rumen cannulated, and four intact wethers in a repeated cross-over design experiment. During a pre-period before each cross-over, the animals received either the silage low in biogenic amines (FAS), or the silage with moderate amine concentrations (WAS). During the first 4 d that FAS + A was fed, the added biogenic amines tended to lower daily dry matter intake (DMI) and lowered significantly the DMI during the principal meal after feeding. This acute effect on daily DMI tended to be reduced when the sheep were previously preconditioned to amines by feeding WAS. The acute DMI depression during the principal meal was significantly reduced by preconditioning. At the end of the 14-d FAS + A feeding-period, daily DMI was similar to that of the FAS treatment. However, the daily pattern of intake remained different, with lower intake of FAS + A during the first 5 h after feeding, being compensated for by end of the day. Rumen fermentation tended to be less during the first 4 d that FAS + A was fed, due to the lower DMI and not due to the acute effect of amines. However, in the sheep unadapted to FAS + A, amine content in the rumen was higher than in sheep adapted for 14 d to FAS + A or WAS. Adaptation to FAS + A and feeding WAS during the pre-periods, increased the amine degrading capacity of rumen fluid. In conclusion, in sheep unadapted to dietary amines, feeding amines will acutely lower DMI through reduced palatability and most probably by stressing intermediary metabolism. Being preconditioned to amines slightly reduces the acute effect on daily DMI. Although the sheep adapted within 14 d to biogenic amines in the diet and increased daily DMI, there was clear evidence that amines have a negative effect on palatability.

Introduction

Biogenic amines are thought to be one of the fermentation products which lower intake of ensiled forages, either through reduced palatability or stress in intermediary metabolism after being absorbed (Beever and Reynolds, 1994). However, studies with sheep (Van Os *et al.* 1995*a*; 1996*a*) and dairy cows (Van Os *et al.* 1995*b*) showed that concentrations of amines (7 g/kg dry matter) comparable to those present in poor-quality grass silages did not affect intake. In those studies, animals were adapted to the dietary treatment for 14 d before intake behaviour was monitored. However, some animals did show reduced silage intake during the first days after the change-over from a silage low in biogenic amine content to the same silage supplemented with biogenic amines. Possibly biogenic amines only affect silage intake in sheep that are not adapted or preconditioned to dietary amines. It was hypothesized that two mechanisms of adaptation to amines may be distinguished. The animals may become accustomed to the taste of amines, and the increased degradation of amines by the rumen microbial mass (Van Os *et al.* 1995*c*) may prevent accumulation in the rumen and absorption.

Therefore an experiment was carried out to investigate the acute effect of biogenic amines on silage intake in sheep not adapted to amines, to find out whether the animals adapt to the dietary amines, and if so to identify the nature of the mechanisms of this adaptation.

Materials and methods

Dietary treatments and feeding

In June 1993, two grass silages were made from the second cut of a single sward of the institutes' permanent pastures of predominantly perennial ryegrass (*Lolium perenne*), fertilized with 450 kg N/ha per year. The grass was pre-wilted and harvested with a precision chop-forage harvester. One part was ensiled untreated, and the other part was ensiled with formic acid (4.0 l/tonne fresh material). Three dietary treatments were used: the silage without additive (WAS), the formic acid preserved silage (FAS) and FAS supplemented with biogenic amines (FAS + A), at the rate of 1.2 g histamine, 2.8 g tyramine, 1.8 g putrescine and 1.4 g cadaverine per kg/dry matter (DM). The amounts of the individual amines added were fixed for FAS + A to achieve an amine content for this silage being slightly

higher than that tested in a previous study by Van Os *et al.* (1996*a*). Histamine and tyramine were added in the hydrochloric form and putrescine and cadaverine as free bases. The amines were purchased from SIGMA Chemical company, St. Louis, USA. All quantities of amines mentioned in this paper refer to real amines and not their hydrochlorides.

For preparation of FAS + A, the amines were dissolved individually in 7.5 l distilled water at concentrations in g/l of 16.8 for histamine, 39.2 for tyramine, 25.2 for putrescine and 19.6 for cadaverine. The total of 30 l of amine solutions was sprayed on 2 tonnes of FAS while mixing the silage in a feed mixing wagon. The WAS and FAS were similarly treated in the mixing wagon. After mixing, the amount of silage needed was stored in portions of 7 kg at -20°. The daily amount of silage needed was placed at room temperature 24 h before feeding. The silage treatments were offered to the sheep once daily (08.00 hours), after collection of the refusals of the previous day.

Animals and experimental design

Four non-fistulated 3-year old (average live weight 80 kg), and four fistulated 2-year old Texel wethers (average live weight 61 kg) were used. None of the wethers had consumed silage before. The fistulated animals were equipped with a PVC rumen cannula (inner diameter 42 mm), 10 weeks before the start of pre-period A.

Pairs were made of one fistulated and one non-fistulated sheep, matching the fistulated sheep being heaviest in bodyweight with the non-fistulated sheep heaviest in body weight. Likewise, three other pairs were made in descending order of body weight. Each pair was randomly assigned to the sequences of diet change-overs according to the experimental design given in Table 8.1. In this repeated cross-over design, the periods 1 and 2, and 3 and 4 concerned the first and second cross-over designs respectively. Measurements were made during the last 4 d of each pre-period (days 21-24), and during the first 4 d (days 1-4) and the last 4 d (days 11-14) of each 14-d experimental period. The acute effect of biogenic amines on intake and rumen fermentation, as well as the extent of adaptation to dietary amines were studied during the 14-d periods.

The **acute effect** was defined as the difference between data measured on days 1-4 that FAS + A was offered and those of FAS on days 11-14, within the same cross-over design. The extent of **adaptation** to biogenic amines in FAS + A during the 14-d period was determined by comparing, within the cross-over design, the data of FAS + A measured on days 11-14 with data of FAS measured on days 11-

Table 8.1 *Experimental design and assignment of pairs of sheep formed of one fistulated and one non-fistulated animal, to the dietary treatments.*

	Pair 1	Pair 2	Pair 3	Pair 4
Experimental period:	Diet:			
Pre-period A (24 d)	WAS	WAS	FAS	FAS
Period 1 (14 d)	FAS	FAS+A	FAS	FAS+A
Period 2 (14 d)	FAS+A	FAS	FAS+A	FAS
	---------- On pasture (35 d) ----------			
Pre-period B (24 d)	FAS	FAS	WAS	WAS
Period 3 (14 d)	FAS	FAS+A	FAS	FAS+A
Period 4 (14 d)	FAS+A	FAS	FAS+A	FAS

WAS, grass silage without additive; FAS, grass silage preserved with formic acid; FAS+A, FAS supplemented with 7.2 g biogenic amines/kg DM.

14. In order to determine the effect of previously having tasted amines (**preconditioning**), the sheep received before each cross-over either the untreated silage (WAS) with a moderate biogenic amine content or FAS with low concentrations for a 24-d pre-period (period A or B). This effect of preconditioning was defined as the difference in the acute effect caused by the pre-period diet.

During the experiment, including the pre-periods, the sheep were kept in metabolism cages placed in an experimental unit where light was provided for 16 h daily. The animals had free access to water. Between the first and second cross-over, the sheep were taken from the metabolism cages and were allowed to pasture for 5 weeks during which they had free access to mineral blocks.

Measurements and sampling

Silage samples were taken from the feed mixing wagon and refusals were sampled each day of measurement. Both the silage and pooled samples of refusals were stored at -20° until analysis.

The daily DM intake (DMI) was calculated as the difference between the amount offered and that refused. During 4 consecutive days on days 1-4 and days 11-14

of each period cumulative intake during the day was measured by re-weighing the manger plus refusals at 0.5, 1, 1.5, 2, 3, 5, 7, 10, 16 and 24 h after feeding. On these days the time spent eating during the principal meal (first meal after feeding) was determined by visual observation and the amount eaten by re-weighing the manger. The principal meal was considered to be finished when the sheep stopped eating and lay down, or withdrew from the manger and did not resume eating for at least 10 minutes.

On days 2, 4, 12 and 14 of each 14-d period and on days 22 and 24 of the pre-periods, rumen fluid was taken from the ventral region by using a 100 ml syringe with a rubber tube (length 50 cm, inner diameter 7 mm). On these days 50 ml was taken just before feeding (08.00 hours), and 400 ml was taken 3 h after feeding (11.00 hours). Immediately after collection, the pH value of the fluid was measured and sub-samples (4.0 ml) were preserved with 4.0 ml 0.5 M sulphuric acid and 0.8 ml orthophosphoric acid (50 ml/l) for NH_3 and volatile fatty acid (VFA) analyses respectively.

The remaining fluid taken at 11.00 hours was used directly to determine *in vitro* the amine degrading capacity of the rumen fluid, by adding biogenic amines and measuring the NH_3 liberated (Van Os *et al.* 1995*c*). Per animal, four 100-ml glass tubes, equilibrated at 39°, were filled with 60 g unfiltered rumen fluid and closed with rubber stoppers permitting overpressure of fermentation gas to escape. Before closing the tubes, 0.8 ml of a freshly prepared solution of biogenic amines was added to the rumen fluid in 2 of the 4 tubes. This solution contained per litre de-ionized water: 5.0 g histamine, 17.0 g tyramine, 8.0 g putrescine and 8.0 g cadaverine. Subsequently, the tubes were incubated in a shaking (100 rpm) waterbath at 39°. Samples (2.0 ml) for NH_3 analysis were taken just before, and 1 h and 6 h after the start of the incubation. The net NH_3 production from the added amines was calculated as the difference between total NH_3 production and the endogenous NH_3 production of the rumen fluid.

Rumen content was sampled for biogenic amine analysis on day 1 and day 13 of each 14-d period and on day 23 of the pre-periods. Three hours after feeding (11.00 h), about 300 g rumen content (liquid and solid phase) was withdrawn using a spring coil (Fadel *et al.* 1987). Exactly 50 g of the rumen content was immediately acidified with 30 ml trichloroacetic acid (TCA, 200 g/l) and stored at -20°. The remaining part was analysed for DM content.

Chemical analysis

Samples of the silages and refusals were dried at 70°C and ground to pass a 1-mm screen. Both were analysed for DM and ash. The silages were additionally analysed for Kjeldahl-N, neutral detergent fibre (NDF) and carbohydrates soluble in 40% ethanol (SC), according to the methods used by Van Vuuren *et al.* (1989). Water extracts were made of silage samples (100 g silage/l) to determine lactic acid, VFA, alcohol, NH_3 concentrations and pH value. These components, as well as VFA and NH_3 content in rumen fluid samples were determined using the same methods as Van Os *et al.* (1996*b*).

The DM content of the silages was corrected for volatile components lost by oven-drying, as recommended by Dulphy *et al.* (1975). *In vitro* digestibility of the silage organic matter was determined by a modified Tilly and Terry method (Van der Meer, 1986). Biogenic amines were extracted from the silage as described by Van Os *et al.* (1996*b*). The amines were extracted from the rumen contents by macerating the sample with the amount of TCA added for preservation. After centrifugation (20 min, 12,000 *g*) of the macerates the amines were determined in the supernatant by ion-exchange chromatography (Van Os *et al.* 1996*b*).

Statistical analysis

The data, normally distributed with homogenous variance, were subjected to analysis of variance using Genstat 5 (Genstat 5 Committee, 1987). The Barlett's test was used for testing homogeneity of the variance (Steel and Torrie, 1980). Because the effect of fistulation on intake variables was not significant, this was excluded from the models used.

Three different effects of biogenic amines were statistically tested; the acute effect, the effect of being preconditioned and the effect of adaptation. The acute effect was tested by analysis of variance on data from FAS + A measured on days 1-4 and data from FAS + A and FAS measured on days 11-14 in the periods 1 to 4 (Table 8.1). Besides these factors (2 df) the effects of animal (7 df for all, and 3 df for fistulated sheep) and period (3 df) were included in the model. Differences between treatment means were compared by the Student's *t*-test. An acute effect of biogenic amines was declared when results for FAS + A measured on days 1-4 differed significantly from FAS measured on days 11-14.

The effect of preconditioning was considered to be present when feeding biogenic amines (WAS) during the pre-period significantly altered the extent of the acute effect in comparison to pre-feeding FAS. In the model used to test the

effects of the pre-feeding treatment (1 df), animal effects (7 df or 3 df) and the effect of repetition of cross-over (1 df) were included.

Adaptation to biogenic amines was derived from the change in measured variables during the 14 d-period that FAS + A was fed. Adaptation was statistically tested by comparing data of the treatments FAS and FAS + A both measured on days 11-14 in periods 1 to 4 (Table 8.1). Sheep were considered to be adapted to biogenic amines when results from FAS + A being given on days 11-14 did not significantly differ from those for FAS being given on days 11-14. The model included the effects of diet (1 df), animal (7 df or 3 df) and period (3 df).

Additionally, we tested whether intake and rumen fermentation on WAS differed from that on FAS at the end of the pre-period. Therefore effects of diet (1 df)was tested in the model including effects of animals (7 df or 3 df) and pre-period (2 df).

Results

Silage composition

Chemical compositions of WAS, FAS and FAS after biogenic amine addition (FAS + A), are presented in Table 8.2. The DM, crude protein and NDF content, and organic matter digestibility did not differ between WAS and FAS. Formic acid treatment limited considerably the formation of lactic acid, acetic acid, NH_3 and biogenic amines, and increased residual SC in the silage. Addition of amines to FAS resulted in a concentration of amines in FAS + A being slightly higher than in the same type of silage used by Van Os *et al.* (1996*a*), without other changes in silage composition when compared to FAS.

Silage intake

Acute effect and preconditioning. Biogenic amines in FAS + A tended to reduce daily DMI during the first 4 d of feeding FAS + A (Table 8.3). This acute effect tended to be smaller when the sheep were preconditioned to biogenic amines by feeding the amine containing WAS. Similar, but significant effects were found for DMI during the principal meal. Duration and intake rate of the principal meal were not significantly affected by biogenic amines. However, a slight acute lower intake rate was observed for FAS + A, whereas being preconditioned to amines slightly lengthened the time spent eating.

Table 8.2 *Chemical composition and fermentation characteristics (g/kg dry matter) of grass silages preserved without additive (WAS) and with formic acid (FAS) and FAS after amine addition (FAS+A).*

Dietary treatment	WAS	FAS	FAS+A
Dry matter (g/kg wet weight)	184	207	210
pH	3.8	3.9	3.9
Crude protein (N x 6.25)	159	162	166
NH_3-N (g/kg N_{total})	84	58	62
Neutral-detergent fibre	476	432	446
Soluble carbohydrates	33	104	95
Ash	137	133	131
OM digestibility (*in vitro*)	68	69	69
Lactic acid	110	65	72
Acetic acid	47.5	20.6	22.2
Propionic acid	2.9	0.4	0.4
Butyric acid	2.2	0.5	0.3
Alcohols	20.5	5.4	5.9
Amines	5.2	1.4	8.9
Histamine	0.7	0.1	1.3
Tyramine	2.1	0.7	3.7
Putrescine	1.0	0.3	1.9
Cadaverine	1.4	0.3	1.9

OM, organic matter expressed as % digested.

The DMI during different time-intervals after feeding (Fig. 8.1) showed that biogenic amines acutely changed the daily intake pattern in sheep that were not preconditioned to amines (Fig 8.1*a*). On days 1-4 of FAS+A feeding, the proportion eaten of the total daily DMI was lower ($P < 0.05$) during the first 5 h after feeding than with FAS on days 11-14. However, this depressed intake was compensated by an increase ($P < 0.05$) of the proportional DMI during the night (14-24 h after feeding). No acute change of the intake pattern was observed in the preconditioned sheep (Fig. 8.1*b*). Although proportional DMI of FAS+A on days 1-4 was slightly lower during the first 5 h after feeding and slightly higher at the

Table 8.3 *The acute effect of biogenic amines (FAS + A on days 1-4 vs FAS on days 11-14) on dry matter intake (DMI) and intake characteristics of the principal meal, and the influence on the acute effect of being preconditioned to amines by pre-feeding the amine-containing WAS compared to FAS.*

Pre-fed diet[a]	WAS			FAS			Statistical significance			
Dietary treatment[a] Measuring days[b]	FAS + A 1-4	FAS + A 11-14	FAS 11-14	FAS + A 1-4	FAS + A 11-14	FAS 11-14	Acute effect[c]	SED	Precon-ditioning[c]	SED
Daily DMI (g)	1331	1354	1368	1333	1352	1386	0.07	34	0.09	17
Principal meal:										
DMI (g)	164	175	188	134	151	182	**	17	*	10
Duration (min)	20	18	19	16	19	20	NS	2.2	NS	2.3
Intake rate (g DM/min)	8.3	10.0	9.9	8.2	8.2	9.6	NS	1.0	NS	1.3

SED, standard error of difference (35 df for the acute effect; 6 df for the effect of preconditioning); NS, not significant ($P > 0.10$); For tendencies ($0.05 < P < 0.10$) probabilities are given. * $P < 0.05$; ** $P < 0.01$.

[a] For dietary treatments, see Table 8.2.

[b] Days that measurements were carried out during the 14-d experimental period the diet was offered.

[c] For definitions of the determined effects, see Statistical analysis.

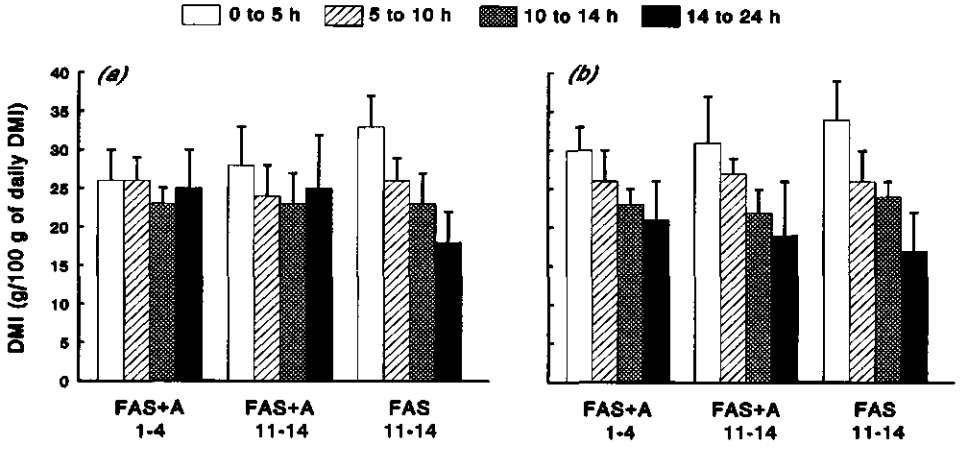

Figure 8.1 *Dry matter intake (DMI; +SE) at different time-intervals after feeding, in sheep fed FAS+A on days 1-4 and days 11-14, or FAS at days 11-14 of the 14-d period the silage was offered. In the pre-period, sheep were fed either silage with (a) low (FAS) or (b) moderate biogenic amine content (WAS). For dietary treatments, see Table 8.2.*

end of the day, the pattern of intake did not significantly differ from that of FAS on days 11-14.

Adaptation. No significant differences in daily DMI were observed between FAS+A and FAS on days 11-14 (Table 8.4). Although the sheep increased the DMI during the principal meal of FAS+A on days 1-4 over the 14-d experimental period, they did not fully adapt to the level of FAS on days 11-14. The DMI of the principal meal of FAS+A on days 11-14 remained lower ($P < 0.05$). The reduced DMI resulted from a combination of both a slightly shorter time spend eating and a slightly lower intake rate. Although similar daily DMI of FAS+A and FAS on days 11-14 suggests full adaptation to amines, the proportional DMI over the day differed between the silages (Fig. 8.2). During the first 5 h after feeding DMI of FAS+A remained slightly depressed, but was compensated for by the end of the day.

Table 8.4 *Daily dry matter intake (DMI) and intake characteristics of the principal meal in sheep on days 11-14 of the 14-period, when adapted to FAS or FAS+A, and on days 21-24 of the pre-period when adapted to FAS or WAS.*

	FAS+A *vs* FAS				WAS *vs* FAS			
Dietary treatment	FAS+A	FAS	Sign	SED	WAS	FAS	Sign	SED
Daily DMI (g)	1353	1378	NS	31	1270	1451	*	53
Principal meal:								
DMI (g)	163	185	*	9	180	188	NS	18
Duration (min)	18	19	NS	0.9	21	20	NS	2.5
Intake rate (g DM/min)	9.1	9.8	NS	0.6	8.5	9.3	NS	1.6

Sign, statistical significance; SED, standard error of difference (6 df for WAS *vs* FAS; 20 df for FAS *vs* FAS+A); NS, not significant ($P > 0.10$); * $P < 0.05$.

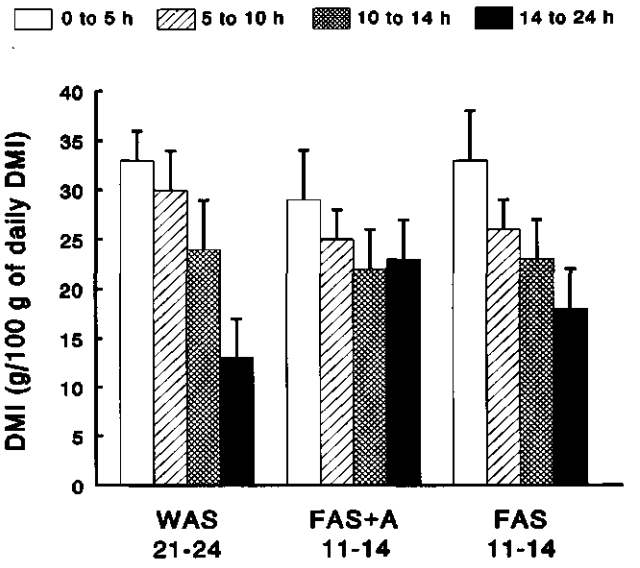

Figure 8.2 *Dry matter intake (DMI; +SE) at different time-intervals after feeding, in sheep on days 11-14 of the 14-d experimental period, when adapted to FAS or FAS+A, and on days 21-24 of the pre-period, when adapted to WAS.*

Intake characteristics of WAS and FAS at the end of the pre-periods (Table 8.4) showed that daily DMI of WAS, with moderate biogenic amine content, was lower than that of FAS. This was mainly caused by the lower ($P < 0.05$) proportional DMI during the night (Fig. 8.2).

Rumen fermentation

Acute effect and preconditioning. Before feeding, no acute effects of biogenic amines were found on rumen fermentation variables during days 1-4 of FAS+A feeding. Values were comparable to those measured before feeding FAS+A on days 11-14 (Table 8.6). In samples taken 3 h after feeding, amines acutely lowered rumen NH_3 content and the proportion of propionic acid in the rumen VFA pool (Table 8.5). Being preconditioned to amines by pre-feeding WAS did not significantly alter the acute effect on NH_3 content or the proportion of propionic acid in the rumen.

Table 8.5 *The acute effect of biogenic amines (FAS+A on days 1-4 vs FAS on days 11-14) on rumen fluid pH, ammonia (NH_3) and volatile fatty acids (VFA) 3 h after feeding.*

Dietary treatment[a] Measuring days	FAS+A 1-4	FAS+A 11-14	FAS 11-14	Significance of the acute effect	SED
NH_3 (mg/l)	175	229	249	*	29
VFA (mmol/l)[b]	89.0	92.0	97.6	NS	5.14
Acetic acid[c]	62.9	61.5	62.1	NS	0.70
Propionic acid	20.9	23.7	23.3	*	1.19
Butyric acid	11.0	10.2	10.4	NS	0.51
pH	6.56	6.50	6.54	NS	0.11

SED, standard error of difference 14 df; NS, not significant ($P > 0.10$); * $P < 0.05$.

[a] For dietary treatments and days of measurements, see Table 8.2 and Table 8.3.

[b] Includes acetic acid, propionic acid, methylpropionic acid, butyric acid, 2- and 3-methylbutyric acid and valeric acid.

[c] In mol/100 mol.

Adaptation. On days 11-14 of feeding FAS + A, no influences of biogenic amines on any of the measured rumen fermentation characteristics were found, either before or 3 h after feeding (Table 8.6).

In sheep pre-fed WAS, rumen fermentation remained different from that of sheep that received only FAS in the pre-period. Notably the pH value before feeding was higher with WAS and the total VFA content was lower, with a lower

Table 8.6 *Rumen fluid pH, ammonia (NH_3) and volatile fatty acids (VFA) before, and 3 h after feeding in sheep on days 11-14 of the 14-period, when adapted to FAS or FAS+A, and on days 21-24 of the pre-period when adapted to FAS or WAS*[a].

	FAS+A *vs* FAS				WAS *vs* FAS			
Dietary treatment	FAS+A	FAS	Sign	SED	WAS	FAS	Sign	SED
Before feeding:								
NH_3 (mg/l)	145	143	NS	10.5	123	120	NS	6.2
VFA (mmol/l)[b]	66.7	72.1	NS	3.3	61.5	77.8	**	9.6
acetic acid[c]	65.8	67.6	NS	1.4	68.4	67.2	NS	0.6
propionic acid	19.8	19.2	NS	1.3	17.1	18.7	**	0.2
butyric acid	10.5	9.9	NS	0.2	10.3	9.8	NS	0.2
pH	6.78	6.82	NS	0.06	6.92	6.69	*	0.05
3 h after feeding:								
NH_3 (mg/l)	229	249	NS	19.1	232	200	*	7.4
VFA (mmol/l)	92.0	97.6	NS	2.8	88.0	97.6	**	0.7
acetic acid	61.5	62.1	NS	0.3	61.6	63.0	NS	0.5
propionic acid	23.7	23.2	NS	0.7	22.0	20.3	NS	1.1
butyric acid	10.1	10.4	NS	0.3	11.8	10.5	*	0.2
pH	6.50	6.54	NS	0.05	6.69	6.63	NS	0.10

Sign, statistical significance; SED, standard error of difference (2 df for WAS *vs* FAS; 8 df for FAS *vs* FAS+A); NS, not significant ($P > 0.10$); * $P < 0.05$; ** $P < 0.01$.

[a] For dietary treatments, see Table 8.2.

[b] Includes acetic acid, propionic acid, methylpropionic acid, butyric acid, 2- and 3-methylbutyric acid and valeric acid.

[c] In mol/100 mol.

molar proportion of propionic acid. In samples taken 3 h after feeding rumen NH_3 was higher with WAS and VFA content was lower, with a higher molar proportion of butyric acid in the VFA pool.

Rumen biogenic amine content

Acute effect and preconditioning. During the first day of FAS + A feeding, a significant rise of all individual amines was observed in the rumen, 3 h after feeding (Table 8.7). The highest increase was found for tyramine followed by cadaverine and histamine. No significant influence of preconditioning was associated with this acute rise of amines in the rumen.

The biogenic amine degrading capacity of the rumen fluid, measured by NH_3 production from added amines, was low in sheep fed FAS on days 11-14 and in those fed FAS + A on days 1-4 (Fig 8.3). The latter was more pronounced in sheep that were not preconditioned (Fig. 8.3*a*). Preconditioning by pre-feeding WAS (Fig. 8.3*b*) tended to increase ($P = 0.08$) amine degradation in the rumen fluid of sheep fed FAS + A on days 1-4.

Adaptation. When the sheep were fed FAS + A, the biogenic amine degrading capacity of the rumen fluid taken on days 11-14, was higher ($P < 0.01$) than that of sheep fed FAS on days 11-14 (Fig. 8.4). On day 14 only traces (< 35 mg/kg DM) of the four biogenic amines were found in the rumen content of sheep fed FAS + A on day 14.

The additional comparison of WAS and FAS at the end of the pre-periods showed a slightly higher amine degrading capacity of the rumen fluid on WAS than on FAS (Fig. 8.4). Nevertheless, rumen biogenic amine content on WAS was similar to that on FAS and contained only traces of amines.

Discussion

The acute effect of biogenic amines

Our previous studies on the influence of biogenic amines on grass silage intake in sheep and dairy cows showed that amines apparently had no impact on chemostatic intake control and only tended to impair silage palatability (Van Os *et al.* 1995*a*; 1995*b*; 1996*a*). In those studies measurements were carried out with animals that had been adapted to the diets containing amines for 14 d. In the present study, a grass silage containing biogenic amines was fed to sheep that had

Table 8.7 *The acute effect of biogenic amines (FAS+A on days 1-4 vs FAS on days 11-14) on rumen amine content (mg/kg DM) in sheep, 3 h after feeding, and the influence on the acute effect of being preconditioned to amines by pre-feeding the amine-containing WAS compared to FAS.*

Pre-fed diet[a]	WAS			FAS			Statistical significance			
Dietary treatment[a] Measuring days	FAS+A 1	FAS+A 14	FAS 14	FAS+A 1	FAS+A 14	FAS 14	Acute effect[b]	SED	Precon-ditioning[b]	SED
Cadaverine	254	16	22	229	18	16	**	56	NS	49
Histamine	295	17	14	340	11	12	**	63	NS	43
Putrescine	46	31	32	61	34	30	*	7	NS	16
Tyramine	468	15	34	382	18	21	**	67	NS	151

SED, standard error of difference (14 df for the acute effect; 2 df for the effect of being preconditioned); NS, not significant ($P > 0.10$). * $P < 0.05$; ** $P < 0.01$.

[a] For dietary treatments and days of measurements, see Table 8.2 and Table 8.3.

[b] For definitions of the determined effects, see Statistical analysis.

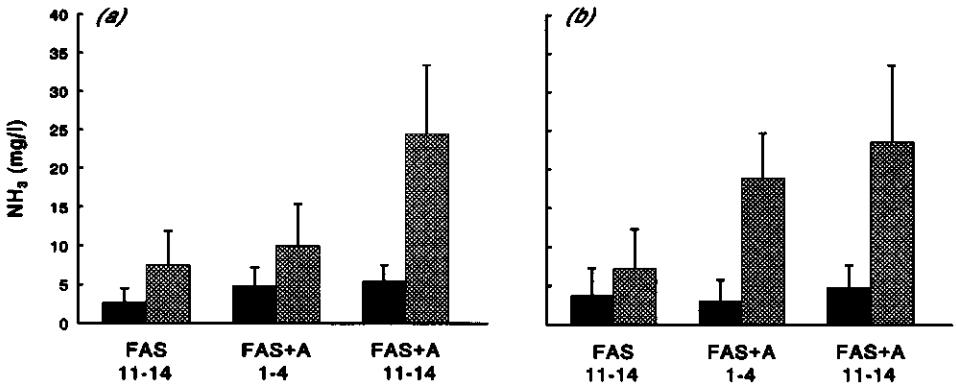

Figure 8.3 *The NH_3 production (+ SE) in rumen fluid from added biogenic amines after 1 (■), and 6 h (▒) of* in vitro *incubation. Rumen fluid was withdrawn 3 h after feeding from sheep fed FAS + A days 1-4 and days 11-14, or FAS on days 11-14 of the 14-d period the silage was offered. In the pre-period, sheep were fed either silage with (a) low (FAS) or (b) moderate biogenic amine content (WAS).*

never been fed silage before and were assumed to be unadapted and not preconditioned to biogenic amines. In these sheep, it was found that a sudden increase of biogenic amines in the diet depressed DMI, tended to depress rumen fermentation rate and increased biogenic amine concentrations in the rumen. This acute effect on DMI is attributed to negative effects on palatability of this combination of amines (Van Os *et al.* 1995*a*; 1996*a*) together with feed-back signals from chemostatic intake regulation as a result of the high amine concentrations in the rumen.

The low capacity of rumen microbiota to degrade amines in unadapted sheep resulted in the accumulation of biogenic amines in the rumen during the first 3 h after feeding FAS + A. This accumulation possibly continued for some days during which the rumen microbiota were not fully adapted to be able to degrade the ingested amines. Tveit *et al.* (1992) found increasing concentrations of amines in the rumen of dairy cows 3 d after a change-over from hay to a silage containing amines. Therefore, the increase in biogenic amines in the rumen may possibly act

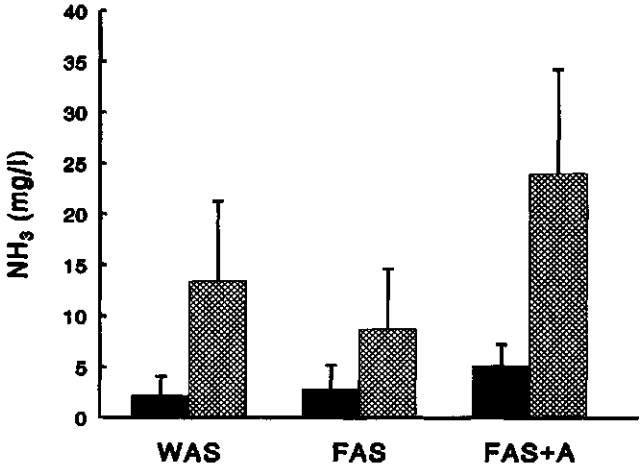

Figure 8.4 *The NH_3 production (+SE) in rumen fluid from added biogenic amines after 1 (■), and 6 h (▒) of* in vitro *incubation. The rumen fluid was withdrawn 3 h after feeding from sheep on days 11-14 of the 14-d experimental period, when adapted to FAS or FAS+A, and on days 21-24 of the pre-period, when adapted to WAS.*

on the chemostatic intake control through local effects in the rumen, or after being absorbed, by their impact on the intermediary metabolism.

In the present study, the inhibition of rumen fermentation in sheep not adapted to FAS+A, deduced from lower rumen NH_3 and slightly lower rumen VFA concentrations, was the result of the lower DMI rather than that of an acute negative effect of amines on microbial activity. This could be concluded from increased NH_3 and VFA productions and unaltered gas production found during *in vitro* fermentation upon adding biogenic amines to rumen content of unadapted, hay-fed sheep (Van Os *et al.* 1995*c*).

It is possible that signals of satiation are generated through chemoreceptors in the rumen, detecting the increased concentrations of amines. However, no chemoreceptors for biogenic amines have been reported to be present in the rumen

as for acetic acid, propionic acid (Martin and Baile, 1972) or osmolality (Carter and Grovum, 1990). Nevertheless, negative effects of tyramine on the development of rumen epithelium have been found (Kutas *et al.* 1986), while Dain *et al.* (1955) observed a decrease in rumen motility by histamine and tyramine, but this decreased motility effect was found in combination with rumen acidosis. Histamine is not absorbed by the rumen epithelium (Kay and Sjaastad, 1974), but whether the other biogenic amines are absorbed is unknown. The acute effects of biogenic amines on intake at the ruminal level are probably limited. It is more likely that the high concentrations of amines in the rumen resulted in an increased flow of amines to the lower stomachs and intestines. Possibly, amines lead to increased gastrin secretion the sheep abomasum, such as observed in the rat stomach (Lichtenberger *et al.* 1982). This gastrointestinal hormone was reported to reduce rumen motility and depress intake in sheep (Grovum, 1981). Furthermore, histamine infused into the abomasum of sheep depressed feed intake and increased their respiration rate (Neumark, 1967). It must however be noticed, that the quantity of histamine infused by Neumark was about double that expected to be found in the abomasum of our unadapted sheep fed on FAS + A.

If concentrations of amines in the intestines exceed the capacity of the amine oxidizing enzymes in the intestinal wall, they will be absorbed causing stress in intermediary metabolism (Joosten, 1988; Sattler *et al.* 1988). In goats, intravenously injected tyramine stimulates the release of catecholamines and corticoides (Forbes *et al.* 1994). The sheep in the present study did not exhibit symptoms of clinical intoxication such as increased respiration upon sudden intake of biogenic amines. A subclinical effect on chemostatic regulation of DMI through increased absorbtion of biogenic amines however can not be excluded. This is supported by the depressed DMI upon rumen infusion of the same combination amines in sheep adapted to silages with a low content of biogenic amines (Buchanan-Smith and Phillip, 1986) and by the lower DMI in cows upon the introduction to the rumen of a high dose of putrescine (Lingaas and Tveit, 1992).

Preconditioning to biogenic amines

Buchanan-Smith (1990), suggested that sheep might become accustomed to the taste of biogenic amines. In the present study, the sheep were preconditioned to amines by pre-feeding WAS. Preconditioning was deduced from a decrease in the acute effect of amines lowering daily DMI and the DMI during the principal meal, and from the similarity of the daily intake pattern of FAS + A on days 1-4 with that

of FAS on days 11-14, when pre-feeding WAS. That this effect of preconditioning was only due to getting used to the taste of amines is doubtful, because pre-feeding amines also slightly increased the amine degrading capacity of the rumen microbiota. A faster degradation of amines should lower the amount of amines in the rumen during days 1-4 that FAS + A is fed, and with that the flow of amines to the lower intestines and possibly to intermediary metabolism. Although expected, pre-feeding WAS did not lower the acute high concentrations of amines in the rumen on the first day of feeding FAS + A. This, however, can not be ruled out on days 2-4 of feeding FAS + A, because no measurements on rumen amine content were made.

Adaptation to biogenic amines

The present study confirmed that sheep adapt to biogenic amines in the diet. The acute effects of biogenic amines found on DMI, rumen fermentation and the rumen amine content disappeared within the 14 d-period of feeding FAS + A. The key role in adaptation is probably the increase of the amine degrading capacity of the rumen microbiota, preventing accumulation of amines in the rumen which may affect metabolic intake control.

Although daily DMI was similar for sheep adapted to FAS + A and FAS, the DMI during the principal meal of FAS + A and during the first 5 h after feeding remained lower in animals not preconditioned, and slightly lower in preconditioned animals. Since no influence of amines on metabolic intake control was expected in sheep adapted to FAS + A, this indicates a persistence of negative effects of the amines on palatability also found by Van Os *et al.* (1995*a*; 1996*a*). A similar intake pattern, with reduced intake just after feeding and compensation at the end of the day have also been reported by Deswysen *et al.* (1991) with grass silage supplemented with a methionine hydroxy analog. These authors also attribute this shift in intake towards the later part of the day to the unpalatability of the added agent.

In conclusion, we demonstrated that in sheep which are not adapted to dietary biogenic amines, feeding a silage containing amines depresses DMI directly through reduced palatability and most probably through their effects on chemostatic intake regulation. In sheep preconditioned to amines by pre-feeding a silage containing amines, this acute effect was less. When a diet containing biogenic amines is fed for a longer period, the sheep adapt quickly by increasing the rate of degradation of amines in the rumen and partly by becoming accustomed to their taste.

Therefore, the generally persistent lower DMI of untreated silages (type WAS) compared to hay or formic acid preserved silage (type FAS) can not be attributed to the effect of biogenic amines *per se* on intake control.

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Chapter 9

General discussion

General discussion

Introduction

The process of ensiling is based on preserving the forage by acidification through a naturally microbial fermentation of sugars into the preserving acids, among which lactic acid is most desirable. After filling and sealing the silo, the initial stage of fermentation is characterized by proteolysis of plant protein by plant enzymes (McKersie, 1981; Heron *et al.* 1989) and vigorous growth of microorganisms (Beck, 1965; Pahlow, 1991). In this initial stage of fermentation, populations of aerobic and facultative aerobic microorganisms develop, among which lactic acid bacteria, enterobacteria and yeasts. When anaerobiosis is attained, and when the initial pH value of the herbage (on average 6.5) decreases, the undesirable enterobacteria die off (Spoelstra, 1987; Rauramaa *et al.* 1987; Lindgren *et al.* 1988). The population of active lactic acid bacteria develops and acidify the herbage until pH is sufficiently low to prevent the development of clostridia (Wieringa, 1958; Whittenbury *et al.* 1967).

Formation of ammonia and biogenic amines during ensiling

In extensively, well-fermented grass silages a large part of the plant protein is degraded by plant enzymes to peptides and amino acids. A part of the amino acids is fermented by the microorganisms that are active during fermentation (Heron *et al.* 1986). They either deaminate amino acids to ammonia (NH_3) and volatile fatty acids (VFA) or decarboxylate them to amines and CO_2 (Ohshima *et al.* 1979). In extensively fermented silage, NH_3-N and amine N are substantial components (about 14 and 17%, respectively) of the non protein nitrogen (NPN) fraction, which can amount up to 70% of the total N content (Chapter 4; Ohshima *et al.* 1979).

Plant enzymes

Plant enzymic activity solubilises grass protein into peptides and free amino acids after cutting. Plant enzymes are in general not involved in amino acid decarboxylation. This with exception of the decarboxylation of glutamic acid to γ-aminobutyric acid (GABA) (MacPherson and Slater, 1959; Ohshima *et al.* 1979). This may explain the relatively high amount of GABA (2 g/kg DM) compared to the

other amines measured in the formic acid preserved silage (FAS; Chapter 7).

An important factor determining the amounts of NH_3 and amines formed is the availability of free amino acids (Joosten, 1987; Ohshima *et al.* 1979). The impact of a rapid pH fall during the first stage of ensiling (Chapter 3) in limiting the amount of free amino acids is only small. Although the optimum pH value for plant proteolytic enzymes in ryegrass is between 7.0 and 5.0, proteolysis by plant enzymes still continues at a pH value of 3.9, though at a lower rate (McKersie, 1981; Heron *et al.* 1989). Most effective in limiting the amount of free amino acids in ensiled herbage is reducing the proteolysis through wilting. A rapid increase of DM content of the herbage to 400 g/kg ceases activity of the plant proteolytic enzymes (Kemble and MacPherson, 1954) and results in lower amounts of amines and NH_3 (Chapter 3) and a higher true protein content of the silage (Muck, 1987; Kowalski *et al.* 1993). Proteolysis, however, takes place when wilting occurs under humid conditions (Kemble and MacPherson, 1954; Carpintero *et al.* 1979). Such a prolonged wilt increases NH_3 content of the herbage in the field up to 6 g/kg N_{total} per day (Carpintero *et al.* 1979; Spoelstra and Hindle, 1989). This NH_3 may be formed during proline synthesis from arginine deamination by plant enzymes (McDonald *et al.* 1991), or is the result of epiphytic bacteria deaminating the free amino acids (Spoelstra and Hindle, 1989).

No data are available in the literature about amines formed during wilting. Results from the experiment described in Chapter 3, however, shows no increase of the concentrations of amines in grass during wilting. The relative high amounts of putrescine, spermine and spermidine in grass before ensiling can be explained by these di- and polyamines being intermediates of the cell-protein metabolism, necessary for RNA and DNA synthesis and cell growth (Tabor and Tabor, 1984). High concentrations of these amines are also present in fresh fruit and vegetables (Bardócz *et al.* 1993)

Microbial enzymes

In silage prepared from γ-irradiated sterile herbage, recovery of total amino acid N (free amino acids plus amino acids from true protein) is higher than in the same herbage after natural fermentation (Gouet *et al.* 1970; Heron *et al.* 1986). The higher NH_3 and amine-N fractions in the naturally fermented silage proves the role of microorganisms in amino acid breakdown.

The rate of acidification during the initial stage of ensilage is important in determining the ultimate amounts of NH_3 and amines formed in the silage (Chapter

3). In this stage, enterobacteria and yeasts compete with the lactic acid bacteria for the available sugars, and convert them mainly to acetic acid and alcohols which slows down acidification in comparison to formation of lactic acid (Pahlow, 1991). Additionally to this competition for substrate, enterobacteria are also undesired because of their deaminating (Heron *et al.* 1987) and decarboxylating (Goldschmidt *et al.* 1971; Joosten and Northolt, 1987) activities. The NH_3 and amines formed, are basic substances that counteract acidification in the silage and delay the elimination of these bacteria. Though lactic acid bacteria are non-proteolytic, some species, particularly the heterofermentative ones, have weak deaminating (Brady, 1966) and decarboxylating (Table 9.1) properties. The reason for these bacteria to deaminate and decarboxylate is most probably to derive energy from the organic acid moiety at lower carbohydrate availability (Brady, 1966; Molenaar *et al.* 1993) or to counteract intracellular acidification (Morris and Fillingame, 1974). The lower rates of deamination and decarboxylation when carbohydrates are abundantly available is clearly demonstrated in the low DM molasses treated silage and to a lesser extent in the cell wall degrading enzyme treated silage (Chapter 3). In these silages lower amounts of amines and NH_3 could not be attributed to a more rapid acidification as compared to the control.

Homofermentative lactic acid bacteria are the most desired in silage fermentation because of their efficient production of lactic acid (Whittenbury *et al.* 1967). The following species are found to be active during fermentation: *Lactobacillus curvatus*, *L. acidophilus*, *L. coryniformis*, *L. plantarum*, *L. casei*, *Pediococcus acidilactici* and *P. pentosaceus* (Beck, 1972; Dellaglio and Torriani, 1986; Masuko *et al.* 1988). Although representatives of these species are able to deaminate amino acids (Brady, 1966), no decarboxylative properties have been detected in these species (Rodwell, 1953; Joosten and Northolt, 1987).

In this thesis, the decarboxylating capacity and the type of amines formed by the individual bacteria predominantly present in silage have not been determined. But comparing species of enterobacteria (Spoelstra, 1987) and lactic acid bacteria (Masuko *et al.* 1988) isolated from silage with literature on amine formation by representatives of the same species isolated from other sources (Table 9.1), indicate that both enterobacteria and lactic acid bacteria are responsible for amine formation in the silage (Chapter 3). The individual bacteria are capable of producing a limited number of amino acid specific decarboxylases (Table 9.1). Given the presence of various decarboxylase producing bacteria, the type and amount of amines formed in silage depend on the type and concentration of substrate and on

Table 9.1 *Types of amines formed by individual microorganisms generally present in extensively, well-fermented grass silage.*

Micro-organisms	Amine[a]	Reference
Lactic acid bacteria		
Homofermentative:		
Lactococcus lactis	Hi, Ty, Try	Chander *et al.* 1988
Facultative heterofermentative:		
Streptococcus faecium	Hi	Tham, 1988
Streptococcus faecalis	Ty, Phea	Joosten and Northolt, 1987
	Hi	Tham, 1988
	Ty, Try	Del Huerto-Garat *et al.* 1989
Heterofermentative:		
Lactobacillus brevis	GABA	Weiler and Radler, 1976
	Ty	Joosten and Northolt, 1987
Lactobacillus buchneri	Hi	Joosten and Northolt, 1987
	Ty	Choudhury *et al.* 1990
Leuconostoc mesenteroides	Hi	Edwards *et al.* 1987
Enterobacteria:		
Hafnia alvei	Ca, Pu	Joosten and Northolt, 1987
	Ca, Pu	Goldschmidt *et al.* 1971
Klebsiella pneumoniae	Ca	Goldschmidt *et al.* 1971
	Hi	Taylor *et al.* 1979
Enterobacter sakazakii	Pu	Goldschmidt *et al.* 1971
	Hi	Taylor *et al.* 1979
Escherichia coli	Pu, Ca	Joosten and Northolt, 1987
	Ca	Goldschmidt *et al.* 1971
	Hi	Taylor and Nancy, 1982
Citrobacter freundii	Pu	Goldschmidt *et al.* 1971
Proteus morganii	Pu	Goldschmidt *et al.* 1971

[a] Ca, cadaverine; GABA, γ-aminobutyric acid; Hi, histamine; Phea, phenyl-ethylamine; Pu, putrescine; Try, tryptamine; Ty, tyramine.

environmental pH (Morris and Fillingame, 1974). The high amounts of GABA, tyramine, putrescine, cadaverine and histamine in silage are in line with the high proportion of their precursor amino acids, being glutamic acid, tyrosine, arginine, lysine and histidine in the grass protein (Ohshima *et al.* 1979; Heron *et al.* 1986). Enterobacteria are active at pH values near to neutral. They produce mainly decarboxylases with optimum activity at pH values between 7 and 5.5, yielding putrescine and cadaverine (Dainty *et al.* 1986). The more acid tolerant lactic acid bacteria produce mainly decarboxylases having optimum activity at lower pH values yielding histamine and tyramine (Edwards *et al.* 1987).

During fermentation also clostridia might develop when pH does not fall rapidly enough or sufficiently low for stable preservation (Whittenbury *et al.* 1967). Though not mentioned in Table 9.1, the proteolytic species deaminate and are able to produce a wide variety of specific decarboxylases (Ohshima and McDonald, 1978). When active, they contribute significantly to the amount of NH_3 and amines formed in the silage (Hughes, 1971; Tveit *et al.* 1992)). However, in the silages prepared in Chapter 3 acidification has been sufficiently rapid to prevent amine formation by clostridial activity.

The rapid pH fall during the initial stage of fermentation limits the amount of amines and NH_3 formed, mainly by preventing the development of excessive numbers of the amine and NH_3 producing microorganisms, and by disfavouring environmental conditions for decarboxylative (Joosten, 1987) and probably deaminative enzymic activity. The pH fall at natural fermentation and the final quality of the silage is determined by factors related to the silage making technique (Kibe *et al.* 1977; Wilson and Flynn, 1979; Seale *et al.* 1982), and by the microbial (Müller *et al.* 1991) and chemical composition (Wilkinson *et al.* 1981; Pettersson and Lindgren, 1990) of the forage ensiled.

Other N-containing fermentation products formed in silage are volatile amines and sometimes nitrosamines. Their concentrations in silage are expected to be low. In good, but also in poor-quality silages the amount of the volatile ethylamine and trimethylamine in the total volatile N-fraction is not higher than 0.7 and 1.5% respectively, whereas there has been no evidence for other volatile bases present (Jackson, 1964). Nitrosamines are formed in the silage by the reaction between amines and nitrite, a degradation product of nitrate (Spoelstra, 1985). In grass silage, only traces (0-20 μg/kg DM) of N-nitrosodimethylamine could be detected (Van Broekhoven and Davies, 1980). Probably, there are also other compounds

present having nitroso structures, but concentrations are expected to be low (Van Broekhoven and Davies, 1980). The formation of nitrosamines is not related to the rate of nitrate reduction in the silage (Van Broekhoven and Davies, 1985).

Ammonia and amines in silage intake regulation.

The biogenic amines histamine, tyramine, cadaverine and putrescine which are predominantly present in grass silage (Chapters 3, 4, 5 and 8), are in human nutrition often causing agents of food poisoning outbreaks (Edwards and Sandine, 1980). At low amounts the ingested amines are degraded by amine oxidases located in the intestinal epithelium. At higher doses of especially tyramine (2 mg/kg body weight; BW) and histamine (8 mg/kg BW) the degrading capacity of the amine oxidases will be exceeded, resulting in absorption of the amines (Marley and Blackwell, 1970). The absorbed amines cause various physiological effects like headache, fever, swelling, hypertension etc. as reviewed by Joosten (1988). Though threshold levels for adverse physiological effects of putrescine and cadaverine are much higher than those for tyramine and histamine, are these amines also of importance in food poisoning. Combined with tyramine and histamine, they facilitate the absorption of these and other hazardous amines like tryptamine and phenyl-ethylamine (Marley and Blackwell, 1970).

Direct effect

The amount of amines ingested daily (g/kg body weight) by ruminants consuming naturally fermented silage (Chapters 3, 4 and 8) is higher than the toxic amounts for humans. For histamine this is about 20-30 mg/kg BW and for tyramine 50-80 mg/kg BW. Therefore the suggested depressive effect of amines on silage DMI is likely. However, the results of this thesis show that the amount of amines present in extensively fermented silage (Chapters 3, 4, 5 and 8) are not responsible for the lower daily DMI in sheep and dairy cows offered this type of silage for a period longer than 14 d. Additionally, no negative effect of NH_3 on daily DMI could be detected (Chapters 4 and 5). Similar amounts and types of amines and ammonia as present in poor-quality silage added to a formic acid preserved silage did not depress its DMI (Chapters 4, 5, 7 and 8).

Unlike ammonia addition, amine addition results in a modification of intake behaviour in sheep (Chapters 4, 7 and 8). In sheep adapted to the amine

supplemented silage, intake rates of the principal meal after feeding and the average intake rate of the subsequent small meals remain slightly lower than that of the unsupplemented control silage (Chapters 4 and 7). Additionally, amines in the silage modify its daily intake pattern by lowering the amount consumed directly after feeding and increasing DMI towards the end of the day (24 h period; Chapter 8). The lower intake rates may originate from both low palatability of the amines and satiation induced by chemostatic intake control at rumen or metabolic level (Chapters 2). In animals adapted to the amine rich diet, no impact of chemostatic control on lowering intake rate is expected, because of 1, the presence of only traces of amines in the rumen and 2, rumen fermentation and motility being similar to that with the unsupplemented control silage (Chapters 4, 7 and 8). So, controversially to the findings of Buchanan-Smith (1990), testing a combination of putrescine, cadaverine and GABA, the combination of tyramine, putrescine, cadaverine and histamine negatively affects silage palatability.

The amines tested are degraded in the rumen mainly by microbial deamination (Chapter 6). In addition to NH_3, other degradation products are expected to be VFA. However, in rumen content of sheep adapted to an amine rich diet, formation of VFA from amines could not be observed. Probably are other, not measured, organic acids formed, which are analogous to those of fermentation of the precursor amino acids (Prins, 1977).

The amine degrading capacity of the rumen microbiota, thus results in only traces (<30 mg/kg DM, equivalent to <3.6 mg/l rumen fluid) of amines being present in the rumen of animals offered a grass silage rich in amines. In this situation, maximum flow of the individual amines to the lower intestines (rumen fluid flow, 0.75 l/h; Chapter 7) is 2.7 mg/h, equivalent to 0.05 mg/kg BW per h in a 60 kg sheep. This low rate of amines passing the rumen in ruminants fed the amine supplemented silage is similar to that when consuming a naturally fermented silage (Chapters 4, 5 and 8).

During normal fermentation, volatile amines and nitrosamines are present in the rumen in traces only. The content of volatile methylamine and ethylamine is about 50 mg/kg DM (Broudiscou and Papon, 1994) and dimethyl-nitrosamine, the only nitrosamine found in rumen contents, is about 0.01 μg/kg DM (Van Broekhoven and Davies, 1981). The threshold level for toxicity of this nitrosamine for sheep is 0.15 mg/kg body weight per day (Koppang, 1974), which is higher than ingested during silage intake (Van Broekhoven and Davies, 1980).

The increased rate of amine breakdown by the rumen microbiota on a diet rich in

amines (Chapter 6), implicates that during a change-over from a low to amine rich diet a certain period is needed for the microorganisms to increase their amine degrading capacity. During this period of adaptation amines accumulate in the rumen and depress intake directly by their effect on chemostatic intake control at rumen or metabolic level (Chapter 8). The impact of amines on mechanisms involved in chemostatic intake control at rumen level are unknown (Forbes, 1995), but direct effects in intermediary metabolism affecting DMI after being absorbed are conceivable (Chapter 2). Three hours after the change-over to the amine supplemented silage, the rumen passage rate of especially tyramine and histamine is increased, whereas a further increase might be expected. Additionally to metabolic effects of the amines, taste effects are probably involved in DMI depression after a change-over from a low amine to an amine rich diet. In the experiment described in Chapter 8, taste and metabolic effects could not be separated. However, the effect of taste is conceivable because after adaptation, indicated by low amine levels in the rumen, the DMI during the principal meal remains depressed. Although preconditioning to amines lowers the acute effect of amines after the diet change-over on daily DMI and during the principal meal (Chapter 8), intake behaviour remained different. Even when the animals are adapted to amines in the diet. So, in spite of the positive effects of preconditioning on DMI by lowering negative taste effects and increasing rumen degradation rate of amines, a direct effect of amines in lowering silage palatability is consistent.

Indirect effect

The lower intake of extensively fermented silage can not be explained by direct effects of NH_3 and amines present in the silage. Studies on the influence of fermentation on silage DMI usually focus on the effects of individual fermentation products in the silage to explain intake depression. At levels present in the silage, fermentation products appear to have no or only minor direct effects on intake. More pronounced effects on intake goes mainly through the amounts formed upon silage digestion (Table 9.3). Additionally it is possible that compounds normally not detected being present in small amounts, and cumulative effects of the wide variety of fermentation products in silage, are responsible for intake depression as postulated by Dulphy and Van Os (1996).

Referring to metabolic disregulation, caused by insufficient nutrient supply to organs and tissues, a new hypothesis is developed by Chamberlain and Choung (1993), which tries to explain indirect effects of NH_3 and amines on silage intake.

Table 9.3 *Fermentation characteristics of medium- to poor-quality silages, and their possible effects on intake.*

Fermentation product	Level in silage[a]	Effect on intake	Level of regulation	Reference
		Direct effect:		
Amines	4-15	Weakly by taste	OP	This thesis
Acetic acid	40-75	Weakly by taste	OP	Buchanan-Smith, 1990
pH	3.8-5	By taste at pH < 4.0	OP	Grovum and Chapman, 1988 Erdman, 1988
		Indirect effect upon digestion:		
Ammonia	1-5	Intoxication at energy shortage,	IM	Choung *et al.* 1990
Amines	4-15	shortage energy supply to IM,	IM	Reynolds, 1992
Soluble nitrogen	10-40	increase osmolality in IM	IM	Carter and Grovum, 1990
Lactic acid	50-140	Rumen osmolality and via effect of propionic acid	RU, IM	Morgan and L'Estrange, 1977
Acetic acid	40-75	Rumen acetate, osmolality, pH and acetate in IM	RU, IM	Anil *et al.* 1993 Gill *et al.* 1988
Propionic acid	1-8	Rumen and liver propionate, rumen osmolality and pH, body glucose supply	RU, IM	Anil, *et al.* 1993 Van Houtert, 1993
Butyric acid	5-40	Rumen butyrate, osmolality, pH, body glucose	RU, IM	Van Houtert, 1993
Osmolality (mosmol/kg)	500-1100	Rumen osmolality	RU	Phillip *et al.* 1981
pH	3.8-5	Rumen acidity	RU	Martin and Baile, 1972

[a] g/kg dry matter, unless otherwise mentioned. OP, oro-pharyngeal; RU, rumen; IM, intermediary metabolism

They state that not fermentation products *per se*, but the ratio of NPN to easily fermentable energy in the silage is an important factor in determining silage DMI. During extensive silage fermentation the formation of NPN (amino acids, amines and NH_3) is high. Sugars are fermented to organic acids (Carpintero *et al.* 1979; Cushanahan and Gordon, 1995) being of low or no energetic value for microorganisms in the rumen (Chamberlain, 1987). The excessive NPN supply and the low energy supply gives a high average rumen NH_3 (Chapters 4, 5 and 8), and reduces microbial protein flow to the intestines (Chamberlain *et al.* 1993; Sinclair *et al.* 1995). The high rumen NH_3 leads to a high rate of absorption which increases the rate of its detoxification by the liver (Morris and Payne, 1970). An increased NH_3 detoxification requires increased supply of energy and amino acids to the liver (Reynolds, 1992). This increased nutrient requirement at the liver lowers the amount of energy and amino acids available for liver gluconeogenesis, reducing body glucose supply (Reynolds, 1992). With extensively fermented silages (medium to poor quality), metabolic processes may thus be disturbed by shortage of energy supply or NH_3 toxicosis. Both may lead to depression of DMI (Chapter 2). The lower NPN to energy ratio in silage with restricted fermentation (Carpintero *et al.* 1979; Chapter 8) having higher DMI, lowers urinary N secretion (Grenet, 1983). This indicates lower NH_3 burden for intermediary metabolism which saves nutrients for metabolic homeostasis. So, NH_3 and amines in the silage might depress DMI indirectly through shortage of energy supply.

Conclusions

Biogenic amines are formed in good-quality grass silages. They are mainly formed during the initial stage of fermentation, and the amount formed is negatively related to the rate of acidification. Tyramine, cadaverine, putrescine and histamine are, in descending order, the principal amines formed.

At concentrations normally present in poorly preserved grass silage, neither amines nor ammonia, nor the combination of amines and ammonia lower daily DMI in sheep and cows which are adapted to the diets containing these products added. For amines a slightly negative effect on palatability is observed, depressing intake rate and altering the intake pattern by decreasing the amount eaten directly after feeding.

Ingested amines are deaminated in the rumen. Histamine and tyramine are most

rapidly degraded. The deamination rate is higher when the animals are adapted to a diet rich in amines.

In sheep that are not adapted to amines in the diet, ingestion of amines depress daily DMI. Preconditioning, however, decreases this acute effect.

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Summary

Summary

In Northern- and Western-Europe, grass silage is a major component in winter feeding rations for ruminants. The intake of ensiled grass is often lower than the intake of hay or the fresh grass of similar digestibility. This intake depression is attributed to the fermentation products present in the silage. These include organic acids (lactic acid, volatile fatty acids) and N-containing fermentation products (ammonia (NH_3) and amines). The impact of N-containing fermentation products, notably amines, in lowering silage intake is suggested, because of their physiological activity in intermediary metabolism.

Chapter 2 reviews the impact of fermentation in silage on silage dry matter intake and the controlling mechanisms involved. More extensively fermented silage shows lower intake when compared with hay or fresh forage from the same sward, especially when high amounts of volatile fatty acids and NH_3 are present. According to literature, this lower intake is mainly due to impaired palatability and earlier satiation induced by chemostatic control mechanisms at rumen level or in the intermediary metabolism. With silage rations, rumen fill is not a factor controlling intake.

Supra-physiological doses of individual fermentation products (acids and NH_3) added to the diet, or intra-ruminally infused depress intake. No intake depression is observed at physiological doses. Only few studies have been carried out on the impact of amines on the control of silage intake.

In Chapter 3 the type and amounts of amines formed in grass silage in relation to the rate of fermentation were investigated. Silages were made from direct cut and wilted perennial ryegrass in laboratory silos. Differences in the rate of fermentation were induced by addition of molasses, cell wall degrading enzymes and inoculation with lactic acid bacteria. Fermentation was restricted by formic acid, while the influence of enterobacteria on the formation of amines was determined by inoculation with a strain identified as belonging to the species *Enterobacter sakazakii*. This strain was isolated from silage. The total amine content of fresh grass was low (0.1 - 0.2 g/kg DM). The silages, of good quality, contained considerable amounts of amines, ranging from 0.1 g/kg DM in the wilted and formic acid treated silages to 7.4 g/kg DM in a low DM, untreated silage. Irrespective of the total amount of amines in the silage, tyramine, cadaverine, putrescine and histamine were, in descending order, the principal biogenic amines present. The amines were mainly formed during the first 10 d of fermentation, and formation was highest in silages with a slow acidification rate. In this study, no

additional amines were formed upon inoculation with the enterobacterium. The amount of amines in the silages correlated with amounts of NH_3, indicating amine formation being related to overall protein degradation.

The amines predominantly present in silage (Chapter 3), and NH_3 were subsequently tested for their influence on silage intake in sheep (Chapter 4) and dairy cows (Chapter 5). A mixture of amines, similar in composition to the amines found in silage at a total rate of 2.8 g/kg DM and NH_3 (2.9 g/kg DM) were added to a formic acid treated silage of good quality. These treatments were compared to the unsupplemented silage and to the same grass ensiled untreated. In the sheep and dairy cows, intake of the untreated silage was lower than the formic acid treated silage. The lower daily intake mainly resulted from the lower intake during the principal meal after feeding, due to palatability effects and earlier satiation. Addition of NH_3 altered neither daily intake nor intake behaviour in sheep and cows. The amines added, tended to depress daily intake in the sheep, but not in cows. The slightly lower intake resulted from lower intake rates during both the principal meal and the subsequent small meals. The depressed intake rate was attributed to reduced palatability as indicated by the depressed initial intake rate during the principal meal. In sheep, no treatment effects were observed on total rumen pool size, DM and neutral-detergent fibre content, and rumen motility. These variables were not measured in cows. Furthermore, NH_3 and amines did not alter rumen pH, NH_3 and volatile fatty acid concentrations in the sheep and cows. Amine addition, however, increased slightly the amount of rumen fluid in sheep, which was concomitant with the lower osmolality in the rumen. This suggested a diluting effect of amines, which was also observed to a slight extent in cows. For all treatments, in sheep as well as in cows, only traces of amines were present in the rumen.

From these studies, was concluded that at the added concentrations, NH_3 and amines are not a direct cause of lower silage intake. The combination of amines, however, tended to have a negative effect on palatability.

In Chapter 6, the degradation of histamine, tyramine, cadaverine and putrescine in the rumen contents was studied *in vitro*. A solution containing a mixture of the amines previously mentioned, was added to rumen content taken from sheep. These sheep were adapted to either a silage diet containing high amounts of amines, low amounts of amines, or to hay containing no amines. Subsequently, the rumen contents to which the amines had been added were incubated for 5 h. Amine addition increased NH_3 concentration in the rumen contents. The highest

rate of NH_3 production was found in rumen contents of sheep adapted to high amounts of amines in the diet. Addition of amines did not affect gas production. After 5 h a part of the added amines was degraded in all types of rumen contents. Highest amine degradation took place in rumen content of sheep adapted to the amine rich diet. The amount of amines degraded agreed stoichiometrically with the amount of NH_3 produced, indicating that amines in the rumen are degraded by deamination. In general, the breakdown was highest for histamine, followed by tyramine, putrescine and cadaverine. These results indicate that in animals adapted to grass silage with high concentrations of biogenic amines, the accumulation of amines in the rumen is prevented by an increase of the amine-degrading capacity of the rumen microbiota.

In Chapter 7, a comparison was made between effects of addition of amines (4.9 g/kg DM) and addition of a combination of amines (2.7 g/kg DM), NH_3 (3.0 g/kg DM) and γ-aminobutyric acid (GABA; 5.0 g/kg DM) on intake in sheep. These products were again added to a formic acid treated silage of good quality. The combination of amines alone did not alter daily intake when compared to the unsupplemented control silage. The combination of amines, NH_3 and GABA slightly increased intake. The amines, however lowered slightly the intake rate of the principal meal and the average intake rate of the small meals. Nevertheless, rumen NH_3 content was the highest after the principal meal for the silage supplemented with the combination of amines, NH_3 and GABA. No effects of amines or the mixture of amines, NH_3 and GABA were observed on rumen pH, osmolality, rumen pool size and liquid content or flow rate. It is concluded that amines alone or amines combined with NH_3 and GABA in concentrations normally found in medium- to poor-quality silages do not affect intake by direct effects on chemostatic regulation. A slight negative effect of amines on silage palatability, however, cannot be excluded.

Bearing in mind the adaptation of the rumen microorganisms to degrade dietary amines reported in Chapter 6, it is conceivable that during the first days of feeding an amine rich silage the amines accumulate in the rumen. They may than pass the rumen and cause a direct effect on intake through their activity in intermediary metabolism. In Chapter 8, this direct effect of amines was studied during a diet change-over experiment with a silage having a low amine content (1.4 g/kg DM) to the same silage supplemented with 7.2 g amines/kg DM. In addition, the effect of preconditioning on the acute effect during the diet change-over was tested by previously feeding a silage containing 5.2 g naturally formed amines/kg DM. The

first 4 d after the change-over, amines tended to lower daily intake and depressed significantly the intake during the principal meal. Preconditioning lowered this acute effect of amines. After 14 d, the sheep were adapted to the dietary amines. The rate of amine degradation in the rumen was increased, resulting in only traces of amines being present in the rumen. The daily pattern of intake, however, remained different with lower intake during the first 5 h after feeding. This confirmed the negative effect of amines on palatability.

In Chapter 9 the microbiological background of the formation of biogenic amines in silage is discussed. Based on literature, it is likely that enterobacteria and heterofermentative lactic acid bacteria are responsible for the formation of amines in medium- to good-quality silages.

This thesis allows to conclude that after adaptation, neither biogenic amines nor ammonia directly reduce intake by effects on chemostatic regulating mechanisms. However, a slight negative effect of amines on palatability is probable. So, amines only slightly influence intake pattern during the day, but without lowering total daily intake. The amines in silage, thus, appear to be one of the factors that has a slight, or possibly in extreme situations, a more pronounced negative effect on taste. Other factors with effect on intake include negative palatability by pH and acetic acid, or disbalance in nutrient supply at rumen or liver level. Although a possible negative effect on intake of each these factors is difficult to demonstrate and to quantify, together by cumulative effects they are likely to reduce silage intake.

Samenvatting

Samenvatting

In Noord- en West-Europa is grassilage een belangrijk bestanddeel van het winterrantsoen voor herkauwers. De opname van grassilage door de dieren is echter variabel en meestal lager dan dat van hooi of het verse gras met dezelfde verteerbaarheid. De verlaagde opname wordt toegeschreven aan de aanwezigheid van fermentatieproducten in de silage, waaronder de organische zuren (melkzuur en vluchtige vetzuren) en de stikstof-(N)-houdende fermentatieproducten (ammoniak (NH_3) en aminen). De N-houdende fermentatieproducten, met name biogene aminen, zouden de opname van silage negatief kunnen beïnvloeden, daar het fysiologisch actieve stoffen zijn.

Hoofdstuk 2 geeft een literatuuroverzicht van de invloed van de mate van fermentatie in silage op de opname ervan en de mechanismen die de opname reguleren. Een hoge mate van fermentatie verlaagt de silage-opname. Dit geldt met name wanneer de fermentatie resulteert in hoge gehalten aan vluchtige vetzuren en NH_3. De lagere opname wordt vooral veroorzaakt door een slechte smaak en een snellere verzadiging welke geïnduceerd wordt door de chemostatische regulatiemechanismen vanuit de pens en het intermediaire metabolisme. De hoeveelheid pensvulling is geen oorzaak van de lagere opname van grassilage.

Wanneer de individuele fermentatieproducten (zuren en NH_3) in zeer hoge doses aan het voer toegevoegd worden of worden geïnfuseerd in de pens, leidt dit tot een verlaagde voeropname. Gebeurt dit in hoeveelheden die overeenkomen met die tijdens het eten van grassilage, dan leidt dit niet tot een opnameverlaging. De invloed van aminen op de opname van silage is echter nooit uitvoerig onderzocht.

Hoofdstuk 3 beschrijft welke aminen en in welke concentraties ze gevormd worden in grassilage. Tevens is onderzocht wat het effect is van de mate van fermentatie op de aminenvorming. Hiervoor zijn, op laboratoriumschaal, silages gemaakt van direct ingekuild en voorgedroogd Engels raaigras. Naast een natuurlijke fermentatie, werd de mate van fermentatie beïnvloed door toevoeging van melasse, celwand-afbrekende enzymen en inoculatie met melkzuurbacteriën. De fermentatie werd geremd door toevoeging van mierezuur, terwijl de invloed van enterobacteriën op de vorming van aminen werd bepaald door inoculatie met een bacteriestam geïdentificeerd als *Enterobacter sakazakii*. Deze stam werd geïsoleerd uit grassilage. Het totale aminengehalte van het verse gras was laag (0,1 - 0,2 g/kg droge stof; ds). De silages, van goede kwaliteit, bevatten allen aminen, variërend van 0,1 g/kg ds in de voordroogsilages en de met mierezuur behandelde silages tot 7,4 g/kg ds in de natuurlijk gefermenteerde silage met een laag ds-gehalte.

Onafhankelijk van de gevormde hoeveelheid, waren tyramine, cadaverine, putrescine en histamine, in volgorde van afnemende concentratie, de meest voorkomende aminen in de silages. Aminen werden vooral gevormd tijdens de eerste 10 d van de fermentatie en in de silages met de langzaamste verzuring. In dit experiment produceerde de geïnoculeerde enterobacterie geen extra aminen. Het aminengehalte in de silage was gecorreleerd aan het NH_3-gehalte, wat aangeeft dat aminenvorming gelijke tred houdt aan de algemene eiwitafbraak.

De meest voorkomende aminen in de silages (Hoofdstuk 3), zijn vervolgens getest op hun effect op de opname van grassilage door schapen (Hoofdstuk 4) en melkkoeien (Hoofdstuk 5). Een combinatie van aminen met een totale hoeveelheid van 2,8 g/kg ds en NH_3 (2,9 g/kg ds) zijn toegevoegd aan een met mierezuur behandelde grassilage van goede kwaliteit. Deze twee voeders zijn vergeleken met de mierezuursilage zonder toevoeging en de natuurlijk gefermenteerde silage van hetzelfde gras. Bij zowel schapen als koeien was de dagelijkse opname van de natuurlijk gefermenteerde silage lager dan die van de mierezuursilage. Deze lagere opname werd veroorzaakt door een lagere opname tijdens de eerste maaltijd na voeren als gevolg van een slechtere smaak en een snellere verzadiging. De toevoeging van NH_3 had geen negatief effect op de dagelijkse voeropname en het voeropnamegedrag van schapen en koeien. De aminentoevoeging verlaagde een weinig de dagelijkse voeropname door de schapen. Dit was niet het geval bij koeien. De opnamedepressie bij schapen werd veroorzaakt door een lagere opname tijdens de eerste maaltijd na voeren als gevolg van een lagere opnamesnelheid. De opnamesnelheid bleef ook lager tijdens de daaropvolgende maaltijden. De lagere opnamesnelheid is mogelijk het gevolg van een slechte smaak, welke te herleiden is uit de lagere opnamesnelheid tijdens het begin van de eerste maaltijd. Bij de schapen hadden de aminen- en NH_3-toevoeging geen effect op de totale pensvulling, de hoeveelheid ds in de pens, de hoeveelheid *neutral-detergent fibre* (NDF) in de pens en de pensmotoriek. Deze variabelen zijn echter niet gemeten bij de koeien. Aminen en NH_3 hadden ook geen effect op pH, NH_3 en vluchtige vetzuurconcentraties in de pens bij zowel schapen als koeien. Als gevolg van de aminentoevoeging leek de hoeveelheid pensvloeistof bij beide diersoorten hoger, wat samen ging met een verlaging van de osmolaliteit in de pens. Bij alle rantsoenen werden in de pens slechts sporen van aminen gevonden. De conclusie van beide experimenten is dat de toegevoegde hoeveelheden NH_3 en aminen geen directe oorzaak zijn van een lagere silage-opname. De resultaten wijzen erop dat de gebruikte combinatie van aminen een negatief effect op de smaak heeft.

Hoofdstuk 6 beschrijft een *in vitro* studie naar de afbraak van tyramine, histamine, putrescine en cadaverine in de pens. Een oplossing met een mengsel van de eerder genoemde aminen werd hiervoor toegevoegd aan pensinhoud van schapen. Deze schapen waren achtereenvolgens gewend aan een silage met een hoog aminengehalte, een laag aminengehalte en aan een rantsoen van hooi dat geen aminen bevatte. Vervolgens werd de pensinhoud, waaraan de aminen toegevoegd waren, gedurende 5 uur geïncubeerd. De toevoeging van aminen verhoogde de NH_3-productie in de pensinhoud. De NH_3-productie was het hoogst in de pensinhoud van schapen die gewend waren aan een aminenrijke silage. Aminentoevoeging had geen effect op de gasproductie. Na 5 uur was in de drie typen pensinhoud een gedeelte van de toegevoegde aminen afgebroken. De grootste afbraak vond plaats in de pensinhoud van dieren gewend aan een aminenrijke silage. De hoeveelheid afgebroken aminen kwam stoichiometrisch overeen met de hoeveelheid gevormde NH_3. Dit wijst op afbraak van aminen in de pens door deaminering. De afbraak van histamine was het hoogst, gevolgd door die van tyramine, putrescine and cadaverine. De resultaten van deze proef geven aan dat bij herkauwers, die gewend zijn aan een aminenrijk voer, een verhoging van aminenafbraakcapaciteit door de pensmicroben voorkomt dat ze in de pens accumuleren.

In Hoofdstuk 7 is het effect van een toevoeging van 4,9 g aminen/kg ds en een toevoeging van een combinatie van aminen (2,7 g/kg ds), NH_3 (3,0 g/kg ds) en γ-aminoboterzuur (GABA; 5,0 g/kg ds) op de voeropname door schapen onderzocht. De stoffen werden toegevoegd aan een mierezuursilage van goede kwaliteit. De toevoeging van aminen alleen had, vergeleken met de mierezuursilage, geen effect op de dagelijkse opname. De combinatie van aminen, NH_3 en GABA verhoogde een weinig de opname van silage. De aminen verlaagden echter enigszins de voeropnamesnelheid tijdens de eerste maaltijd na voeren en tijdens de daaropvolgende maaltijden. In de pens was de NH_3-concentratie het hoogst aan het eind van de eerste maaltijd van de silage met de toevoeging van de combinatie van aminen, NH_3 en GABA. Zowel de toevoeging van aminen alleen, als de combinatie van aminen, NH_3 en GABA veranderde niet de pH en osmolaliteit in de pens, de pensinhoud, het vloeistofvolume en de vloeistofpassage door de pens. De conclusie is dat noch aminen alleen, noch een combinatie van aminen, NH_3 en GABA, in de concentraties zoals aanwezig in silages van middelmatige tot slechte kwaliteit, de opname van silage verlagen als gevolg van een effect op chemostatische opnameregulatie. Een klein negatief effect van aminen op de smaak kan echter niet

worden uitgesloten.

Gelet op de mogelijkheid van het verhogen van de aminenafbraakcapaciteit door de pensmicroben, zoals beschreven in Hoofdstuk 6, is het aannemelijk dat gedurende de eerste dagen dat een aminenrijke silage gevoerd wordt, de aminen in de pens accumuleren. Mogelijk stromen ze dan door naar de darmen, waar ze na resorptie, door hun fysiologische activiteit, een direct effect hebben op de opname.

In Hoofdstuk 8 is dit directe effect bestudeerd bij schapen tijdens een rantsoenwisseling van een silage met weinig aminen (1,4 g kg ds) naar dezelfde silage met een aminentoevoeging van 7,2 g/kg ds. Tevens is gekeken of een gewenning aan aminen vooraf, middels een silage met 5,2 g natuurlijk gevormde aminen/kg ds, het acute effect tijdens de rantsoenwisseling vermindert. Na de rantsoenwisseling was gedurende de eerste 4 d de dagelijkse voeropname iets lager en die tijdens de eerste maaltijd veel lager. Een gewenning aan aminen verminderde deze acute daling van de opname. Na 14 d waren de dieren aangepast aan het hoge aminengehalte in het voer. De dagelijkse opname was gelijk aan die van de mierezuursilage en de afbraakcapaciteit van de pensmicroben was verhoogd. Dit resulteerde in de aanwezigheid van slechts sporen van aminen in de pens. Het dagelijkse opnamepatroon van silage bleef echter verschillend. De opname aan het begin van de dag (eerste 5 uur na voeren) bleef lager. Dit benadrukt het negatieve effect van de aminen op de smaak.

Hoofdstuk 9 bediscussieert de microbiële achtergrond van de aminenvorming in silage. Gezien de informatie uit de literatuur, is het hoogstwaarschijnlijk dat enterobacteriën en heterofermentatieve melkzuurbacteriën verantwoordelijk zijn voor de vorming van aminen in silages van goede tot middelmatige kwaliteit.

De conclusie van dit proefschrift is dat na aanpassing, noch NH_3, noch biogene aminen de opname van silage verlagen als gevolg van een direct effect op chemostatische opnameregulatie. Een verminderde smaak als gevolg van aanwezigheid van aminen is echter mogelijk, waardoor het opnamepatroon beïnvloed wordt, zonder dat het een effect heeft op de dagelijkse opname van silage. Aminen in silage is dus één van de factoren die een klein effect heeft op de opname. Andere factoren van invloed op de opname zijn een verminderde smaak door een lage pH en door azijnzuur, alsmede een niet evenwichtige nutriëntenvoorziening in de pens of op niveau van de lever. Hoewel de negatieve invloed op de opname per genoemde factor moeilijk aan te tonen en te kwantificeren is, is het aannemelijk dat ze gezamenlijk door mogelijk cumulatieve effecten de silage-opname verlagen.

Résumé

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En Europe du Nord et de l'Ouest l'ensilage d'herbe constitue une part principale des rations de base hivernales des ruminants. Or l'ingestion d'ensilage d'herbe est souvent inférieure à l'ingestion de foin ou de fourrage vert de digestibilité identique. Cette diminution d'ingestion est attribuée aux produits de fermentation présents dans l'ensilage. Ces produits comprennent des acides organiques (acide lactique, acides gras volatils) et des composés azotés (ammoniac; NH_3 et amines). Le rôle des composés azotés, en particulier celui des amines, dans le diminution de l'ingestion d'un fourrage après son ensilage, a été avancé il y a longtemps à cause de leur activité physiologique au niveau du métabolisme intermédiaire.

Le Chapitre 2 fait la synthèse des conséquences des fermentations entraînées par l'ensilage sur le niveau d'ingestion des fourrages et sur mécanismes impliqués. Les ensilages riches en produits fermentaires sont relativement mal ingérés par comparaison au fourrage vert et au foin (même prairie et même date de coupe), en particulier lorsqu'ils contiennent des quantités élevées d'acides gras volatils et d'ammoniac. Selon la littérature, cette ingestion plus faible serait due principalement à une mauvaise appétibilité et à un rassasiement rapide de l'animal consommateur induit par des mécanismes de contrôle ayant leur point de départ au niveau du rumen ou du métabolisme intermédiaire. Parallèlement, pour les rations à base d'ensilage, l'encombrement du rumen ne semble pas un facteur contrôlant le niveau d'ingestion.

Des doses supra-physiologiques de composés fermentaires (acides et NH_3) administrés individuellement, via la ration, ou directement par voie intra-ruminale, diminuent l'ingestion. Cependant aucune diminution notable n'est observée avec des doses physiologiques. En outre, très peu d'études ont été effectuées sur le rôle des amines au niveau du contrôle de l'ingestion d'ensilage.

Le Chapitre 3 rapporte les résultats concernant la nature et les quantités d'amines formées dans des ensilages d'herbe et de quantités d'amines formées dans des ensilages d'herbe en relation avec l'intensité des fermentations. Les ensilages étudiés ont été préparés à partir d'un fourrage de "ray-grass" anglais ensilé, soit directement après la coupe, soit après préfanage, dans des silos de laboratoire. L'addition d'enzymes cellulolytiques, de mélasse et de bactéries lactiques a permis d'induire des différences dans la vitesse de fermentation, et celle d'acide formique de limiter ces fermentations. Quant à l'influence des entérobactéries sur la formation des amines, elle a été étudiée en inoculant une souche appartenant à l'espèce *Enterobacter sakasakii*, souche isolée à partir

d'autres ensilages. La quantité totale d'amines trouvée dans le fourrage frais est très faible (0,1 à 2,0 g/kg matière sèche; MS). Les ensilages de bonne qualité contiennent des quantités notables d'amines, de 0,2 g/kg MS pour les ensilages préfanés ou traités à l'acide formique à 7,4 g/kg MS dans un ensilage sans conservateur et pauvre en matière sèche. Quelles que soient les quantités totales d'amines des ensilages, tyramine, cadavérine, putrescine et histamine ont été, par ordre décroissant, les principales amines présentes. Les amines ont été produites avant tout durant les dix premiers jours de fermentation et leur production est d'autant plus élevée que la vitesse d'acidification est plus lente. Dans cette étude, l'inoculation par les entérobactéries n'a pas entraîné la formation de plus d'amines. Les quantités d'amines trouvées dans les ensilages sont liées avec les quantités de NH_3, ce qui montre qu'elles résultent de la dégradation générale des protéines.

Par la suite, les amines les plus importantes trouvées dans l'ensilage (Chapitre 3), ainsi que l'ammoniac, ont été utilisés pour étudier leur influence sur l'ingestion chez les moutons (Chapitre 4) et les vaches laitières (Chapitre 5). Ainsi une mélange d'amines en portion semblable à celui trouvé en moyenne précédemment (à hauteur de 2,8 g/kg de MS) ou de l'ammoniac (2,9 g/kg de MS), ont été ajoutés à un ensilage de bonne qualité, préparé par l'addition d'acide formique. Les trois ensilages ainsi obtenus ont été comparés à l'ensilage de la même prairie, mais sans conservateur. A la fois chez les moutons et les vaches laitières, les quantités de MS ingérées d'ensilage sans conservateur ont été plus faibles que celles d'ensilage avec acide formique. L'ingestion plus faibles d'ensilage sans conservateur résulte d'une ingestion plus faible lors des repas principaux suivant la distribution de fourrage, causée apparemment par une plus faible appétibilité et un rassasiement plus rapide des animaux. L'addition de NH_3 n'a modifié ni le niveau d'ingestion, ni le comportement alimentaire des animaux. Les amines ajoutées ont eu tendance à faire diminuer l'ingestion journalière chez les moutons, mais pas chez les vaches. Cette ingestion légèrement plus faible, a été due à une vitesse d'ingestion plus faible durant les grands, mais aussi les petits repas. La baisse observée de la vitesse d'ingestion a été probablement causée par une plus faible appétibilité, compte tenu d'une plus faible vitesse d'ingestion au début des grands repas. Chez les moutons les traitements étudiés n'ont pas eu de conséquence sur le contenu ruminal, ses teneurs en MS et parois végétales, ainsi que sur la motricité de la paroi ruminale. Ces paramètres n'ont pas été mesurés chez les vaches. Par ailleurs, l'ammoniac et les amines ajoutés n'ont pas modifié le pH du contenu ruminal, ainsi que ses teneurs en NH_3 et acides gras volatils, chez les moutons et les vaches.

L'addition d'amines a, cependant, fait augmenter légèrement la quantité de liquide dans le rumen des moutons. Cette observation est cohérente avec une plus faible pression osmotique du contenu ruminal, suggérant un effet diluant des amines, retrouvé aussi, mais faiblement, chez les vaches. Avec tous les fourrages utilisés, chez les moutons comme chez les vaches, seules des quantités négligeables d'amines ont été retrouvées dans le contenu ruminal. A partir de ce résultas on peut conclure que, aux concentrations utilisées, l'ammoniac et les amines, ne diminuent pas l'ingestion des ensilages. Le mélange d'amines, cependant, a tendance à avoir un effet négatif sur l'appétibilité des fourrages.

La dégradation des amines par le contenu ruminal a ensuite été étudiée *in vitro* (Chapitre 6). Pour cela, une solution, contenant un mélange d'amines mentionnées plus haut, a été ajouté à des contenus ruminaux de moutons. Ces moutons avaient été adaptés à trois régimes différentes: ensilage contenant une quantité élevée d'amines, ensilage contenant peu d'amines, ou foin ne contenant pas d'amines. Les contenus ruminaux, auxquels la solution d'amines avait été ajoutée, ont été mis en incubation pendant 5 heures. L'addition d'amines a augmenté la concentration en NH_3 des contenus ruminaux. L'augmentation la plus rapide a été trouvée avec les contenus des moutons adaptés à l'ensilage contenant une quantité élevée d'amines. Par contre, l'addition de la solution d'amines n'a pas modifié la production de gaz des contenus mis en incubation. Au bout de 5 heures une partie des amines a été détruite quelle que soit la nature de contenu ruminal utilisé. La destruction la plus élevée ayant lieu dans le contenu des moutons adaptés au régime riche en amines. La quantité d'amines dégradée correspond tout à fait stoechiométrique à la quantité d'ammoniac produit, ce qui indique que les amines sont dégradées dans le rumen par désamination. En général, la dégradation est la plus élevée pour l'histamine, suivie par la tyramine, la putrescine, puis la cadavérine. Ces résultats montrent que, chez les animaux adaptés à des ensilages d'herbe riches en amines, l'augmentation de la capacité de désamination des microbes de contenu ruminal empêche l'accumulation de ces amines dans le rumen.

Le Chapitre 7 rapporte les résultats d'une étude comparant les effets d'une addition d'amines (4,9 g/kg MS) et d'une addition d'un mélange d'amines (2,7 g/kg MS), d'ammoniac (3,0 g/kg MS) et d'acide γ amino-butyrique (GABA; 5.0 g/kg MS), sur les quantités de MS ingérées par les moutons. Ces produits furent, de nouveau, ajoutés à un ensilage de bonne qualité préparé avec l'addition d'acide formique. Le mélange d'amines seules n'a pas modifié l'ingestion journalière

lorsqu'il est ajouté à l'ensilage témoin. Le mélange amines, ammoniac, et GABA a légèrement fait augmenter le niveau d'ingestion. Cependant, l'addition d'amines a fait baisser légèrement la vitesse d'ingestion lors des repas principaux et, également lors des petits repas. Néanmoins, la teneur en NH_3 de contenu ruminal a été la plus élevée après les repas principaux pour l'ensilage additionné du mélange amines, NH_3 et GABA. Aucun effet des amines seules, ou du mélange amines, NH_3 et GABA, n'a été observé sur le pH du contenu ruminal, la pression osmotique, la quantité de contenu ruminal et sa vitesse de renouvellement. On conclut de cet essai que les amines seules ou le mélange amines, NH_3 et GABA, à des concentrations normalement trouvées dans les ensilages de qualité moyenne à mauvaise, ne modifient pas l'ingestion par le biais de mécanismes de contrôle chimiostatique de l'appétit. Seul un léger effet négatif des amines sur l'appétibilité des ensilages est possible.

Compte tenu de l'adaptation des microorganismes du rumen à dégrader les amines (Chapitre 6) on peut supposer que ces dernières ne s'accumulent dans le rumen, pour les ensilages qui en sont riches, que pendant quelques jours durant lesquels elles pourraient alors franchir la parois du rumen et avoir un effet direct sur l'appétit, via leur action au niveau du métabolisme intermédiaire des animaux. Dans le Chapitre 8 cet effet direct des amines a été étudié, au travers de changements des régimes, en utilisant un ensilage pauvre en amines (1,4 g/kg MS) et le même additionné de 7,2 g d'amines/kg de MS. De plus, l'effet d'un préconditionnement des animaux sur les effets à court terme observés lors du changement de régime a été testé en distribuant auparavant un ensilage contenant 5,2 g d'amines naturellement formées par kg de MS. Les amines ont eu tendance à faire baisser les quantités ingérées par jour et pendant le repas principal lors des 4 jours qui ont suivi le changement de régime. Le préconditionnement a diminué l'effet à court terme des amines. Au bout de 14 jours les moutons étaient parfaitement adaptés à l'ingestion des amines présentes dans l'ensilage. La vitesse de dégradation des amines dans le rumen était alors telle qu'il n'en restait plus que des traces dans le contenu ruminal. Cependant le profil journalier de l'ingestion est resté différent, avec une ingestion plus faible pendent les 5 heures suivant la distribution d'ensilage. Ceci confirmerait l'effet négatif des amines sur l'appétibilité des ensilages.

Dans le Chapitre 9, les circonstances dans lesquelles sont formées les amines dans les ensilages sont discutées. Compte tenu de la littérature, il est probable que les entérobactéries et les bactéries lactiques hétéro fermentaires sont responsables

de la formation de ces amines dans les ensilages de qualité moyenne à bonne.

Les résultats obtenus dans cette thèse permettent de conclure que, dès que les animaux sont adapté, ni les amines ni l'ammoniac ne réduisent directement l'appétit via des mécanismes de contrôle chimiostatiques. Cependant, un léger effet négatif des amines sur l'appétibilité des ensilages est probable. Dans ces conditions, les amines influencent faiblement le comportement alimentaire des animaux, mais sans faire diminuer le niveau d'ingestion. Les amines présentes dans les ensilages ne sont donc que certains composés, parmi d'autres qui ont un très léger effet voire, mais rarement, un effet notable dans des situations extrêmes, avec alors un effet négatif plus prononcé sur l'appétit. Parmi les autres facteurs qui ont un effet sur l'ingestion on peut citer une mauvaise appétibilité liée à certains, pH et à l'acide acétique, ou des déséquilibres entre nutriments au niveau de rumen ou du foie. Bien que les effets de ces facteurs sur l'ingestion soient difficiles à quantifier, leurs conséquences négatives sont tout à fait probables.

Curriculum Vitae

Monique van Os werd geboren op 21 december 1963 te Delft. Na MAVO en HAVO, behaalde zij in 1984 het VWO diploma aan de Rijksscholengemeenschap te Oud-Beijerland. In datzelfde jaar begon zij met de studie Zoötechniek aan de toenmalige Landbouwhogeschool in Wageningen, welke zij in augustus 1990 afsloot met als hoofdvakken Veevoeding en Dierfysiologie. In november van datzelfde jaar werd zij aangesteld als wetenschappelijk onderzoeker in tijdelijke dienst bij het DLO-Instituut voor Veevoedingsonderzoek (IVVO-DLO) te Lelystad. Bij ditzelfde instituut begon zij in 1991 als assistent in opleiding (AIO), verbonden aan de vakgroep Fysiologie van Mens en Dier van de Landbouwuniversiteit te Wageningen, aan het onderzoek dat resulteerde in dit proefschrift. Daar het onderzoek een samenwerking betrof met het franse *Institut National de la Recherche Agronomique* werd zij in 1992 en 1993 gedetacheerd bij het *INRA-Station de la Recherches sur la Nutrition des Herbivores - Theix* te Clermont Ferrand. Vervolgens werd in januari 1994 het onderzoek voortgezet bij het IVVO-DLO te Lelystad, welk sindsdien opgegaan is in het DLO-Instituut voor Dierhouderij en Diergezondheid (ID-DLO). Sinds september 1995 is zij werkzaam als post-doc onderzoeksmedewerker bij de sectie Dierlijke Productie Systemen binnen de Vakgroep Veehouderij van de Landbouwuniversiteit.