Institut für Tierwissenschaften

Impact of ensiling conditions on formation of biogenic amines in grass and legume silages and feed intake behaviour of goats

Dissertation
zur
Erlangung des Grades

Doktorin der Agrarwissenschaften (Dr. agr.)

der Landwirtschaftlichen Fakultät
der Rheinischen Friedrich-Wilhelms-Universität Bonn

von

Master of Science

Rebecca Scherer

aus Bonn

Bonn 2019

Referent: Prof. Dr. Karl-Heinz Südekum

Korreferent: Prof. Dr. Martin Gierus

Tag der mündlichen Prüfung: 01.02.2019

Angefertigt mit Genehmigung der Landwirtschaftlichen Fakultät der Universität Bonn

Die vorliegende Arbeit wurde gefördert durch die Deutsche Forschungsgemeinschaft (DFG, SU 124/31-1).

SUMMARY

Impact of ensiling conditions on formation of biogenic amines in grass and legume silages and feed intake behaviour of goats

Silage produced from small-grained legumes can contribute to the protein supply of ruminants. However, the susceptibility to protein degradation during harvest and the entire conservation process impedes to achieve high feed quality. The changes in the composition of crude protein fractions from protein nitrogen to non-protein nitrogen, which encompasses amongst others biogenic amines, are assessed negatively for several reasons, as they may result in a reduction of feed intake.

The aim was to determine the effect of ensiling conditions on formation of fermentation products with focus on biogenic amines and the resulting feed intake behaviour and the short-term dry matter intake (DMI) of silages by goats. Moreover it was examined whether the preference for one of two forages on offer develops immediately at the beginning of feeding based on the emanating odors or whether the feed has to be ingested in order to activate the postingestive feedback. Six silages with different treatments were produced each from lucerne (*Medicago sativa* L.), red clover (*Trifolium pratense* L.) and Italian ryegrass (*Lolium multiflorum* LAM.). After ensiling of at least 90 days preference trials with Saanen-type goats (n = 8 for lucerne and red clover silages and n = 6 for grass silages) and a comprehensive chemical characterisation were carried out. During the experimental phase, each possible two-way combination of the six silages and lucerne hay, which served for comparison of different runs, was offered as free choice for 3 h. The first three minutes of feeding were filmed to analyse the behaviour towards the two offered forages.

The results revealed that intense proteolysis did not result in high contents of biogenic amines. Within the determined concentration range (1.2–4.1 g/kg DM) no influence on feed intake behavior was found. In addition, the crude protein fractions and the fermentation products could not clearly be attributed to influence the DMI. The DMI of the treatments differed significantly within the plant species. However, the DMI rankings of the three plant species were very similar. The preference of goats for one of the two freely selectable silages was already shown during the first few minutes of feeding. The behavioural observation by means of video recordings over a very short period of time thus seems to be suitable for assessing the development of preference when presenting two silages.

KURZFASSUNG

Einfluss der Silierbedingungen auf die Bildung biogener Amine in Leguminosen- und Grassilagen und das Futteraufnahmeverhalten von Ziegen

Aus kleinkörnigen Leguminosen hergestellte Silage kann zur Proteinversorgung von Wiederkäuern beitragen. Unter anderem die Anfälligkeit für Proteinabbau bereits während der Ernte und während des gesamten Konservierungsprozesses erschwert jedoch das Erreichen einer hohen Futterqualität. Die mit der Proteolyse einhergehenden Veränderungen in der Zusammensetzung der Rohproteinfraktionen vom Proteinstickstoff zum Nichtproteinstickstoff sind aus verschiedenen Gründen negativ zu beurteilen, da sie unter anderem in einer Reduzierung der Futteraufnahme resultieren können.

Ziel dieser Arbeit war es, die Auswirkungen unterschiedlicher Silierbedingungen auf die Bildung verschiedener Fermentationsprodukte mit Fokus auf biogene Amine und das Präferenzverhalten sowie die kurzzeitige Trockenmasseaufnahme von Ziegen zu untersuchen. Außerdem wurde die Hypothese überprüft, ob sich die Präferenz für ein Futter unmittelbar zu Beginn der Fütterung basierend auf den von den Silagen ausgehenden Gerüchen entwickelt und das Futter nicht erst gefressen werden muss, um das postingestive Feedback zu aktivieren. Dazu wurden jeweils sechs Silagen aus Luzerne (*Medicago sativa* L.), Rotklee (*Trifolium pratense* L.) und Welschem Weidelgras (*Lolium multiflorum* LAM.) hergestellt. Nach einer mindestens 90-tägigen Silierdauer wurde mit den Luzerne-, Rotklee- und Grassilagen jeweils ein Präferenzversuch mit Ziegen (Weiße Deutsche Edelziege, n = 8 bei den Luzerne- und Rotkleesilagen bzw. n = 6 bei den Grassilagen) sowie eine umfassende chemische Charakterisierung durchgeführt. Das Design der Versuchsphase sah vor, jeder Ziege jede mögliche Kombination zweier Silagen und eines Luzerneheus, welches als Standardfutter zur Vergleichbarkeit der drei Durchgänge diente, für 3 h zur freien Wahl anzubieten. Die ersten drei Minuten der Futteraufnahme wurden gefilmt, um die Futteraufnahme beobachten und analysieren zu können.

Die Ergebnisse zeigen, dass während der Silierung eine intensive Proteolyse stattfand, die jedoch nicht zu einem hohen Gehalt an biogenen Aminen führte. Innerhalb des ermittelten Konzentrationsbereiches (1,2–4,1 g/kg TM) wurde kein Einfluss auf das Futteraufnahmeverhalten festgestellt. Zwar unterschieden sich die Trockenmasseaufnahmen

der einzelnen Behandlungen innerhalb der einzelnen Spezies teilweise signifikant, jedoch konnte den einzelnen Rohproteinfraktionen sowie den Fermentationsprodukten keine eindeutige Bedeutung für die Beeinflussung der Trockenmasseaufnahme zugeschrieben werden. Es zeigte sich, dass sich die Präferenz für eine der zwei frei wählbaren Futtermittel bereits während der ersten Minuten der Fütterung entwickelt. Die Verhaltensbeobachtung mittels Videoaufzeichnungen über einen sehr kurzen Zeitraum scheint demnach geeignet, die Entwicklung der Präferenz bei Vorlage zweier Silagen zu beurteilen.

TABLE OF CONTENTS

FIGURES

Chapter 3

Chapter 5

TABLES

Chapter 3

Chapter 4

Chapter 5

ABBREVIATIONS

A	CP fraction, non-protein N multiplied by 6.25, soluble in tungstic acid
ADFom	Acid detergent fibre expressed exclusive residual ash
aNDFom	Neutral detergent fibre analyzed with heat stable amylase and expressed exclusive residual ash
ADL	Acid detergent lignin
B1	CP fraction, true protein soluble in borate-phosphate buffer
B2	CP fraction, CP insoluble in borate-phosphate buffer minus neutral detergent insoluble CP
B3	CP fraction, neutral detergent insoluble CP minus acid detergent insoluble CP
BW	Body weight
C	CP fraction, corresponds to acid detergent insoluble CP
cfu	Colony forming unit
CL	Crude lipid
CP	Crude protein
d	Day
DM	Dry matter
DM_{cor}	Dry matter corrected
DMI	Dry matter intake
e.g.	exempli gratia
Fig.	Figure
GC	Gas chromatography
GP	In vitro gas production after 24 h of incubation
IR	Italian ryegrass
LU	Lucerne
ME	Metabolisable energy
MDS	Multi dimensional scaling

MPN	Most probable number
MSD	Minimum significant difference
MS	Mass spectrometry
n.a.	Not analysed
n.d.	Not detected (below detection limit)
NH_3-N	Ammonia N
NPN	Non-protein nitrogen
RC	Red clover
SD	Standard deviation
VFA	Volatile fatty acid/s
WSC	Water soluble carbohydrates
Silage treatments	
CON	Control
CHE1	Chemical silage additive (2.5 L/t), based on sodium nitrite and hexamine (hexamethylenetetramine)
CHE2	Chemical silage additive (4 L/t), based on 75% formic acid buffered with sodium hydroxide to pH 2.5
BIO	Biological additive (1 g/t), based on homofermentative lactic acid bacteria (*Lactobacillus plantarum*; $3.0 \cdot 10^{11}$ cfu/g)

CHAPTER 1

General introduction

Feed costs represent a significant proportion of the total costs of cattle farming. A decisive factor in optimising economics is therefore the provision of high-quality feed, which enables high livestock productivity at reasonable costs. A large part of preserved feed in many areas of the world is provided by silage. Beside hay-making ensiling is a principal form of feed preservation, especially in regions of the world where humidity, often combined with bad weather conditions, leads to excessive dry matter (DM) and quality losses during hay-making (Pahlow et al., 2003). The aim of silage preparation and the subsequent storage is the protection of feed from spoilage while maintaining the feed characteristics as best as possible (Pahlow and Hünting, 2011). The intension of ensiling is based on lactic acid fermentation. As a consequence, the pH declines and harmful anaerobic microbes are suppressed or eliminated (Pahlow, 2007).

Forage crops used for ensiling in European countries are silage maize, grasses, legumes and whole-crop cereals other than maize. These plant species differ in quality characteristics which can make them either easy or difficult to ensile. Grass is one of the most common crops for ensiling not only in many parts of Europe but also in North America, Australia and New Zealand (Keady et al., 2012). Less common is legume silage made from red clover or lucerne, although legumes have attained considerable political and public attention since the so called “Eiweißpflanzenstrategie” (strategy for protein crops) of the German Federal Ministry of Food and Agriculture (BMEL) aims to promote the cultivation and utilisation of legumes (BMEL, 2018). Legume cultivation has significantly declined, although it has several beneficial effects. The inclusion of legumes in cultivation systems and crop rotations may lead to a positive carbon balance, improved soil fertility and a decreased usage of nitrogen fertilisers. Moreover the emission of greenhouse gases in agriculture can be significantly reduced. Last but not least the cultivation of legumes can contribute to the biological diversity of agricultural landscapes. The strategy aims to reduce competitive disadvantages of local protein crops compared to imported protein crops like soybean or its co-product soybean meal, to close research gaps and to implement the necessary measures to put them into practice (BMEL, 2018).

Through the symbiosis with nitrogen binding bacteria legumes are self-sustaining with nitrogen and are therefore beneficial especially in organic farms (Dazzo and Brill, 1978). In addition to nitrogen fixation, they are characterised by a number of other ecosystem services including improvement of soil properties and phytosanitary effects. For ruminant nutrition they can also be favourable. Voluntary intake of legume forage can be 10–15% greater than that of grasses of similar digestibility. This difference is attributed to a lower resistance of legumes to chewing, a faster rate of digestion and a faster rate of clearance from the rumen (Waghorn et al., 1989; Dewhurst et al., 2009), which in turn reduce rumen fill (Lüscher et al., 2014). Ruminants are able to compensate for the relatively low energy content and the lower digestibility of lucerne rations by increasing their DM intake. The usage of lucerne silage as an alternative to grass silage for up to 30% of DM seems therefore recommendable for maize-based rations (Bulang et al., 2006). Further, the demand for sufficient structural fibre can be met. Especially lucerne is characterised by a good structural effect and thereby contributes together with the high buffer capacity compared to maize silage to the stabilisation of the ruminal environment (McBurney et al. 1983; Lüscher et al., 2014). An additional benefit of white clover is that the rate of decline in nutritive value throughout the plant-ageing process is much lower than for grasses. This has been known for a long time (Ulyatt, 1970 in Lüscher et al., 2014). However, legumes also have unfavourable ensilage characteristics which need consideration. High concentrations of crude protein and organic acids and, at the same time, low concentrations of water-soluble carbohydrates result in a high buffering capacity which hampers a rapid and strong pH decline to produce a stable product (McDonald et al., 1991). To achieve legume silage high in quality a fast and intensive wilting after harvest and application of silage additives is recommendable (Pahlow et al., 2003).

Voluntary silage intake by ruminants is mostly determined by fermentation products, DM concentration, aerobic stability and microbial counts, e.g., yeasts, moulds and aerobic mesophilic bacteria. All these variables can have a significant effect on the acceptance of silage by ruminants and thus, directly or indirectly, on performance and health status. A strong colonization with microorganisms should be avoided. Clostridial activity for example can lead to extreme quality decline and end up in complete deterioration of the ensiled forage (Weiß, 2001). In addition to clostridia, microbes like enterobacteria may survive in spoiled silage, which are also related to the accumulation of undesirable metabolites. Another mechanism of generation of unfavourable degradation products is proteolysis. During proteolysis high-molecular proteins are broken down by plant and microbial peptidases to

peptides, amino acids and amides, resulting in a loss of true protein and hence an increase in non-protein nitrogen (NPN). This increase is associated with a decrease in silage protein value, which can be expressed as utilisable crude protein (CP) at the duodenum (uCP; Hoedtke et al., 2010; Richardt et al., 2011). Desmolysis further degrades amino acids to ammonia, organic acids and biogenic amines. This process is predominantly catalysed by microbial enzymes and especially proteolytic clostridia (Hoedtke et al., 2010).

The development of biogenic amines in silage is discussed in connection with deleterious effects on the animal (Křížek, 1991). Biogenic amines are organic nitrogenous bases with low molecular weight and an aliphatic, aromatic or heterocyclic structure (Santos, 1996). Those with several terminal amino groups, called polyamines like putrescine, spermidine and spermine are indispensable for human and animal cells being part of the regulation of nucleic acid and protein synthesis (Bardócz et al., 1993; Halász et al., 1994). In addition, they serve as hormones, neurotransmitters and mediators in cell metabolism or as building blocks of phospholipids and other cell components. They can be formed endogenously or are introduced from exogenous sources (Pietrzak et al., 2002). However, if their concentration or activity exceeds certain limits, they indicate spoilage and are considered as anti-nutritional components in food and in feed (Smith, 1981). For decades, there has been little progress in research on occurrence of biogenic amines in silage and their relevance to animal health and productivity. In silage mainly putrescine, cadaverine, spermidine, spermine, histamine, tyramine and tryptamine occur. Křížek (1993) found considerable levels of biogenic amines in laboratory silage made from orchardgrass, red clover and oats. The highest concentrations were typically found for putrescine and cadaverine, and therefore those two amines were particularly indicative of the extent of proteolysis and putrefaction.

Data and explanatory notes on the influence of biogenic amines which were formed during ensiling on feed intake and the feed preference behaviour of ruminants are lacking. In addition, no findings were available on the interplay of biogenic amines with other fermentation products in silages. To address these issues adequately, comprehensive chemical analysis should be combined with measurements on animals. Preference trials as a special form of choice feeding experiments can be used to assess the willingness of animals to ingest feeds in a choice situation, i.e., when different feeds are offered separately at the same time and preference or palatability are determined (Meier et al., 2012). Preference trials with varying objectives using maize, grass and lucerne silages were conducted at the University of Bonn (Gerlach et al., 2013, 2014; Brüning et al., 2018). By means of these experiments

including comprehensive chemical analyses the complex structure of compounds with their dependencies among one another can hardly be determined, but the impact of ensiling conditions and plant species on the formation of biogenic amines and feed intake behaviour can be assessed. To unbundle these influences and to determine which impact a feed has on the DMI in the short and in the long term is a forward-looking goal.

References

Bardócz, S., Grant, G., Brown, D.S., Ralph, A., Pusztai, A., 1993. Polyamines in food – implications for growth and health. J. Nutr. Biochem. 4, 66–71.

BMEL, 2018. Eiweißpflanzenstrategie. https://www.bmel.de/DE/Landwirtschaft/Pflanzenbau/Ackerbau/_Texte/Eiweisspflanzenstrategie.html (accessed 18 January 2018).

Bulang, M., Kluth, H., Engelhard, T., Spilke, J., Rodehutscord, M., 2006. Zum Einsatz von Luzernesilage bei Kühen mit hoher Milchleistung. J. Anim. Physiol. Anim. Nutr. 90, 89–102.

Brüning, D., Gerlach, K., Weiß, K., Südekum, K.-H., 2018. Effect of compaction, delayed sealing and aerobic exposure on forage choice and short-term intake of maize silage by goats. Grass Forage Sci. 73, 392–405.

Dazzo, F.B., Brill, W.J., 1978. Regulation by fixed nitrogen of host-symbiont recognition in the *Rhizobium*-clover symbiosis. Plant Physiol. 62, 18–21.

Dewhurst, R.J., Delaby, L., Moloney, A., Boland, T., Lewis, E., 2009. Nutritive value of forage legumes used for grazing and silage. Irish J. Agric. Food Res. 48, 167–187.

Gerlach, K., Roß, F., Weiß, K., Büscher, W., Südekum, K.-H., 2013. Changes in maize silage fermentation products during aerobic deterioration and effects on dry matter intake by goats. Agric. Food Sci. 22, 168–181.

Gerlach, K., Roß, F., Weiß, K., Büscher, W., Südekum, K.-H., 2014. Aerobic exposure of grass silages and its impact on dry matter intake and preference by goats. Small Ruminant Res. 117, 131–141.

Halász, A., Baráth, A., Simon-Sarkadi, L., Holzapfel, W., 1994. Biogenic amines and their production by microorganisms in food. Trends Food Sci. Technol. 5, 42–48.

Hoedtke, S., Gabel, M., Zeyner, A., 2010. Der Proteinabbau im Futter während der Silierung und Veränderungen der Zusammensetzung der Rohproteinfraktion. Übers. Tierernährg. 38, 157–179.

Keady, T.W.J., Marley, C.M., Scollan, N.D., 2012. Grass and alternative forage silages for beef cattle and sheep: effects on animal performance. In: Kuoppala, K., Rinne, M. & Vanhatalo, A. (Eds). Proceedings XVI Int. Silage Conf. Hämeenlinna, Finland. MTT Agrifood Research Finland and University of Helsinki, Finland, pp. 152–165.

Křížek, M., 1991. The determination of biogenic amines in silage. Arch. Anim. Nutr. 41, 97–104.

Křížek, M., 1993. Biogenic amines in silage. 1. The occurrence of biogenic amines in silage. Arch. Anim. Nutr. 43, 169–177.

Lüscher, A., Mueller-Harvey, I, Soussana, J.F., Rees, R.M., Peyraud, J.L., 2014. Potential of legume-based grassland–livestock systems in Europe: a review. Grass Forage Sci. 69, 206–228.

McBurney, M.I., Van Soest, P.J., Chase, L.E., 1983. Cation exchange capacity and buffering capacity of neutral detergent fibres. J. Sci. Food Agric. 34, 910–916.

McDonald, P., Henderson, A.R., Heron, S.J.E., 1991. The Biochemistry of Silage. Chalcombe Publications, Marlow, UK.

Meier, J.S., Kreuzer, M., Marquardt, S., 2012. Design and methodology of choice feeding experiments with ruminant livestock. Appl. Anim. Behav. Sci. 97, 206–220.

Pahlow, G., Muck, R.E., Driehuis, F., Oude Elferink, S.J.W.H., Spoelstra, S.F., 2003. Microbiology of ensiling. In: Silage Science and Technology. Eds.: D.R. Buxton, R.E. Muck, J.H. Harrison. American Society of Agronomy, Crop Science Society of America, Soil Science of America, Madison, Wisconsin, pp. 1–93, 305–360.

Pahlow, G., 2007. Grundlagen und Grundsätze der Silierung. Übers. Tierernährg. 35, 1–11.

Pahlow, G., Hünting, K., 2011. Gärungsbiologische Grundlagen und biochemische Prozesse der Silagebereitung. In: Praxishandbuch Futter- und Substratkonservierung. DLG-Verlags-GmbH, Frankfurt am Main, Germany.

Pietrzak, T., Schad, A., Zentek, J., Mosenthin, R., 2002. Biogene Amine in der Tierernährung: Entstehung, Stoffwechsel und physiologische Aspekte. Übers. Tierernährg. 30. 37–64.

Richardt, W., Wein, R., Steinhöfel, O., Pries, M., 2011. Untersuchungen zur Veränderung der Proteinqualität während der Silierung von Grassilagen unter Berücksichtigung biogener Amine. VDLUFA-Schriftenr. 67, 541–549.

Santos, S.M.H., 1996. Biogenic amines: their importance in foods. Int. J. Food Microbiol. 29, 213–231.

Smith, T.A., 1981. Amines in food. Food Chem. 6, 169–200.

Waghorn, G.C., Shelton, I.D., Thomas, V.J., 1989. Particle breakdown and rumen digestion of fresh ryegrass (*Lolium perenne* L.) and lucerne (*Medicago sativa* L.) fed to cows during a restricted feeding period. Br. J. Nutr. 61, 409–423.

Weiß, K., 2001. Gärungsverlauf und Gärqualität von Silagen aus nitratarmen Grünfutter. Doctoral Thesis, Humboldt-Universität zu Berlin, Germany.

CHAPTER 2

Scope of the thesis

Silage research mostly focuses on the chemical composition while disregarding the resulting feed intake, although there is still a lack of findings concerning the interplay between the chemical composition of silages and the effects on feed intake and preference behaviour of ruminants. The aim of this thesis was (1) to clarify the relationships between ensiling conditions on the formation of fermentation products including biogenic amines in grass and legume silages and feed intake behaviour of goats. Moreover (2) knowledge was generated regarding the memory process of ruminants concerning two known feeds on offer during a preference trial.

The prepared silages were comprehensively characterised. All samples included in this study were analysed for chemical composition including proximate constituents, fibre fractions, crude protein composition, fermentation acid analyses comprising twenty variables and biogenic amines. In vitro gas production was measured and energy values were estimated. Furthermore, the microbiological stocking was determined. By using short-time feeding trials, the preference behaviour of goats when forages are offered in choice situations was described. For lucerne, red clover and grass silages attempts were made to find silage characteristics being responsible for preference or avoidance during feed intake. The focus was set on protein degradation products like ammonia and biogenic amines. Levels of amines which arise in legume and grass silages under varying ensiling conditions were compared to each other and the influence on feed intake was determined. Furthermore, it was examined whether the preference for one of two offered forages develops in the initial moments of feeding, and this is led by recognition via smell linked to the postingestive feedback. To this video recordings of the first three minutes of feeding were analysed.

The first manuscript (Chapter 3) provides a literature review of studies dealing with biogenic amines in silage and their formation, occurrence and influence on dry matter intake and ruminant production. Literature was summarised and critically evaluated. Moreover the continuing need for research was determined and discussed. The second manuscript (Chapter 4) deals with the impact of forage species and ensiling conditions on silage composition and quality and the feed choice behaviour of goats. The third manuscript (Chapter 5) handles the question whether recognition of goats concerning known feeds in a choice situation is mainly

based on sensory characteristics linked to the postingestive feedback or whether the latter is activated each time before a choice is made.

The aforementioned third, fourth and fifth chapter are manuscripts published in scientific journals. They are formatted according to the layout and the instructions of the respective journal. However, font has been adjusted to make appearance of this thesis consistent. Moreover, the numbering of tables in this document is continuous and does not correspond to the numbering of the respective manuscripts.

CHAPTER 3

Biogenic amines and gamma-amino butyric acid in silages: Formation, occurrence and influence on dry matter intake and ruminant production

R. Scherer, K. Gerlach, K.-H. Südekum

Institute of Animal Science, University of Bonn, Endenicher Allee 15, 53115 Bonn, Germany

Published in **Animal Feed Science and Technology** 210 (2015), 1–16.

Abstract

The concentration of biogenic amines (mono-, di- and polyamines) in silage and in the rumen, body tissues and body fluids mainly depends on the crop at harvest, the ensiling process, the silage and the digestion in the animal. Both the synthesis and the chemical structure of mono- and diamines are well documented. The basis for their formation is proteolysis, a naturally occurring process during ensilage, comprising the enzymatic decarboxylation of amino acids by the action of plant proteases and peptidases and of enzymes of various lactic acid bacteria (LAB), clostridia and other genera. Recent research has already delivered detailed knowledge about proteolysis, but the biochemical effects of biogenic amines in ruminants, including their impact on dry matter (DM) intake (DMI) by influencing sensory characteristics or post-ingestive feedback of the feed has not been elucidated. Data on effects of biogenic amines on palatability and their possible impacts on animal performance are scarce. However, some studies have been performed concerning the influence of biogenic amines on DMI, mainly with the quantitatively most relevant amines histamine, tyramine, putrescine and cadaverine. Studies differed greatly with regard to type of administration (supplemented to silage, provided orally in capsules or infused in the gastrointestinal tract via ruminal cannulas), dosages (2–40 g amine/kg DM) and mixtures (single doses or various combinations of amines; combinations of amines with aldehydes, organic acids or keto-acids). Single doses and low-level doses showed no effect on DMI, whereas higher concentrations naturally not occurring in silage and combinations like histamine and formaldehyde revealed an appetite-reducing effect. Gamma-amino butyric acid (GABA), a non-protein amino acid often classified into the category of amines has also been studied. The conflicting results in terms of the impact of GABA on DMI may result from the administration of non-protected versus rumen-protected GABA and the differing modes of action of the two forms in the hypothalamus. In this review the results of research into the effects of different levels of amines in silages from different crops and ensiling treatments including the use of additives as well as the consideration of possible reasons for variation in the concentration of amines in silage are evaluated. Approaches for elucidating the possible impact on ruminant feed intake and level of production are also discussed. For an overall understanding of amine formation during ensilage further investigations with emphasis on correlations between the impact of ensiling and storage conditions and extent of amine formation are recommended to reveal relations between cause and effect.

1. Introduction

Ensiled forage offered to ruminants often results in a lower voluntary dry matter (DM) intake (DMI) as compared to the corresponding fresh (Donaldson and Edwards, 1976) or dried feed (Thiago et al., 1992). Intake may be decreased compared to hay and fresh forage by more than half (Campling, 1966). However, silage is important for productive and efficient ruminant livestock farms, especially in humid and temperate areas, where DM and quality losses in making hay may be excessive due to wet weather between cutting and harvesting the crop (Pahlow et al., 2003). Advantages of silage compared with hay are commonly higher digestible energy and lower hemicellulose concentration in the DM (Thomas et al., 1969). Often this is due to earlier dates of harvest. In general, ensiling is less weather dependent than hay production due to the shorter period of time between cutting and harvesting. A disadvantage of both types of forage conservation is loss of feed value compared to that of the original crop. The extent of the loss depends on the crop management, resulting in large variation in nutritional value and fermentation quality (McDonald et al., 1991). The quality of silage correlates with the pattern of fermentation, which may be the primary cause for decreased DMI in ruminants offered silage-based diets (Eisner et al., 2006). Factors contributing to decreased silage DMI including fermentation acids, pH and ammonia (NH_3), the influence of silage fermentation on utilization of protein and energy by the ruminant and methods for improving voluntary feed intake, protein quality and carbohydrate fermentation were summarized by Charmley (2001). It was presumed that fermentation acids act retrospectively by determining the balance of volatile fatty acids (VFA) produced in the rumen instead of influencing silage intake directly. The same might apply for NH_3 as it is supposed to exert a greater negative influence on silage intake by increasing total nitrogen solubility than by being detrimental per se since NH_3 is produced in the rumen by the microbial degradation of protein and amino acids. The most widespread and promising methods to improve silage intake are effective wilting and rapid acidification. A meta-analysis confirmed the significance of digestible organic matter (OM) in DM and the total concentration of fermentation acids in affecting silage intake by dairy cows (Huhtanen et al., 2007). Another meta-analysis found a negative relationship between DMI and the concentration of organic acids, NH_3-N and soluble-N compounds (Südekum and Eisner, 2009). Moreover, the degradation products resulting from proteolysis may impair animal health (Hoedtke et al., 2010); particularly biogenic amines have been given consideration (Křížek, 1995; Saarinen, 2002). Biogenic amines as N-containing compounds of low

molecular weight (Křížek, 1993a) have been found in silage and were associated with lowered DMI in sheep (Buchanan-Smith and Phillip, 1986) and acute and subacute toxicity in rats (Til et al., 1997).

Biogenic amines arise from decarboxylation of amino acids (Table 1), based on the action of either plant enzymes or microbial enzymes of various species of lactic acid bacteria (LAB) (*Lactobacillus*, *Pediococcus* and *Streptococcus*) and species of the genera *Clostridia*, *Bacillus*, *Klebsiella*, *Escherichia*, *Pseudomonas*, *Citrobacter*, *Proteus*, *Salmonella*, *Shigella* and *Photobacterium* (Křížek, 1991, 1993a; Santos, 1996). Determining amine concentrations in silage may help to indicate undesirable changes in forages and could prevent possible toxicity for livestock (Křížek, 1991). However, until now amine analyses are not included in the standard chemical analyses of forages.

This review provides a comprehensive view of likely causes of different levels of amines in silages and approaches for elucidating their possible impact on feed intake and performance of ruminants. Due to conflicting perspectives on functions and effects of gamma-amino butyric acid (GABA) in the metabolism, the current state of research is described and gaps in knowledge are identified. For some time there has been an increase in studies dealing with rumen-protected GABA, arguing that this compound is a stimulating factor on feed intake and nutrient digestibility and that it has a positive effect on milk production and on the antioxidative status of the animal. This is in contrast to previous investigations that focused on non-protected GABA and on presumed negative effects on DMI and health status. The underlying research questions as well as the study designs and concomitant results will be scrutinized. Despite the apparent contradiction, both perceptions have validity since GABA can act simultaneously as an appetite stimulant and suppressor (Morley, 1980). In which way it exerts its neuronal activity depends on the site of its action in the hypothalamus (Section 4.2).

Table 1 Amino acids occurring in silages and their corresponding biogenic amines (Santos, 1996)

Amino acid	Corresponding biogenic amine	Classification
	Aliphatic	
Arginine	Putrescine	Diamine/Polyamine
Lysine	Cadaverine	Diamine/Polyamine
Arginine	Spermidine	Polyamine
Arginine	Spermine	Polyamine
	Aromatic	
Tyrosine	Tyramine	Monoamine
Phenylalanine	Phenylethylamine	Monoamine
	Heterocyclic	
Tryptophan	Tryptamine	Monoamine
Histidine	Histamine	Monoamine

2. Proteolysis, amino acid degradation and formation of biogenic amines

The basic objective during ensilage is to minimize the degradation of OM and preserve as much as possible of the crop's original feed value. Reduction of proteolysis is essential since the preservation of protein has great importance for high quality silage (Hoedtke et al., 2010). Specifically, this means preventing a reduction of true protein as a proportion of crude protein (CP) and thus a decrease in ruminally undegraded feed CP (RUP) and utilizable CP at the duodenum (uCP), a precursor to metabolisable protein. However, the extent of plant enzymatic proteolysis and influence of the ensiling process on proteolysis are poorly understood (Hoedtke et al., 2010; Gresner et al., 2014). Nishino et al. (2007), working with silages made of grass and maize and a total mixed ration (wet brewer's grains, lucerne hay, cracked maize, sugar beet pulp, soybean meal and molasses) found a decrease in histamine, tyramine, putrescine and cadaverine when the crops were inoculated with *L. casei* regardless of crop type. The effect of *L. buchneri* on amine concentration was less clear since tyramine and putrescine were lowered in grass silage but increased in maize silage as compared to the uninoculated control. Histamine also decreased in grass silage but was not affected in maize silage. When inoculated in vitro amine production occurred in both bacterial species, indicating that amines develop to a lesser extent when silages were inoculated, whereas the

bacterial species per se produce them. Hence, it could be suggested that not the species themselves inhibit amine production but their effects on acetic acid, ethanol and yeasts in the silage (Nishino et al., 2007).

Activity of plant enzymes leads to a rapid and extensive degradation of protein immediately after harvesting (Ohshima and McDonald, 1978) and in the early stages of the ensiling process (Kemble, 1956). Furthermore, slow acidification resulting in a slow pH drop and a prolonged wilting in humid conditions intensifies proteolysis (Carpintero et al., 1979), whereas protein stability is favoured by a high DM content of the wilted forage, a rapid pH drop to ≤ 4.0 (Papadopoulos and McKersie, 1983) and rapid wilting (Edmunds et al., 2014). The latter increased concentrations of true protein, RUP and uCP in silage (Edmunds et al., 2014). However, even under favourable conditions a certain amount of protein is always decomposed (Makoni et al., 1997; Slottner and Bertilsson, 2006; Lorenz and Udén, 2011). Depending on the activity of protein-cleaving plant and microbial enzymes, proteins are degraded to amino acids and other N compounds (Charmley, 2001) and these changes in CP composition may result in a reduction of feed value and feed intake (Buchanan-Smith and Phillip, 1986; Südekum and Eisner, 2009).

High quality grass silages have less than 50 g NH_3-N/kg total N (Huhtanen et al., 2002), grass silage with amounts greater than 150 g NH_3-N/kg total N is seen as spoiled (Thaysen, 2004). Increased NH_3 due to elevated desmolysis (sum of deamination and decarboxylation) correlates negatively to DMI (Huhtanen et al., 2002). Microorganisms in the rumen form another source of amines. Amino acids are deaminated to organic acids and NH_3 or decarboxylated to amines and CO_2. Pre-conditions for microbial biogenic amine formation are availability of free amino acids (Marklinder and Lönner, 1992), presence of decarboxylase-positive microorganisms (Brink et al., 1990; Huis in't Veld et al., 1990) and conditions allowing bacterial growth, decarboxylase synthesis and decarboxylase activity (Brink et al., 1990).

As mentioned above (Section 1), clostridia are not the only source of amines. Van Os et al. (1996b) reported that considerable amounts of amines were formed in well-preserved silages during the first ten days of ensiling, which could not be attributed to clostridia. Nevertheless, contamination with soil-borne clostridia during harvest should be avoided. Activity of clostridial species, such as *C. sporogenes*, *C. sphenoides* and *C. bifermentans* (Pahlow et al., 2003), results in high DM losses and unstable poor-quality silage. Furthermore, the concentration of butyric acid as a metabolite and breakdown of protein can rise considerably

(McDonald et al., 1991). Products of amino acid breakdown such as NH_3, amines and amides are considered to be responsible for lowering silage DMI. As butyric acid per se has not significantly decreased DMI, it may be used as a marker for protein degradation products (Hoedtke et al., 2010). Wilting to DM concentrations > 300 g/kg and a pH <4.5 during ensilage may prevent survival of clostridial spores in silage and spore germination later on in the feedout phase (Pahlow et al., 2003).

3. Amines in animal feedstuffs

3.1. Amine levels in silages

Varying concentrations of amines in silages made from the same crop species can have various causes such as weather conditions before harvest, stage of crop maturity, DM concentration and hygiene during harvest (McDonald et al., 1991). The single steps during ensilage and storage as well as the quality of sealing to prevent entry of oxygen for a rapid decrease of pH play an important role in influencing the extent of proteolysis and desmolysis, resulting in different concentrations of amines. Differences in amine concentrations between various crops result from different concentrations and composition of CP per se and differing extents of naturally occurring proteolysis within these crops. The influence of factors such as crop species and variety, type of tissue, germination, conditions of growth, stage of development, degree of ripening, processing and storage conditions on proteolysis and desmolysis is not clear (Glória et al., 2005). Due to the vast number of influencing factors, amine concentrations in silages differ widely, which complicates their prediction even when DM is known.

More data are available for levels of biogenic amines in foods as compared to animal feeds and silages in particular (Bardócz et al., 1993, 1995; Zaman et al., 2009). The DM and amine concentrations of silages of different crops and various pre-treatments are summarized in Table 2. Amines arising during ensilage and the subsequent storage mainly include histamine, tyramine, putrescine and cadaverine with concentrations up to 2 g/kg DM of each amine (Ohshima et al., 1979; Hole, 1985). It is conspicuous that within the same crop an increasing DM concentration led to decreased amine levels, which is also obvious for green oats (Fig. 1). However, no statistically significant correlation between both variables could be demonstrated. Contents of histamine, tyramine, putrescine, cadaverine, tryptamine, phenylethylamine, spermidine and spermine (mg/kg DM; with standard deviations (SD) in

parentheses) of silages without additives were averaged based on the data of Table 2 across studies without considering silo scales. They were 165 (300.6), 672 (861.8), 615 (909.4), 666 (1081.3), 91 (113.6), 68 (80.9), 28 (21.8) and 13 (30.3), respectively. Means (with SD) of histamine, tyramine, putrescine, cadaverine, tryptamine, phenylethylamine, spermidine and spermine of silages treated with additives were 163 (271.3), 506 (681.1), 360 (582.3), 380 (717.7), 50 (121.4), 53 (70.9), 17 (13.9) and 6 (6.6), respectively. The untreated and additive-treated silages partially had very similar amine concentrations. The additive-treatment only showed an effect with tryptamine, putrescine, cadaverine and spermine. Moreover, these concentrations were much lower than the forage-added (Dawson and Mayne, 1995; Van Os et al., 1995a,b, 1996a, 1997; Fusi et al., 2004) or rumen-infused concentrations (Neumark et al., 1964; Dawson and Mayne, 1995, 1996, 1997; Section 4). Although a reasonable number of in vivo studies have been published, they vary largely in terms of crop species, study designs and research methods, and, even more important, response variables studied. This extreme heterogeneity, which left only few and sometimes just one single number for a given variable related to the concentrations of amines, precluded a statistical data analysis using quantitative methods. It became obvious that more systematic and comparative studies are needed before this goal can be achieved. As an example, the apparent differences in amine concentrations between farm silos and laboratory silos need comparative evaluation.

Van Os et al. (1995b) suggested that high-quality silages are low in pH and NH_3, VFA, alcohol and amines. Křížek (1993a) examined whether addition of formic acid or wilting could selectively suppress the formation of some amines and whether the formation of certain amines may be influenced by the type of forage (cocksfoot (*Dactylis glomerata*), red clover (*Trifolium pratense*) and oats (*Avena sativa*)). Except for spermidine, the method and efficiency of the preserving treatment were more influential on amine formation than the type of ensiled crop. When formic acid was applied, on average only 0.14 histamine, 0.18 putrescine and 0.25 cadaverine developed relative to the untreated control (=1), whereas the proportionate formation of spermine and spermidine in the formic acid-treated silage was 0.35 and 0.59, respectively. The formation rate of spermine and spermidine was greater compared to other amines, possibly because these polyamines do not evolve directly from amino acids but from putrescine (Křížek, 1991). The action of putrescine (synthesized from arginine) on decarboxylated S-adenosyl methionine produces spermidine, which attacks another decarboxylated S-adenosyl methionine molecule that is converted to spermine.

Křížek (1993b) also examined the rate of amine formation in ensiled cocksfoot, red clover and oats and observed the dynamics of amine development in two consecutive years (1988 and 1989) at 6, 8, 14, 30, 60, 90 and 210 days in the first year and 7, 14, 30, 62, 90, 152, 230 and 300 days after filling and sealing the laboratory silos in the second year. The great dependency on the year of harvest was the most striking observation. While putrescine doubled during the first 30 days of ensiling in 1988 (from about 150–300 mg/kg DM), followed by a slight decrease and afterwards a small increase, in the following year, 1989, it tripled from a first peak of 400 mg/kg DM after 62 days to a second peak of 1200 mg/kg DM by day 230. However, the two peaks only occurred in poor-quality silages. Formic acid showed greater effects in suppressing amine formation than wilting as generally less amines were formed. Degradation of plant protein during wilting and ensiling is inevitable whereas formic acid as a strong organic acid (power for decreasing pH: formic > lactic > acetic > propionic) immediately decreases silage pH and inhibits the growth of acid-producing microorganisms. This results in reduction of less acidifying lactic acid, acetic acid, butyric acid and furthermore NH_3-N and increases water-soluble carbohydrates (Pahlow et al., 2003), resulting altogether in an inhibited proteolysis. The pattern of putrescine formation in oat silages was diverse after preservation with formic acid or wilting (Křížek, 1993b). Treatment with formic acid resulted in a putrescine maximum at day 30 of ensiling and in a stable forage, whereas with wilting putrescine increased steadily throughout the entire storage period. In contrast, the concentration of histamine rose markedly after the first maximum on day 60 until day 300 of ensiling.

Table 2 Amine levels in silages of different plant species, sorted by size of silo

Crop species	Additive (g/kg commodity); [L/t, unless stated]	DM [g/kg]	Biogenic amines [mg/kg DM]								Reference
			Him	Tym	Put	Cad	Trm	Pea	Spd	Spm	
Farm silo											
Grass (species n. s.)	No additives	262	120	1310	414	642	-	-	33.6	139	Křížek et al. (1993)
	No additives	364	48	635	122	194	-	-	30.4	16	
	No additives	450	12.4	262	51.5	102	-	-	90.7	7.8	
	No additives	549	ND	20.8	30.3	25.8	-	-	74.5	ND	
Maize (species n. s.)	No additives	186	62.2	1090	495	593	-	-	20.4	5.2	
	No additives	226	70.8	292	308	225	-	-	29.6	5.7	
	No additives	271	79.1	455	387	355	-	-	18.3	4.9	
	No additives	314	54.1	368	634	390	-	-	33.6	ND	
Maize (species n. s.)	See notes below table[a]	449 (year 1999)	2.6	482	97.8	48.3	4.2	-	16.6	0.06	Steidlová and Kalač (2002)
		378 (year 2000)	3	145	136	96.2	2.5	-	37.9	2.8	
Timothy (*Phleum pratense*; 0.81); meadow fescue (*Festuca pratensis*; 0.16); red clover (*Trifolium pratense*; 0.02) (remaining 0.01 n.s.)	Based on LAB [3.98]	212	739	1500	1460	1910	66	99	-	-	Krizsan and Randby (2007)
	Molasses [56.0]	204	477	1480	1270	1350	117	119	-	-	
	FA (645) + NH_3 (60) [7.13]	232	64	1640	950	650	19	19	-	-	
	No additives	178	1430	2680	3730	5410	205	374	-	-	
	FA (645) + NH_3 (60) [4.14]	189	1057	1880	2980	3680	257	290	-	-	
	Molasses (20.6) + additive based on LAB [20.6]	166	990	2230	2400	3730	156	643	-	-	

Table 2 Continued

Crop species	Additive (g/kg commodity); [L/t, unless stated]	DM [g/kg]	Biogenic amines [mg/kg DM]								Reference
			Him	Tym	Put	Cad	Trm	Pea	Spd	Spm	
Farm silo											
Timothy (*P. pratense*; 0.81); meadow fescue (*F. pratensis*; 0.16); red clover (*T. pratense*; 0.02) (remaining 0.01 n.s.)	FA (645) + NH_3 (60) [4.13]	223	150	470	170	170	11	ND	-	-	Krizsan and Randby (2007)
	No additives	213	166	2040	1970	1190	14	166	-	-	
	FA (645) + NH_3 (60) [8.17]	221	136	520	540	790	5	9	-	-	
	FA (645) + NH_3 (60) [1.68]	211	514	2210	1600	2090	47	206	-	-	
	Molasses [11.7]	207	85	1460	980	1100	14	102	-	-	
	Molasses [10.2]	211	669	2220	1800	2400	112	230	-	-	
	Based on LAB [2.72]	211	23	290	400	430	7	84	-	-	
	Based on LAB [4.49]	199	680	2370	1950	2300	105	155	-	-	
	No additives	213	470	1760	950	1210	82	221	-	-	
	Fermented lactose permeate [4.28]	235	263	1230	1000	1040	11	106	-	-	
	Based on chemicals [2.03]	228	191	1490	1620	810	18	129	-	-	
	Based on chemicals and LAB [2.7]	233	60	1680	730	330	ND	83	-	-	
	FA (645) + NH_3 (60) [4.39]	237	21	770	630	210	ND	34	-	-	
	No additives	203	70	1620	2810	600	60	46	-	-	
	FA (641) + NH_3 (54) + PA (93) + benzoic acid (19) [3.78]	218	ND	690	670	120	ND	ND	-		
	Based on chemicals [3.43]	217	ND	1200	1260	220	ND	ND	-	-	

Table 2 Continued

Crop species	Additive (g/kg commodity); [L/t, unless stated]	DM [g/kg]	Biogenic amines [mg/kg DM]								Reference
			Him	Tym	Put	Cad	Trm	Pea	Spd	Spm	
Farm silo											
Timothy (*P. pratense*; 0.81); meadow fescue (*F. pratensis*; 0.16); red clover (*T. pratense*; 0.02) (remaining 0.01 n.s.)	Fermented lactose permeate [3.34]	228	33	1640	2080	640	ND	21	-	-	Krizsan and Randby (2007)
	FA (780) + NH_3 (70) + lactose (20) [3.14]	227	48	720	600	240	ND	9	-	-	
Laboratory silo											
Cocksfoot (*Dactylis glomerata*)	Means of amines for wilted silage and silage treated with and without FA (not wilted) for two years of cultivation	-	28.4	-	551	591	-	-	26.8	15.7	Křížek (1993a)
Red clover (*T. pratense*)		-	36.8	-	199	338	-	-	26.7	6.6	
Oats (*Avena sativa*)		-	37.8	-	354	643	-	-	61.7	21.4	
Means of amines for cocksfoot (*D. glomerata*), red clover (*T. pratense*) and oats (*A. sativa*) for two years of cultivation	FA (not wilted)	-	9.1	-	107	191	-	-	30.6	8.7	
	No additives (wilted)	340	29.9	-	374	621	-	-	33.1	10.5	
	No additives (not wilted)	-	65.3	-	611	759	-	-	51.5	24.8	
Perennial ryegrass (*Lolium perenne*)	No additives	200	313	2215	2138	2559	-	7	ND	1	Van Os et al. (1996b)
	No additives	250	62	1930	157	663	-	41	3	4	
	No additives	320	139	3197	1598	2043	-	7	7	2	
	No additives	370	94	342	94	124	-	17	3	4	
	No additives	450	17	409	77	40	-	14	2	2	
	No additives	550	3	99	83	220	-	12	1	1	

Table 2 Continued

Crop species	Additive (g/kg commodity); [L/t, unless stated]	DM [g/kg]	Biogenic amines [mg/kg DM]								Reference
			Him	Tym	Put	Cad	Trm	Pea	Spd	Spm	
Laboratory silo											
Perennial ryegrass (*L. perenne*)	FA [5]	200	1	29	97	95	-	2	3	1	Van Os et al. (1996b)
	FA [5]	250	26	142	97	22	-	19	4	3	
	FA [5]	320	36	125	79	168	-	2	14	13	
	FA [5]	370	3	48	39	7	-	11	1	36	
	FA [5]	450	3	42	70	5	-	11	2	2	
	FA [5]	550	ND	30	30	66	-	1	1	1	
	L. plantarum (10^7 CFU/g)	200	1	77	99	35	-	2	ND	1	
		250	16	116	107	306		5	17	11	
		320	76	622	328	590	-	2	15	11	
		370	2	29	51	8	-	12	15	3	
		450	2	44	78	22	-	4	2	2	
		550	ND	21	33	15	-	6	ND	1	
	E. sakazakii (6 x 10^6 CFU/g)	250	44	2086	162	621	-	22	2	4	
		450	34	498	81	39	-	8	1	2	
	LPSF (10^5 CFU/g)	250	31	1007	125	575	-	10	3	4	
		450	2	649	82	36	-	11	1	2	
	Cell wall degrading Enzymes	250	47	1649	139	516	-	15	2	4	
		450	13	350	82	32	-	6	1	2	
	Molasses (50)	250	36	1674	113	465	-	17	1	3	
		450	31	360	69	23	-	6	1	2	

Table 2 Continued

Crop species	Additive (g/kg commodity); [L/t, unless stated]	DM [g/kg]	Biogenic amines [mg/kg DM]								Reference
			Him	Tym	Put	Cad	Trm	Pea	Spd	Spm	
Laboratory silo											
Maize (Felicia (FAO 260))	No additives	410	ND	134	154	27.3	3.5	-	37.9	8.3	Steidlová and Kalač (2003)
	L. buchner [b]	410	ND	149	148	35.3	3.45	-	35.8	6.7	
	L. plantarum[b]	410	ND	143	166	39.6	3.7	-	34	5.9	
	Based on various LAB[b]	410	ND	104.9	147.5	28.8	2.1	-	32.2	7.1	
Maize (Calimera (FAO 250))	No additives	373	ND	122	121	21.1	ND	-	29.3	1.7	
	L. buchneri[b]	373	ND	134	130.5	29.3	ND		25.9	ND	
	L. plantarum[b]	373	ND	159.5	129.5	31.3	ND	-	26.2	2.4	
	Based on various LAB[b]	373	ND	120.5	113.5	28.2	5	-	30.8	ND	
Maize (CE-210S (FAO 210; 0.8) and Cester (FAO 220; 0.2))	No additives	300	ND	153	147	94	1.8	-	28.9	4.5	
	L. buchneri[b]	300	ND	83.1	54.9	35.6	ND	-	27	2.6	
	L. plantarum[b]	300	ND	34.9	57.6	35.6	ND	-	25.1	2.7	
	Based on various LAB[b]	300	ND	105.9	126	21.5	ND	-	28.5	ND	
Maize (Tereza (FAO 240))	No additives	275	3	145	97.8	113	14.4	-	36.4	6.9	
	L. buchneri[b]	275	ND	62	33.6	81.4	7.9	-	36	6.7	
	L. plantarum[b]	275	ND	53.8	47.4	80.4	3.8	-	30	5.4	
	Based on various LAB[b]	275	ND	76.2	63.6	106.5	6.4	-	34	9.9	
	L. buchneri[b]	285	ND	78.8	92.1	85.7	2.7	-	28.4	3.9	
Maize (Markíza (FAO 290))	No additives	285	ND	185	112	105	7	-	33.4	ND	
	L. plantarum[b]	285	7.8	93.4	81.8	114.4	5.5	-	32.7	5.1	
	Based on various LAB[b]	285	ND	87.1	74.9	102.8	4.8	-	34.2	4.7	

Table 2 Continued

Crop species	Additive (g/kg commodity); [L/t, unless stated]	DM [g/kg]	Biogenic amines [mg/kg DM]								Reference
			Him	Tym	Put	Cad	Trm	Pea	Spd	Spm	
Laboratory silo											
Grass (*D. glomerata*, *Trisetum flavescens*, *F. pratensis*; 0.64); clover (mainly *T. pratense*; 0.08); forbs (mainly *Taraxacum officinale* and *Achillea millefolium*; 0.28)	No additives	235	ND	102	111	210	ND	-	11.2	ND	Steidlová and Kalač (2004)
	FA	235	ND	47.1	9.8	26.2	ND	-	6.8	ND	
	L. buchneri[b]	235	ND	38.6	13.6	41.1	ND	-	10.3	1.6	
	L. plantarum[b]	235	ND	97.7	40.8	97.7	ND	-	8.4	ND	
	Based on various LAB[b]	235	ND	80.8	49.7	99.4	ND	-	9.8	ND	
Grass (*D. glomerata*, *Agrostis vulgaris*, *T. flavescens*; 0.77); clover (0.1); forbs (mainly *T. officinale*; 0.13)	No additives	180	ND	75.8	88	140	ND	-	7.7	3.7	
	FA	180	ND	59.6	14.4	35	ND	-	9.2	ND	
	L. buchneri[b]	180	ND	40.1	18.8	51.6	ND	-	6.2	ND	
	L. plantarum[b]	180	ND	46.1	27.9	72.1	ND	-	6.9	ND	
	Based on various LAB[b]	180	ND	49.1	55.3	83.2	ND	-	6.2	ND	
Perennial ryegrass (*L. perenne*)	No additives	212	ND	82.1	11.7	83.9	4.4	-	34.7	ND	
	FA	212	ND	18.8	7.7	21.1	5.9	-	26.1	ND	
	L. buchneri[b]	212	ND	53.8	7.5	31.6	2	-	11.5	ND	
	Based on various LAB[b]	212	ND	68.7	6.5	41.1	ND	-	11.8	ND	
False oat (*Arrhenatherum elatius*)	No additives	230	7.4	120	182	218	-	ND	16	ND	
	FA	230	ND	112	94.4	102	-	1.6	23.8	ND	
	L. buchneri[b]	230	ND	75.9	73.7	55.2	ND	-	19	ND	
	L. plantarum[b]	230	ND	83.3	151	87.2	2.1	-	13	ND	
	Based on various LAB[b]	230	ND	111	103	102	ND	-	19.7	ND	

Table 2 Continued

Crop species	Additive (g/kg commodity); [L/t, unless stated]	DM [g/kg]	Biogenic amines [mg/kg DM]								Reference
			Him	Tym	Put	Cad	Trm	Pea	Spd	Spm	
Laboratory silo											
Grass (*Poa pratensis*, *L. perenne*, *Alopecurus pratensis*; 0.69); clover (0.6); forbs (mainly *Achillea millefolium*, *T. officinale* and *Galium verum*; 0.25) Perennial ryegrass (*L. perenne*; 0.68); clover (0.08); forbs (mainly *T. officinale*; 0.24)	No additives	325	ND	53.4	46.2	115	ND	-	53	1.8	Steidlová and Kalač (2004)
	FA	325	ND	19.8	36	27.2	9.1	-	49	1.5	
	L. buchner [b]	325	ND	65.8	72.2	55	1.3	-	23.2	ND	
	L. plantarum[b]	325	ND	39.6	58	46.2	ND	-	17.6	ND	
	Based on various LAB[b]	325	ND	86.8	59.2	82.6	ND	-	23	ND	
	No additives	255	ND	70.6	87.5	106	ND	-	12.6	ND	
	FA	255	ND	42.9	133	87	ND	-	5.7	ND	
	L. buchneri[b]	255	ND	102	153	156	ND	-	8.7	ND	
	L. plantarum[b]	255	ND	99.6	129	167	ND	-	3.6	ND	
	Based on various LAB[b]	255	ND	76.2	125	107	ND	-	7.1	ND	
Scale not specified											
Green oats (species n. s.)	No additives	155	90	260	-	-	200	40	-	-	Neumark et al. (1964)
		200	60	400	-	-	200	40	-	-	
		223	80	120	-	-	140	20	-	-	
		271	15	78	-	-	Traces[c]	78	-	-	
Pearl millet (species n. s.)	No additives	211	78	446	-	-	19	227	-	-	
		238	16	120	-	-	Traces[c]	10	-	-	
Sugar beet tops (species n. s.)	No additives	286	0.3	23	-	-	ND	ND	-	-	
Vetch and sugar cane (species n. s.)	No additives	245	48	131	-	-	128	12	-	-	

Table 2 Continued

Crop species	Additive (g/kg commodity); [L/t, unless stated]	DM [g/kg]	Biogenic amines [mg/kg DM]								Reference
			Him	Tym	Put	Cad	Trm	Pea	Spd	Spm	
Scale not specified											
Cocksfoot (*D. glomerata*)	No additives	199	900	2100	1800	2400	-	-	-	-	Van Os et al. (1995a)
	FA [4.5]	209	200	500	200	300	-	-	-	-	
Grass (species n. s.)	FA [4.5]	230	400	1200	400	400	-	-	-	-	Van Os et al. (1995c)

Him: Histamine; Tym: Tyramine; Put: Putrescine; Cad: Cadaverine; Trm: Tryptamine; Pea: Phenylethylamine; Spd: Spermidine; Spm: Spermine; FA: formic acid; PA: propionic acid; FM: Fresh matter; LAB: Lactic acid bacteria; *E. sakazakii*: *Enterobacter sakazakii*; *L. buchneri*: *Lactobacillus buchneri*; *L. plantarum*: *Lactobacillus plantarum*; LPSF: *Lactobacillus plantarum* + *Streptococcus faecium*; CFU: colony forming unit; ND: not detected; -: not analysed.

[a] Most of the silages were preserved by spontaneous fermentation, different biological additives (LAB inoculants and/or hydrolytic enzymes) were applied in 18 silages of 113 in total; the mean values are given.

[b] Two LAB concentrations were used and averaged for calculating the amine contents.

[c] „Traces“ were not defined in the publication.

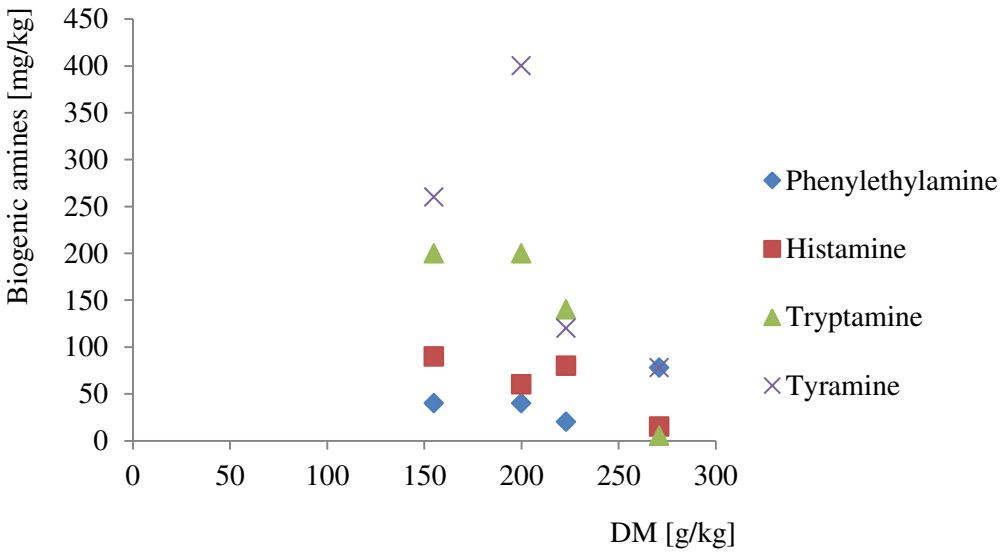

Fig. 1 Biogenic amines [mg/kg] in relation to DM [g/kg] in green oats; DM: dry matter; after Neumark et al. (1964)

The pH affects amino acid decarboxylase (EC 4.1.1.28) activity to a considerable degree as it is stronger in an acidic environment, having the optimum within the pH range of 4.0–5.5 (Teodorovic et al., 1994). In addition, rapidly fermentable carbohydrates such as glucose influence bacterial growth and also the activity of amino acid decarboxylase in bacteria. Concentrations of glucose of 5–20 g/kg DM promoted amino acid decarboxylase activity, whereas concentrations greater than 30 g/kg DM were inhibiting (Halász et al., 1994). Fermentation of fructose and other hexoses lowers the pH of ensiled crops: after completion of silo filling, sugars are converted to lactic acid during the fermentation, resulting in a reduction in pH to between 4.5 and 4.0. By adding glucose in amounts of 5–20 g/kg DM the pH remains in the optimal range of 4.0–4.5. Santos (1996) reviewed the impact of storage temperature on the synthesis of biogenic amines in vitro and in food (Table 3) and concluded that bacterial activity declined with decreasing temperature. The lowest limiting temperature varied depending on the bacterial species. For example *Klebsiella pneumoniae* UH-2 ceased amine production between 2°C and 10°C and *Enterobacter cloacae* between 10°C and 20°C. It may be assumed that these results are transferable to ensiling conditions although no experimental evidence is available.

3.2. Analytical methods

In this section common analytical methods for detecting amines are briefly mentioned. For detailed information hints are given to relevant literature. Amine analyses are dominated by chromatographic methods like thin layer chromatography (TLC), gas chromatography (GC), capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC) (Önal, 2007). Moreover, Dadáková et al. (2009) mentioned ion exchange chromatography (IEC) and capillary zone electrophoresis (CZE). When using HPLC pre clean-up procedures by means of extraction of the samples with suitable solvents are strongly recommended since they improve the analytical recoveries (Önal, 2007). Numerous studies on food items have addressed sample pre-treatment, e.g. the solvents being used for the extraction of amines from solid food samples, the stationary and mobile phase and its flow rate and the derivatization (Calbiani et al., 2005; Saccani et al., 2005; Lavizzari et al., 2006). Perchloric acid is often used as extraction agent for foods as this extremely strong acid reacts well with the basic amines (Moret and Conte, 1996). Amines are separated as derivatives, mostly dansyl chlorides, which is attributable to a lack of chromophoric groups. Önal (2007) rated derivatization as the decisive step in the analysis for obtaining adequate recoveries for all amines. Since this step is time-consuming, time-saving methods are required. In agricultural research studies related to these issues are needed. The analysis of volatile amines in intestinal digesta and faecal samples can be achieved without derivatization by means of GC, the analysis of non-volatile amines by IEC with ultraviolet detection (Smith and Macfarlane, 1996). Application of these techniques in the field of animal nutrition would be highly desirable for a better understanding of amine-related physiological processes.

Table 3 Influence of duration and temperature of incubation on growth of amine-producing bacteria

Biogenic amine	Bacteria	Time (h unless stated)	Temp. (°C)	Result *	Reference
Histamine	*Klebsiella pneumoniae* UH-2	0–24	37	+	Baranowski et al. (1985)
Histamine	*Klebsiella pneumoniae* UH-2	0–24	25	+	Baranowski et al. (1985)
Histamine	*Klebsiella pneumoniae* UH-2	12–72	10	+	Baranowski et al. (1985)
Histamine	*Klebsiella pneumoniae* UH-2	24–144	2	- **	Baranowski et al. (1985)
Putrescine	*Enterobacter cloacae*	24	20	+	Halász et al. (1994)
Putrescine	*Enterobacter cloacae*	24	10	-	Halász et al. (1994)
Cadaverine	*Klebsiella pneumoniae*	24	20	+	Halász et al. (1994)
Cadaverine	*Klebsiella pneumoniae*	24	10	+ ***	Halász et al. (1994)
Histamine	*Pseudomonas morganis*	1 month	1	-	Halász et al. (1994)
Histamine	*Pseudomonas vulgaris*	1 month	1	-	Halász et al. (1994)
Histamine	*Hafnia* spp.	1 month	1	-	Halász et al. (1994)

* Biogenic amine biosynthesis detected: +; no biogenic amine biosynthesis detected: -.

** Histamine was found: resting cells were capable of producing histamine during storage at low temperature although *Klebsiella pneumoniae* UH-2 did not grow at 2°C.

*** Less extensive than at 20°C.

4. Impact of biogenic amines and gamma-amino butyric acid on dry matter intake

The hypothesis that biogenic amines affect DMI in ruminants has been examined in the past with various forms of administration, dosages and mixtures which complicate the comparison of results. Amines were either added to the silages (Dawson and Mayne, 1995; Van Os et al., 1995a,b, 1996a, 1997; Fusi et al., 2004), directly given orally (Fusi et al., 2004) or via rumen infusion (Neumark et al., 1964; Dawson and Mayne, 1995, 1996, 1997). Daily oral administration of histamine in gelatin capsules (0.5 g/sheep, McDonald et al., 1963) and daily intraruminal infusions of tyramine (3.0 g/heifer, Thomas et al., 1961) had no effect on physiological conditions or well-being of the animals. Feeding silage spiked with histamine in increments up to 1 g/day to sheep over a period of seven days (McDonald et al., 1963) and 5 g/day to heifers had no impact on DMI (Okamoto et al., 1964). In a series of experiments

using varying study designs, Van Os et al. (1995a,b,c, 1996, 1997) observed a negative impact of amines on silage DMI. Obviously data about biogenic amines as potential feed intake depressants are contradictory. This stems mainly from the difficulty of assigning specific effects to a single group of compounds as they always depend on the set of interactions.

4.1. Closer considerations of two studies concerning the impact of biogenic amines on silage dry matter intake

A summary of studies dealing with the impact of biogenic amines on DMI is shown in Table 4. Two of the studies are considered in more detail to identify possible approaches of examining the impact of biogenic amines on feed intake and to demonstrate causal relationships. Neumark et al. (1964) conducted one of the first systematic studies. They evaluated silages from different crops for their amine (histamine, tyramine, tryptamine and phenylethylamine), aldehyde (formaldehyde, acetaldehyde, propionaldehyde and butyraldehyde) and keto-acid (pyruvic acid) content in the context of DMI by ruminants. Silages from green oats, pearl millet, sugar beet tops, vetch and sugar cane were divided into two groups (A and B, each with different crop species), depending on their preference among the animals, which had been tested in preliminary tests. For the trials silages eaten in high amounts were allocated to group A, silages eaten in small amounts to group B. It was striking that the less-favoured silages (B) contained higher levels of biogenic amines and aldehydes, whereas the DM and keto-acid content as well as the acidity were higher in group A silages. Tryptamine, a typical amine in low-quality silages, did not occur in group A. Because silages of both groups comprised certain concentrations of tyramine, it was assumed that all plants already contained small concentrations of tyramine before ensiling. Recent research on stress-induced changes in polyamine and tyramine levels confirm this assumption (Aziz et al., 1998; Németh et al., 2002).

None of the analysed amines (tryptamine, tyramine and histamine) were related to the reduced intake, either singly or in combination (Neumark et al., 1964). Only histamine exerted a synergistic effect in combination with formaldehyde. Intake decline was stronger when formaldehyde and histamine were combined compared with a single dose of formaldehyde at the same amount. When only one third of formaldehyde and the same amount of histamine were fed the intake depression was much weaker, indicating that indeed formaldehyde was primarily responsible for the lowered intake (Neumark et al., 1964). This is in contrast to results of Barry et al. (1973), who demonstrated an increased intake of

formaldehyde-treated silages. These conflicting results may be explained by the assumption that the appetite-depressing effect of formaldehyde was counteracted by the appetite-promoting effect of a decreased concentration of NH_3, organic acids and VFA (Barry and Fennessy, 1972), the protective effect on protein degradation in the rumen and reduced proteolysis during ensiling (Thompson et al., 1981), which might counteract the feed intake depressing effect of amines. Last but not least formaldehyde added as an additive might not reach the reticulo-rumen in the free form but bound to protein and this raises the question if it also binds to amines.

Moreover, a synergy between formic acid and histamine occurred (Neumark et al., 1964). Feed was rejected completely when formic acid and histamine were added to the feed together with formaldehyde. However, there was no influence on feed intake of formic acid in combination with formaldehyde or of infusions of either formic acid or histamine. Single doses of acetaldehyde or butyraldehyde had no appreciable effect on DMI. In total a positive relationship existed between DM content and acidity, while negative correlations were found between DM content and pyruvic acid, formaldehyde and biogenic amines.

Table 4 Overview of effects of biogenic amines and gamma-amino butyric acid on DMI (sorting according to plants and harvest time, additives, number and type of animals, treatments and duration, way of administration, time of measurement and effect)

Plants, harvest time	Additives [L/t, unless stated]	Number/ type of animals	Treatments and duration	Way of administration	Time of measurements	Effect	Reference
Green oats, pearl millet, sugar beet tops, vetch/sugar cane	No additives	Eight Merino rams, two Saanen goats	Treatments were not explicitly illustrated; duration not stated	Introduced into rumen through plastic tube and a fistula	Not stated	No effect of Trm, Tym and Him on DMI; Him + formal-dehyde: DMI ↓	Neumark et al. (1964)
Perennial ryegrass sward; third regrowth	"Maxgrass", based on aliphatic organic acids: [6]; sucrose: 10 kg/t fresh grass	Four steers	Six treatments: Put, Cad, GABA, Put+Cad+GABA, juice expressed from silage, water (control); application of amines at a rate of 2 g/kg DM; each period lasted one week (including four days preliminary period)	Addition to the diet and intra-ruminal infusion (infusion at the same time as the animals were offered their daily portion of food)	Daily: effects on intake: 3 and 24 h post feeding; effect on eating rate: 30, 60, 120 h post feeding; on second day: recording intake every five minutes for the first 3 h after feeding and every 2 h for the next 10 h	Neither addition of amines nor method of application had effect on DMI and eating rate	Dawson and Mayne (1995)
Cocksfoot; first cut of a single sward	Formic acid [4.5]	Eight sheep (Texel wethers)	Four dietary treatments: WAS; FAS; FAS+N; FAS+A; one week	Application to the sheep via polyamide rumen cannula	Intake behaviour: monitored during five consecutive days; pattern of intake registered by feed dispensers	DMI reduced for WAS compared to FAS	Van Os et al. (1995b)

Table 4 Continued

Plants, harvest time	Additives [L/t, unless stated]	Number/ type of animals	Treatments and duration	Way of administration	Time of measurement	Effect	Reference
Perennial ryegrass sward; first regrowth	No additives	Four steers	Ten different treatments: Put, Tym, GABA: application of amines each with a rate of 2, 4 and 6 g/kg DM, water (control); each period lasted one week (including four days preliminary period)	Intraruminal infusion; infusion started when animals were offered their daily portion of food at a constant rate of 0.5 L/h for 8 h	Daily: DMI: 1, 2, 3, 5, 7, 9, 11, 13 and 24 h post feeding; on second day of treatment: recording intake every five minutes for the first 3 h after feeding; from this information calculation of eating rates and daily cumulative DMI; on third day of treatment: effects on rumen fermentation parameters: -1, 0, 1, 2, 3, 5, 8, 11, 15, 23 h post feeding	No effects on DMI; Tym infusion increased pH and isovalerate proportion in rumen fluid	Dawson and Mayne (1996)
Perennial ryegrass sward; primary growth	*Lactobacillus plantarum*, strain MTD /1 [2.5]	Four steers	Nine treatments: Put, GABA, water (control), each combined with three basal diets containing different levels of lactic acid; application of amines at a rate of 6 g/kg DM; each period lasted two weeks	Intraruminal infusion; infusion started when animals were offered their daily portion of food at a constant rate of 1.0 L/h; infusion carried out for 50, 40, 30 min in each h for the first 3 h post feeding; 20 min in each h for the next 3 h, 10 min in each h until 12 h post feeding	Silage was offered in feeding boxes placed on weigh platforms in front of each steer; continuously monitoring throughout the day; by this calculation of daily DMI and eating rate; number and average size and eating rate of secondary meals	Infusion of Put and GABA had no effect on DMI and feeding behaviour; Put infusion reduced ruminal N degrade-ability	Dawson and Mayne (1997)

DM: dry matter; DMI: dry matter intake; Cad: Cadaverine; Him: Histamine; Put: Putrescine; Trm: Tryptamine; Tym: Tyramine; GABA: Gamma-amino butyric acid; N: Nitrogen; P: Phosphate; WAS: silage without an additive; FAS: silage with formic acid; FAS+N: FAS with the addition of 2.9 g NH_3/kg DM; FAS+A: FAS with the addition of 2.8 g amines/kg DM

Van Os et al. (1995b) investigated four different diets consisting of untreated silage, silage treated with formic acid before ensiling, the latter with the addition of 2.9 g NH_3/kg DM and 2.8 g amines/kg DM (cadaverine, 0.6 g; histamine, 0.5 g; putrescine, 0.7 g; tyramine, 1.0 g). The added level of amines reflected the lower part of the range of amine concentrations found in grass silages of medium quality. All silages were prepared from a first cut of a cocksfoot sward in June. The investigation included assessment of daily DMI by sheep, average intake rate, duration, intake and intake rate of the main meal, the number, intake and intake rate of small meals in between the main meals as well as duration, lag time and efficiency of rumination and mastication. Significant effects were revealed only for daily intake rate, initial intake rate of the main meal, number of small meals and average daily duration of rumination and mastication. Altogether, treatments revealed no clear trend. Formic acid compensated for the loss of quality which silage suffered when no additive was added, resulting in low levels of fermentation products (NH_3, VFA, alcohols, amines). This approach undeniably constituted an advance in the evaluation of the influence of amines on feeding behaviour, because previous studies did not try to exclude the effects of other fermentation products on feed intake. Feeding amines versus infusing them intraruminally can be regarded to be the more realistic study design, yet no significant effect on DMI was observed (Dawson and Mayne, 1995). Data on the influence of naturally occurring biogenic amines in silage on DMI and preference behaviour in ruminants are lacking, and results ofsilages spiked with pure amines and intraruminal infusions of biogenic amines are inconsistent and conflictive. This maybe partially due to the experimental conditions in the different studies, which never coincided, e.g. added dosages varied substantially.

4.2. The role of gamma-amino butyric acid in feed intake and animal production

Besides the amines typically occurring in silage, some other nitrogenous compounds are supposed to be important for influencing feed intake and performance of ruminants. Gamma-amino butyric acid is a non-protein amino acid acting as a neurotransmitter. Derived from decarboxylation of l-glutamic acid catalyzed by glutamate decarboxylase (EC 4.1.1.15; Oh and Oh, 2004) it is widely distributed in the central nervous system, peripheral nervous tissue and non-neural tissue (Martin and Rimvall, 1993) and moreover was found in appreciable quantities in grass silage (Ohshima et al., 1979). Besides being part in the regulation of cardiovascular functions (among others blood pressure and heart rate), the sensations of pain and anxiety and playing a role in various neurological diseases (Oh and Oh, 2004) it is involved in the hypothalamic regulation of intake behaviour (Wang et al., 2013b).

Research on effects of GABA on variables concerning feed intake has been conducted for a long time but without showing clear trends. Buchanan-Smith (1982) infused 40 g of GABA into the rumen of sheep fed with lucerne pellets and found a decreased DMI. In subsequent studies rumen infusions of combinations of cadaverine, histamine, putrescine, tyramine and GABA also caused a reduced DMI by sheep (Buchanan-Smith and Phillip, 1986). Furthermore, Buchanan-Smith (1990) examined the effect of GABA in combination with putrescine and cadaverine (0, 1.2, 2.3, 3.5, 4.6 g amine- and GABA-N/kg DM) on DMI of sham fed sheep (intake of feed with a surgically created esophageal fistula that prevents the ingested food from reaching the forestomach). A positive relationship between medium levels of addition and feed intake was observed, while no effect could be noticed with the highest addition. Cole (1992) infused putrescine and GABA to steers intraruminally. Single and combined doses of 6 g and 24 g/kg DM, respectively (corresponding to 1.86 g putrescine-N/kg DM and 3.26 GABA-N/kg DM), based on the average intake during preliminary trials, were used. A trend was observed for a greater intake reduction when putrescine and GABA were given in combination as compared to separate infusions. Lingaas and Tveit (1992) observed a marked reduction in silage DMI by early lactation cows (7.2 kg DM/day compared to the control (8.4 kg DM/day)), when single doses of 100 g/day of putrescine were infused intraruminally. Dawson and Mayne (1995) reported that neither single nor mixed doses of up to 6 g/kg DM of GABA, putrescine and cadaverine, added to the diet or infused intraruminally to steers, resulted in reduced DMI. Infusions of putrescine, tyramine and GABA into the rumen of steers in doses of 2, 4 and 6 g/kg DM (Dawson and Mayne, 1996) and intraruminal infusions of GABA and putrescine (each at 6 g/kg DM) over a period of 14 days did not affect DMI or feeding behaviour of steers (Dawson and Mayne, 1997). However, more than one compound may have a role in intake control and thus, amines and GABA may have greater effects when applied as a mixture with other amines than those mentioned above or with other fermentation products.

4.2.1. Rumen-protected versus non-protected gamma-amino butyric acid

In contrast to the aforementioned studies which revealed negative effects of GABA on DMI recent studies showed a more nuanced picture. Studies dealing with supplementation of non-protected and rumen-protected GABA were conducted to examine effects on DMI and on energy balance, performance, serum metabolites and nutrient digestibility in transition cows (Wang et al., 2013a), early lactating cows (Wang et al., 2013b) and heat-stressed dairy cows (Cheng et al., 2014). Wang et al. (2013a) added doses of 0.6 g/day of non-protected GABA

and 0.6 g/day and 1.2 g/day, respectively, of rumen-protected GABA in a period of six weeks (two weeks before calving until four weeks after calving). Degradation by ruminal microorganisms might be the cause for the lack of effect of non-protected GABA. Doses of 1.2 g/day of rumen-protected GABA resulted in higher DMI in the third and fourth week after calving ($P < 0.05$) and increased DMI and CP intake ($P < 0.01$) in week 4 after calving. Cholecystokinin (CCK; peptide hormone of the gastro-intestinal tract, involved in satiety mechanisms; increments result in reduced feed intake) concentrations in the serum decreased within the second and third week after calving ($P < 0.05$). None of the treatments had an effect on serum concentrations of GABA, neuropeptide Y (NPY; peptide involved in the central nervous control of hunger: increases feed intake and decreases physical activity in response to a plummeting blood glucose level) and leptin (regulates appetite and the feeling of being hungry). The contrary effects of GABA concerning the regulation of appetite likely correspond to regulatory processes of GABA in the hypothalamus (Morley, 1980). The neuroendocrine control of appetite in humans is based on a stimulation of food intake when GABA is injected into the ventromedial hypothalamus, whereas food intake decreases when GABA is injected into the lateral hypothalamus (Morley, 1980). Injections of GABA or GABA analogues into the ventromedial hypothalamus increase appetite by decreasing the serotonin activity, resulting in an inhibition of the ventromedial satiety centre. Decreased serotonin-levels are accompanied with reduced CCK-levels, resulting in an increased feed intake. In contrast, the inhibiting effect of GABA on the lateral hypothalamic dopaminergic system results in decreased feed intake. Obviously CCK plays a major role in regulating appetite and DMI, whereas it is not proven for NPY, which was considered to play a role in the regulation of feed intake (Pu et al., 1999), so that a GABA-related connection between increased DMI and NPY seemed obvious. Serum concentrations of GABA and NPY quadratically increased with rumen-protected GABA (0.8, 1.6 and 2.4 g/day) compared to the control (Wang et al., 2013b). However, despite increased DMI serum concentrations of NPY did not change when adding non-protected (0.6 g/day) and rumen-protected GABA (0.6 g/day and 1.2 g/day) to the feed (Wang et al., 2013a). Thus, other mechanisms than GABA-NPY-mediated processes may be involved in intake regulation. The lack of an effect of NPY might be due to constant serum concentrations of GABA which at least would confirm their synergism. Because DMI increased in the third and fourth week after calving but GABA serum concentrations were unchanged, a direct impact of GABA on DMI is questionable.

Summing up it appears that the contradictory and sometimes conflictive results on the impact of GABA on DMI may result from different modes of action of GABA in the hypothalamus, the administration of non-protected versus rumen-protected GABA or interactions among these factors. Designing experiments that allow investigating the mode of action of GABA on a whole-animal basis are required for a better understanding of its overall role in animals.

4.2.2. Modes of action of gamma-amino butyric acid and effects on rumen fermentation variables

From the previous section it becomes obvious that few data on the modes of action of GABA in ruminants are available but observed effects on DMI may be related to oropharyngeal (smell, taste) or metabolic (satiation) processes (Baile and Della-Fera, 1988). Greenhalgh and Reid (1967) already suggested that the combination of digestibility and palatability is responsible for the level of feed intake since palatability is based on the chemical composition and physical structure of the feed which in turn affect digestibility. Dawson and Mayne (1995) infused GABA into the rumen of steers and added GABA to the diet to elucidate whether it affects DMI via oropharyngeal or feedback mechanisms in the rumen and small intestine but neither route of application decreased DMI.

It was supposed that the animals‘ physiological state played an important role in the reaction to amines including GABA, e.g. growth and stage of lactation change the metabolic situation considerably (Dawson and Mayne, 1995). A study on intra-venous amine infusions in humans revealed increases in blood pressure, respiration rate and blood sugar levels. Furthermore, decreased gut motility and effects on the nervous system were detected (Joosten, 1988). Intravenous injections of histamine also reduced rumen motility (Thomas et al., 1980). However, no effect was observed on DMI when histamine was added to the feed. McDonald et al. (1963) investigated the impact of 0.5 g histamine/day in sheep via capsules on rumen movements, respiration and pulse rates but found no changes compared with pre-treatment values. This was not surprising since no histamine was found in blood samples after the administration. The lack of effect might be due to low absorption of histamine from the gastrointestinal tract as the concentration of histamine in blood may be critical for an impact on physiology and DMI.

The degradation of biogenic amines in rumen contents of wether sheep adapted to diets with different levels of biogenic amines (high: 7.4 g/kg DM, low: 2.4 g/kg DM and without: 0

g/kg DM) was investigated by Van Os et al. (1995c). A mixture of putrescine, cadaverine, histamine and tyramine was added to 200 g rumen contents, followed by 5 h lasting incubation. Amine degradation occurred, but to varying extents. Due to better adaptation of the rumen microflora, the highest proportion of the ruminally added amount of amines was degraded in vitro when animals were adapted to the highest level of amines: 709 g/kg amines versus 542 g/kg amines and 253 g/kg amines, respectively, for the high, low and zero treatments. Higher effectiveness of microorganisms stimulates amine degradation and thus prevents amine accumulation in the rumen. In practice amines may be relevant especially in the short term when changing the feeds since in adapted animals amines are rapidly degraded. Histamine was degraded to the greatest extent, followed by tyramine, putrescine and cadaverine. Ammonia increments were highest in rumen contents from sheep adapted to silage with the highest amine content. Gas production in the fermenters, containing rumen content plus amines, was not affected by amine addition. However, gas production was significantly higher in rumen contents from diets containing amines in comparison to those without (Van Os et al., 1995c).

Changes in rumen pH have also been studied. Silage with added amines prior to feeding led to an increased rumen pH in sheep (Van Os et al., 1995b), whereas the influence of amine and GABA infusion (various levels) was not significant (Dawson and Mayne, 1996), although the concentrations applied to the animals via infusion were higher than those added to the diet. These results confirm it is not the concentration of biogenic amines but the route of application that is crucial for their effect. Moreover, Van Os et al. (1995b) reported an elevated rumen fluid volume, resulting in a lowered osmolality of the rumen fluid. No significant differences were detected in rumen fill between silages without formic acid, added formic acid + NH_3 and added formic acid + amines. Furthermore, treatments did not affect chewing efficiency, rumen pool size, DM and neutral-detergent fibre concentrations in ruminal ingesta or rumen motility.

Intake of large amounts of amines can affect the health status of animals. Křížek (1991, 1993a) discussed that many liver and kidney disorders relate to the detoxification and catabolism of biogenic amines. Histamine and tyramine may also damage rumen mucosa and ruminal microorganisms. High concentrations of orally administered biogenic amines showed negative effects on various organs of goat kids (Fusi et al., 2004) and caused ketonemia – biogenic amines in general and putrescine in particular (Lingaas and Tveit, 1992; Phuntsok et

al., 1998) – and histaminosis (histamine) in ruminants with acidosis (Aschenbach and Gäbel, 2000).

5. Final considerations and conclusions

For an overall understanding of amine formation during ensilage further investigations with emphasis on correlations between the impact of ensiling and storage conditions and extent of amine formation are recommended to reveal relations between cause and effect. Many factors affect quality of forages before, during and after ensilage and many substances are produced due to ensiling, forming a set of interactions that influences the flavour profile and palatability of silage. Therefore, studies about the effects of single groups of substances should be conducted in the context of their environment, i.e. the external conditions and other chemical constituents. Hence, concentrations of biogenic amines in forages always have to be considered in connection with the concentration of other fermentation products like lactic acid, VFA and esters. On the other hand, this situation impedes the attribution of defined effects to biogenic amines.

Histamine is assumed to have the greatest impact on DMI and preference, however, mainly in connection with formaldehyde, which does not occur in silages naturally and has fallen into desuetude compared with the 1980´s. Consequently, investigations should be focused on histamine and the formation and impact of active complexes containing this amine in combination with other fermentation products being present in silage. As the extent of histamine breakdown in the rumen is greater than that of other amines it would be challenging to examine if it is degraded preferentially.

Adaptation of the rumen microflora to amine degradation is facilitated in the presence of high concentrations of biogenic amines in the forage. When these silages are offered to the animals, DMI declines for a short period and returns to a normal range after a few days. Thus, microbial adjustment mechanisms seem plausible. The protection of animals against excessive amine load is one positive side effect of the increased activity of microorganisms. Yet it is still unknown why they proliferate.

Chemical analysis of the feed in order to identify all related substances should be combined with investigations of physiological responses by the animal at the time of feed intake. For the future it would be desirable to include biogenic amines, especially histamine, in routine forage analysis to obtain more data about possible relationships.

The available literature is related to biochemical and physiological relationships between feed intake, performance and amine content of silages. To elaborate more profound causalities the respective features need to be better characterized. Additionally, new features should be created. Studies involving integration of physical and physiological regulation would be an important step forward, for example the possible impacts of silage chop length on proteolysis as well as physiological processes.

References

Aschenbach, J.R., Gäbel, G., 2000. Effect and absorption of histamine in sheep rumen: significance of acidotic epithelial damage. J. Anim. Sci. 78, 464–470.

Aziz, A., Martin-Tanguy, J., Larher, F., 1998. Stress-induced changes in polyamine and tyramine levels can regulate proline accumulation in tomato leaf discs treated with sodium chloride. Physiol. Plantarum 104, 195–202.

Baile, C.A., Della-Fera, M.A., 1988. Physiology of control of food intake and regulation of energy balance in dairy cows, in: Garnsworthy, P.C. (Ed.), Nutrition and Lactation in the Dairy Cow. Butterworths, London, pp. 251–261.

Baranowski, J.D., Brust, P.A., Frank, H.A., 1985. Growth of *Klebsiella pneumoniae* UH-2 and properties of its histidine decarboxylase system in resting cells. J. Food Biochem. 9, 349–360.

Bardócz, S., Duguid, T.J., Brown, D.S., Grant, G., Pusztai, A., White, A., Ralph, A., 1995. The importance of dietary polyamines in cell regeneration and growth. Br. J. Nutr. 73, 819–828.

Bardócz, S., Grant, G., Brown, D.S., Ralph, A., Pusztai, A., 1993. Polyamines in food – implications for growth and health. J. Nutr. Biochem. 4, 66–71.

Barry, T.N., Fennessy, P.F., 1972. The effect of formaldehyde treatment on the chemical composition and nutritive value of silage. I. Chemical composition. N. Z. J. Agric. Res. 15, 712–722.

Barry, T.N., Fennessy, P.F., Duncan, S.J., 1973. The effect of formaldehyde treatment on the chemical composition and nutritive value of silage. III. Voluntary intake, live-weight gain, and wool growth in sheep fed the silages with and without intraperitoneal supplementation with d-l methionine. N. Z. J. Agric. Res. 16, 64–68.

Brink, B., Damink, C., Joosten, H.M.L.J., Huis in't Veld, J.H.J., 1990. Occurrence and formation of biologically active amines in foods. Int. J. Food Microbiol. 11, 73–84.

Buchanan-Smith, J.G., 1982. Voluntary intake in ruminants affected by silage extracts and amines in particular, in: Thomson, D.J., Beever, D.E., Gunn, R.G. (Eds.), Forage Protein in Ruminant Animal Production. Occ. Publ. Br. Soc. Anim. Prod. 6, pp. 180–183.

Buchanan-Smith, J.G., 1990. An investigation into palatability as a factor responsible for reduced intake of silage by sheep. Anim. Prod. 50, 253–260.

Buchanan-Smith, J.G., Phillip, L.E., 1986. Food intake in sheep following intraruminal infusion of extracts from lucerne silage with particular reference to organic acids and products of protein degradation. J. Agric. Sci. 106, 611–617.

Calbiani, F., Careri, M., Elviri, L., Mangia, A., Pistarà, L., Zagnoni, I., 2005. Rapid assay for analyzing biogenic amines in cheese: matrix solid-phase dispersion followed by liquid chromatography-electro-spray-tandem mass spectrometry. J. Agric. Food Chem. 53, 3779–3783.

Campling, R.C., 1966. The intake of hay and silage by cows. Grass Forage Sci. 21, 41–48.

Carpintero, C.M., Henderson A.R., McDonald, P., 1979. The effect of some pre-treatments on proteolysis during the ensiling of herbage. Grass Forage Sci. 34, 311–315.

Charmley, E., 2001. Towards improved silage quality - A review. Can. J. Anim. Sci. 81, 157–168.

Cheng, J.B., Bu, D.P., Wang, J.Q., Sun, X.Z., Pan, L., Zhou, L.Y., Liu, W., 2014. Effects of rumen-protected γ-aminobutyric acid on performance and nutrient digestibility in heat-stressed dairy cows. J. Dairy Sci. 97, 5599–5607.

Cole, M.A., 1992. Effects of fermentation products of silage on its intake by cattle. Ph.D. Dissertation. University of Leeds.

Dadáková, E., Křížek, M., Pelikánová, T., 2009. Determination of biogenic amines in foods using ultra-performance liquid chromatography (UPLC). Food Chem. 116, 365–370.

Dawson, L.E.R., Mayne, C.S., 1995. The effects of either dietary additions or intraruminal infusion of amines and juice extracted from grass silage on the voluntary intake of steers offered grass silage. Anim. Feed Sci. Technol. 56, 119–131.

Dawson, L.E.R., Mayne, C.S., 1996. The effect of intraruminal infusions of amines and gamma-amino butyric acid on rumen fermentation parameters and food intake of steers offered grass silage. Anim. Feed Sci. Technol. 63, 35–49.

Dawson, L.E.R., Mayne, C.S., 1997. The effect of infusion of putrescine and gamma-amino butyric acid on the intake of steers offered grass silage containing three levels of lactic acid. Anim. Feed Sci. Technol. 66, 15–29.

Donaldson, E., Edwards, R.A., 1976. Feeding value of silage: silages made from freshly cut grass, wilted grass and formic acid treated wilted grass. J. Sci. Food Agric. 21, 536–544.

Edmunds, B., Spiekers, H., Südekum, K.-H., Nussbaum, H., Schwarz, F.J., Bennett, R., 2014. Effect of extent and rate of wilting on nitrogen components of grass silage. Grass Forage Sci. 69, 140–152.

Eisner, I., Südekum, K.-H., Kirchhof, S., 2006. Beziehungen zwischen Fermentationscharakteristika von Silagen und der Futteraufnahme von Milchkühen. Übers. Tierernährg. 34, 197–221.

Fusi, E., Rossi, L., Rebucci, R., Cheli, F., Di Giancamillo, A., Domeneghini, C., Pinotti, L., Dell`Orto, V., Baldi, A., 2004. Administration of biogenic amines to Saanen kids: effects on growth performance, meat quality and gut histology. Small Rumin. Res. 53, 1–7.

Glória, M.B.A., Tavares-Neto, J., Labanca, R.A., 2005. Influence of cultivar and germination on bioactive amines in soybeans (*Glycine max* L. Merril). J. Agric. Food Chem. 53, 7480–7485.

Greenhalgh, J.F.D., Reid, G.W., 1967. Separating the effects of digestibility and palatability on food intake in ruminant animals. Nature 214, 744.

Gresner, N., Südekum, K.-H., Höltershinken, M., 2014. Umsetzungen von Stickstoffverbindungen des Futters im Pansen. Übers. Tierernährg. 42, 27–80.

Halász, A., Baráth, A., Simon-Sarkadi, L., Holzapfel, W., 1994. Biogenic amines and their production by microorganisms in food. Trends Food Sci. Technol. 5, 42–48.

Hoedtke, S., Gabel, M., Zeyner, A., 2010. Der Proteinabbau im Futter während der Silierung und Veränderungen der Zusammensetzung der Rohproteinfraktion. Übers. Tierernährg. 38, 157–179.

Hole, J.R., 1985. The nutritive value of silage made from *Poa pratensis* spp. *aligeria* and *Phleum pratense*. V. Amines in silage made from first cut of *Poa pratensis* or *Phleum pratense*. Sci. Rep. Agric. Univ. Norway 64, 1–9.

Huhtanen, P., Khalili, H., Nousiainen, J.I., Rinne, M., Jaakkola, S., Heikkilä, T., Nousiainen, J., 2002. Prediction of relative intake potential of grass silage by dairy cows. Livest. Prod. Sci. 73, 111–130.

Huhtanen, P., Rinne, M., Nousiainen, J., 2007. Evaluation of the factors affecting silage intake of dairy cows: a revision of the relative silage dry-matter intake index. Animal 1, 758–770.

Huis in't Veld, J.H.J., Hose, H., Schaafsma, G.J., Silla, H., Smith, J.E., 1990. Health aspects of food biotechnology, in: Zeuthen, P., Cheftel, J.C., Ericksson, C., Gormley, T.R., Link, P., Paulus, K. (Eds.), Processing and Quality of Foods Vol. 2. Food Biotechnology: Avenues to Healthy and Nutritious Products. Elsevier Applied Science, London and New York, pp. 273–297.

Joosten, H.M.L.J., 1988. The biogenic amine contents of Dutch cheese and their toxicological significance. Neth. Milk Dairy J. 42, 25–42.

Kemble, A.R., 1956. Studies on the nitrogen metabolism of the ensilage process. J. Sci. Food Agric. 7, 125–130.

Krizsan, S.J., Randby, Å.T., 2007. The effect of fermentation quality on the voluntary intake of grass silage by growing cattle fed silage as the sole feed. J. Anim. Sci. 85, 984–996.

Křížek, M., 1991. The determination of biogenic amines in silage. Arch. Anim. Nutr. 41, 97–104.

Křížek, M., 1993a. Biogenic amines in silage. 1. The occurrence of biogenic amines in silage. Arch. Anim. Nutr. 43, 169–177.

Křížek, M., 1993b. Biogenic amines in silage. 2. The dynamics of the formation of biogenic amines in silage. Arch. Anim. Nutr. 43, 179–187.

Křížek, M., Kalač, P., Peterka, J., 1993. Biogenic amines in silage. 3. The occurrence of six biogenic amines in farm-scale grass and maize silages. Arch. Anim. Nutr. 45, 131–137.

Křížek, M., 1995. Possible ways of affecting biogenic-amine content in silages (in Czech). Vet. Med. 40, 111–115.

Lavizzari, T., Veciana-Nogués, M.T., Bover-Cid, S., Mariné-Font, A., Vidal-Carou, M.C., 2006. Improved method for the determination of biogenic amines and polyamines in vegetable products by ion-pair high-performance liquid chromatography. J. Chromatogr. A 1129, 67–72.

Lingaas, F., Tveit, B., 1992. Etiology of acetonemia in Norwegian cattle. 2. Effect of butyric acid, valeric acid and putrescine. J. Dairy Sci. 75, 2433–2439.

Lorenz, M.M., Udén, P., 2011. Influence of formic acid and dry matter on protein degradation in the tanniniferous legume sainfoin. Anim. Feed Sci. Technol. 164, 217–224.

Makoni, N.F., Broderick, G.A., Muck, R.E., 1997. Effect of modified atmospheres on proteolysis and fermentation of ensiled alfalfa. J. Dairy Sci. 80, 912–920.

Marklinder, I., Lönner, C., 1992. Fermentation properties of intestinal strains of *Lactobacillus,* of a sour dough and of a yoghurt starter culture in an oat-based nutritive solution. Food Microbiol. 9, 197–205.

Martin, D.L., Rimvall, K., 1993. Regulation of gamma-aminobutyric acid synthesis in the brain. J. Neurochem. 60, 395–407.

McDonald, P., Macpherson H.T., Watt, J.A., 1963. The effect of histamine on silage dry-matter intake. Grass Forage Sci. 18, 230–232.

McDonald, P., Henderson, A.R., Heron, S.J.E., 1991. The Biochemistry of Silage, second ed. Chalcombe Publications, Marlow.

Moret, S., Conte, L.S., 1996. High-performance liquid chromatographic evaluation of biogenic amines in foods – an analysis of different methods of sample preparation in relation to food characteristics. J. Chromatogr. A 729, 363–369.

Morley, J.E., 1980. The neuroendocrine control of appetite: the role of endogenous opiates, cholecystokinin, TRH, GABA and the diazepam receptor. Life Sci. 27, 355–368.

Neumark, H., Rondi, A., Volcani, R., 1964. Amines, aldehydes and keto-acids in silages and their effect on food intake by ruminants. J. Sci. Food Agric. 15, 487–492.

Németh, M., Janda, T., Horváth, E., Páldi, E., Szalai, G., 2002. Exogenous salicylic acid increases polyamine content but may decrease drought tolerance in maize. Plant Sci. 162, 569–574.

Nishino, N., Hattori, H., Wada, H., Touno, E., 2007. Biogenic amine production in grass, maize and total mixed ration silages inoculated with *Lactobacillus casei* and *Lactobacillus buchneri*. J. Appl. Microbiol. 103, 325–332.

Oh, C.-H., Oh, S.-H., 2004. Effects of germinated brown rice extracts with enhanced levels of GABA on cancer cell proliferation and apoptosis. J. Med. Food 7, 19–23.

Ohshima, M., McDonald, P., 1978. A review of the changes in nitrogenous compounds of herbage during ensilage. J. Sci. Food Agric. 29, 491–505.

Ohshima, M., McDonald, P., Acamovic, T., 1979. Changes during ensilage in the nitrogenous components of fresh and additive treated ryegrass and lucerne. J. Sci. Food Agric. 30, 97–106.

Okamoto, M., Waldo, D.R., Miller, R.W., Moore, L.A., 1964. Histamine levels in forages and dry matter intake of heifers. J. Dairy Sci. 47, 1231–1236.

Önal, A., 2007. A review: current analytical methods for the determination of biogenic amines in foods. Food Chem. 103, 1475–1486.

Pahlow, G., Muck, R.E., Driehuis, F., Elferink, S.J.W.H.O., Spoelstra, S.F., 2003. Microbiology of ensiling, in: Buxton, D.R., Muck, R.E., Harrison, J.H. (Eds.), Silage Science and Technology. American Society of Agronomy Inc., Crop Science Society of America Inc., Soil Science of America Inc., Madison, WI, pp. 31–93, 128, 328–330.

Papadopoulos, Y.A., McKersie, B.D., 1983. A comparison of protein degradation during wilting and ensiling of six forage species. Can. J. Plant Sci. 63, 903–912.

Phuntsok, T., Froetschel, M.A., Amos, H.E., Zheng, M., Huang, Y.W., 1998. Biogenic amines in silage, apparent postruminal passage, and the relationship between biogenic amines and digestive function and intake by steers. J. Dairy Sci. 81, 2193–2203.

Pu, S., Jain, M.R., Horvath, T.L., Diano, S., Kalra, P.S., Kalra, S.P., 1999. Interactions between neuropeptide Y and γ-aminobutyric acid in stimulation of feeding: A morphological and pharmacological analysis. Endocrinology 140, 933–940.

Saarinen, M.T., 2002. Determination of biogenic amines and dansyl derivatives in intestinal digesta and feces by reversed phase HPLC. Chromatographia 55, 297–300.

Saccani, G., Tanzi, E., Pastore, P., Cavalli, S., Rey, M., 2005. Determination of biogenic amines in fresh and processed meat by suppressed ion chromatography-mass spectrometry using a cation-exchange column. J. Chromatogr. A 1082, 43–50.

Santos, S.M.H., 1996. Biogenic amines: their importance in foods. Int. J. Food Microbiol. 29, 213–231.

Slottner, D., Bertilsson, J., 2006. Effect of ensiling technology on protein degradation during ensilage. Anim. Feed Sci. Technol. 127, 101–111.

Smith, E.A., Macfarlane, G.T., 1996. Studies on amine production in the human colon: enumeration of amine forming bacteria and physiological effects of carbohydrate and pH. Anaerobe 2, 285–297.

Steidlová, Š., Kalač, P., 2002. Levels of biogenic amines in maize silages. Anim. Feed Sci. Technol. 102, 197–205.

Steidlová, Š., Kalač, P., 2003. The effects of using lactic acid bacteria inoculants in maize silage on the formation of biogenic amines. Arch. Anim. Nutr. 57, 359–368.

Steidlová, Š., Kalač, P., 2004. The effects of lactic acid bacteria inoculants and formic acid on the formation of biogenic amines in grass silages. Arch. Anim. Nutr. 58, 245–254.

Südekum, K.-H., Eisner, I., 2009. Silagen: Voraussetzungen für eine hohe Akzeptanz und maximale Trockenmasse-Aufnahme. Übers. Tierernährg. 37, 133–140.

Teodorovic, V., Buncic, S., Smiljanic, D., 1994. A study of factors influencing histamine production in meat. Fleischwirtschaft 74, 170–172.

Thaysen, J., 2004. Die Produktion von qualitativ hochwertigen Grassilagen. Übers. Tierernährg. 32, 57–102.

Thiago, L.R.S., Gill, M., Dhanoa, M.S., 1992. Studies of method of conserving grass herbage and frequency of feeding in cattle. 1. Voluntary feed intake, digestion and rate of passage. Br. J. Nutr. 67, 305–318.

Thomas, J.W., Moore, L.A., Okamoto, M., Sykes, J.F., 1961. A study of factors affecting rate of intake of heifers fed silage. J. Dairy Sci. 44, 1471–1483.

Thomas, J.W., Brown, L.D., Emery, R.S., Benne, E.J., Huber, J.T., 1969. Comparison between alfalfa silage and hay. J. Dairy Sci. 52, 195–204.

Thomas, P.C., Kelly, N.C., Chamberlain, D.G., 1980. Silage. Proc. Nutr. Soc. 39, 257–264.

Thompson, D.J., Beever, D.E., Lonsdale, C.R., Haines, M.J., Cammell, S.B., Austin, A.R., 1981. The digestion by cattle of grass silage made with formic acid and formic acid-formaldehyde. Br. J. Nutr. 46, 193–207.

Til, H.P., Falke, H.E., Prinsen, M.K., Willems, M.I., 1997. Acute and subacute toxicity of tyramine, spermidine, spermine, putrescine and cadaverine in rats. Food Chem. Toxicol. 35, 337–348.

Van Os, M., Dulphy, J.P., Baumont, R., 1995a. The influence of ammonia and amines on grass silage intake and intake behaviour in dairy cows. Ann. Zootech. 44, 73–85.

Van Os, M., Dulphy, J.P., Baumont, R., 1995b. The effect of protein degradation products in grass silages on feed intake and intake behaviour in sheep. Br. J. Nutr. 73, 51–64.

Van Os, M., Lassalas, B., Toillon, S., Jouany, J.P., 1995c. In vitro degradation of amines by rumen micro-organisms. J. Agric. Sci. 125, 299–305.

Van Os, M., Dulphy, J.P., Baumont, R., 1996a. The influence of ammonia, biogenic amines and gamma-aminobutyric acid on grass silage intake in sheep. Br. J. Nutr. 76, 347–358.

Van Os, M., Wikselaar Van, P.G., Spoelstra, S.F., 1996b. Formation of biogenic amines in well fermented grass silages. J. Agric. Sci. 127, 97–107.

Van Os, M., Van Vuuren, A., Spoelstra, S., 1997. Mechanisms of adaptation in sheep to overcome silage intake depression induced by biogenic amines. Br. J. Nutr. 77, 399–415.

Wang, D.M., Liu, H.Y., Wang, C., Liu, J.X., Ferguson, J.D., 2013a. Effects of rumen-protected gamma-aminobutyric acid on feed intake, performance and antioxidative status in transition cows. Livest. Sci. 153, 66–72.

Wang, D.M., Wang, C., Liu, H.Y., Liu, J.X., Ferguson, J.D., 2013b. Effects of rumen-protected gamma-aminobutyric acid on feed intake, performance and antioxidative status in early lactating cows. J. Dairy Sci. 96, 3222–3227.

Zaman, M.Z., Abdulamir, A.S., Abu Bakar, F., Selamat, J., Bakar, J., 2009. Review: Microbiological, physicochemical and health impact of high level of biogenic amines in fish sauce. Am. J. Appl. Sci. 6, 1199–1211.

CHAPTER 4

Impact of forage species and ensiling conditions on silage composition and quality and the feed choice behaviour of goats

R. Scherer[1], K. Gerlach[1], J. Taubert[2], S. Adolph[2], K. Weiß[3], K.-H. Südekum[1]

Institute of Animal Science, University of Bonn, Endenicher Allee 15, 53115 Bonn, Germany

[1] Institute of Animal Science, University of Bonn, Bonn, Germany

[2] Institute of Animal Nutrition, Nutrition Diseases and Dietetics, University of Leipzig, Leipzig, Germany

[3] Albrecht Daniel Thaer-Institute of Agricultural and Horticultural Sciences, Humboldt-Universität zu Berlin, Berlin, Germany

Published in **Grass and Forage Science** 74 (2019), 297–313.

Abstract

The hypothesis was that crop species and ensiling conditions have an impact on the formation of biogenic amines and the feed choice of goats in short-term preference trials. At ensiling, lucerne (*Medicago sativa* L., first cut), red clover (*Trifolium pratense* L., first cut), and Italian ryegrass (*Lolium multiflorum* LAM., second cut) were treated differently to obtain a range of fermentation qualities. Six treatments of each forage species were prepared and included different dry-matter concentrations, chemical and biological silage additives, and additions of soil. Silages were sampled for chemical analyses (proximate constituents, fermentation products and other volatile compounds, crude protein fractions and biogenic amines) and stored anaerobically in vacuum-sealed plastic bags for use in preference trials (one for each forage species) with Saanen-type wethers (n = 8 or 6). Each possible two-way combination of the six silage treatments and a standard hay (n = 21 combinations) was offered for ad libitum intake for 3 h. Data were analysed using multidimensional scaling, analysis of variance and correlation analysis between silage characteristics and dry-matter intake (DMI). For each forage species, fermentation characteristics and crude protein fractions revealed only small differences among treatments. Although the degree of proteolysis, as measured by non-protein nitrogen, of all silages was high, the biogenic amine and butyric acid concentrations were low. The different treatments apparently had no direct influence on the formation of biogenic amines and feed choice. The preference behaviour within one forage species was strongly divergent, but the DMI rankings of the three species were very similar.

1. Introduction

Forage grass and legume species are frequently ensiled, causing different degrees of protein and amino acid degradation. Legumes especially are vulnerable to proteolysis due to their low content of water-soluble carbohydrates (WSC), high buffering capacity and high moisture content (McDonald, Henderson, & Heron, 1991). As a result of proteolysis by plant or bacterial proteases, an accumulation of amino acids may occur during all the steps of ensiling and these can further be degraded by desmolysis into biogenic amines, ammonia (NH_3) and butyric acid. Depending on the concentrations, biogenic amines may decrease the dry-matter (DM) intake (DMI) of ruminants (Křížek, 1991, 1993) and give clinical findings (Phuntsok, Froetschel, Amos, Zheng, & Huang, 1998). The studies showing a reduction of DMI were conducted with various forms of administration, dosages and mixtures of amines

(Van Os, Dulphy, & Baumont, 1995), making a sound comparison among studies and a quantitative comparative analysis impossible (Scherer, Gerlach, & Südekum, 2015). Data on the impact of amines that were produced during ensiling on the silage DMI of ruminants are still scarce. In addition, findings on the relationship between amines and fermentation products like fermentation acids, WSC and NH_3-N are lacking. This study aimed to investigate whether amines are formed naturally during ensiling in concentrations such that an influence on feed intake may occur. For this purpose, various ensiling conditions were set to produce grass and legume silages with varying chemical compositions, particularly regarding the levels of amines. The silages were then offered to goats in a feed choice experiment. The hypothesis was that amines affect the short-term DMI of goats.

2. Materials and methods

2.1 Silage preparation

Silages were prepared from pure stands of lucerne (*Medicago sativa* L., first cut, LU), red clover (*Trifolium pratense* L., first cut, RC) and Italian ryegrass (*Lolium multiflorum* LAM., second cut, IR). Lucerne and IR were grown at the Educational and Research Centre Frankenforst of the Faculty of Agriculture, University of Bonn (Königswinter, Germany, 50°43`14``N and 7°12`22``E). Lucerne was harvested on 20 May 2015 and IR on 9 June 2016 at the late vegetative stage. To produce silages with two different DM concentrations, one half was ensiled immediately after reaching a rather low DM (274 g/kg and 251 g/kg, respectively) and the other half after overnight wilting (DM at 380 g/kg and 381 g/kg). Red clover was grown at the Educational and Research Centre Hofgut Neumühle, Münchweiler an der Alsenz, Germany (49°32`18``N and 7°53`0``E) and was also harvested at the late vegetative stage. Harvesting took place twice: in the morning of 2 June 2015 for the silage higher in DM and in the evening of 3 June 2015 for the silage lower in DM. The plant material was ensiled in the morning of 3 June with a DM of 301 g/kg and in the morning of 4 June with a DM of 232 g/kg, respectively. For chemical analyses, a sample (1000 g) of the forages was taken and immediately frozen (-18°C). Another sample (500 g) was prepared for immediate microbiological analyses.

For each plant species, six silage treatments were prepared, each in quadruplicate. The treatments included two different DM levels, three different silage additives and the targeted addition of soil. Detailed information about the DM concentrations and the treatments is

summarized in Table 5. In the following, the silages higher in DM are named CON1, CHE1 and BIO and those lower in DM are referred to as CON2, CHE2 and SOIL (Table 5).

Refined sugar (sucrose) (Diamant Zucker, Pfeifer & Langen, Cologne, Germany) was added to LU and RC at ensiling to ensure adequate substrate availability. The amount was determined based on the average reported levels in LU, RC and IR to bridge the expected differences between the lower sugar concentrations in legumes (65 and 115 g/kg DM, respectively) and the higher sugar concentrations in IR (190 g/kg DM) (Bundesarbeitskreis Futterkonservierung, 2011). For this reason, 125 g/kg DM and 75 g/kg DM sucrose were added to LU and RC, respectively.

The forage was processed in a clean dry place immediately after unloading from the loader wagon. For this purpose, the appropriate quantities were spread on a tarpaulin (3 × 4 m) and treated accordingly. For detailed information about the dosing of the additives, see Table 5. The CHE1 additive was applied with a compressed air spray gun. The BIO additive was applied with spray bottles. For the silage additive based on formic acid (CHE2), an acid-proof pressure sprayer (P5si Industry Drucksprüher 5 L, Hamm, Germany) was used. Soil was evenly distributed as a top layer on the forage and then thoroughly mixed with the forage. After preparing the respective treatment, the forage was compacted in 120-L barrels with a lid in accordance with the recommended densities (for low-DM LU, RC and IR: 210 kg DM/m^3; high-DM: 170 kg DM/m^3 (Honig, 1987)).

After 120 days (d) of ensiling, the barrels were opened. For chemical analyses, a composite sample (1000 g) was taken and immediately frozen (-18°C). Another sample (50.0 g, weighed to the nearest 0.1 g) for the analysis of fermentation variables was taken and also frozen (-18°C). After sampling the four replicates of each treatment, they were combined and thoroughly mixed on a tarpaulin and then stored anaerobically in polyethylenbags (170 µm, 400 mm x 600 mm; Frey Serviceverpackungen, Dombühl, Germany) for subsequent use in the preference trials with goats. About 2.5 kg were packed per bag so that small portions could be thawed each day for feeding.

Table 5 Treatments of lucerne (LU), red clover (RC) and Italian ryegrass (IR), the associated dry matter (DM, %) and the abbreviations of treatments. Sucrose was added at 125g//kg DM to LU and 75 g/kg DM to RC before ensiling

	Substrate		
	LU	RC	IR
Treatment	DM and abbreviation of treatment		
CON1	38 (LU38CON1)	30 (RC30CON1)	38 (IR38CON1)
CHE1	38 (LU38CHE1)	30 (RC30HE1)	38 (IR38CHE1)
BIO	38 (LU38BIO)	30 (RC30BIO)	38 (IR38BIO)
CON2	27 (LU27CON2)	23 (RC23CON2)	25 (IR25CON2)
CHE2	27 (LU27CHE2)	23 (RC23CHE2)	25 (IR25CHE2)
SOIL	27 (LU27SOIL)	23 (RC23SOIL)	25 (IR25SOIL)

BIO: biological additive (1 g/t) based on homofermentative lactic acid bacteria (*Lactobacillus plantarum*; $3.0 \cdot 10^{11}$ colony-forming units/g); CON1 and CON2: untreated; CHE1: chemical silage additive (2.5 L/t) based on sodium nitrite and hexamine (hexamethylenetetramine); CHE2: chemical silage additive (4 L/t) based on 75% formic acid buffered with sodium hydroxide to pH 2.5; SOIL: addition of 7,600 g soil/t.

2.2 General analyses

Silage samples were freeze-dried (P18K-E-6, serial number 20100305; Dieter Piatkowski, Munich, Germany) in duplicate. After pre-drying at 60°C, the DM of the silages was estimated by oven-drying a triplicate subsample overnight at 105°C. A correction of DM (DM_{cor}) for the loss of volatiles during drying was conducted using the following equation (Weißbach & Strubelt, 2008): $DM_{cor} = DM + (1.05 – 0.059 \cdot pH) \cdot$ total volatile fatty acids (VFA, C2 – C6) + 0.08 · lactic acid + 0.77 · 1,2-propanediol + 0.87 · 2,3-butanediol + 1.00 · total of other alcohols (C2 – C4). All concentrations are expressed as g/kg.

Proximate analyses of the silage samples and of the LU and grass hays were performed according to VDLUFA (2012), and method numbers given below. Ash and crude lipids (CL) were analysed using methods 8.1 and 5.1. Crude protein (CP) was determined by Dumas combustion (4.1.2, Elementaranalysator rapid micro N cube, Elementar Analysensysteme GmbH, Langenselbold, Germany). Crude protein fractionation was conducted according to the Cornell net carbohydrate and protein system (Licitra, Hernandez, & Van Soest, 1996), comprising fractions A (non-protein N (NPN)), B1 (buffer-soluble true protein), B2 (buffer-insoluble true protein), B3 (cell-wall associated true protein) and C (insoluble in acid

detergent, indigestible). The concentrations of neutral detergent fibre (NDF) (6.5.1; assayed with heat-stable amylase (aNDF)), acid detergent fibre (ADF) (6.5.2) and acid detergent lignin (ADL) (6.5.3) were analysed using the Fiber Analyzer Ankom A2000 (Ankom Technology, Macedon, NY, USA).

In accordance with point 8.8 of method 6.5.2, the analysis of ADFom was performed sequentially for pectin-containing LU and RC samples. The NDF and ADF values are expressed exclusive of residual ash (aNDFom, ADFom).

The Hohenheim gas test (25.1) was conducted to measure the 24 h in vitro gas production (GP) and estimate the concentration of metabolizable energy (ME) of the grass silages using the following equations:

Grass silages (GfE, 2008): ME (MJ/kg DM) = 7.81 + 0.07559 · GP – 0.00384 · ash + 0.00565 · CP + 0.01898 · CL – 0.00831 · ADFom.

Lucerne and RC silages (GfE, 2017): ME (MJ/kg organic matter) = 12.49 – 0.0114 · ADFom + 0.00425 · CP + 0.0269 · CL + 0.01683 · GP; ME (MJ/kg DM) = ME (MJ/kg organic matter) · [1000 – ash]/1000. Ash, CP, CL, and ADFom are in g/kg DM and GP is in mL/200 mg DM.

2.3 Chemical analyses of fermentation variables

Frozen subsamples (50.0 g) of silages were used for the determination of lactic acid, pH, volatile fatty acids (VFA), alcohols (methanol, ethanol, propanol, 1,2-propanediol, 2,3-butanediol, 1-butanol, and 2-butanol), acetone, NH_3-N, and WSC. Furthermore, the silages were analysed for ethyl lactate, ethyl acetate and propyl acetate. Cold-water extracts were prepared by blending the frozen samples with a mixture of 300 ml distilled water and 1 ml toluol, kept in a refrigerator overnight and afterwards filtered with a folded filter paper (MN 615; Macherey-Nagel, Düren, Germany). The pH in the extract was determined potentiometrically using a calibrated pH electrode. The extract was filtered through a Minisart syringe filter (pore size 0.45 µm; Sartorius, Göttingen, Germany) according to Weiß and Kaiser (1995). Volatile fatty acids and alcohols were determined by gas chromatography (flame ionization detector, Shimadzu Deutschland, Duisburg, Germany), as described by Weiß (2001). The analysis of ethyl esters as well as acetone, propanol, methanol, 1-butanol and 2-butanol was performed following Weiß and Sommer (2012). The lower detection limit for VFA and alcohols was 0.01%, and for esters it was 0.001%. The NH_3-N concentration was analysed colourimetrically, based on the Berthelot reaction, using a continuous flow

analyser (Skalar Analytical, Breda, the Netherlands). The concentration of WSC was determined with the anthrone method according to Von Lengerken and Zimmermann (1991). The fermentation quality of the silages was assessed by the DLG scheme (DLG, 2006), based on the concentrations of acetic acid and butyric acid and the pH.

2.4 Sample preparation and chromatographic analyses of biogenic amine determination

The freeze-dried samples were analysed for histamine, alpha-amino butyric acid (AABA), beta-amino butyric acid (BABA), gamma-amino butyric acid (GABA), cadaverine, putrescine and tyramine. An exactly weighed (0.5 g) of a lyophilized and homogenized sample was dissolved in 20 ml 5% trichloroacetic acid (TCA) and stirred for 30 min. A volume of 1 ml extract was centrifuged at 23,184 · g at 4°C for 10 min after sedimentation of undissolved material. The supernatant was twofold to sevenfold diluted with 5% TCA. The internal standard (10 µM 1,7-diaminoheptane), containing samples and calibration standards were dried with a SpeedVac concentrator without heating procedures. A calibration standard contained 25 µM histamine, 25 µM tyramine, 10 µM putrescine, 10 µM cadaverine, 25 µM GABA, 25 µM BABA, 25 µM AABA and 10 µM 1.7-diaminoheptane dissolved in 0.1 M hydrochloric acid. Pre-column derivatization was achieved as described elsewhere, including small modifications (Ebert, 1986). The derivatization mixture was prepared in a ratio of 7:1:1:1 (96% ethanol:H_2O:triethylamine:phenylisothiocyanate), and 20 µl were added to a solvent-evaporated sample or standard mixture. The reaction mixture was incubated for 20 min in the dark followed by a vaporization step via SpeedVac concentrator. Samples and standards were redissolved in 100% acetonitrile and filtered through a 0.2 µm membrane. Amine determination was performed through high-performance liquid chromatography as phenylthiocarbamyl derivatives using a reversed phase column (Kinetex® 5 µM, C18; Phenomenex, Aschaffenburg, Germany) tempered at 40°C. The separation of amine derivatives was conducted with mobile phase solvent A, consisting of 20 mM KH_2PO_4 (pH 2.0, adjusted with H_3PO_4) and 10% acetonitrile, and solvent B, consisting of 75% acetonitrile. The linear gradient elution programme was configured from 20% solvent B to 100% solvent B in 20 min with a flow rate of 1.0 ml/min. Analytes were detected through the measurement of absorbance at 254 nm.

2.5 Microbiological analyses

A composite sample (500 g) of each forage species directly after harvesting and for each silage treatment after silo opening was put into a polyethylene bag using sterile gloves, then sealed anaerobically and sent immediately in a chilled box to a laboratory (Wessling Laboratorien, Altenberge, Germany). Aerobic mesophilic bacteria, yeasts and moulds were determined according to VDLUFA (2012; method 28.1.1–28.1.4). Anaerobic lactate utilising sporulating microbes was determined according to WEC (028) (CNERNA, 1986), lactic acid bacteria according to ISO 15214 (1998), and clostridial endospores according to Pahlow (1986). All microbial counts were log 10-transformed to obtain log-normal distributed data and presented on a wet-weight basis. In order to calculate the averages, the values below detection level were assigned as values corresponding to half of the detection level (Tabacco, Piano, Cavallarin, Bernardes, & Borreani, 2009).

2.6 Preference trials

Feed choice behaviour was evaluated by conducting a preference trial design according to Burns, Fisher, & Mayland (2001). The trial was divided into three runs (one for each forage species) and was conducted with Saanen-type wethers (German Improved White Goat breed, mean (SD) body weight 104 kg (± 3.2), 105 kg (± 2.2) and 93 kg (± 7.8 kg) respectively). Animal care and handling were undertaken following the official German regulations. Eight wethers were used for the legume silages and six wethers for the IR silages. Two wethers shared an indoor pen of approximately 2 x 3 m bedded with straw. Every morning, before offering the silages, the animals were tethered for the duration of experimental feeding, and it was ensured that they could drink and lie down.

During an adaptation period prior to the experimental phase (Kyriazakis, Emmans, & Whittemore, 1990), single meals of each silage treatment of LU, RC, and IR, respectively, and LU hay were offered once to allow the animal to associate the silage with post-ingestive metabolic response, taste and smell. For each run, the adaptation period lasted 7 days (six silage treatments and LU hay). Lucerne hay was used as a standard in each run so that quantitative comparisons between the three runs were possible. The forages were offered in randomized order. During the subsequent experimental phase, each possible two-way combination of the six silages and the LU hay (n = 21 combinations) was presented. Forages were offered in plastic boxes (400 x 340 x 250 mm), and the forage pairs were presented side by side. The forages were randomly allocated each day to prevent a habit reflex, as one

position might be perceived to be more convenient by the animal than the other (Meier, Kreuzer, & Marquardt, 2012). Goats had free access to both feeding boxes so that free choice between the two forages could be guaranteed. The boxes were weighed 30 min after starting to feed (initial feed intake) and after 3 hr (Buntinx, Pond, Fisher, & Burns, 1997). To ensure *ad libitum* feed intake, the respective forage was refilled as soon as less than 300 g remained in the box. Each day, the experimental meal was offered for 3 hr, starting at 7:30. In the afternoon, starting at 15:30, grass hay was offered for *ad libitum* consumption for two hours. The concentrations (g/kg DM) of ash, CP, CL, ADFom, aNDFom and ADL of the grass hay were on average 53.8, 85.9, 15.4, 379, 660 and 54.5. The concentration of ME (MJ/kg DM) was 8.6. Each run lasted for 28 days, consisting of 7 days for adaptation and 21 days for experimental measurements.

2.7 Statistical analyses

All the data were analysed using SAS 9.4 (SAS®, 2010). The experimental design allowed statistical analysis of the preference trial by multidimensional scaling (MDS), as previously described by Buntinx et al. (1997) and Burns et al. (2001). This procedure was used to develop a spatial arrangement representing the differences expressed as selective forage intake by the animals. The difference in preference between a pair of silages was expressed by subtracting the amount of the least-preferred forage from the most-preferred forage and dividing the difference by the sum of both intakes. In this way, preference was expressed numerically as a relative difference or distance. If an animal consumed equal quantities in one pair, the difference ratio was equal to zero and no preference or distance between the silages was expressed. If only one of the pairs was consumed, the difference ratio was equal to one and the maximum difference in preference between forages was expressed (Buntinx et al., 1997). PROC MDS is an iterative fitting procedure for data with the aim of expressing distances or relative differences between stimuli (e.g., forages) in an unknown number of orthogonal dimensions, as described by Burns et al. (2001). A least-squares fit is approximated using an array of points representing the different stimuli. The coordinates of the points are adjusted iteratively until the reduction in the residual sum of squares is below a specified level. The residual sum of squares is calculated by comparing the “distance” between the points representing the stimuli and the observed distances or differences between the stimuli. Subsequently, a map is developed with points representing each stimulus (Burns et al., 2001). Forages with coordinates that are similar in the dimensional space are modelled as similar in preference and, conversely, coordinates that are far from each other in the

dimensional space indicate forages differing in preference (Buntinx et al., 1997). The order of fit is dimension one first, which will generally include the most important variables (most sums of squares), followed by dimension two (Burns et al., 2001). The forage with the highest DMI in each run was used as a positive control by assigning it positive coordinates (Burns et al., 2001). Consequently, forage with two positive dimensions represents preference, while two negative dimensions indicate avoidance.

Each run was also tested through a two-factorial analysis of variance after averaging the DMI of each forage (averaged across each combination, $n = 6$). The analysis of variance only included terms for the animal and forage. Within the forage treatments, the means were separated using the minimum significant difference (MSD) from the Waller–Duncan *k*-ratio *t*-test ($k = 100$) (Burns et al., 2001). For each of the three forage species, a one-factorial analysis of variance was performed with the SAS MIXED procedure using the restricted maximum-likelihood algorithm (Schabenberger & Pierce, 2002) to test the effect of the silage treatments on proximate constituents, fermentation characteristics and biogenic amines. When significant effects were detected by the global F-test, pairwise comparisons were performed using the Tukey test. Furthermore, correlation coefficients of LU, RC and IR silage characteristics and DMI were calculated ($n = 48$ (LU and RC silages, respectively) and $n = 36$ (IR silages)). Significance was declared at $p < 0.05$, whereas a trend towards a significant effect was noted when $0.05 \leq p \leq 0.10$. The simple linear regression technique was used to associate the amine concentrations with the 3 hr DMI.

3. Results

3.1 General chemical composition

The chemical composition of the crops before ensiling is shown in Table 6, and the results of the chemical analyses of the LU silages are presented in Table 7. Lucerne silages showed lactic acid concentrations in the range of 44 to 88 g/kg DM. Moderate acetic acid concentrations (<20 g/kg DM) were found in four of the six silages; only the untreated low-DM silage and the soil-treated low-DM silage had greater concentrations (>30 g/kg DM). Butyric acid was found in the formic acid-treated low-DM silage, the untreated high-DM silage and the high-DM silage treated with homofermentative lactic acid bacteria, but only in negligible concentrations. Ethanol was detected in all the LU silages, but the concentration exceeded 10 g/kg DM only in the soil-treated low-DM silage. Propanol was found only in the

low-DM LU silages, and the greatest values were observed in the soil-treated low-DM silage. The fermentation quality according to the DLG scoring (DLG, 2006) showed the high-DM silage treated with the biological additive to be the best silage (total score 100 points, i.e. "very good"). The low-DM silage treated with formic acid, the high-DM untreated control and the high-DM silage treated with the chemical additive were also given the classification "very good", but with minimal reductions (total score 95) due to a slightly increased pH. Due to an increased pH and increased acetic acid concentrations, the untreated low-DM control only scored 75 points. The very high acetic acid concentration in the soil-treated low-DM silage resulted in the judgement "bad" and only 50 points.

The chemical composition of RC silages is shown in Table 8. The lactic acid concentrations in the RC silages, like the LU silages, ranged from low to normal. Except for the soil-treated low-DM silage, acetic acid remained below 30 g/kg DM. Traces of butyric acid were found only in the high-DM untreated control. Ethanol was present in all the silages in low concentrations. According to the DLG scoring, five of the silages received the classification "very good" with total scores of 95 (RC23CON2 and RC23CHE2) and 100 points (RC30CON1, RC30CHE1 and RC30BIO). Due to the high content of acetic acid, the soil-treated low-DM silage was classified as "needs to be improved".

The results of the chemical analyses of the IR silages are presented in Table 9. The lactic acid concentrations were in the same range as for the legume silages (42–83 g/kg DM). Acetic acid ranged within typical values for well-fermented silages. Butyric acid was not present in any of the silages to a notable extent except for the untreated low-DM control (1.5 g/kg DM), although the critical value of 3 g/kg DM was not reached. All the IR silages were classified as "very good", with the high-DM silage treated with the biological additive being the best with 100 points, followed by the other treatments with 95 points.

3.2 Nitrogen compounds

3.2.1 Crude protein fractions

Detailed information about the CP fractions of the forages before ensiling is shown in Table 6. The CP fractions of the resulting LU silages are presented in Table 7 and those of the RC silages in Table 8 and the IR silages in Table 9. Within the crop species, the treatments had varying effects on the fractions, ranging from moderate to strong. The biggest differences between the treatments occurred for fractions B1 and B3. This was observed consistently for all three crop species. The highest concentrations of NPN were found in the LU silages (784 g

kg/CP in LU38CON1) followed by the IR silages, in which the NPN concentrations of all the treatments were around 600 g/kg CP. The NPN concentrations in the RC silages were lower, more variable, and ranged between 395 and 513 g/kg CP. The correlation coefficients between NPN and NH_3-N of silages showed a wide range, with positive values for the LU (0.41, $p < 0.0001$) and RC silages (0.34, $p < 0.0001$) and a negative value for the IR silages (-0.15, $p < 0.01$).

3.2.2 Biogenic amines and ammonia

The concentrations of total biogenic amines (TBA) were between 1.2 and 4.1 g/kg DM (Table 10). Lucerne silages had the greatest TBA concentrations; they all contained > 2.0 g/kg DM, with the highest concentrations in the soil-treated low-DM silage. In the RC silages, the TBA concentrations were <2.0 g/kg DM; in the IR silages, they ranged from 1.7 to 2.5 g/kg DM. In the RC and IR silages, the highest amine concentrations were found in the untreated low-DM control. All the analysed amines were detected only in the soil-treated low-DM LU silage. In general, the concentrations of amines in the silages differed greatly. Putrescine was only found in the untreated and the soil-treated low-DM LU and IR silages and the soil-treated low-DM RC silages. Cadaverine was not found in the RC silages, but in LU27CON2, LU27SOIL, IR25CON2 and IR25SOIL as well as in IR38CON1. Histamine was the most widespread amine followed by GABA and BABA. In LU silages, it accounted for 48, 59, 54, 17, 56 and 7%, respectively, of TBA in CON1, CHE1, BIO, CON2, CHE2 and SOIL. In IRC silages, it represented 54, 68, 54, 55, 77 and 23% of TBA, respectively, and 34, 47, 41, 14, 37 and 12% of TBA in the IR silages, respectively. Alpha-amino butyric acid was only found in the soil-treated low-DM silage. The correlation coefficient between NH_3 and TBA of the 18 silages was $r = 0.89$. The share of ammonia of NPN in the LU silage was in the range of 12 to 18%; in the RC silages, it accounted for 9 to 17%, and in the IR silages, it was between 9% and 15%.

Table 6 Chemical composition and crude protein fractions of the substrates before ensiling (g/kg DM unless stated) and the associated DM

	Lucerne		Red clover		Italian ryegrass	
Dry matter	274	380	232	301	251	381
Ash	102	115	105	117	104	113
Crude protein (CP)	193	171	151	142	119	116
Crude lipids	19.1	20.3	23.3	24.1	24.3	23.2
aNDFom	446	448	399	402	550	552
ADFom	327	313	256	241	339	331
ADL	93.0	82.4	91.1	81.6	39.8	40.8
ME (MJ/kg DM)	10.9	11.0	12.8	12.2	9.5	9.4
CP fractions (g/kg CP)						
A	341	407	289	279	278	312
B1	112	21	-*	-	50	34
B2	463	474	493	502	564	453
B3	39	44	172	177	75	158
C	46	54	53	50	33	43

Notes: ADF: Acid detergent fibre, expressed exclusive of residual ash; ADL: acid detergent lignin; A: non-protein N, enzymatic degradation not applicable; B1: buffer-soluble true protein, quickly degraded; B2: buffer-insoluble true protein, intermediately degraded; B3: cell wall-associated true protein, intermediately to slowly degraded; C: insoluble in acid detergent, indigestible; DM: dry matter; ME: metabolisable energy; NDF: neutral detergent fibre, assayed with a heat-stable amylase and expressed exclusive of residual ash.

* No data available.

Table 7 Density and chemical composition of lucerne (LU) silages (g/kg DM unless stated) (n = 4)

Variable	Treatment[‡]						HSD[§]
	LU38CON1	LU38CHE1	LU38BIO	LU27CON2	LU27CHE2	LU27SOIL	
Density (g DM/m^3) [¶]	170	170	170	210	210	210	
Dry matter (g/kg)	374	384	382	283	264	276	
Ash	106^{a}	112^{ab}	116^{ab}	120^{b}	121^{b}	144^{c}	10.64
Crude protein (CP)	171^{ab}	174^{ab}	168^{a}	169^{ab}	170^{ab}	178^{b}	8.9
Crude lipids	25^{ab}	27^{ab}	28^{ab}	30^{ab}	25^{a}	30^{b}	4.96
Neutral detergent fibre[**]	393^{a}	380^{a}	385^{a}	446^{b}	398^{a}	439^{b}	41.42
Acid detergent fibre[††]	326^{a}	310^{a}	300^{a}	322^{a}	320^{a}	324^{a}	28.65
Acid detergent lignin	79^{a}	75^{a}	76^{a}	82^{a}	81^{a}	85^{a}	10.3
24 h gas production (mL/g DM)	243^{b}	243^{b}	242^{b}	227^{ab}	239^{ab}	218^{a}	23.85
Metabolisable energy (MJ/kg DM)	11.0^{a}	10.9^{a}	11.3^{a}	11.1^{a}	11.0^{a}	11.4^{a}	0.88
pH	4.57^{b}	4.81^{c}	4.31^{a}	4.28^{a}	4.27^{a}	4.55^{b}	0.12
Lactic acid	59^{ab}	44^{a}	74^{bd}	88^{d}	78^{cd}	66^{bc}	15.86
Acetic acid	19^{a}	17^{a}	15^{a}	42^{b}	20^{a}	62^{c}	9.12
Butyric acid[‡‡]	0.1^{a}	-[*]	0.1^{a}	-	0.2^{a}	-	0.53
Propionic acid	-	-	-	-	-	3.0	-
1,2-Propanediol	0.3^{ab}	0.1^{a}	-	0.9^{c}	0.6^{bc}	-	0.49
2,3-Butanediol	0.1^{a}	0.2^{a}	0.1^{a}	-	-	-	0.15
Ethanol	7.6^{bc}	2.2^{a}	6.2^{b}	9.7^{c}	4.8^{ab}	14.8^{d}	3.42
Methanol	2.1^{ac}	1.8^{a}	2.0^{ab}	1.8^{a}	2.8^{c}	2.7^{bc}	0.86
Propanol	-	-	-	7.0^{b}	0.8^{a}	16.8^{c}	4.84

Table 7 Continued

Variable	Treatment[‡]						HSD[§]
	LU38CON1	LU38CHE1	LU38BIO	LU27CON2	LU27CHE2	LU27SOIL	
1-Butanol (mg/kg DM)	-[†]	-	-	-	-	16	-
2-Butanol (mg/kg DM)	-	-	-	167^{b}	28^{a}	423^{c}	92.5
Ethyl lactate (mg/kg DM)	63^{a}	-	97^{ab}	174^{bc}	51^{a}	207^{c}	96.06
Ethyl acetate (mg/kg DM)	96^{ab}	-	54^{ab}	261^{b}	25^{a}	659^{c}	225
Propyl acetate (mg/kg DM)	-	-	-	383^{a}	-	1427^{b}	508.34
Ammonia-N (g/kg N)	112^{ab}	107^{ab}	88^{a}	129^{bc}	105^{ab}	147^{c}	29.3
Water-soluble carbohydrates	61^{ab}	67^{ab}	91^{b}	14^{ab}	86^{ab}	11^{a}	81.26
CP fractions (g/kg CP)							
A	784^{d}	666^{a}	668^{a}	751^{c}	696^{b}	766^{cd}	20.86
B1	13^{a}	58^{c}	68^{d}	26^{b}	25^{b}	10^{a}	8
B2	141^{a}	200^{c}	190^{c}	161^{b}	205^{c}	160^{b}	18.52
B3	8^{b}	-	3^{a}	5^{ab}	12^{c}	7^{b}	3.38
C	54^{a}	80^{e}	70^{d}	57^{ab}	62^{c}	59^{bc}	4.37

Notes. Means within a row with different superscripts (a–d) differ ($p < 0.05$); $n = 4$.

A: non-protein N, enzymatic degradation not applicable; B1: buffer-soluble true protein, quickly degraded; B2: buffer-insoluble true protein, intermediately degraded; B3: cell wall-associated true protein, intermediately to slowly degraded; C: insoluble in acid detergent, indigestible; DM: dry matter. [*]Below detection limit (0.01%). [†]Below detection limit (0.001%). [‡]BIO: around 38% DM and biological additive (1 g/t), based on homofermentative lactic acid bacteria (*Lactobacillus plantarum*; $3.0 \cdot 10^{11}$ colony-forming units/g); CON1: untreated and around 38% DM; CHE1: around 38% DM and chemical silage additive (2.5 L/t), based on sodium nitrite and hexamine (hexamethylenetetramine); CON2: untreated and around 27% DM; CHE2: around 27% DM and chemical silage additive (4 L/t), based on 75% formic acid buffered with sodium hydroxide to pH 2.5; SOIL: around 27% DM and addition of 7,600 g soil/t. [§]Tukey's honestly significant difference ($\alpha = .05$). [¶]Mean substrate density before ensiling, $n = 4$. [**]Analysed with heat-stable amylase and expressed exclusive of residual ash. [††]Expressed exclusive of residual ash. [‡‡]Sum of i-/n-butyric acid, i-/n-valerian acid, and n-caproic acid.

Table 8 Density and chemical composition of red clover (RC) silages (g/kg DM unless stated) (n = 4)

Variable	Treatment[‡]						HSD[§]
	RC30CON1	RC30CHE1	RC30BIO	RC23CON2	RC23CHE2	RC23SOIL	
Density (g DM/m^3) [¶]	170	170	170	210	210	210	
Dry matter (g/kg)	301	301	301	230	233	234	
Ash	103^{a}	104^{a}	105^{a}	104^{a}	103^{a}	130^{b}	21.23
Crude protein (CP)	159^{b}	161^{b}	153^{ab}	159^{b}	147^{a}	162^{b}	10.35
Crude lipids	28^{ab}	30^{bc}	24^{a}	28^{ac}	24^{a}	32^{c}	4.44
Neutral detergent fibre[**]	335^{ab}	349^{ab}	352^{ab}	333^{a}	335^{a}	365^{b}	29.96
Acid detergent fibre[††]	241^{a}	246^{a}	243^{a}	238^{a}	245^{a}	248^{a}	18.67
Acid detergent lignin	42^{a}	49^{a}	51^{a}	46^{a}	43^{a}	49^{a}	15.31
24 h gas production (mL/g DM)	275^{b}	269^{b}	265^{b}	271^{b}	277^{b}	243^{a}	17.21
Metabolisable energy (MJ/kg DM)	12.1^{a}	12.1^{a}	11.9^{a}	12.1^{a}	12.0^{a}	12.0^{a}	0.24
pH	4.36^{e}	4.48^{f}	3.98^{a}	4.12^{c}	4.25^{d}	4.03^{b}	0.04
Lactic acid	65^{b}	60^{ab}	87^{c}	87^{c}	55^{a}	95^{c}	9.3
Acetic acid	24^{b}	26^{c}	17^{a}	29^{d}	17^{a}	46^{e}	2.39
Butyric acid[‡‡]	0.3	-[*]	-	-	-	-	-
Propionic acid	-	-	-	-	0.1^{a}	1.2^{b}	0.24
1,2-Propanediol	0.1^{a}	-	-	0.3^{a}	-	-	0.37
2,3-Butanediol	0.1^{a}	1.3^{c}	0.6^{b}	0.5^{ab}	0.2^{ab}	0.5^{ab}	0.43
Ethanol	6.9^{b}	1.5^{a}	7.1^{b}	9.6^{c}	2.5^{a}	11.5^{d}	1.06
Methanol	3.0^{a}	3.6^{ab}	3.2^{a}	3.4^{ab}	3.8^{b}	3.4^{ab}	0.53
Propanol	-	-	-	-	-	9.7	-

Table 8 Continued

Variable	Treatment[‡]						HSD[§]
	RC30CON1	RC30CHE1	RC30BIO	RC23CON2	RC23CHE2	RC23SOIL	
1-Butanol (mg/kg DM)	-[†]	-	-	-	-	-	-
2-Butanol (mg/kg DM)	-	-	-	-	-	649	-
Ethyl lactate (mg/kg DM)	59^{a}	-	124^{b}	85^{a}	-	240^{c}	34.28
Ethyl acetate (mg/kg DM)	24^{a}	-	15^{a}	76^{ab}	-	138^{b}	94.79
Propyl acetate (mg/kg DM)	-	-	-	-	-	194	-
Ammonia-N (g/kg N)	72^{d}	83^{e}	42^{a}	67^{c}	54^{b}	67^{c}	3.36
Water-soluble carbohydrates	127^{bc}	155^{c}	130^{bc}	87^{b}	237^{d}	31^{a}	50.68
CP fractions (g/kg CP)							
A	478^{c}	396^{ab}	395^{a}	493^{cd}	417^{b}	513^{d}	20.84
B1	21^{b}	25^{b}	17^{ab}	84^{c}	20^{ab}	11^{a}	9.5
B2	336^{b}	351^{b}	375^{c}	287^{a}	339^{b}	343^{b}	19.51
B3	115^{c}	151^{e}	130^{d}	64^{a}	146^{e}	92^{b}	11.04
C	50^{a}	77^{cd}	83^{d}	72^{c}	78^{cd}	62^{b}	8.18

Notes. Means within a row with different superscripts (a–f) differ ($p < 0.05$); $n = 4$.

A: non-protein N, enzymatic degradation not applicable; B1: buffer-soluble true protein, quickly degraded; B2: buffer-insoluble true protein, intermediately degraded; B3: cell wall-associated true protein, intermediately to slowly degraded; C: insoluble in acid detergent, indigestible; DM: dry matter. [*]Below detection limit (0.01%). [†]Below detection limit (0.001%). [‡]BIO: around 30% DM and biological additive (1 g/t), based on homofermentative lactic acid bacteria (*Lactobacillus plantarum;* $3.0 \cdot 10^{11}$ colony-forming units/g); CON1: untreated and around 30% DM; CHE1: around 30% DM and chemical silage additive (2.5 L/t), based on sodium nitrite and hexamine (hexamethylenetetramine); CON2: untreated and around 23% DM; CHE2: around 23% DM and chemical silage additive (4 L/t), based on 75% formic acid buffered with sodium hydroxide to pH 2.5; SOIL: around 23% DM and addition of 7,600 g soil/t. [§]Tukey's honestly significant difference ($\alpha = .05$). [¶]Mean substrate density before ensiling, $n = 4$. [**]Analysed with heat-stable amylase and expressed exclusive of residual ash. [††]Expressed exclusive of residual ash. [‡‡]Sum of i-/n-butyric acid, i-/n-valerian acid, and n-caproic acid.

Table 9 Density and chemical composition of Italian ryegrass (IR) silages (g/kg DM unless stated) (n = 4)

Variable	Treatment[‡]						HSD[§]
	IR38CON1	IR38CHE1	IR38BIO	IR25CON2	IR25CHE2	IR25SOIL	
Density (g DM/m^3)[¶]	170	170	170	210	210	210	
Dry matter (g/kg)	369	395	378	244	257	252	
Ash	121^{a}	118^{a}	121^{a}	125^{ab}	122^{a}	138^{b}	7.92
Crude protein (CP)	134^{a}	135^{a}	133^{a}	137^{a}	137^{a}	132^{a}	8.05
Crude lipids	27^{b}	23^{a}	23^{a}	29^{b}	29^{b}	28^{b}	3.14
Neutral detergent fibre[**]	536^{a}	549^{a}	556^{a}	545^{a}	545^{a}	543^{a}	43.77
Acid detergent fibre[††]	346^{a}	331^{a}	329^{a}	342^{a}	342^{a}	341^{a}	51.83
Acid detergent lignin	46^{a}	58^{a}	60^{a}	48^{a}	54^{a}	54^{a}	17.66
24 h gas production (mL/g DM)	212^{a}	212^{a}	212^{a}	205^{a}	213^{a}	201^{a}	13.09
Metabolisable energy (MJ/kg DM)	8.9^{a}	9.0^{a}	9.0^{a}	8.9^{a}	9.0^{a}	8.8^{a}	0.5
pH	4.53^{c}	4.62^{d}	4.05^{a}	4.17^{b}	4.14^{b}	4.11^{ab}	0.07
Lactic acid	48^{b}	42^{a}	79^{d}	79^{b}	67^{c}	83^{d}	5.25
Acetic acid	14^{bc}	15^{bc}	9^{a}	21^{d}	10^{ab}	17^{cd}	5.58
Butyric acid[‡‡]	0.4^{ab}	-[*]	0.1^{a}	1.5^{b}	-	0.2^{ab}	1.34
Propionic acid	0.1^{a}	0.04^{a}	0.1^{a}	0.5^{b}	0.2^{ab}	0.4^{b}	0.31
1,2-Propanediol	1.0	0.8	0.3^{a}	3.7^{c}	0.7^{ab}	1.8^{b}	1.35
2,3-Butanediol	-	-	-	-	-	-	-
Ethanol	6.5a^{b}	3.4^{a}	5.5^{ab}	8.8^{b}	5.2^{a}	9.2^{b}	3.11
Methanol	0.4^{bc}	0.4^{c}	0.3^{a}	0.5^{c}	0.3^{ab}	0.4^{c}	0.12
Propanol	0.3^{a}	-	-	2.4^{a}	0.2^{a}	0.9^{a}	2.95

Table 9 Continued

Variable	Treatment[‡]						HSD[§]
	IR38CON1	IR38CHE1	IR38BIO	IR25CON2	IR25CHE2	IR25SOIL	
1-Butanol (mg/kg DM)	-[†]	-	-	15[a]	-	6[a]	25.19
2-Butanol (mg/kg DM)	-	-	-	31[a]	-	39[a]	85.6
Ethyl lactate (mg/kg DM)	63[b]	31[a]	116[c]	103[c]	61[b]	186[d]	27.53
Ethyl acetate (mg/kg DM)	80[c]	63[bc]	32[a]	80[c]	45[ab]	73[b]	29.69
Propyl acetate (mg/kg DM)	-	-	-	20[a]	-	10[a]	55.42
Ammonia-N (g/kg N)	90[b]	93[bc]	63[a]	103[d]	88[b]	99[cd]	8.19
Water-soluble carbohydrates	36[b]	50[bc]	42[b]	9[a]	57[c]	7[a]	14.23
CP fractions (g/kg CP)							
A	648[e]	590[a]	610[b]	633[c]	645[d]	649[f]	-
B1	4[a]	20[c]	32[e]	28[d]	13[b]	34[f]	-
B2	240[d]	246[f]	215[a]	245[e]	230[c]	216[b]	8.13
B3	53[c]	82[f]	81[e]	14[a]	63[d]	43[b]	3.88
C	53[b]	63[d]	63[e]	80[f]	49[a]	58[c]	-

Notes. Means within a row with different superscripts (a–f) differ ($p < 0.05$); $n = 4$.

.

A: non-protein N, enzymatic degradation not applicable; B1: buffer-soluble true protein, quickly degraded; B2: buffer-insoluble true protein, intermediately degraded; B3: cell wall-associated true protein, intermediately to slowly degraded; C: insoluble in acid detergent, indigestible; DM: dry matter. [*]Below detection limit (0.01%). [†]Below detection limit (0.001%). [‡]BIO: around 38% DM and biological additive (1 g/t), based on homofermentative lactic acid bacteria (*Lactobacillus plantarum*; $3.0 \cdot 10^{11}$ colony-forming units/g); CON1: untreated and around 38% DM; CHE1: around 38% DM and chemical silage additive (2.5 L/t), based on sodium nitrite and hexamine (hexamethylenetetramine); CON2: untreated and around 27% DM; CHE2: around 27% DM and chemical silage additive (4 L/t), based on 75% formic acid buffered with sodium hydroxide to pH 2.5; SOIL: around 27% DM and addition of 7,600 g soil/t. [§]Tukey's honestly significant difference ($\alpha = .05$). [¶]Mean substrate density before ensiling, $n = 4$. [**]Analysed with heat-stable amylase and expressed exclusive of residual ash. [††]Expressed exclusive of residual ash. [‡‡]Sum of i-/n-butyric acid, i-/n-valerian acid, and n-caproic acid.

Table 10 Biogenic amine concentrations of lucerne (LU), red clover (RC) and Italian ryegrass (IR) silages (mg/kg DM)

Plant-species	Biogenic amines	Silage treatment†						HSD‡
		CON1	CHE1	BIO	CON2	CHE2	SOIL	
LU	Histamine	1277^{bc}	1287^{bc}	1183^{bc}	473^{ab}	1763^{c}	280^{a}	0.89
	Gamma-amino butyric acid	420^{ab}	293^{a}	328^{a}	530^{bc}	380^{ab}	695^{c}	0.19
	Beta-amino butyric acid	783^{ab}	587^{a}	660^{a}	1117^{b}	838^{ab}	1845^{c}	0.42
	Alpha-amino butyric acid	-*	-	-	-	-	373	-
	Tyramine	150^{a}	-	-	313^{b}	195^{a}	360^{b}	0.11
	Putrescine	-	-	-	157^{a}	-	258^{b}	0.07
	Cadaverine	-	-	-	137^{a}	-	275^{b}	0.12
	Total biogenic amines	2627^{a}	2163^{a}	2173^{a}	2733^{a}	3183^{ab}	4083^{b}	1.2
RC	Histamine	828^{bc}	950^{bc}	635^{ab}	1043^{c}	945^{bc}	358^{a}	0.33
	Gamma-amino butyric acid	225^{b}	135^{a}	205^{b}	185^{ab}	-	248^{b}	0.06
	Beta-amino butyric acid	405^{bc}	303^{ab}	338^{ab}	495^{cd}	288^{a}	588^{d}	0.11
	Alpha-amino butyric acid	-	-	-	-	-	-	-
	Tyramine	83^{a}	-	-	183^{b}	-	228^{c}	0.04
	Putrescine	-	-	-	-	-	118	-
	Cadaverine	-	-	-	-	-	-	-
	Total biogenic amines	1615^{ab}	1390^{a}	1175^{a}	1905^{b}	1233^{a}	1610^{ab}	0.45
IR	Histamine	640^{b}	873^{c}	708^{b}	350^{a}	678^{b}	273^{a}	0.11
	Gamma-amino butyric acid	413^{b}	337^{ab}	380^{ab}	738^{d}	320^{a}	643^{c}	0.08
	Beta-amino butyric acid	663^{a}	593^{a}	630^{a}	815^{b}	690^{a}	780^{b}	0.09
	Alpha-amino butyric acid	-	-	-	-	-	-	-
	Tyramine	105^{a}	50^{a}	-	305^{c}	133^{b}	265^{c}	0.04

Table 10 Continued

Plant-species	Biogenic amines	Silage treatment[†]						HSD[‡]
		CON1	CHE1	BIO	CON2	CHE2	SOIL	
IR	Putrescine	-	-	-	113	-	100	0.05
	Cadaverine	48[a]	-	-	148[b]	-	105[b]	0.05
	Total biogenic amines	1868[a]	1857[a]	1718[a]	2473[c]	1815[a]	2163[b]	0.22

Notes. Means within a row with different superscripts (a–f) differ ($p < 0.05$); $n = 4$.

[*]Below detection limit (0.01%). [†] BIO: biological additive (1 g/t), based on homofermentative lactic acid bacteria (*Lactobacillus plantarum*; $3.0 \cdot 10^{11}$ colony-forming units/g); CON1 and CON2: untreated; CHE1: chemical silage additive (2.5 L/t), based on sodium nitrite and hexamine (hexamethylenetetramine); CHE2: chemical silage additive (4 L/t), based on 75% formic acid buffered with sodium hydroxide to pH 2.5; CON1, CHE1 and BIO, respectively, 38% DM (LU silages), 30% DM (RC silages), 38% DM (IR silages); CON2, CHE2 and SOIL, respectively, 27% DM (LU silages) 23% DM (RC silages) and 25% DM (IR silages); DM: dry matter; SOIL, addition of 7,600 g soil/t. [‡]Tukey's honestly significant difference ($\alpha = .05$). [§]Histamine, gamma-amino butyric acid, beta-amino butyric acid, alpha-amino butyric acid, tyramine, putrescine, cadaverine.

3.3 Microbiological analyses

The results of the microbiological examinations of freshly cut LU, RC and IR immediately after harvesting and of the silages are summarized in Table 11. The anaerobic lactate-utilizing sporulating microbes ranged from 93 to $1.1 \cdot 10^3$ most probable numbers (MPN)/g and 28 to $1.1 \cdot 10^4$ MPN/g in the LU and IR silages, whereas in the RC silages the values ranged only from 15 to 460 MPN/g. The yeast counts did not exceed 500 colony-forming units (cfu)/g in most silages. The exceptions were the untreated low-DM RC silage ($5.0 \cdot 10^3$ cfu/g), the untreated high-DM IR silage ($5.0 \cdot 10^3$ cfu/g) and the high-DM IR silage treated with the biological additive ($6.0 \cdot 10^5$ cfu/g). The content of clostridial endospores in the silages was on average 228 (LU), 149 (RC) and 320 MPN/g (IR).

3.4 Animal preference and short-time dry-matter intake

Multidimensional scaling revealed that goats' selection between forages was associated with two dimensions. The results of the three preference trial runs, consisting of 30 min and 3 hr DMI data and coordinates of both dimensions of the 3 hr DMI, are presented in Table 12. According to MDS, LU38BIO, RC30CON1, RC30BIO, IR38CON1 and IR38CHE1 were preferred. The most-avoided forages in the first run with the LU silages were the formic acid-treated low-DM LU silage and LU hay, and in the second and third runs, the low-DM RC and IR silages treated with formic acid and the high-DM IR silage treated with the biological additive.

The DMI (g/3 hr) was highest for the high-DM silage treated with the chemical additive in run 1 (LU silages). In runs 2 and 3 (RC and IR silages), goats showed the highest DMI for the untreated high-DM silage ($p < 0.05$). In all three runs, the lowest DMI was shown for the soil-treated low-DM silages and LU hay. With two exceptions, the 3 hr DMI ranking of the silages was the same for the LU, RC and IR silages (Table 12); in decreasing order it was CON1, CHE1 (CON1 and CHE1 were reversed for the LU silages), BIO, CHE2, CON2 (CHE2 and CON2 were reversed for the IR silages) and SOIL.

3.5 Silage characteristics influencing short-time dry-matter intake

The results of the correlation analysis between silage characteristics, DM concentration and DMI are presented in Table 13. The concentration of most fermentation products was negatively correlated with silage DM concentration. The lucerne silages showed the closest

associations for lactic and acetic acid, propanol, 2-butanol and NH_3-N (<-0.6, $p < 0.001$). For the RC silages, correlations were of comparable strength for methanol ($p < 0.001$), and for IR silages lactic acid, propionic acid, ethanol and 1- and 2-butanol and 1,2-propandiol were closely correlated with silage DM ($p < 0.001$).

Most of the fermentation products were negatively correlated with preference when expressed as DMI (g/3 hr). For the LU and RC silages, most correlation coefficients ranged between -0.3 and -0.5. For the IR silages, no relationship was found between the fermentation products and DMI. Ethyl lactate was negatively associated with DMI in all three plant species. Ethyl acetate showed a weak negative association with the DMI of the LU and RC silages. No relationship was found between ethyl acetate and the DMI of the IR silages. Averaged across the three forage species, the regression of TBA on DMI did not reveal a relation between feed intake and amines ($R^2 = 0.07$). However, within forage species all individual amines had negative correlations of between 0.4 and 0.51 for LU and most had weaker correlations for RC. The correlations for IR were lower, but still significant.

Table 11 Microbiological analyses of freshly cut lucerne (LU), red clover (RC) and Italian ryegras (IR) immediately after harvesting and of the silages immediately after silo opening

Substrate	Anaerobic lactate utilising sporulating microbes (MPN/g)	Yeasts (cfu/g)	Moulds (cfu/g)	Aerobic mesophilic bacteria (cfu/g)	Lactic acid bacteria (cfu/g)	Clostridial endo-spores (MPN/g)
Lucerne*	23	$1.8 \cdot 10^6$	$5.5 \cdot 10^4$	$5.0 \cdot 10^6$	$5.0 \cdot 10^4$	43
Red clover*	< 3	$3.8 \cdot 10^5$	$1.3 \cdot 10^4$	$2.0 \cdot 10^8$	$3.0 \cdot 10^7$	930
Italian ryegrass*	43	$8.0 \cdot 10^4$	$2.0 \cdot 10^5$	$1.2 \cdot 10^8$	$4.0 \cdot 10^4$	9
LU38CON1	93	< 500	< 500	$2.0 \cdot 10^3$	$1.6 \cdot 10^7$	93
LU3CHE1	150	< 500	< 500	$1.7 \cdot 10^6$	$7.0 \cdot 10^6$	460
LU38BIO	$1.1 \cdot 10^3$	< 500	< 500	$6.0 \cdot 10^5$	$4.0 \cdot 10^5$	240
LU27CON2	$1.1 \cdot 10^3$	< 500	< 500	$1.5 \cdot 10^6$	$6.0 \cdot 10^7$	93
LU27CHE2	$1.1 \cdot 10^3$	< 500	< 500	$7.0 \cdot 10^4$	$3.0 \cdot 10^7$	240
LU27SOIL	460	< 500	< 500	$8.0 \cdot 10^5$	$3.6 \cdot 10^7$	240
RC30CON1	43	< 500	500	$5.5 \cdot 10^3$	$1.9 \cdot 10^7$	43
RC30CHE1	15	< 500	500	$3.0 \cdot 10^3$	$1.3 \cdot 10^7$	15
RC30BIO	93	500	500	$3.0 \cdot 10^3$	$1.0 \cdot 10^3$	93
RC23CON2	460	$5.0 \cdot 10^3$	$1.0 \cdot 10^3$	$4.0 \cdot 10^3$	$4.4 \cdot 10^7$	460
RC23CHE2	240	< 500	500	$3.5 \cdot 10^3$	$7.9 \cdot 10^6$	240
RC23SOIL	460	< 500	< 500	$5.5 \cdot 10^3$	$6.4 \cdot 10^7$	43
IR38CON1	$1.1 \cdot 10^3$	$5.0 \cdot 10^3$	< 500	$1.0 \cdot 10^6$	$7.1 \cdot 10^7$	240

Table 11 Continued

Substrate	Anaerobic lactate utilizing sporulating microbes (MPN/g)	Yeasts (cfu/g)	Moulds (cfu/g)	Aerobic mesophilic bacteria (cfu/g)	Lactic acid bacteria (cfu/g)	Clostridial endo-spores (MPN/g)
IR38CHE1	$2.4 \cdot 10^3$	< 500	< 500	$1.4 \cdot 10^6$	$5.3 \cdot 10^8$	$1.1 \cdot 10^3$
IR38BIO	$1.1 \cdot 10^4$	$6.0 \cdot 10^5$	< 500	$1.6 \cdot 10^6$	$5.4 \cdot 10^7$	43
IR25CON2	$1.1 \cdot 10^4$	< 500	< 500	$2.4 \cdot 10^6$	$3.8 \cdot 10^8$	460
IR25CHE2	28	500	< 500	$1.3 \cdot 10^5$	$1.5 \cdot 10^6$	75
IR25SOIL	$1.1 \cdot 10^3$	< 500	< 500	$2.6 \cdot 10^5$	$6.8 \cdot 10^6$	< 3

Notes. BIO: biological additive (1 g/t) based on homofermentative lactic acid bacteria (*Lactobacillus plantarum*; $3.0 \cdot 10^{11}$ colony-forming units (cfu)/g); CON1 and CON2: untreated; CHE1: chemical silage additive (2.5 L/t) based on sodium nitrite and hexamine (hexamethylenetetramine); CHE2: chemical silage additive (4 L/t) based on 75% formic acid buffered with sodium hydroxide to pH 2.5; IR: Italian ryegrass; the numbers behind LU, RC, and IR indicate dry matter (in %); LU: lucerne; MPN: most probable number; <: detectable but not quantifiable; RC: red clover; SOIL: addition of 7,600 g soil/t. *Forages immediately after harvesting.

Table 12 Dry-matter intake (DMI) (g DMI/3 hr and g DMI/30 min respectively) and stimulus coordinates (for g DMI/3 hr) for the two-dimensional solution to the preference among eight (lucerne (LU) and red clover (RC) silages) and six respectively (Italian ryegrass (IR) silages) goats (n = 48 (LU and RC silages respectively) and n = 36 (IR silages))

Forage species		Silage treatment*						LU hay	MSD†
		CON1	CHE1	BIO	CON2	CHE2	SOIL		
LU	DMI (30 min)	376^{ab}	419^{a}	336^{b}	188^{d}	261^{c}	68^{e}	91^{e}	57
	DMI (3 hr)	748^{b}	860^{aA}	744^{bA}	480^{d}	622^{cA}	226^{e}	221^{e}	107
	Dimension 1	1.04	-0.15	1.06	-0.39	-1.92	0.84	-0.49	
	Dimension 2	-0.63	1.18	0.79	1.26	-0.29	-0.82	-1.49	
RC	DMI (30 min)	434^{a}	402^{a}	327^{b}	220^{c}	320^{b}	87^{d}	65^{d}	52
	DMI (3 hr)	858^{a}	704^{bB}	647^{bcAB}	559^{c}	666^{bA}	283^{d}	207^{d}	93
	Dimension 1	-0.03	-0.34	1.26	1.39	-1.72	-0.61	0.04	
	Dimension 2	1.13	0.99	0.37	-0.38	-0.94	0.63	-1.79	
IR	DMI (30 min)	293^{a}	223^{ab}	159^{bc}	166^{bc}	146^{bcd}	87^{cd}	65^{d}	86
	DMI (3 hr)	763^{a}	606^{abB}	540^{bcB}	460^{bc}	402^{cdB}	262^{de}	197^{e}	158
	Dimension 1	1.33	1.38	-1.22	-1.19	-0.12	0.35	-0.53	
	Dimension 2	0.97	0.41	-0.55	1.07	-1.30	-1.43	0.84	
All species	DMI (30 min)	374^{a}	360^{a}	285^{b}	194^{c}	251^{b}	80^{d}	74^{d}	37
	DMI (3 hr)	793^{a}	734^{a}	653^{b}	503^{d}	578^{c}	256^{e}	209^{e}	67

Notes. Means within a row with different superscripts (a–e) and within a column with different superscripts (A, B) differ ($p < 0.05$).

[*] BIO: biological additive (1 g/t), based on homofermentative lactic acid bacteria (*Lactobacillus plantarum*; $3.0 \cdot 10^{11}$ colony-forming units/g); CON1 and CON2: untreated; CHE1: chemical silage additive (2.5 L/t), based on sodium nitrite and hexamine (hexamethylenetetramine); CHE2: chemical silage additive (4 L/t), based on 75% formic acid buffered with sodium hydroxide to pH 2.5; CON2, CHE2 and SOIL: respectively, 27% DM (LU silages), 23% DM (RC silages) and 25% DM (IR silages); SOIL: addition of 7,600 g soil/t; CON1, CHE1 and BIO, respectively: 38% dry matter (DM) (LU silages), 30% DM (RC silages) and 38% DM (IR silages); [†]Minimum significant difference (Waller–Duncan *k*-ratio *t*-test).

Table 13 Correlation (Pearson coefficients) between lucerne (LU), red clover (RC) and Italian ryegrass (IR) silage characteristics, DM concentrations of silages and DMI of goats (n = 48 (LU and RC silages respectively) and n = 36 (IR silages) respectively)

	LU silages		RC silages		IR silages		All forage species	
	DM	DMI	DM	DMI	DM	DMI	DM	DMI
DM	1	0.41***	1	0.34***	1	0.29***	1	0.28***
DMI	0.41***	1	0.34***	1	0.29***	1	0.28***	1
Ash	-0.69***	-0.48***	-0.40***	-0.46***	-0.63***	-0.29***	-0.17***	-0.4***
Crude protein (CP)	-0.2**	-0.26***	0.11Ψ	-0.18**	-0.39***	0.02	0.04	0.06Ψ
Crude lipids	-0.29***	-0.36***	-0.16**	-0.28***	-0.87***	-0.16*	0.14***	-0.13***
Acid detergent fibre†	-0.57***	-0.27***	-0.03	-0.24***	-0.56***	-0.01	-0.02	0.1**
Neutral detergent fibre‡	-0.75***	-0.46***	0.08	-0.35***	0.27***	-0.07	0.06Ψ	-0.11**
Acid detergent lignin	-0.89***	-0.48***	0.04	-0.34***	0.35***	-0.09	0.24***	-0.13***
24 h gas production	0.71***	0.51***	0.22***	0.45***	0.60***	0.33***	-0.17***	0.16***
Metabolisable energy	0.55***	0.19**	-0.01	0.01	0.61***	0.34***	0.13***	-0.13***
pH	0.51***	0.12*	0.39***	0.34***	0.61***	0.27***	0.59***	0.21***
Lactic acid	-0.63***	-0.22***	-0.27***	-0.38***	-0.64***	-0.28***	-0.32***	0.01
Acetic acid	-0.67***	-0.5***	-0.43***	-0.42***	-0.43***	-0.07	-0.35	-0.18
Propionic acid	-0.43***	-0.44***	-0.46***	-0.45***	-0.9***	-0.27***	-0.28***	-0.32***
Butyric acid	-0.29***	0.14*	0.45***	0.32***	-0.45***	-0.012	0.89*	-0.13***
Ethanol	-0.47***	-0.47***	-0.39***	-0.34***	-0.76***	-0.2**	-0.33***	-0.18***
Methanol	-0.38***	-0.44***	-0.66***	-0.29***	0.18**	0.13Ψ	-0.29***	-0.19***
Propanol	-0.61***	-0.50***	-0.43***	-0.45***	-0.59***	-0.11	-0.28***	-0.33***
1,2-Propanediol	-0.55***	-0.06	-0.34***	0.05	-0.63***	-0.11	-0.38***	0.01
2,3-Butanediol	0.88***	-0.39***	0.41***	-0.02	-	-	-0.11**	0.08*

Table 13 Continued

	LU silages		RC silages		IR silages		All forage species	
	DM	DMI	DM	DMI	DM	DMI	DM	DMI
1-Butanol	-0.43***	-0.44***	-	-	-0.65***	-0.15*	-0.31***	-0.27***
2-Butanol	-0.63***	-0.5***	-0.42***	-0.45***	-0.69***	-0.26***	-0.36	-0.38***
Ethyl lactate	-0.58***	-0.46***	-0.27***	-0.42***	-0.47***	-0.26***	-0.28***	-0.32***
Ethyl acetate	-0.54***	-0.49***	-0.57***	-0.45***	-0.25***	0.04	-0.18***	-0.27***
Ammonia-N	-0.64***	-0.43***	0.12*	0.05	-0.55***	-0.1	0.14***	0.02
Water-soluble carbohydrates	0.5***	0.42***	0.15*	0.34***	0.5***	0.16*	-0.19***	0.26***
Histamine	0.31***	0.40***	0.02	0.33***	0.77***	0.24***	0.1**	0.28***
Gamma-amino butyric acid	-0.67***	-0.50***	0.27***	-0.14*	-0.62***	-0.17*	0.13***	-0.25***
Beta-amino butyric acid	-0.67***	-0.51***	-0.50***	-0.39***	-0.87***	-0.23***	-0.09*	-0.12***
Alpha-amino butyric acid	-0.43***	-0.44***	-	-	-	-	-0.25***	-0.31***
Tyramine	-0.85***	-0.47***	-0.59***	-0.37***	-0.84***	-0.22**	-0.44***	-0.29***
Putrescine	-0.62***	-0.49***	-0.42***	-0.45***	-0.71***	-0.23***	-0.34***	-0.36***
Cadaverine	-0.61***	-0.50***	-	-	-0.62***	-0.13Ψ	0.07*	-0.16***
Total biogenic acids§	-0.79***	-0.47***	-0.42***	-0.12*	-0.67***	-0.15*	0.001	-0.18***
CP fractions								
A	-0.18***	-0.2***	-0.75***	-0.29***	-0.66***	-0.11†	-0.03	0.03
B1	0.63***	0.33***	-0.38***	-0.005	-0.25***	-0.25***	0.09*	0.11**
B2	0.02	0.19***	0.61***	0.06	0.12Ψ	0.2**	-0.39***	0.07*
B3	-0.4***	0.1	0.53***	0.26***	0.74***	0.12Ψ	-0.17***	0.14***
C	0.49***	0.26***	-0.04	-0.04	-0.15*	-0.03	-0.07Ψ	0.09**

Notes: A: non-protein N, enzymatic degradation not applicable; B1: buffer-soluble true protein, quickly degraded; B2: buffer-insoluble true protein, intermediately degraded; B3: cell wall-associated true protein, intermediately to slowly degraded; C: insoluble in acid detergent, indigestible; DM: dry matter; DMI: DM intake; IR: Italian ryegrass; LU: lucerne; RC: red clover.

† Expressed exclusive residual ash. ‡ Analysed with heat-stable amylase and expressed exclusive residual ash. § Histamine, gamma-amino butyric acid, beta-amino butyric acid, alpha-amino butyric acid, tyramine, putrescine, cadaverine. ᵠ $p < 0.10$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

4. Discussion

4.1 Silage characteristics and short-time dry matte- intake

The proximate constituents were in the typical range of grass silages (Thaysen, 2004) and legume silages (Kung & Shaver, 2001). There were a few large differences in the fermentation patterns of LU, RC and IR silages treated differently before ensiling, but most of them differed only moderately. The concentrations of acetic acid ranged from 9 g/kg DM in the high-DM IR silage treated with homofermentative lactic acid bacteria to 62 g/kg DM in the soil-treated low-DM LU silage. The acetic acid concentrations were greater in the soil-treated low-DM legume silages than in the soil-treated low-DM IR silages. However, the upper limit of acceptable values of 30 g/kg DM (Kung & Shaver, 2001) was exceeded only by a few LU and RC silages. The acetic acid concentrations of the IR silages were all below this limit. Some studies have found a negative correlation between acetic acid and DMI (Huhtanen et al., 2002; Krizsan & Randby, 2007). Eisner, Südekum, and Kirchhof (2006) conducted a meta-analysis and reported that with increasing acetic acid concentrations a considerable decrease in silage DMI was observed, especially in the low-concentration range (< 10 g/kg DM). However, the current data did not reveal an influence of acetic acid on short-time DMI, because the treatments within crop species often had similar acetic acid concentrations but largely differing DMI values. Moreover, the correlation analysis revealed only a weak negative relationship for LU and RC silages between acetic acid and DMI, and no correlation for IR silages. However, when comparing the results of Eisner et al. (2006) with the current data, it must be kept in mind that our data are based on short-time DMI, whereas the meta-analysis used data of DMI measurements over longer periods of time without allowing the animals to choose between forages.

During ensiling, different alcohols can be formed. Ethanol usually predominates (Weiß, 2001), which was corroborated in the present study. The upper limit for high-quality grass silage was reported at 10 g ethanol/kg DM (Thaysen, 2004), which was only exceeded by the soil-treated low-DM LU silage, indicating increased activity of yeasts and enterobacteria (Rooke & Hatfield, 2003), although the values for aerobic mesophilic bacteria, which include enterobacteria, did not show conspicuous values compared with the other silages. Ethanol can also be formed by heterofermentative lactic acid bacteria, resulting in elevated ethanol and acetic acid concentrations, which were observed for the soil-treated LU, RC and IR silages.

4.2 Nitrogen compounds

4.2.1 Proteolysis and desmolysis

During ensiling, herbage proteins are extensively degraded into oligopeptides and free amino acids, the latter being further degraded into NH_3 and other forms of NPN (Ohshima & McDonald, 1978). Extensive proteolysis in ensiled legume forages reduces the protein quality for ruminants, since the rapid degradation of a high proportion of silage NPN in the rumen does not result in energy-yielding substrates for protein synthesis by rumen microorganisms, leading to less efficient utilization of N by rumen microbes in silages than in fresh or dried forages (Givens & Rulquin, 2004). Around 70% of true protein was broken down during ensiling in the LU and IR silages, which was shown to be at the upper limit of the observed extent of proteolysis by Hoedtke, Gabel, and Zeyner (2010). The lower levels in the RC silages were probably due to the protection of protein by polyphenol oxidase (PPO) (McDonald et al., 1991; Lee et al., 2004). The largest share of NPN may have been contributed by oligo- and dipeptides as well as by free amino acids and acid amides of various organic acids, which were not degraded into ammonia.

The most rapid proteolysis in ensiled lucerne occurs within 24–48 h of ensiling (Fairbairn, Alli, & Baker, 1988; Guo, Zhou, & Zhu, 2007). Proteolysis in ensiled forage is mainly affected by plant proteolytic enzymes (Ohshima & McDonald, 1978; McKersie, 1981). In lucerne, at least three proteolytic enzymes are relevant – carboxypeptidase, aminopeptidase, and acid proteinase – each differing in its pH and temperature optima and sensitivity to inhibitors (McKersie, 1981).

Only weak relationships were observed between NH_3-N and NPN in LU and RC silages, and there was no relationship in IR silages. This indicates that, although NPN comprises NH_3-N, the latter does not seem to be suitable for assessing total NPN compounds. However, NH_3-N may still be related to the overall fermentation quality (Huhtanen et al., 2002; Thaysen, 2004). Italian ryegrass silages of high quality have less than 50 g NH_3-N/kg N, whereas IR silages with more than 150 g NH_3-N/kg N are considered as spoiled (Huhtanen et al., 2002; Thaysen, 2004). The NH_3-N concentrations of the LU silages ranged from 88 (LU38BIO) to 147 g/kg N (LU27SOIL), with LU27SOIL almost reaching the spoiled class. The red clover silages ranged from 42 to 83 g/kg N, which is related to lower proteolysis per se, possibly due to PPO (Lee et al., 2004). Despite the lower susceptibility of grass silages to proteolysis, they showed a relatively high degree of protein and amino acid degradation, with

NH_3-N ranging from 63 to 103 g/kg N. Surprisingly, a relationship between DMI and NH_3-N was not found in the RC and IR silages but only in the LU silages, as already shown by Sánchez-Duarte and García (2017).

The correlations between CP fractions and DMI were weak and partly inconsistent between the crop species. However, an effect of the individual fractions would have been surprising, because the relationships between total CP and DMI were weak as well. Thus, the effect of N compounds is ambiguous and difficult to attribute clearly to preference data, as shown by the lack of significant correlation in this study. The influence of N compounds is even less clearly explained than that of acids (Dulphy & Van Os, 1996). In line with this, no impact of CP fractions on DMI was shown here.

4.2.2 Biogenic amines and their relation to feed intake

Substrates with different suitability for ensiling were used to produce silages differing in fermentation patterns and amine concentrations. This should be kept in mind when the results are interpreted.

The ensiling treatments generally had a greater effect on amine formation than the ensiled forage species. However, untreated (control) and additive-treated silages still sometimes had very similar amine concentrations. This was consistent with the findings of Křížek (1993), Krizsan and Randby (2007), Van Os et al. (1995), and Van Os, Van Wikselaar, and Spoelstra (1996). In silages made from orchard grass (*Dactylis glomerata*), red clover (*Trifolium pratense*) and oats (*Avena sativa*), which were preserved by wilting to approximately 34% DM and formic acid addition, respectively, Křížek (1993) observed putrescine and cadaverine as the amines present in greatest concentrations. Contrary to expectations, in our study, these amines were detected in only 5 or 6 of the 18 silage treatments. Křížek (1991) had shown that formic acid suppressed histamine, putrescine and cadaverine considerably, but this later findings (Křížek, 1993) indicated that neither the application of formic acid nor wilting suppressed the formation of any of the amines in silage selectively. However, formic acid and wilting, respectively, did decrease the total concentrations of amines (Křížek, 1993). The current data did not reveal the same effect of formic acid and wilting except for GABA in the IR silages. Here, the concentration of GABA was higher in the untreated low-DM IR silage than in the untreated high-DM IR silage followed by the formic acid-treated low-DM IR silage. Effects of formic acid and wilting could not be demonstrated for the other amines. The TBA in LU silages was even higher in the formic acid-treated low-DM LU silage than in the

high- and low-DM silages. The amount and type of amines formed depends on various factors, like the epiphytic microorganisms in the crop with their varying amino acid decarboxylase activity, the temperature of the silages during storage and the oxygen supply. *Enterobacter cloacae* produced about 50% less putrescine under anaerobic conditions than in an aerobic situation and *Klebsiella pneumonia* produced significantly less cadaverine, but gained the ability to produce putrescine under anaerobic conditions. Moreover, strains of the same bacteria species showed wide variations in histamine formation (Halász, Baráth, Simon-Sarkadi, & Holzapfel, 1994). This illustrates that the formation of biogenic amines can vary widely depending on the surrounding conditions despite the application of the same treatments.

In a concentration range of 1.2 to 4.1 g TBA/kg DM, and with the given amine pattern, no reduction in feed intake was observed in a choice feeding situation. The actual intake of amines varied with DMI and ranged on average from 0.9 to 2.0 g/d for the LU silages, 0.5 to 1.3 g/d for the RC silages and 0.9 to 1.4 g/d for the IR silages. Because the DM concentration was not related to the amine concentrations, the lower DMI of the moist silages compared with the silages higher in DM was probably not related to amines. It may be concluded that the lower feed intake of low-DM silages was not caused by amines in the concentration range studied here. In line with this assumption, we supposed that the DMI would be lowest with the soil-treated low-DM silages, because the inadvertent, unavoidable consumption of soil with the forage is associated with a number of detrimental consequences, like increased tooth wear, physical impaction in the digestive tract and indirect effects of harmful chemicals adsorbed into soil particles (Mayland, Shewmaker, & Bull, 1977). Indeed, the DMI of the SOIL treatment was the lowest for all three crop species. Although the regression of the individual amines on DMI showed weak relationships, it is possible that the relationships would be stronger at higher amine concentrations.

Poly- and oligomers of carbohydrates are normally predominant compared with monomers in all forages, including silage. The addition of sucrose to the legumes at ensiling as a low-molecular weight carbohydrate (dimer) raises two issues. On the one hand, it may have promoted bacteria that perform butyric acid formation (clostridia) and proteolysis (Rooke & Hatfield, 2003); on the other hand, it could have served as a substrate for lactic acid bacteria for a rapid and stable pH drop (Muck, 1990). Halász et al. (1994) showed that rapidly fermentable carbohydrates like glucose affect the activity of amino acid decarboxylase in

bacteria. However, they did not specify whether this is true for aerobic or anaerobic substrates. Depending on the concentrations, the carbohydrates promoted (5–20 g/kg DM) or inhibited (> 30 g/kg DM) the amino acid decarboxylase activity. This procedure may have led to the protection of amino acids. Since the NPN was quite high, possibly the true protein was split into oligopeptides, dipeptides and amino acids, and the latter were still present when the silos were opened, resulting in high levels of NPN.

According to Weiss, Chamberlain, and Hunt (2003), poorly fermented silages are characterized not only by butyric acid concentrations of more than 25 g/kg DM and NH_3-N concentrations of more than 200 g/kg N, but also by TBA concentrations > 2 g/kg DM. The current data show that amine concentrations in this range occur even when butyric acid and NH_3-N are below the mentioned values. The development of amine concentrations > 2 g/kg DM is obviously not always accompanied by visibly increased butyric acid and NH_3-N concentrations. Thus, amines in the mentioned range occur not only in poorly fermented but also in well-fermented silages. However, the NH_3-N concentrations were in the range of 50–150 g NH_3-N/kg N. Van Os et al. (1996) showed high correlations between NH_3-N and TBA ($r = 0.86$, $p < 0.01$). Richardt, Wein, Steinhöfel, and Pries (2011) proposed to use NH_3-N as an indicator for biogenic amines ($r = 0.67$, $p < 0.05$). This can be supported by the current data. Although the development of NH_3-N and amines is summarized under the term desmolysis, they arise from two different reactions – deamination and decarbocxylation. The close correlation between NH_3-N and biogenic amines indicates that both reactions are concomitant processes of amino acid degradation during ensiling.

High concentrations of NH_3-N and amines were found in clostridial silages (Tveit, Lingaas, Svendsen, and Sjaastad, 1992), but these compounds were reported by Macpherson and Violante (1966) to occur in substantial amounts in non-clostridial silages with low pH and without butyric acid, indicating that, during fermentation, microorganisms other than clostridia deaminate and decarboxylate amino acids, for example entero- and lactic acid bacteria (Van Os et al., 1996). Thus, we do not consider butyric acid or high clostridial levels to be reliable markers for biogenic amine formation.

5. Conclusions

The silages were predominantly well fermented, as measured by low butyric and acetic acid concentrations. Although extensive proteolysis was observed in the legume and the IR

silages, unexpectedly, there were no elevated concentrations of biogenic amines. Moreover, biogenic amines did not account for a large proportion of NPN and, within the determined concentration range, had a moderate influence on feed intake behaviour. In addition, no significance could be attributed to the individual crude protein fractions in influencing DMI. These observations suggest that even with a range of ensiling conditions across forage species and application of extensive laboratory measurements and study of animal response characteristics, there were no clear relationships between forage species, ensiling conditions and biogenic amine concentrations. Other unidentified compounds, including protein degradation products such as oligo- and dipeptides, may have exerted an effect on appetite. A relation between NH_3-N and amine concentrations was found. Although further validation is required there is promise that NH_3-N may be used as a marker for amine levels. Consideration should also be given to long-term studies to determine further the impact of biogenic amines on feed intake. This study has provided further evidence for a complex relationship between forage species, ensiling conditions and the formation of biogenic amines during ensiling and their combined effects on the feed choice behaviour of ruminants.

Acknowledgements

This study was financially supported by the “Deutsche Forschungsgemeinschaft” (DFG, German Research Foundation, SU 124/31-1). We thank Nadja Wahl and Petra Jacquemien, University of Bonn, and the team of Central Analytical Laboratory, Humboldt-Universität zu Berlin, for support in chemical analyses. We further thank Christian Koch, Susanne Schraut, Thomas Hartinger, Anika Busch, Daniela Klasen and Heinrich Scherer for their support during harvesting.

Conflict of interest

The authors declare that there is no conflict of interest.

References

Bundesarbeitskreis Futterkonservierung (2011). *Praxishandbuch Futter- und Substratkonservierung*. Frankfurt am Main, Germany: DLG-Verlags-GmbH (in German).

Buntinx, S. E., Pond, K. R., Fisher, D. S., & Burns, J. C. (1997). The utilization of multidimensional scaling to identify forage characteristics associated with preference in sheep. *Journal of Animal Science*, *75*, 1641–1650. https://doi.org/10.2527/1997.7561641x

Burns, J. C., Fisher, D. S., & Mayland, H. F. (2001). Preference by sheep and goats among hay of eight tall fescue cultivars. *Journal of Animal Science*, *79*, 213–224. https://doi.org/10.2527/2001.791213x

CNERNA (Centre national d'études et de recommandations sur la nutrition et l'alimentation, Commission Qualité Bactériologique du lait) (1986). Recommandations pour l'estimation de la contamination du lait en spores de Clostridia par la méthode de culture en milieu liquide. *Revue Laitière Française*, *451*, 39–45 (in French).

DLG (2006). *Grobfutterbewertung Teil B. DLG-Schlüssel zur Beurteilung der Gärqualität von Grünfuttersilagen auf Basis der chemischen Untersuchung. DLG-Information 2/2006*. Frankfurt am Main, Germany: DLG (in German).

Dulphy, J. P., & Van Os, M. (1996). Control of voluntary intake of precision-chopped silages by ruminants: A review. *Reproduction Nutrition Development*, *36*, 113–135.

Ebert, R. F. (1986). Amino acid analysis by HPLC: Optimized conditions for chromatography of phenylthiocarbamyl derivatives. *Analytical Biochemistry*, *154*, 431–435. https://doi.org/10.1016/0003-2697(86)90010-2

Eisner, I., Südekum, K.-H., & Kirchhof, S. (2006). Beziehungen zwischen Fermentationscharakteristika von Silagen und der Futteraufnahme von Milchkühen. *Übersichten zur Tierernährung*, *34*, 197–221 (in German).

Fairbairn, R., Alli, I., & Baker, B. E. (1988). Proteolysis associated with the ensiling of chopped alfalfa. *Journal of Dairy Science*, *71*, 152–158. https://doi.org/10.3168/jds.S0022-0302(88)79536-3

GfE (2008). New equations for predicting metabolisable energy of grass and maize products for ruminants. *Proceedings of the Society of Nutrition Physiology*, *17*, 168–181.

GfE (2017). Equations for predicting metabolisable energy and digestibility of organic matter in forage legumes for ruminants. *Proceedings of the Society of Nutrition Physiology*, *26*, 186–193.

Givens, D. I., & Rulquin, H. (2004). Utilization by ruminants of nitrogen compounds in silage-based diets. *Animal Feed Science and Technology*, *114*, 1–18. https://doi.org/10.1016/j.anifeedsci.2003.09.005

Guo, X. S., Zhou, H., & Zhu, Y. (2007). Changes in the distribution of N and plant enzymatic activity during ensilage of lucerne treated with different additives. *Grass and Forage Science*, *1*, 35–43. https://doi.org/10.1111/j.1365-2494.2007.00559.x

Halász, A., Baráth, A., Simon-Sarkadi, L., & Holzapfel, W. (1994). Biogenic amines and their production by microorganisms in food. *Trends in Food Science and Technology*, *5*, 42–48. https://doi.org/10.1016/0924-2244(94)90070-1

Hoedtke, S., Gabel, M., & Zeyner, A. (2010). Der Proteinabbau im Futter während der Silierung und Veränderungen der Zusammensetzung der Rohproteinfraktion. *Übersichten zur Tierernährung*, *38*, 157–179 (in German).

Honig, H. (1987). Influence of forage type and consolidation on gas exchange and losses in silo. In *8th Silage Conference* (pp. 51–52), Hurley, UK.

Huhtanen, P., Khalili, H., Nousiainen, J. I., Rinne, M., Jaakkola, S., Heikkilä, T., & Nousiainen, J. (2002). Prediction of relative intake potential of grass silage by dairy cows. *Livestock Production Science*, *73*, 111–130. https://doi.org/10.1016/S0301-6226(01)00279-2

ISO 15214 (1998). Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of mesophilic lactic acid bacteria – Colony-count technique at 30 degrees C. Geneva, Switzerland: International Organization for Standardization.

Křížek, M. (1991). The determination of biogenic amines in silage. *Archives of Animal Nutrition*, *41*, 97–104. https://doi.org/10.1080/17450399109424736

Křížek, M. (1993). Biogenic amines in silage. 1. The occurrence of biogenic amines in silage. *Archives of Animal Nutrition*, *43*, 169–177. https://doi.org/10.1080/17450399309386032

Krizsan, S. J., & Randby, Å. T. (2007). The effect of fermentation quality on the voluntary intake of grass silage by growing cattle fed silage as the sole feed. *Journal of Animal Science*, *85*, 984–996. https://10.2527/jas.2005-587

Kung, L., & Shaver, R. (2001). Interpretation and use of silage fermentation analysis reports. *Focus Forage*, *3*, 1–5.

Kyriazakis, I., Emmans, G. C., & Whittemore, C. T. (1990). Diet selection in pigs: Choices made by growing pigs given foods of different protein concentrations. *Animal Science*, *51*, 189–199. https://doi.org/10.1017/S0003356100005298

Lee, M. R. F., Winters, A. L., Scollan, N. D., Dewhurst, R. J., Theodorou, M. K., & Minchin, F. R. (2004). Plant-mediated lipolysis and proteolysis in red clover with different polyphenol oxidase activities. *Journal of the Science of Food and Agriculture*, *84*, 1639–1645. https://doi:10.1002/jsfa.1854

Licitra, G., Hernandez, T. M., & Van Soest, P. J. (1996). Standardization of procedures for nitrogen fractionation of ruminant feeds. *Animal Feed Science and Technology*, *57*, 347–358. https://doi.org/10.1016/0377-8401(95)00837-3

Macpherson, H. T., & Violante, P. (1966). Ornithine, putrescine and cadaverine in farm silage. *Journal of the Science of Food and Agriculture*, *17*, 124–127. https://doi.org/10.1002/jsfa.2740170305

Mayland, H. F., Shewmaker, G. E., & Bull, R. C. (1977). Soil ingestion by cattle grazing crested wheatgrass. *Journal of Range Management*, *30*, 264–265.

McDonald, P., Henderson, A. R., & Heron, S. J. E. (1991). *The biochemistry of silage*. Marlow, UK: Chalcombe Publications.

McKersie, B. D. (1981). Proteinases and peptidases of alfalfa herbage. *Canadian Journal of Plant Science*, *61*, 53–59. https://doi.org/10.4141/cjps81-008

Meier, J. S., Kreuzer, M., & Marquardt, S. (2012). Design and methodology of choice feeding experiments with ruminant livestock. *Applied Animal Behavior Science*, *97*, 206–220. https://doi.org/10.1016/j.applanim.2012.04.008

Muck, R. E. (1990). Dry matter level effects on alfalfa silage quality II. Fermentation products and starch hydrolysis. *Transactions of the ASAE*, *30*, 7–14. https://doi: 10.13031/2013.31340

Ohshima, M., & McDonald, P. (1978). A review of the changes of in nitrogenous compounds in herbage during ensiling. *Journal of the Science of Food and Agriculture*, *29*, 497–505. https://doi.org/10.1002/jsfa.2740290602

Pahlow, G. (1986). *Nachweis und Bestimmung von Clostridium Endosporen. Description of Methods M3* (pp. 1–8). Braunschweig, Germany: Institute of Grassland and Forage Research, Federal Research Center of Agriculture (FAL).

Phuntsok, T., Froetschel, M. A., Amos, H. E., Zheng, M., & Huang, Y. W. (1998). Biogenic amines in silage, apparent postruminal passage, and the relationship between biogenic amines and digestive function and intake by steers. *Journal of Dairy Science*, *81*, 2193–2203. https://doi.org/10.3168/jds.S0022-0302(98)75798-4

Richardt, W., Wein, R., Steinhöfel, O., & Pries, M. (2011). Untersuchungen zur Veränderung der Proteinqualität während der Silierung von Grassilagen unter Berücksichtigung biogener Amine. *VDLUFA-Schriftenreihe*, *67*, 541–549 (in German).

Rooke, J. A., & Hatfield, R. D. (2003). Biochemistry of ensiling. In D. R. Buxton, R. E. Muck, & J. H. Harrison (Eds.), *Silage science and technology* (pp. 95–139). Madison, WI, USA: American Society of Agronomy.

Sánchez-Duarte, J. I., & García, A. (2017). Ammonia-N concentration in alfalfa silage and its effects on dairy cow performance: A meta-analysis. *Revista Colombiana de Ciencias Pecuarias*, *30*, 175–184. https://doi:10.17533/udea.rccp.v30n3a01

SAS® (2010). *SAS/STAT 9.4 user's guide*. Cary, NC: SAS Institute, Inc.

Schabenberger, O., & Pierce, F. J. (2002). *Contemporary statistical models for the plant and soil sciences*. Boca Raton, FL: CRC Press.

Scherer, R., Gerlach, K., & Südekum, K.-H. (2015). Biogenic amines and gamma-amino butyric acid in silages: Formation, occurrence and influence on dry matter intake and ruminant production. *Animal Feed Science and Technology*, *210*, 1–16. https://doi.org/10.1016/j.anifeedsci.2015.10.001

Tabacco, E., Piano, S., Cavallarin, L., Bernardes, T. F., & Borreani, G. (2009). Clostridia spore formation during aerobic deterioration of maize and sorghum silages as influenced by *Lactobacillus buchneri* and *Lactobacillus plantarum* inoculants. *Journal of Applied Microbiology*, *107*, 1632–1641. https://doi.org/10.1111/j.1365-2672.2009.04344.x

Thaysen, J. (2004). Die Produktion von qualitativ hochwertigen Grassilagen. *Übersichten zur Tierernährung*, *32*, 57–102 (in German).

Tveit, B., Lingaas, F., Svendsen, M., & Sjaastad, O. V. (1992). Etiology of acetonemia in Norwegian cattle. 1. Effect of ketogenic silage, season, energy level and genetic factors. *Journal of Dairy Science*, *75*, 2421–2432. https://doi.org/10.3168/jds.S0022-0302(92)78003-5

Van Os, M., Dulphy, J. P., & Baumont, R. (1995). The influence of ammonia and amines on grass silage intake and intake behaviour in dairy cows. *Annales de Zootechnie*, *44*, 73–85.

Van Os, M., Van Wikselaar, P. G., & Spoelstra, S. F. (1996). Formation of biogenic amines in well fermented grass silages. *Journal of Agricultural Science*, *127*, 97–107. https://doi.org/10.1017/S0021859600077418

VDLUFA (2012). *Methodenbuch, Vol. III. Die Chemische Untersuchung von Futtermitteln*. Darmstadt, Germany: VDLUFA-Verlag (in German).

Von Lengerken, J., & Zimmermann, K. (1991). *Handbuch Futtermittelprüfung* (p. 651). Berlin, Germany: Deutscher Landwirtschaftsverlag (in German).

Weiss, W. P., Chamberlain, D. G., & Hunt, C. W. (2003). *Feeding silages*. In D. R. Buxton, R. E. Muck, & J. H. Harrison (Eds.), *Silage science and technology* (pp. 469–504). Madison, WI, USA: American Society of Agronomy.

Weiß, K. (2001). *Gärungsverlauf und Gärqualität von Silagen aus nitratarmen Grünfutter*. Doctoral Thesis, Humboldt-Universität zu Berlin, Germany (in German).

Weiß, K., & Kaiser, E. (1995). Milchsäurebestimmung in Silageextrakten mit Hilfe der HPLC. *Das wirtschaftseigene Futter*, *41*, 69–80 (in German).

Weiß, K., & Sommer, G. (2012). Bestimmung von Estern und anderen flüchtigen organischen Substanzen (VOC) in Silageextrakten mit Hilfe der Gaschromatographie. *VDLUFA-Schriftenreihe*, *68*, 561–569 (in German).

Weißbach, F., & Strubelt, C. (2008). Correcting the dry matter content of grass silages as a substrate for biogas production. *Landtechnik*, *63*, 210–211.

CHAPTER 5

Decision-making of goats when exposed to choice feeding: triggered by taste or smell?

R. Scherer, K. Gerlach, K.-H. Südekum

Institute of Animal Science, University of Bonn, Endenicher Allee 15, 53115 Bonn, Germany

Published in **Applied Animal Behaviour Science** 210 (2019), 46–51.

Abstract

Animals recognise known forages. However, it is unclear whether recognition is mainly based on sensory characteristics linked to postingestive feedback or whether the latter is activated each time before a choice is made. Moreover, knowledge is scarce regarding the duration of the memory process and if the memory is primarily activated by smell or a combination of smell and taste. The hypothesis of this study was that in choice situations (1) the preference for one of two offered forages develops in the initial moments of feeding, and (2) this is led by recognition via smell linked to postingestive feedback. In order to study the potential relationships between initial feed intake (FI) (3 min) and short-term FI (3 h), a preference trial as a special form of feed choice experiments with Saanen-type wethers was undertaken. Forages comprised lucerne (*Medicago sativa* L., first cut), red clover (*Trifolium pratense* L., first cut) and Italian ryegrass (*Lolium multiflorum* LAM., second cut). Six different treatments of each forage type were ensiled such that silages of varying quality were produced. Each possible two-way combination of silages and standard hay (n = 21 combinations) was offered and complemented with video recordings of the first 3 min of feed consumption in the morning. Data were analysed via correlation and regression analysis using PROC CORR (Pearson correlation coefficients) and PROC REG of SAS for examination of the relationships between dry matter intake (DMI; g/30 min and g/3 h) and the duration (s) of FI in the first 3 min. The head was moved 0.8 to 3.0 times between both boxes until the decision was made, and no differences were observed in the frequency of head movements between the silage treatments. The decision did not seem to occur by chance: the initially selected lucerne silages were consumed in greater amounts than the initially non-selected silages within the first 3 min of feeding in 88.3% of the pairwise comparisons. With the red clover silages the proportion was 90.1%, and with the grass silages, 87.3%, 81.7%, 77.5% and 58.9% of the initially non-selected lucerne, red clover and Italian ryegrass silages, respectively, were not eaten at all (i.e. 0 s). A strong relationship was observed between DMI (g/30 min and g/3 h) and the duration of FI in the first 3 min, indicating a high predictive power of initial FI for the preference that develops within 3 h. Around 90% of the variation in DMI, both expressed as g/3 h and g/30 min, was explained by the duration of FI in the first 3 min of feeding. The results suggest that already in the very first minutes of feeding, recognition of the silages and decision-making processes take place based on the smell emitted by the silages.

1. Introduction

Two aspects play a major role in the regulation of the feed intake (FI) behaviour of ruminants: the sensory input (often summarised as feed flavour), comprising taste, odour and texture, and the postingestive feedback, including the effects of nutrients and antinutritive factors on chemo-, osmo- and mechano-receptors (Provenza et al., 1996). The sensory response in the animal, which is considered to be the corollary of the animal's appetite for the feed, can be termed palatability (Baumont, 1996). Sensory characteristics and postingestive effects are strongly interwoven. In addition, individual sensations and nutrient requirements supervene, which can also contribute to feed selection (Forbes, 2007). Profound insights into the complex relationships can be gained through preference trials as a specific form of choice feeding experiments. They are carried out in order to assess the willingness of animals to ingest feeds in a choice situation, i.e. when different feeds are offered at the same time (Meier et al., 2012). Depending on the model applied, the feeds are fed pairwise for two or more h per day, as it is assumed that the preference for one of the two feeds will only develop within a longer period of time. Larson (1995) stated that 2 to 4 h have been found to be optimal for preference trials.

This study investigated the hypotheses that (1) preference for a feed evolves even at the beginning of feeding based on the smell of the silages emitted and (2) visual capture of FI variables over a few minutes is representative for characterising choice feeding and especially short-term feed preference. Based on video recordings the decision-making processes of the animals were observed to examine if initial FI behaviour in the first 3 min of feeding reflects short-term (3 h) FI and if the animals only use the flavour to remember the forage or if they must taste it first to activate the memory of postingestive feedback mechanisms.

2. Materials and methods

2.1. Silage preparation

Silages were prepared from pure stands of lucerne (*Medicago sativa* L., first cut), red clover (*Trifolium pratense* L., first cut) and Italian ryegrass (*Lolium multiflorum* LAM., second cut). Lucerne and Italian ryegrass were grown at the Educational and Research Centre Frankenforst of the Faculty of Agriculture, University of Bonn (Königswinter, Germany; 50°43`14``N and 7°12`22``E). A first cut was harvested on 20 May 2015 and 9 June 2016

(late vegetative stage). One half was ensiled after moderate wilting at a dry matter (DM) concentration of 274 g/kg (lucerne) and 251 g/kg (Italian ryegrass); the other half was wilted overnight and ensiled at a DM of 380 g/kg and 381 g/kg, respectively. Red clover was grown and also harvested at the late vegetative stage at the Educational and Research Centre for Animal Husbandry, Hofgut Neumühle, Münchweiler an der Alsenz, Germany (49°32`18``N and 7°53`0``E). For the silage higher in DM, red clover was harvested in the morning of 2 June 2015 and ensiled the next morning at a DM of 301 g/kg. In the evening of 3 June 2015 the red clover was harvested for the silage lower in DM and ensiled the next morning at a DM of 232 g/kg.

The treatments were specifically chosen to obtain silages reflecting a wide range of fermentation qualities indicative of a wide range of sensory attributes. The plant material higher in DM was treated with a chemical additive (CHE1, 2.5 L/t) and a biological additive (BIO, 1 g/t). Soil (SOIL, 7,600 g/t) and another chemical silage additive (CHE2, 4 L/t), respectively, were added to the plant material lower in DM. Detailed information about the DM concentrations and the applied treatments are summarised in Table 14. Per forage species, two DM concentrations with three treatments each were prepared, resulting in six treatments per forage species and 18 treatments in total.

Refined sugar (sucrose; Diamant Zucker, Pfeifer & Langen, Cologne, Germany) was added to lucerne and red clover to ensure adequate substrate availability during ensiling. The amount was determined based on the average levels of ‘sugar’ (i.e., water-soluble carbohydrates) in lucerne, red clover and Italian ryegrass to close the gap between the low concentrations in the two legumes (typical values, 65 and 115 g/kg DM, respectively) and the high sugar concentrations in Italian ryegrass (typical value 190 g/kg DM; Bundesarbeitskreis Futterkonservierung, 2011).

Immediately following unloading from the loader wagon onto a clean, dry place, the forage was processed. For this purpose the appropriate quantities were spread on a tarpaulin (3 x 4 m) and treated as assigned. The plant material was subsequently compacted in barrels with a lid (120 L), following the recommendations of Bundesarbeitskreis Futterkonservierung (2011). The density for low-DM lucerne, red clover and Italian ryegrass was 210 kg DM/m^3 and for the high-DM forage 170 kg DM/m^3. Each treatment was ensiled for 120 days in quadruplicate.

Table 14 Silage treatments of lucerne, red clover and Italian ryegrass forage used in the preference trial with goats.

	Forage species		
	Lucerne	Red clover	Italian ryegrass
Treatment		Dry matter (%)	
CON1	38	30	38
CHE1	38	30	38
BIO	38	30	38
CON2	27	23	25
CHE2	27	23	25
SOIL	27	23	25

CON, untreated control; CHE1, chemical silage additive (2.5 L/t) based on sodium nitrite and hexamine (hexamethylenetetramine); CHE2, chemical silage additive (4 L/t) based on 75% formic acid buffered with sodium hydroxide to pH 2.5; BIO, biological additive (1 g/t) based on homofermentative lactic acid bacteria (*Lactobacillus plantarum*; $3.0 \cdot 10^{11}$ colony forming units/g); SOIL, addition of 7,600 g soil/t.

2.2. Preference trials

A preference trial according to Burns et al. (2001) was conducted in order to evaluate feed choice behaviour among silages of the three forage species. The trial was divided into three runs (one for each species) and was undertaken with Saanen-type wethers (German Improved White Goat breed). For the legume silages eight wethers and for the Italian ryegrass silages six wethers were used. Two wethers shared an indoor pen of approximately 2 x 3 m bedded with straw. Every morning before offering the silages, the animals were tied up for the duration of feeding, i.e. 3 h. Animals had ad libitum access to drinking water and were able to lie down. Animal care and handling accorded with official German regulations and was approved by veterinary authorities.

During an adaptation period prior to the experimental phase, single meals of each lucerne, red clover and Italian ryegrass silage and lucerne hay were offered once in randomised order, in order to allow the animals to associate the silage with postingestive metabolic response, taste and smell (Kyriazakis et al., 1990). For each run the adaptation period lasted 7 d (six silage treatments and lucerne hay). Lucerne hay was compared as a standard to each of the six silages. During the experimental phase, each possible 2-way combination of the six silages

and the standard lucerne hay (n = 21 combinations) was presented. Forages were offered in plastic boxes (400 x 340 x 250 mm) and the pairs were presented side by side. Forages were randomly allocated each day in order to prevent a habit reflex as it is possible that a particular position becomes perceived as relatively more convenient by the animal (Meier et al., 2012). Goats had free access to both feeding boxes so that free choice between forages could be guaranteed. The boxes were weighed 30 minutes after the start of feeding, and again after 3 h (Buntinx et al., 1997). In order to ensure ad libitum FI, the respective silage was refilled as soon as less than 300 g remained in the box. Each day, the experimental meal was offered for 3 h, starting at 7:30 h. Grass hay was offered for ad libitum consumption each afternoon for 2 h starting at 15:30 h to meet the animals' overall energy and nutrient requirements. Each run lasted 28 d, consisting of 7 d for adaptation and 21 d for experimental measurements.

2.3. Video recordings

The first 3 min of feeding were recorded. The video recordings were made with two commercial digital cameras (Panasonic DMC-FX30, Panasonic HDC-SD99, Kadoma, Japan) on tripods. During the positioning of the tripods, attention was paid to avoid distracting the animals and to ensure good visibility of each animal. After 3 min the cameras were switched off and the animals continued to eat. The video recordings were evaluated through applying the following foci: time (s) spent with FI and time spent elsewise (which was distinguished in terms of time spent hesitating, sniffing and standing behind the feeding rack). Feed intake was differentiated into the duration (s) of the first contact of the initially selected and the initially non-selected silage and the respective accumulated duration within the 3 min. Moreover, the number of box changes by moving the head before choosing the initially selected silage and the total number of box changes within the 3 min were determined. These observations resulted in the following response variables: duration of the initial FI of the initially selected silage, duration of FI of the initially non-selected silage, number of changes before initial FI, number of changes during 3 min, duration of FI of the initially selected silage during 3 min (s FI/3 min), duration of FI of the initially non-selected silage (s FI/3 min), and duration of hesitation or sniffing and of standing behind the feeding rack.

2.4. Evaluation scheme for sensory characteristics

Based on the concentrations of acetic acid, butyric acid and the pH value, the fermentation quality of the silages was assessed using the DLG scheme (Deutsche

Landwirtschaftsgesellschaft, 2006). Depending on the respective concentrations the silages received points so that they could be graded from 1 to 5 (very good, good, needs improvement, bad and very bad). Increased concentrations of acetic or butyric acid or an insufficient pH decline, all of which can lead to a deterioration of quality, reduced the final score.

2.5. Statistical analyses

Data were analysed using SAS 9.4 (SAS®, 2010). The CORR and REG procedures were used to evaluate the associations between DMI over 30 min and 3 h respectively, and the duration of FI within the first 3 min of feeding. The mean values of each forage combination averaged across animals served as a database for the calculation of mean values across forage combinations. In correlation and regression analysis, significance was declared at $p < 0.05$, whereas a trend towards a significant effect was noted when $0.05 \leq p \leq 0.1$.

Furthermore, each trial was evaluated by analysis of variance. It included terms for animal and forage. Within the silage treatments, means were separated using the minimum significant difference (MSD) from the Waller-Duncan *k*-ratio *t*-test ($k = 100$) (Burns et al., 2001).

3. Results

Detailed laboratory results are given in Scherer et al. (2018). Based on the DLG evaluation scheme (Deutsche Landwirtschaftsgesellschaft, 2006), silages were generally of high quality. Six of the 18 silages had the highest score and another six a score of 95, such that 12 silages were classified as ‘very good’. Three silages were given the grade 2 or ‘well-fermented’. Only the soil-treated silages were assessed with grade 3 and 4 (‘need for improvement’ and ‘bad’), reflecting a deterioration in quality. Despite similar fermentation quality estimates based on the DLG scheme, the DMI of the silages within forage species differed remarkably. The rankings of DMI for 30 min and 3 h respectively, and the duration of FI within the first 3 min were almost identical. Moreover, the 3 min, 30 min and 3 h rankings of the three forage species were very similar (Table 15).

Table 15 Comparison of the means of the duration of feed intake (FI) of the silages made of lucerne, red clover and Italian ryegrass within the first 3 min of feeding (s FI/3 min) and dry matter intake (DMI) within 30 min and 3 h (g DMI/30 min and g DMI/3 h, respectively) of goats (n = 8 for lucerne and red clover silages and n = 6 for Italian ryegrass silages).

Forage-species		Treatment[A]						MSD[B]
		CON1	CHE1	BIO	CON2	CHE2	SOIL	
Lucerne	DMI (g DMI/3 h)	724^{a,b}	829^{a}	670^{b,c}	417^{d}	591^{c,d}	148^{e}	118
	DMI (g DMI/30 min)	369^{a,b}	416^{a}	311^{b,c}	159^{d}	246^{c}	35^{e}	70
	Duration (s FI/3 min)	118^{a,b}	141^{a}	98^{b,c}	54^{d}	82^{c,d}	8^{e}	30
Red clover	DMI (g DMI/3 h)	804^{a}	629^{b}	582^{b,c}	493^{c}	598^{b}	183^{d}	90
	DMI (g DMI/30 min)	416^{a}	385^{a}	307^{b}	183^{c}	297^{b}	39^{d}	59
	Duration (s FI/3 min)	137a	143a	86b	46^{c}	89^{b}	5^{d}	26
Italian ryegrass	DMI (g DMI/3 h)	634^{a}	432^{b}	369^{b}	355^{b}	257^{c}	158^{d}	94
	DMI (g DMI/30 min)	218^{a}	126^{b}	81^{c}	108^{bc}	72^{c,d}	41^{d}	39
	Duration (s FI/3 min)	108^{a}	65^{b}	39^{c,d}	61^{b,c}	36^{d,e}	13^{e}	24

Means within row with different superscripts (a-e) differ ($p < 0.05$).

[A] CON, untreated control; CHE1, chemical silage additive (2.5 L/t), based on sodium nitrite and hexamine (hexamethylenetetramine); CHE2, chemical silage additive (4 L/t), based on 75% formic acid buffered with sodium hydroxide to pH 2.5; BIO, biological additive (1 g/t), based on homofermentative lactic acid bacteria (*Lactobacillus plantarum*; $3.0 \cdot 10^{11}$ colony forming units/g); SOIL, addition of 7,600 g soil/t.

[B] Minimum significant difference (Waller-Duncan *k*-ratio *t*-test).

Although it was strictly observed that the two boxes were placed simultaneously into the feeding troughs, it often happened that the animals were already stretching their heads in the direction of one of the two boxes before they were placed in front of them. In the majority of cases the animals sniffed at each silage either once or several times, and then quickly decided on the initially preferred silage. Moreover, with a few silages it was observed that the animals failed to decide having consumed a small amount of each silage and resigned from the feeding trough. Whether they sniffed first or fed immediately was not related to the silage combination.

The relationships across the silages of the three forage species (n = 18), as well as within one species (n = 6), were very strong (Table 16). When using the individual data, i.e. the 3-

min, 30 min and 3 h data respectively of each animal and each combination, the relationships were partly weaker (Table 17). The durations (s) of initial FI of the initially selected silages and the duration of FI of the initially selected silages (s FI/3 min) were closely related (Table 18).

The silages that were initially selected were proportionally more greatly eaten than the initially non-selected silages: in 88.3% of the pairwise comparisons the duration of FI of the initially selected lucerne silages was longer than the FI of the initially non-selected lucerne silages. In red clover silages the proportion accounted for 90.1% and in grass silages for 87.3% of the pairwise comparisons. Within the first 3 min of FI, 81.7%, 77.5% and 58.9% respectively of the initially non-selected silages were not eaten at all, i.e. 0 s. The head was moved 0.7 to 3.3 times between the two feed boxes until the decision for one of the two lucerne silages was made. In the red clover silages the head was moved 0.4 to 3.3 times and in the grass silages 1.6 to 3.0 times. No differences were observed in the frequency of head movements between the silage treatments. The assumption that the head was rotated less frequently in combinations with the moister silages, i.e. SOIL, CHE2 or CON2, was not confirmed. No head movements before the start of feeding were examined in 28.9% of lucerne silages, in 33.3% of red clover silages, and in 15.1% of grass silages. In the lucerne silages the total number of head movements between both boxes during the 3 min of recording was 1.8 to 5.6, in red clover silages 2.0 to 4.9, and in grass silages 3.3 to 8.3.

Table 16 Pearson correlation coefficients between the duration of feed intake (FI) of 18 silages made of lucerne, red clover and Italian ryegrass within the first 3 min of feeding (s FI/3min) and dry matter intake (DMI) within 30 min and 3 h (g DMI/30 min and g DMI/3 h respectively) of goats[A] (n = 8 for lucerne and red clover silages and n = 6 for Italian ryegrass silages).

	s FI/3 min			
	All lucerne-, red clover and Italian ryegrass silages	Lucerne silages	Red clover silages	Italian ryegrass silages
g DMI/3 h	0.907	0.987	0.921	0.949
g DMI/30 min	0.898	0.991	0.982	0.993

[A] Calculation basis: mean values of DMI (g/3 h and g/30 min respectively) and s FI/3 min of the animals per combination and across all combinations.

Table 17 Pearson correlation coefficients for the duration of feed intake (FI) within the first 3 min of feeding (s FI/3 min) and dry matter intake (DMI) within 30 min and 3 h (g DMI/30 min and g DMI/3 h, respectively) of goats[A] (n = 8 for lucerne and red clover silages and n = 6 for Italian ryegrass silages).

	s FI/3 min					
	Forage species and treatment[B]					
	CON1	CHE1	BIO	CON2	CHE2	SOIL
	Lucerne silages					
g DMI/3 h	0.47	0.34	0.27	0.49	0.57	0.67
g DMI/30 min	0.63	0.62	0.69	0.7	0.69	0.88
	Red clover silages					
g DMI/3 h	0.41	0.15	0.49	0.37	0.53	0.29
g DMI/30 min	0.49	0.29	0.59	0.51	0.63	0.5
	Italian ryegrass silages					
g DMI/3 h	0.77	0.7	0.81	0.74	0.68	0.64
g DMI/30 min	0.79	0.64	0.69	0.7	0.66	0.35
	All lucerne silages					
g DMI/3 h	0.61					
g DMI/30 min	0.74					
	All red clover silages					
g DMI/3 h	0.61					
g DMI/30 min	0.72					
	All Italian ryegrass silages					
g DMI/3 h	0.68					
g DMI/30 min	0.8					
	All lucerne-, red clover and Italian ryegrass silages					
g DMI/3 h	0.64					
g DMI/30 min	0.64					

[A] Based on individual values per animal and forage combination.

[B] CON, untreated control; CHE1, chemical silage additive (2.5 L/t), based on sodium nitrite and hexamine (hexamethylenetetramine); CHE2, chemical silage additive (4 L/t), based on 75% formic acid buffered with sodium hydroxide to pH 2.5; BIO, biological additive (1 g/t), based on homofermentative lactic acid bacteria (*Lactobacillus plantarum*; $3.0 \cdot 10^{11}$ colony forming units/g); SOIL, addition of 7,600 g soil/t.

p-values: < 0.05, except for lucerne, BIO, g DMI/3 h: 0.12; red clover, SOIL, g DMI/3 h: < 0.1; red clover, CHE1, g DMI/30 min: < 0.1.

Table 18 Pearson correlation coefficients between the initial duration (s) of feed intake (FI) of the initially selected silage and the duration of FI of the initially selected silage over 3 min (s/3 min).

Forage species and treatment[A]					
CON1	CHE1	BIO	CON2	CHE2	SOIL
Lucerne silages					
0.76	0.83	0.89	0.96	0.93	1.0
Red clover silages					
0.73	0.83	0.85	0.86	0.76	1.0
Italian ryegrass silages					
0.64	0.8	0.81	0.76	0.71	0.42
All lucerne silages					
0.91					
All red clover silages					
0.87					
All Italian ryegrass silages					
0.68					

[A] CON, untreated control; CHE1, chemical silage additive (2.5 L/t), based on sodium nitrite and hexamine (hexamethylenetetramine); CHE2, chemical silage additive (4 L/t), based on 75% formic acid buffered with sodium hydroxide to pH 2.5; BIO, biological additive (1 g/t), based on homofermentative lactic acid bacteria (*Lactobacillus plantarum*; $3.0 \cdot 10^{11}$ colony forming units/g); SOIL, addition of 7,600 g soil/t.

p-values: < 0.05, except for Italian ryegrass, SOIL: 0.02

Regression analysis for the lucerne, red clover and Italian ryegrass data concerning the duration of FI and DMI revealed linear functions (Figure 2 and 3). Since the F-test as part of the analysis of regression revealed a coefficient of determination in the overall population that was significantly different from zero, the function was suitable for describing the relationship between the data of the initial FI (s/3 min) and the short-term DMI (g/30 min and g/3 h, respectively). The relationship between initial FI and short-term DMI of the initially selected silage was very strong, since 89.2% of the variation in DMI (g/3 h) was explained by the variation of the duration of initial FI in the first 3 min of feeding. The relationship between g DMI/30 min and the initial duration of FI of the initially selected silage was also very strong:

89.5% of the variation in DMI (g/30 min) was explained by the duration of FI in the first 3 min of feeding.

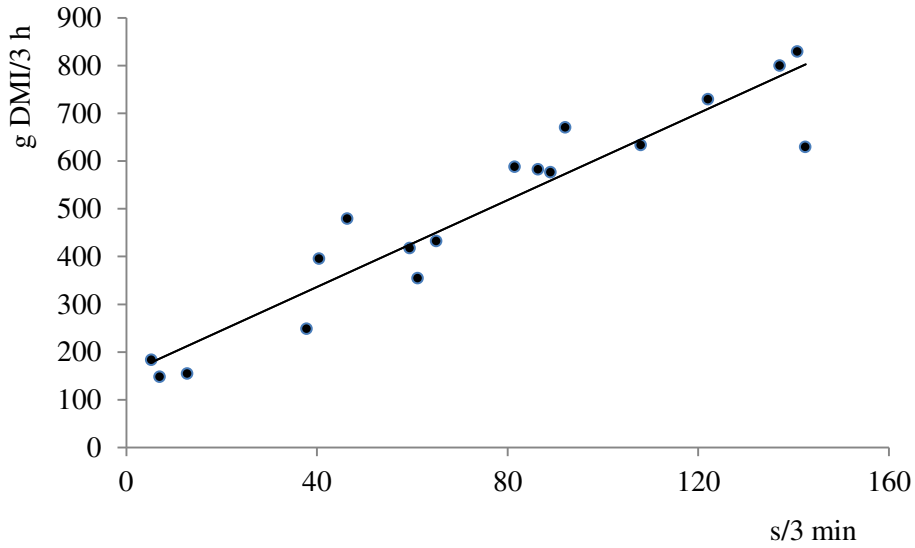

Fig. 2 Relationship between the dry matter intake (DMI) (g DMI/3 h) and the duration (s) of feed intake (FI) (s/3 min) of the initially selected silage [y = 154.08 (SE = 33.13; p = 0.0003) + 4.55 (SE = 0.39; $p < 0.0001$)x; $R^2 = 0.897$].

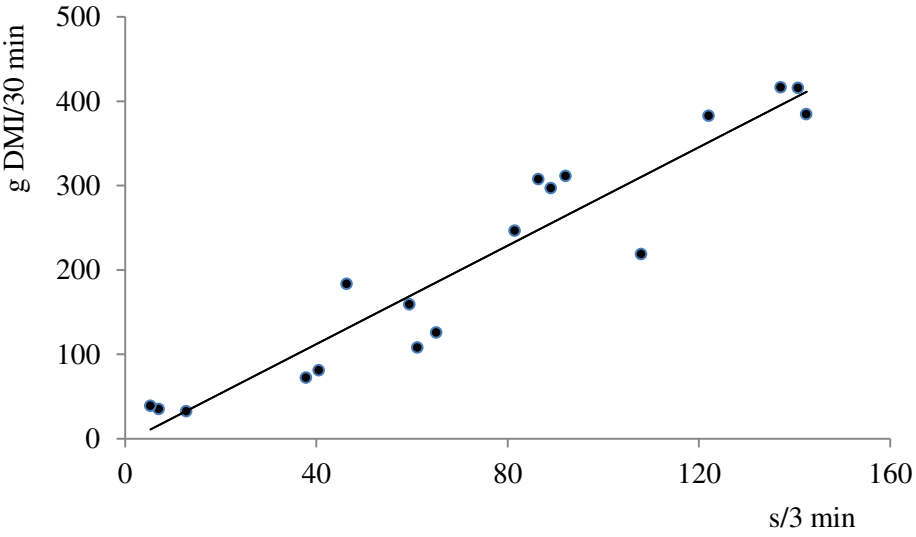

Fig. 3 Relationship between the matter intake (DMI) (g DMI/30 min) and the duration (s) of feed intake (FI) (s/3 min) of the initially selected silage [y = -4.61 (SE = 21.02; p = 0.8291) + 2.92 (SE = 0.25; $p < 0.0001$)x; $R^2 = 0.899$].

4. Discussion

4.1. Physiological considerations

Decision-making in free choice situations is a very complex process and depends on numerous factors, whose interplay links feed and animal properties in response to the feed, including plant morphology (Craigmiles et al., 1964), sensory quality of an offered feed, instinctive or learned responses of the animal to a given feed (Larson, 1995), and learned associations with postingestive feedback effects (Provenza et al., 1994). Ruminants are capable of developing recognition mechanisms for favourable or adverse feed components (Provenza, 1995) and of recognising forages they have already tasted due to the postingestive feedback that develops following feeding (Kyriazakis et al., 1990). This includes the identification of odour and taste (Provenza et al., 1996), which helps animals to select diets that meet their requirements (Görgülü et al., 1996), thus maintaining a good rumen environment (Cooper et al., 1995) and alleviating illness (Villalba and Provenza, 2007; Villalba et al., 2010). However, when forages are similar in their chemical composition such that no remarkable differences in postingestive feedback evolve, the preference for a forage in a choice situation can exclusively be made on the basis of sensory characteristics. In the present case, it is unlikely that only the latter were responsible for recognising the silages, since differences in the chemical composition were found (Scherer et al., 2018), hence suggesting differences in the developed postingestive feedbacks. Thus, recognition was either provoked by the postingestive feedback signals being activated by smell or taste of the silages every time before a decision for or against the two offered forages was made, or it was provoked by the sensory characteristics of the silages being particularly strongly linked to the postingestive feedback so that the animals were able to remember them. In order to examine these relationships and to eliminate the notion that the decision-making process could simply be based on fortune, the initial FI behaviour was evaluated via observation of the goats in the first 3 min of feeding.

In most cases the initially non-selected silage was not eaten at all during the first 3 min, or the initially chosen silage was consumed in greater amounts than the initially non-selected silage. Moreover, the rankings of initial FI (s/3 min) and short-term DMI (g/3 h) were very similar. Taken together, these results render random decisions unlikely. Most of the time the goats had sniffed at both silages before deciding on their preference of silage, which strongly indicates that the choice was based on olfaction. Thus, preference and avoidance seemed to

emerge in the very first moments of feeding, with the sensory characteristics of the silages sufficient to activating the postingestive feedback. Stretching the head in the direction of one of the two boxes before they were placed in the feeding trough also suggests that the odours emitting from the silages are sufficient for their recognition. In contrast, preference for or aversion to one of the two offered silages was not identified when the animals did not decide directly and resigned from the feeding trough having spent some time sniffing.

The strong correlation between initial FI (s/3 min) and short-term DMI (g/3 h) also indicates that the decision-making process of the animals was based on recognition. Indeed, the DMI data show that the initially selected silage was not eaten all the time during the 3 h, a finding that is not expected in a preference trial. For example, the SOIL treatment was hardly ever initially selected, but it was nevertheless eaten during the following 177 min with two feeds on offer. Moving the head between the two boxes might have been caused by boredom or curiosity, especially when moving the head to a silage that was subsequently avoided. Another reason for this observation may be a change in the animals' perception of forage quality over time. Both considerations suggest that preference evolves even from the beginning of feeding and is modified by a longer duration of feeding. Nevertheless, the 3 h preference was well reflected by the 3-min values since 89% of the variation in DMI was explained by the duration of FI in the first 3 min of feeding.

4.2. Methodical considerations

Preference for and avoidance of a feed was deeply shaped by its flavour components: every aspect of the decision-making process is directly or indirectly related to smell or taste. Olfaction has a huge influence on the flavour of a feed: what we think of as taste is in fact predominantly olfaction. Indeed, olfaction is the primary sensory modality for most mammals (Jones and Roper, 1997). The influence of odours forms a major component of the animals' environment. However, airborne chemical stimuli are difficult to work with, measure and control (Nielsen et al., 2015). Therefore, studies on the sense of smell are not as advanced as those of other sensory modalities (Fendt et al., 2017). Tastes may change when odorants vaporise, since taste and smell are inevitably and closely related. This means that the taste of silage can change without the experimenter noticing it, as would contrarily be the case in an auditory or visual stimulus trial. In addition, the changes would not be measurable. To further complicate matters, thousands of molecules that can be detected exist, and their vapour pressure, rate of diffusion and solubility vary considerably. Thus, controlling and measuring

odours is a major problem in studies of olfaction (Fendt et al., 2017), impeding the evaluation of the present results concerning the impact of taste and odour on the decision-making process.

As the experimental design of preference trials is used to determine how the animals react to feedstuffs of differing chemical compositions (Buntinx et al., 1997; Burns et al., 2001), a constant composition of the silages throughout the feeding period must be ensured. During 3 h, however, silages will slightly change in DM concentrations and the concentrations of volatile compounds will diminish (Daniel et al., 2013), such that the perception of the animals towards the silages may also change to an extent. Therefore, the 3-min measurement design may allow for more reliable insights into the relationships between the chemical composition of a feed – especially sensory attributes – and its short-term voluntary intake in a choice situation.

A possible limitation of the 3-min design is that decision-making within the first 3 min can be guided by hunger. Hunger is a major confounding factor in choice feeding experiments: hungry animals tend to start consuming the very first accessible feed irrespective of its real preference (Scharenberg et al., 2007), so that real preference may only develop after the initial hunger is satisfied. Moreover, it is unclear whether the decision to prefer or avoid a feed would have been the same if the animals received the feed for only 3 min daily, since this would not necessarily lead to a postingestive feedback. Although this feedback is already achieved during the adaptation phase, it is certainly also reinforced by the 3–h feeding time during the experimental phase. The uncertainty about the feedback could be eliminated by always presenting the feed for 3 h (Burns et al., 2001), even though only 3 min are recorded.

The method to measure feed preference in free choice situations (Burns et al., 2001) was conducted on-site (Gerlach et al., 2014a,b) in order to examine the effects of aerobic exposure of lucerne and grass silages on short-term DMI and preference amongst goats. It is proposed as a complement to preference trials by recording FI during the initial few minutes, because preference and avoidance develop and are expressed in the very first moments of feeding. Conducting the trials on a smaller scale, both regarding the duration and amount of required forage, would enable testing of a larger number of forages in a given period of time.

5. Conclusion

The preference of ruminants for one of two ensiled forages offered for free choice is formed and expressed from the very first minutes of feeding. Thus, the technique of behavioural observation via video recordings over a very short time period seems suitable for assessing and judging the development of animal preference in a free-choice situation between two known forages. It is recommended to complement preference trials with video recordings of the first minutes of feeding to substantiate current results. Depending on the objective, preference trials may be carried out at a smaller scale than previously seen, hence implementing a procedure that is less time-consuming and costly. Given that the available findings apply to forages that are high in volatile compounds, examination of whether similar results are achieved with feeds others than silage is recommended.

Acknowledgements

This study was supported by the “Deutsche Forschungsgemeinschaft” (DFG, German Research Foundation, SU 124/31-1) and partly conducted in the Center of Integrated Dairy Research (CIDRe), University of Bonn (Bonn, Germany).

References

Baumont, 1996. Palatability and feeding in ruminants. A review. Ann. Zootech. 45, 385–400.

Bundesarbeitskreis Futterkonservierung, 2011. Praxishandbuch Futter- und Substratkonservierung. DLG-Verlags-GmbH, Frankfurt am Main, Germany (in German).

Buntinx, S.E., Pond, K.R., Fisher, D.S., Burns, J.C., 1997. The utilization of multidimensional scaling to identify forage characteristics associated with preference in sheep. J. Anim. Sci. 75, 1641–1650.

Burns, J.C., Fisher, D.S., Mayland, H.F., 2001. Preference by sheep and goats among hay of eight tall fescue cultivars. J. Anim. Sci. 79, 213–224.

Cooper, S.D.B., Kyriazakis, I., Nolan, J.V., 1995. Diet selection in sheep: the role of the rumen environment in the selection of a diet from two feeds that differ in their energy density. Br. J. Nutr. 74, 39–54.

Craigmiles, J.P., Crowder, L.V., Newton, J.P., 1964. Palatability differences in tall fescue using leaf and plant type. Crop Sci. 4, 658–660.

Daniel, J., Weiß, K., Custodio, L., Sá Neto, A., Santos, M., 2013. Occurrence of volatile organic compounds in sugarcane silages. Anim. Feed Sci. Technol. 185, 101–105.

Deutsche Landwirtschaftsgesellschaft, 2006. Grobfutterbewertung-Teil B – DLG-Schlüssel zur Beurteilung der Gärqualität.

Von Grünfuttersilagen auf Basis der chemischen Untersuchung. DLG-Information 2/2006. DLG, Frankfurt am Main, Germany (in German).

Fendt, M., Apfelbach, R., Slotnick, B., 2017. Behavioural tests of olfaction, in: Nielsen, B.L. (Ed.), Olfaction in animal behaviour and welfare. CAB International, Wallingford, UK, pp. 39–60.

Forbes, J.M., 2007. Voluntary food intake and diet selection in farm animals. CAB International, Wallingford, UK.

Gerlach, K., Roß, F.H., Weiß, K., Büscher, W., Südekum, K.-H., 2014a. Aerobic exposure of grass silages and its impact on dry matter intake and preference by goats. Small Ruminant Res. 117, 131–141.

Gerlach, K., Liao, Y., Südekum, K.-H., 2014b. Aerobic exposure of lucerne silages and its impact on preference and dry matter intake by goats. Small Ruminant Res. 121, 308-313.

Görgülü, M., Kutlu, H.R., Demir, E., Öztürkcan, O., Forbes, J.M., 1996. Nutritional consequences among ingredients of free-choice feeding Awassi lambs. Small Ruminant Res. 20, 23–29.

Kyriazakis, I., Emmans, G.C., Whittemore, C.T., 1990. Diet selection in pigs: choices made by growing pigs given foods of different protein concentrations. Anim. Sci. 51, 189–199.

Jones, R.B., Roper, T.J., 1997. Olfaction in the domestic fowl: a critical review. Physiol. Behav. 62, 1009–1018.

Larson, D.A., 1995. Dietary preference and its relationship to feed intake. Pages 312–325 in Symposium: Intake by Feedlot Cattle. Oklahoma Agric. Exp. Stn. P-942. Oklahoma State Univ., Stillwater.

Meier, J.S., Kreuzer, M., Marquardt, S., 2012. Design and methodology of choice feeding experiments with ruminant livestock. Appl. Anim. Behav. Sci. 97, 206–220.

Nielsen, B.L., Jezierski, T., Bolhuis, J.E., Amo, L., Rosell, F., Oostindjer, M., Christensen, J.W., McKeegan, D., Wells, D.L., Hepper, P., 2015. Olfaction: an overlooked sensory modality in applied ethology and animal welfare. Front. Vet. Sci. 2, 1–3.

Provenza, F.D., 1995. Postingestive feedback as an elementary determinant of food preference and intake in ruminants. J. Range Manage. 48, 2–17.

Provenza, F.D., Lynch, J.J., Burritt, E.A., Scott, C.B., 1994. How goats learn to distinguish between novel foods that differ in postingestive consequences. J. Chem. Ecol. 20, 609–624.

Provenza, F.D., Scott, C.B., Phy, T.S., Lynch, J.J., 1996. Preference of sheep for foods varying in flavors and nutrients. J. Anim. Sci. 74, 2355–2361.

SAS®, 2010. SAS/STAT 9.4 user's guide. SAS Institute, Inc., Cary, NC, USA.

Scharenberg, A., Arrigo, Y., Gutzwiller, A., Soliva, C.R., Wyss, U., Kreuzer, M., Dohme, F., 2007. Palatability in sheep and in vitro nutritional value of dried and ensiled sainfoin (*Onobrychis viciifolia*) birdsfoot trefoil (*Lotus corniculatus*), and chicory (*Cichorium intybus*). Arch. Anim. Nutr. 61, 481–496.

Scherer, R., Gerlach, K., Taubert, J., Adolph, S. Weiß, K., Südekum, K.-H., 2018. Effects of plant species and ensiling conditions on the formation of biogenic amines in silage and the preference behaviour of small ruminants. (under review).

Villalba, J.J., Provenza, F.D., 2007. Self-medication and homeostatic behaviour in herbivores: learning about the benefits of natures`s pharmacy. Animal 1, 1360–1370.

Villalba, J.J., Provenza, F.D., Hall, J.O., Lisonbee, L.D., 2010. Selection of tannins by sheep in response to gastrointestinal nematode infection. J. Anim. Sci. 88, 2189–2198.

CHAPTER 6

General discussion and outlook

In most ruminant production systems livestock derive between 40 and 90% of their feed requirements from forages (Charmley, 2001). For more than six decades the preservation of forage crops by ensiling based on lactic acid fermentation is a fundamental element of efficient ruminant livestock nutrition (Virtanen and Miettinen, 1951). Besides haymaking ensiling is the only option for farmers to conserve forage on a large scale (Charmley, 2001) and for extended periods. Especially in humid and cool areas, where dry matter (DM) and quality losses in making hay may be excessive, ensiling plays a major role (Pahlow et al., 2003). At the end of the 1990s a trend over the last 30 years was observed indicating that the proportion of forage conserved as silage has increased, while the proportion of haymaking has declined (Wilkinson et al., 1996). Due to the ever-growing importance of silage, it is crucial to continue research and widen knowledge about all steps of forage production and processing.

It still remains unclear which variables are primarily responsible for affecting voluntary feed intake (Huhtanen et al., 2007; Gerlach et al., 2013). One relevant factor may be that, typically, only common variables including lactic acid, short-chain fatty acids, NH_3-N and other soluble-N compounds are measured and considered. Ward (2011) stated the relevance of the qualitative versus the quantitative evaluation of feeds, including DM, fermentation acid levels, ammonia, ADICP (acid detergent-insoluble crude protein), fiber digestibility, moulds, yeasts and ash when evaluating the nutrient composition of the ration presented to the animals. This represented an advanced point of view but seven years later, it becomes obvious that this approach was not sufficient (Scherer et al., 2018). For future research on silage there is a need to raise awareness that there are still many unexplored chemical compounds that may affect feed intake. The traditional way in research to analyse silage includes the identification and classification of its components in categories such as protein, fat, fibre fractions, mineral elements and ash (Thaysen, 2004). However, first attempts have already been made some years ago to apply metabolomics in feed analyses (Johnson et al., 2004) and its application is extended (Guo et al., 2018). By means of this method deeper insights in the complex network and interactions of bioactive molecules in silage are possible. Metabolomics deals with a high-throuput characterisation of all molecules and chemical compounds present

in biological matrices (Wishart, 2008). The range of application of metabolomics is diverse, resulting in the subdivision into several subgroups – metabolomics, metabolite profiling, metabolic fingerprinting, metabolite target analysis and metabonomics – each with different objectives (Dunn and Ellis, 2005). Generally, a distinction is drawn between targeted and non-targeted analysis. With a non-targeted analysis, thousands of metabolites can be quantitatively measured from minimal amounts of biological material (Patti et al., 2012). At present, implementation in the range of feed analyses is still utopian, but it should be considered for the future. It will still take a while before the methodology is more established in livestock science but when the time has come it is a promising way to make great progress in silage research. For example the combination of metabolomic with microbiome data of the first few days of ensiling, i.e. when the majority of chemical changes are occurring in the silo, may help to identify the roles of different microbial species (Wilkinson and Muck, 2018).

Perspectives for the implementation of metabolomics in livestock nutrition research

Within the scope of the project on which this thesis is based, metabolomics were applied to the legume silages used for the feed choice trial described in Chapter 2. The overall aim was the identification of metabolites of plant and microbial origin in ensiled forage. In order to have the optimum comparison of feed composition with regard to feed compounds provoking either preference or avoidance, lucerne and red clover silages with the greatest differences in DMI values (lowest and highest 3 h DMI by goats) were chosen. Consequently, in total four samples were analysed. Since in both runs of the preference trial the rankings of 3 h DMI were very similar, samples of the same treatments of lucerne and red clover silages were used. This had the side effect of gaining comparability between both plant species. A non-targeted metabolite profiling was conducted based on GC-MS (gas chromatography-mass spectrometry) and LC-QTOF/MS (liquid chromatography-quadrupole-time of flight/MS) analyses. This combination allows detection of metabolites in the atomic mass range of 50–1,700 Da with an accuracy up to 1–2 ppm and a resolution of mass/Δmass = 40,000. In this way 6,403 metabolites were detected. For an initial overview of the metabolites a principal component (PC) analysis (PCA) was conducted: PC1 separated the different plant species, explaining 40.7% of the variation in the data set; PC2 separated the silage treatments explaining 22.9% of total variation. Silages with the greatest differences in 3 h DMI differed in 29% (lucerne) and 15% (red clover) of their metabolites. These results indicate that

metabolomics can serve as a useful complement to conventional analyses to characterise silages profoundly and to clarify relations between silage composition and feed intake behaviour.

A more descriptive approach was the taxonomic classification of the data. Not annotated metabolites could not be considered (n = 4,727). The remaining 1,676 metabolites were assigned to their super class, class, subclass and direct parent to get an overview of the groups of elements they are belonging to. Because a plant-related database was not available, the classification was conducted with version 3.6 of the Human Metabolome Database (HMDB, 2018), a freely available electronic database providing detailed information about metabolites of the human body. Using samples of the same treatments of lucerne and red clover silages offered the possibility to directly compare their metabolites. For this purpose, metabolites were filtered from the entirety of annotated metabolites which differed between the most preferred and most avoided silages of both forage species, lucerne and red clover. These metabolites were then defined as feed intake relevant compounds, which may have contributed to sensory characteristics of the silages and the postingestive feedback of the goats. Volatile compounds could have had the greatest impact on preference and avoidance of silage since they are the most flavourful compounds in silage. Amongst other substances, they are composed of monoterpenes, sesquiterpenes, alcohols (mono- and sesquiterpene alcohols), ketones, phenols, aldehydes, coumarins, esters and oxides (Parker, 2015), which were all annotated in the samples. The 67 metabolites being more concentrated in the avoided compared to the preferred silages are assumed to have a negative impact on feed intake behaviour. These metabolites consisted of oligopeptides (33), dipeptides (3), amino acids (6), lipids (fatty acyls, glycerolipids and glycerophospholipids; 13), indolacetaldehyde, 1-pentenyl glucosinolate, styrene, methylfurane, xanthine, diadenosine tetraphosphate, L-carnitine, hydrocinnamic acid, ethyl 1-(ethylthio)ethyl disulphide, sphingosine, D-threitol and erythritol. Carnitine as a quarternary ammonium salt, for example, accounts for a bitter taste and acts as a taste modifying molecule (Behrens and Meyerhof, 2015). Hydrocinnamic acid has a sweet, floral scent at room temperature (HMDB, 2018) and might have contributed to the flavour of the avoided silages in an olfactory way. Precursors such as oligopeptides and amino acids contribute to the flavour of foods by chemical reactions that occur during food processing (Anonymous, 2008). The same may be the case during ensiling. Aspects concerning protein degradation can also be considered. As could be shown in Chapter 4, only a small part of NPN was composed of biogenic amines and NH_3-N. Although it is known that a high share of

protein is reduced to free amino acids and peptides (Winters et al., 2000, 2001) the question remains to which other compounds protein disappears to. Questions like these as well can be addressed with metabolomics.

The exploration of aroma

In the evaluation of experiments that serve to identify compounds that may have an effect on feed intake of ruminants often the term “flavour” is used (Provenza et al., 1996, 1998; Kristensen et al., 2010). In the English language the term “aroma” can be used synonymously. According to Guichard (2015) aroma compounds are volatile molecules present in the vapour phase at room temperature that are able to reach the olfactory receptors in order to be perceived. Other sources (DIN10950, 2012) define the term more comprehensive, including olfactory, gustatory, trigeminal (mouth-feel characteristics) and haptic (textural) sensations generated by material taken into the mouth. The taste (gustation) and smell (olfaction) cognition of most mammals are very sensitive so that only low concentrations of compounds in foods and feeds are needed to elicit a response. The sense of smell is much more sensitive than the sense of taste, and some compounds can be detected in aqueous solution by the human olfactory organ at concentrations as low as $2 \cdot 10^{-8}$ mg/L. Moreover, the threshold values for aroma compounds perceived by the odour receptor sites in the olfactory epithelium are much lower than the thresholds for taste receptors (Mottram, 2015). Scientists interested in feed intake of ruminants have probably paid less attention to the role of senses than to physical or energy control of intake. It is noteworthy that none of the published feed intake prediction systems takes into account the sensory response to the feed (Baumont, 1996).

There are more than 10,000 different molecules that can be detected in a food or feed matrix by most mammals, and their vapour pressure, rate of diffusion and solubility varies considerably (Fendt et al., 2017). At least it is known that certain functional groups can be linked to classes of aroma. For example, esters usually have fruity aroma, aliphatic aldehydes and alcohols with six carbons have green aroma (Mottram, 2015). In livestock nutrition it has not to be prioritised which aroma are represented by the various functional groups, especially since it would be difficult to assess whether ruminants have the same sensations towards an aroma like humans. Instead, it would be interesting to see the reactions of the animals to the functional groups. In this respect it would be advantageous to know how the functional groups react in the overall composition of a feed, although there is no general rule for the relationships between chemical structure and aroma (Mottram, 2015). Feed matrices are

complex multi-component systems composed of volatile and non-volatile substances (Guichard, 2015). The release of the aroma compounds from the feed matrix into the vapour phase highly depends on their interaction with non-volatile compounds (Guichard, 2002). Thus, it should be kept in mind that depending on the profile of volatiles and non-volatiles in a feed, a particular compound may differ in its contribution to the aroma. Moreover, the interactions between the main components such as lipids, protein, fibre fractions and hydrocolloids should not be disregarded. Not only the amount, but also the nature of the different macromolecules influences aroma release (Druaux and Voilley, 1997; Guichard, 2006). Hence, both the qualitative and quantitative profiles of aroma compounds are relevant when determining the aroma (Mottram, 2015).

Initial approaches for assessing aroma related compounds of silage were already made some years ago by integrating the examination of esters in the fermentation acid analyses (Weiß et al., 2009, 2016). Esters are known to be odorant, which is why they probably have an effect on the aroma of silage (Mo et al., 2001). According to Figueiredo et al. (2007) esters are the most abundant class of volatile compounds in red clover silages with ethyl esters being the predominant subclass of all esters. Until now the effect of different ethyl esters in silages on voluntary feed intake by ruminants has not been studied intensively (Gerlach et al., 2013, 2014).

Final conclusions

It is advisable to take the aforementioned considerations into account in future experiments although implementation would be very costly since it requires a lot of analytical and technical effort. An alternative option to these very complex approaches would be filming the first minutes of feeding during preference trials like shown in Chapter 5. It revealed the 3 h DMI data and the resulting preference rankings to be very well reflected by the 3 min feed intake duration. When the duration of daily feeding is reduced from several hours to a few minutes a higher turnover of preference trials would be possible than up to date. It would enable to test a larger range of silages in one trial with the same amount of forage. Alternatively, more animals could be fed in order to achieve a higher significance of the data. Given the possibility of having a much greater number of preference trials, the preferences and aversions of young and old animals could be compared. There are no limits for raising new questions. A first step must be to verify that video data can serve for predicting the preference of animals towards two feeds offered for a defined period of time.

In order to generate new knowledge regarding the preference and feed intake behaviour of farm animals more effective than before, it may be conceivable to perform a fast-track screening of various silage treatments via application of video recording the animals in the first minutes of feed intake and to re-test afterwards the silage treatments with the most extreme DMI characteristics with a conventional preference trial. The DMI data can then serve as a basis for selecting samples for metabolomics to analyse silages systematically. It is noteworthy that ideas of this kind to advance silage research have not been part of the XVIII International Silage Conference 2018, Bonn, Germany, where experienced and well-known scientists are presenting and discussing new insights and future perspectives.

References

Anonymous, 2008. Regulation (EC) No 1334/2008 of the European Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council regulation (EEC) No 1601/91, regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC. Off. J. Eur. Union 50, 34–50.

Baumont, R (1996). Palatability and feeding behaviour in ruminants. A review. Annales de zootechnie 45, 385–400.

Behrens, M., Meyerhof, W., 2015. Taste receptors, in: Parker, J.K., Elmore, J.S., Methven, L. (Eds.), Flavour development, analysis and perception in food and beverages. Woodhead Publishing, Cambridge, UK, pp. 297–329.

Charmley, E., 2001. Towards improved silage quality – a review. Can. J. Anim. Sci. 81, 157–168.

DIN 10950, 2012. Sensory analysis – basic principles. https://www.beuth.de/de/norm/din-10950/154727508 (accessed 19 March 2018).

Druaux, C., Voilley, A., 1997. Effect of food composition and microstructure on volatile flavour release. Trends Food Sci. Technol. 8, 364–368.

Dunn, W.B., Ellis, D.I., 2005. Metabolomics: Current analytical platforms and methodologies. Trends Anal. Chem. 24, 285–294.

Fendt, M., Apfelbach, R., Slotnick, B., 2017. Behavioural tests of olfaction, in: Nielsen, B.L. (Ed.), Olfaction in animal behaviour and welfare. CAB International, Boston, MA, USA, pp. 39–60.

Figueiredo, R., Rodrigues, A.I., do Céu Costa, M., 2007. Volatile composition of red clover (*Trifolium pratense* L.) forages in Portugal: The influence of ripening stage and ensilage. Food Chem. 104, 1445–1453.

Gerlach, K., Ross, F.H., Weiß, K., Büscher, W., Südekum, K.-H., 2013. Changes in maize silage fermentation products during aerobic deterioration and effects on dry matter intake by goats. Agri. Food Sci. 22, 168–181.

Gerlach, K., Roß, F.H., Weiß, K., Büscher, W., Südekum, K.-H., 2014. Aerobic exposure of grass silages and its impact on dry matter intake and preference by goats. Small Ruminant Res. 117, 131–141.

Guichard, E., 2002. Interactions between flavor compounds and food ingredients and their influence on flavor perception. Food Rev. Int. 19, 49–70.

Guichard, E., 2006. Flavor retention and release from protein solutions. Biotechnol. Adv. 24, 226–229.

Guichard, E., 2015. Interaction of aroma compounds with food matrices, in: Parker, J.K., Elmore, J.S., Methven, L. (Eds.), Flavour development, analysis and perception in food and beverages. Woodhead Publishing, Cambridge, UK, pp. 273–295.

Guo, X.S., Ke, W.C. Ding, W.R., Ding, L.M., Xu, D.M., Wang, W.W., Zhang, P., Yang, F.Y., 2018. Profiling of metabolome and bacterial community dynamics in ensiled *Medicago sativa* inoculated without or with *Lactobacillus plantarum* or *Lactobacillus buchneri*. Scientific Reports 8 (published online).

HMDB, 2018. The human metabolome database. http://www.hmdb.ca/metabolites/HMDB0000764 (accessed 12 March 2018).

Huhtanen, P., Rinne, M., Nousiainen, J., 2007. Evaluation of the factors affecting silage intake of dairy cows: a revision of the relative silage dry-matter intake index. Animal 1, 758–770.

Johnson, H.E., Broadhurst, D., Kell, D.B., Theodorou, M.K., Merry, R.J., Griffith, G.W., 2004. High-throughput metabolic fingerprinting of legume silage fermentations via Fourier

transform infrared spectroscopy and chemometrics. Appl. Environ. Microbiol. 70, 1583–1592.

Kristensen, N.B., Sloth, K.H., Højberg, O., Spliid, N.H., Jensen, C., Thøgersen, R., 2010. Effects of microbial inoculants on corn silage fermentation, microbial contents, aerobic stability, and milk production under field conditions. J. Dairy Sci. 93, 3764–3774.

Mo, M., Selmer-Olsen, I., Randby, Å.T., Aakre, S.E., Asmyhr, A., 2001. Fermentation products in grass silage and their effects on feed intake and milk taste. Proceedings of the 10th International Symposium on Forage Conservation. Brno, Czech Republic, p. 98–99.

Mottram, D. S., 2015. Introduction, in: Parker, J. K., Elmore, J. S., Methven, L. (Eds.), Flavour development, analysis and perception in food and beverages. Woodhead Publishing, Cambridge, pp. xxvii–xxx.

Pahlow, G., Muck, R.E., Driehuis, F., Oude Elferink, S.J.W.H., Spoelstra, S.F., 2003. Microbiology of ensiling, in: Buxton, D.R., Muck, R.E., Harrison, J.H. (Eds.), Silage Science and Technology. American Society of Agronomy Inc., Crop Science Society of America Inc., Soil Science of America Inc., Madison, WI, USA, pp. 328–330.

Parker, J.K., 2015. Introduction to aroma compounds in foods, in: Parker, J.K., Elmore, J.S., Methven, L. (Eds.), Flavour development, analysis and perception in food and beverages. Woodhead Publishing, Cambridge, UK, pp. 3–30.

Patti, G.J., Yanes, O., Siuzdak, G., 2012. Metabolomics: the apogee of the omics triology. Nat. Rev. Mol. Cell Biol. 13, 263–269.

Provenza, F.D., Scott, C.B., Phy, T.S., Lynch, J.J., 1996. Preference of sheep for foods varying in flavors and nutrients. J. Anim. Sci. 74, 2355–2361.

Provenza, F.D, Villalba, J.J., Cheney, C.D., Werner, S.J., 1998. Self-organization of foraging behaviour: From simplicity to complexity without goals. Nutr. Res. Rev. 11, 199–222.

Scherer, R., Gerlach, K., Taubert, J., Adolph, S. Weiß, K., Südekum, K.-H., 2018. Effects of plant species and ensiling conditions on the formation of biogenic amines in silage and the preference behaviour of small ruminants. (under review).

Thaysen, J., 2004. Die Produktion von qualitativ hochwertigen Grassilagen. Übers. Tierernährg. 32, 57-102.

Virtanen, A.I., Miettinen, J.K., 1951. Estimation of volatile fatty acids and ammonia in silage by means of paper chromatography. Nature 168, 294–295.

Ward, R.T., 2011. Analyzing silage crops for quality: what is most important?, in: Proceedings, Western Alfalfa and Forage Conference. Las Vegas, NV: California Alfalfa Workgroup, University of California, 20 pp.

Weiß K., Kalzendorf, C., Zittlau, J., Auerbach, H., 2009. Novel results on the occurrence of volatile compounds in maize silage. Proc. 15th Int. Silage Conf., Madison, WI, USA, pp. 33–34.

Weiß, K., Kroschweski, B., Auerbach, H., 2016. Effects of air exposure, temperature and additives on fermentation characteristics, yeast count, aerobic stability and volatile organic compounds in corn silage. J. Dairy. Sci. 99, 8053–8069.

Wilkinson, J.M., Wadephul, F., Hill, J., 1996. Silage in Europe – A survey of 33 countries. Chalcombe Publications, Bucks, UK.

Wilkinson, J.M., Muck, R.E. The future of ensiling: challenges and opportunities, in: Gerlach, K., Südekum, K.-H. (Eds), Proceedings *XVIII International Silage Conference*. Bonn, Germany, pp. 2–15.

Winters, A.L., Cockburn, J.E., Dhanoa, M.S., Merry, R.J., 2000. Effects of lactic acid bacteria in inoculants on changes in amino acid composition during ensilage of sterile and non-sterile ryegrass. J Appl. Microbiol. 89, 442–451.

Winters, A.L., Fychan, R., Jones. R., 2001. Effect of formic acid and a bacterial inoculant on the amino acid composition of grass silage and on animal performance. Grass Forage Sci. 56, 181–192.

Wishart, D., 2008. Metabolomics: applications to food science and nutrition research. Trends Food Sci. Technol. 19, 482–493.

DANKSAGUNG

Die ersten Worte gelten Herrn Prof. Karl-Heinz Südekum, der mich die Tierernährung hat entdecken lassen. Ich danke ihm ganz herzlich für das in mich gesetzte Vertrauen und die fachlich sehr gute Betreuung und die damit verbundenen wertvollen Diskussionen.

Ein weiterer Dank gilt dem Zweitgutachter, Herrn Prof. Martin Gierus, der sich gerne dazu bereit erklärt hat, diese Rolle zu übernehmen. Außerdem möchte ich Herrn Prof. Wolfgang Büscher und Herrn Prof. Jens Léon für ihre Funktion als fachnahes Mitglied bzw. die Übernahme des Prüfungsvorsitzes danken.

Dr. Katrin Gerlach danke ich von Herzen für ihre große Unterstützung während aller Phasen der Arbeit. Dr. Kirsten Weiß und ihrem Team danke ich für die gute Zusammenarbeit während meiner Aufenthalte in Berlin und darüber hinaus. Ein großes Dankeschön gilt auch Dr. Dave Brüning, der mir während der gesamten Zeit mit Rat und Tat zur Seite stand.

Ich danke den Mitarbeiterinnen des Labors der Tierernährung für die Unterstützung bei den nicht enden wollenden Analysen. Ein weiterer Dank geht an die zahlreichen Erntehelfer und die Tierpfleger. Ganz besonders möchte ich Alexandra von Hodenberg zum einen für ihre zuverlässige Hilfe bei den Laborarbeiten und allen weiteren angefallenen Tätigkeiten und zum anderen für die wunderbare Freundschaft, die daraus entstanden ist, danken.

Meinen Dank richte ich auch an meine Bürokollegen, vor allem Ahmed Abdel-Wareth, Anna Rauen, Thomas Hartinger, Bernd Hilgers, Jan-Helge Deitmers, Ziade Abraha Kahsay und Daniela Latzke, für die gute gemeinsame Zeit. Mohammed Zidane danke ich für die vielen wunderbaren Worte, die er an mich gerichtet hat, die schönen gemeinsamen Feierabende und nicht zuletzt die verlässliche Freundschaft, die uns verbindet.

Danken möchte ich außerdem allen Mitarbeitern des Institutes für Tierernährung sowie den Mitarbeitern der anderen Arbeitsgruppen, die mich während meiner Zeit dort begleitet und auf unterschiedliche Art und Weise weitergebracht haben.

Meiner Familie danke ich dafür, dass sie immer für mich da ist. Ohne sie wäre ich nicht die, die ich bin. Meinem Vater bin ich besonderen Dank schuldig für die Unterstützung bei den Erntearbeiten.

Zuletzt danke ich meinem Freund Lars dafür, dass er an meiner Seite steht, für seine unendliche Geduld und seine gute Art, die mich immer wieder nach vorne hat schauen lassen.