

**Effects of hydrolysable tannins and dry matter
content on nitrogen metabolism in different ensiled
forage species and feed intake behaviour of goats**

Dissertation

zur

Erlangung des Grades

Doktor der Agrarwissenschaften (Dr. agr.)

der Agrar-, Ernährungs- und Ingenieurwissenschaftlichen Fakultät

der Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

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Bonn, 2026

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Tag der mündlichen Prüfung: 17.04.2026

Angefertigt mit Genehmigung der Agrar-, Ernährungs- und Ingenieurwissenschaftlichen
Fakultät der Universität Bonn

Die Förderung des Vorhabens erfolgte aus Mitteln des Bundesministeriums für Ernährung und Landwirtschaft (BMEL) aufgrund eines Beschlusses des deutschen Bundestages. Die Projektträgerschaft erfolgte über die Bundesanstalt für Landwirtschaft und Ernährung (BLE) im Rahmen des Programms zur Innovationsförderung (Förderkennzeichen 2818300116).

Meinen Eltern

SUMMARY

Effects of hydrolysable tannins and dry matter content on nitrogen metabolism in different ensiled forage species and feed intake behaviour of goats

Ensiled grass and legume forages are key components of ruminant diets, providing locally available, resource-efficient sources of crude protein (CP), energy and fibre. However, inevitable degradation and conversion processes occur during the ensiling process, due to plant-derived and microbial enzyme activity, leading to proteolysis of CP. An extensive degradation of CP must be critically considered, as this process results in reduced feed intake and nitrogen use efficiency. The objective of the present thesis was to ascertain to which extent a treatment of forages with a hydrolysable tannin (HT) extract influenced fermentation and protein quality, as well as the feed choice behaviour of goats, under varying ensiling conditions. The initial focus was therefore on a comprehensive chemical characterisation of the prepared lucerne, Italian ryegrass and red clover silage treatments. It was demonstrated that increasing the dry matter (DM) content of plant material from 200 to 350 g/kg prior to ensiling significantly improved fermentation quality and gas production. Meanwhile, adding HT up to 40 g/kg DM was found to have a positive impact on protein quality due to a restricted accumulation of non-protein-nitrogen (NPN) compounds. The impact of the pre-ensiling treatments on the feed choice behaviour and short-term DM intake of goats was subsequently investigated in three independent feeding trials. The animals showed a significantly higher preference and feed consumption for treatments with a higher DM content, whereby this observation was particularly pronounced in legume silages. This was due to the enhanced fermentation quality of high DM silages, as the formation of volatile fermentation products such as acetic acid and ammonia-nitrogen ($\text{NH}_3\text{-N}$), which were negatively associated with silage quality and short-term DM intake of goats, were considerably reduced due to field wilting. On the other hand, the application of HT prior to the ensiling only led to a higher feed intake in low DM silages. The final study involved a detailed examination of the formation of free amino acids (AA) and biogenic amines (BA) in all pre-ensiling treatments. The highest amounts of individual and total BA were consistently detected in low DM silages, with the highest concentrations in lucerne and lowest in red clover silages. In addition to increasing the DM content, HT treatment also significantly impaired proteolytic activity during the ensiling process, as the accumulation of free AA, γ -aminobutyric acid and BA was markedly reduced. Furthermore, multiple regression analysis clarified that NPN compounds, such as $\text{NH}_3\text{-N}$ and BA were valid indicators for the acceptance and DM intake of silages in feed choice behaviour trials. Overall, the findings indicate that both the fermentation and protein quality of ensiled forages and the subsequent feed intake can be systematically enhanced by pre-ensiling treatments, which offers potential for further research and practical use.

ZUSAMMENFASSUNG

Einfluss von hydrolysierbaren Tanninen und Trockenmassegehalt auf den Stickstoffmetabolismus von verschiedenen silierten Futterpflanzen und das Futteraufnahmeverhalten von Ziegen

Silierte Grünlandaufwüchse stellen für Wiederkäuer häufig die wichtigste Futtergrundlage dar und dienen als regional verfügbare und ressourcenschonende Energie-, Faser- und Rohproteinquellen. Im Verlauf der anaeroben Lagerphase treten jedoch unvermeidliche Ab- und Umbauprozesse auf. In diesem Kontext ist die Proteolyse von entscheidender Bedeutung, da sie zur Anreicherung von Nicht-Protein-Stickstoff-Verbindungen (NPN) führt und somit eine Verringerung der Proteinqualität zur Folge hat. Ein umfangreicher Abbau von Rohprotein ist in diesem Zusammenhang aus mehreren Gründen kritisch zu bewerten, da dieser in einer reduzierten Futteraufnahme und Stickstoff-Nutzungseffizienz resultieren kann. Ziel der vorliegenden Arbeit war die Untersuchung der Auswirkungen einer Behandlung verschiedener Gräser und Leguminosen mit einem Extrakt aus hydrolysierbaren Tanninen (HT) auf die Gär- und Proteinqualität sowie das Futterwahlverhalten von Ziegen bei unterschiedlichen Trockenmasse-(TM)-Gehalten. Der erste Teil dieser Arbeit fokussiert sich auf die umfassende chemische Charakterisierung der erzeugten Luzerne-, Weidelgras- und Rotkleesilagen. Während sich die Erhöhung der TM-Konzentration des Anwelkguts von 200 auf 350 g/kg vorteilhaft auf die *in vitro*-Fermentation und die Gärqualität auswirkte, resultierte die Zugabe von HT mit zunehmender Konzentration (bis 40 g/kg TM) in einer reduzierten Bildung von NPN-Verbindungen. Des Weiteren wurde der Einfluss unterschiedlicher Silage-Behandlungen auf das Futterwahlverhalten und die kurzzeitige TM-Aufnahme von Ziegen untersucht. Die Erhöhung der TM-Konzentration hatte insbesondere bei den silierten Leguminosen einen positiven Einfluss auf die Futteraufnahme der Tiere. Dieser Zusammenhang konnte anhand einer verbesserten Gärqualität begründet werden. Die Applikation von HT vor dem Silierprozess resultierte lediglich in Silagen mit niedrigen TM-Konzentrationen in einer gesteigerten Futteraufnahme. Der dritte Teil dieser Arbeit widmet sich der detaillierten Untersuchung der Bildung von freien Aminosäuren (AA) und biogenen Aminen (BA) in den behandelten Silagen. Die höchsten Konzentrationen an BA wurden in Silagen mit niedrigen TM-Konzentrationen ermittelt. Es konnte zudem festgestellt werden, dass neben der Erhöhung der TM-Konzentration auch die Applikation von HT zu einer substantiellen Reduktion von proteolytischen Aktivitäten während des Silierprozesses führte. Darüber hinaus konnte der Nachweis erbracht werden, dass $\text{NH}_3\text{-N}$ und BA als valide Indikatoren für die Akzeptanz beziehungsweise die TM-Aufnahme von Silagen in Futterwahlversuchen fungieren. Die Ergebnisse lassen die Schlussfolgerung zu, dass die Gär- und Proteinqualität von silierten Grünlandaufwüchsen und die anschließende Futteraufnahme gezielt beeinflusst und auch verbessert werden kann, was Potenzial für nachfolgende Forschungsarbeiten bietet.

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ABBREVIATIONS

Ψ	P < 0.1
*	P < 0.05
**	P < 0.01
***	P < 0.001
0TLO	0 g/kg DM hydrolysable tannins and ≈ 200 g/kg
2TLO	20 g/kg DM hydrolysable tannins and ≈ 200 g/kg
4TLO	40 g/kg DM hydrolysable tannins and ≈ 200 g/kg
0THI	0 g/kg DM hydrolysable tannins and ≈ 350 g/kg
2THI	20 g/kg DM hydrolysable tannins and ≈ 350 g/kg
4THI	40 g/kg DM hydrolysable tannins and ≈ 350 g/kg
A	CP fraction, non-protein-nitrogen, calculated as CP- true protein
AA	Amino acids
ADFom	Acid detergent fibre expressed exclusive residual ash
ADL	Acid detergent lignin
aNDFom	Neutral detergent fibre after incineration and amylase treatment
B1	CP fraction, buffer-soluble protein, quickly degradable
B2	CP fraction, buffer-insoluble protein, moderately degradable
B3	CP fraction, neutral-detergent insoluble protein, slowly degradable
BW	Body weight
C	CP fraction, acid detergent insoluble CP
cfu	Colony forming unit
CT	Condensed Tannins

Abbreviations

CP	Crude protein
d	Day
DM	Dry matter
DM _{cor}	Dry matter corrected
DMI	Dry matter intake
DML	Dry matter level
e.g.	Exempli gratia
GABA	Gamma-aminobutyric acid
GC	Gas chromatography
GP	Gas production
h	Hour
HI	Silages with an approximate dry matter content of 350 g/kg
HT	Hydrolysable tannins
IR	Italian ryegrass
Lin	Linear effect of different hydrolysable tannin concentrations
LO	Silages with an approximate dry matter content of 200 g/kg
LU	Lucerne
ME	Metabolisable Energy
MDS	Multi dimensional scaling
MSD	Minimum significant difference
N	Nitrogen
NH ₃ -N	Ammonia-nitrogen
NPN	Non-protein nitrogen,

Abbreviations

NUE	Nitrogen use efficiency
NS	Not significant
OM	Organic matter
OMD	Organic matter digestibility
PPO	Polyphenol oxidase
Quad	Quadratic effect of different hydrolysable tannin concentrations
R ²	Coefficient of determination, R-square
R ² _{adj}	Adjusted coefficient of determination
RC	Red clover
RUP	Rumen-undegraded dietary crude protein
SEM	Standard error of the mean
TP	True protein
VDLUFA	Association of German Agricultural Analytic and Research Institutes
VFA	Volatile fatty acid
WSC	Water-soluble carbohydrates

CHAPTER 1

General introduction

Despite the increasing prevalence of alternative plant-based protein sources, livestock farming and the consumption of animal-source food continue to play a crucial role in ensuring food and income security on a global scale (Randolph et al., 2007). Although agriculture, and notably livestock farming, is of considerable importance, these sectors are encountering an increasing number of challenges and criticism (Eisler et al., 2014; Schader et al., 2015). The most significant challenge will be to ensure food security for the growing world population, while simultaneously addressing changing consumer behaviour without disregarding resource and environmental protection. Projections indicate that the global population will reach 9.15 billion by the year 2050, while the average daily calorie availability is anticipated to rise to approximately 3,000 kilocalories per person. This corresponds to an increase in agricultural production of over 60% based on the years 2005/2007. Furthermore, it can be hypothesised that the diet will contain less starch, but more protein and fat (FAO, 2022). While the demand for food and feed is constantly increasing, the amount of agricultural land is limited, and expansion at the expense of deforestation is becoming more and more politically opposed (Berning and Sotirov, 2023). In Europe, the amount of arable land available for agricultural use decreased by approximately 6% between 2000 and 2023 (FAOSTAT, 2025). A comparable decline can also be observed in Germany, where more than one million hectares have been excluded from agricultural production since 1992 due to urbanisation (Deutsche Nachhaltigkeitsstrategie, 2025).

In order to counteract this growing conflict situation surrounding limited resources, a variety of approaches are required throughout the entire food supply system. Promising strategies include reducing food waste and consumption of animal products, as well as the sustainable intensification of agriculture. This can be achieved through the genetic modification of crops or the improvement of feeding and feed efficiency of livestock (Godfray et al., 2010; Schader et al., 2015). Another key factor is reducing the use of food-competing feed ingredients in livestock diets. Currently, approximately one third of arable land worldwide is dedicated to the production of animal feed. In developed regions such as Europe, this proportion exceeds even 60% (Eisler et al., 2014; FAO, 2018).

Grassland, on the other hand, which accounts for around one third of European agricultural land. It is often unsuitable for growing crops and therefore does not pose a potential conflict with human nutrition, but still provides an ideal food source for ruminants, which can convert roughage into high-quality animal-sourced food, due to their unique digestive system (Chaudhry, 2008; van Zanten et al., 2016; Schils et al., 2022). Properly managed grass and legume stands provide ruminants with sufficient structured fibre and offer a considerable basis for the supply of energy, crude protein (CP) and minerals in milk and beef production systems (Guyader et al., 2016; Wilson et al., 2020). Several authors described annual dry matter (DM) and CP yields within a range from 6,000 to 32,000 kg DM/ha and more than 1,500 kg CP/ha for various grasses and legumes (Redfearn et al., 2002; Tabacco et al., 2002; Albrecht and Beauchemin, 2003; Callow et al., 2003; Tongel and Ayan, 2010). Legumes, such as lucerne (*Medicago sativa* L.), are able to produce high quantities DM and CP without requiring nitrogen (N) fertiliser due to their ability to fix atmospheric N (Albrecht and Beauchemin, 2003; Lüscher et al., 2014). Therefore, more intensive forage production systems can contribute significantly to reducing dependence on imported protein feed while reducing feed costs and environmental impact (Tabacco et al., 2018; Steinshamm et al., 2021).

Although fresh, unpreserved grass is the main type of forage produced in many countries worldwide, including some European regions, the geographical location and long north-south extent of Europe often means that most of the grassland biomass harvested is preserved as hay or silage. In Nordic countries and regions with a maritime climate, little hay is produced and the vast majority of harvested forage is preserved as silage for year-round availability. Silage making is less weather-dependent than hay production, and leaf material is less likely to be lost through shattering (McDonald et al., 1991; Bernardes et al., 2018). However, good management is still required to prevent excessive losses and minimise reductions in the nutritive value of the harvested crop. In other words, the aim of ensiling is to maintain the nutritional value of fresh plant material as much as possible.

A key measure for successful silage production is to avoid undesirable fermentation processes and minimise the inevitable degradation of CP (Hoedtke et al., 2010). The composition of forage CP undergoes qualitative changes during the wilting and ensiling processes due to proteolysis, which varies greatly depending on various influencing factors such as plant species, the rate and extent of pH change, DM content, temperature, and the presence of proteolytic microorganisms (Pahlow et al., 2003; Hoedtke et al., 2010).

The CP composition in plant material mostly consists of a heterogeneous mixture of proteins and 10-25% non-protein nitrogen (NPN) compounds, including peptides, free amino acids, amides, nucleotides, chlorophyll and nitrates (Fijałkowska et al., 2015). Variation in NPN proportion of total N in fresh plant material can be caused by maturity, species, amount of applied N-fertilizer and analytical methods (Licitra et al., 1996; Yu et al., 2003; Hoekstra et al., 2008). However, anaerobic fermentation processes cause significant changes to XP composition, resulting in an accumulation of NPN compounds at the detriment of proteins. According to a research study of 26 farm silos, the proportion of NPN compounds increased to an average of 60% due to protein degradation (Wyss et al., 2018). Other authors have reported an even more extensive proteolytic processes during ensiling, with an increase in NPN compounds reaching 80% of total N; in some cases this value has been even exceeded (Tremblay et al., 2005; Contreras-Govea et al., 2006; Hartinger et al., 2019).

While proteins are mainly hydrolysed to peptides and free amino acids (AA) by plant proteases under anaerobic and aerobic conditions, the subsequent degradation in the silo is primarily attributed to proteolytic bacteria (Winters et al., 2000; Pahlow et al., 2003; Dong et al., 2019). The microbial degradation process of free AA can be categorised into two distinct pathways, each with characteristic end products. The enzymatic deamination of AA produces volatile fatty acids and ammonia (NH_3), whereas decarboxylation is associated with the formation of biogenic amines (BA) (Hoedtke et al., 2010; Huang et al., 2020). Biogenic amines are a group of low-molecular-weight, nitrogen-containing organic substances that are structurally related to alkaloids. In animals and plants, BAs play vital roles in bioregulating processes such as cell growth, gene expression, membrane-linked enzymes, and protein synthesis (Kusano et al., 2008; Igarashi and Kashiwagi, 2010). However, elevated concentrations of BA formed in silage are considered harmful if ingested by ruminants. They induce immunological diseases and promote carcinogenic reactions. They also impair the rumen mucosa and microflora, which can potentially cause metabolic disorders and inflammations. Furthermore, BA can negatively affect feed intake and palatability and compromise food safety, particularly in dairy products under defined circumstances (Scherer et al., 2015; Benkerroum, 2016). Fortunately, the rumen microflora can metabolise BA at certain concentrations, enabling ruminants to adapt to and withstand adverse effects to some extent (van Os et al., 1995; van Os et al., 1997). In terms of quantity, the most abundant BA found in poorly fermented forages are tyramine, cadaverine, putrescine, spermidine and histamine, with putrescine, cadaverine and tyramine being the most relevant BA in most silage, regardless of the plant species. (Steidlová and Kalač, 2002; Scherer et al., 2015; Mlejnkova et al., 2016; Jia

et al., 2021; Li et al., 2022; Mao et al., 2024). Under the anaerobic conditions in a silo, a variety of bacteria characterised by AA decarboxylase activity are able to convert amino acids into BA. The most significant producers are found within the *Clostridium* and *Enterobacteriaceae* (*Citrobacter*, *Klebsiella* and *Escherichia*) genera. However, some individual proteolytic lactic acid bacteria, such as those belonging to the *Weissella* and *Pediococcus* genera, are also potential BA producers (Li et al., 2022; Mao et al., 2024).

A key consequence of a high proportion of NPN in total CP is the inefficient utilization of N by ruminants, due to the low efficiency of microbial CP synthesis in the rumen (Broderick, 1995). For example, Nguyen et al. (2005) observed that N retention in sheep decreased from 29.5% for fresh orchardgrass (*Dactylis glomerata* L.) to 17% for a high moisture silage made from the same plant material. The diminished utilisation of CP leads to higher concentrations of N in urine (>50% of N intake) and consequently elevated environmental pollution (Nguyen et al., 2004, 2005). Extensive CP degradation, which limits the nutritive value of ensiled forages, is particularly associated with legumes and grasses that are harvested at an early maturity stage and that have received a high level of N fertilization, due to their higher buffer capacity (McKersie, 1985; Tremblay et al., 2005). Moreover, legumes characteristically contain comparatively low levels of water-soluble carbohydrates (WSC). When combined with elevated buffer capacity, this makes them less suitable for ensiling. This results in a slower and insufficiently deep acidification rate, along with an insufficient suppression of proteolytic activities by plant and microbial enzymes. The optimal pH range for enzymes such as acid proteinase, aminopeptidase and carboxypeptidase is between 5 and 7. However, even reducing the pH to below 4 does not completely prevent protein hydrolysis (Guo et al., 2007; Martens et al., 2019). The largest proportion of enzymatic protein hydrolysis is assigned to the action of proteolytic plant enzymes before and during the fermentation. This conclusion is based on studies in which plant material was irradiated prior to the ensiling process, in order to suppress microbial activity (Dong et al., 2019). However, it was also evident in this context that the extent of protein degradation is influenced by other internal plant factors, as the accumulation of NPN compounds was significantly lower in both unirradiated and irradiated red clover (*Trifolium pratense* L.) than in lucerne under the same conditions. This observation was attributed to the presence of the enzyme polyphenol oxidase (PPO) in red clover, which serves as a natural protein-protective mechanism (Winters et al., 2008; van Ranst et al., 2011).

Another group of protein-protecting compounds present in various forage crops are tannins. Tannins are complex water-soluble secondary plant metabolites, belonging to the group of

polyphenols with a variable but generally high molecular weight (500 to 20,000 Da) (Frutos et al., 2004; Mueller-Harvey, 2006). These bioactive compounds are particularly prevalent in forage and shrub legumes, as well as in trees, where tannins serve as an ecological defence mechanism against herbivores, insects and pathogenic microorganisms. This explains the characteristic functions of tannins: binding proteins to ward off predators and providing antioxidant and antimicrobial activities that protect plant tissue from oxidative stress and infection. (Barbehenn and Constabel, 2011). The tannins used in this publication are derived from the bark of the chestnut tree (*Castanea sativa* L.), which contains an average of 60-100 g/kg DM, making it a natural by-product of wood processing that is obtained through hot water extraction and subsequent spray drying (Santos-Buelga and Scalbert, 2000).

Based on their chemical structure and properties, tannins can be divided into two main groups: condensed and hydrolysable tannins (HT) (Makkar, 2003). Hydrolysable tannins consist of a central polyol core, such as glucose, with hydroxyl groups esterified with phenolic groups. Due to the presence of a phenol group, HT can be further subdivided into gallotannins and ellagitannins. Condensed tannins (CT), also known as procyanidins and prodelphinidins, are mostly polymers of catechin units (flavan-3-ols). These monomers are linked by C-C bonds, making CT more chemically stable and resistant to hydrolysis than HT (Mueller-Harvey, 2006; Patra and Saxena, 2011). In contrast to CT, HT are not fully rumen-stable, as they can be bio-transformed and fermented by microbes, producing, among other things, the metabolite pyrogallol, which is considered toxic (Lotfi, 2020). The wide variation in the chemical structure of this group of active substances (degree of polymerisation, stereochemistry and C-C bonding) means that their properties also vary considerably, making direct comparisons difficult (Patra and Saxena, 2011). Despite these variations, CT and HT have the ability to form complexes with dietary proteins in common, which can reduce degradation both during ensiling and in the rumen, inducing a shift in N excretion from urine to faeces and possibly enhancing N retention (Jayanegara et al., 2019; Zhang et al., 2019; Niderkorn and Jayanegara, 2021). Complex formation with proteins occurs via four types of bonds, which are either reversible and pH-dependent (hydrogen bonds, ionic bonds and hydrophobic interaction) or considered to be highly stable and insoluble (covalent bonds by oxidation of polyphenols) (Frutos et al., 2004). The pH-dependent complexes are generally considered stable within the pH range of 3 to 7 and therefore do not dissociate in the rumen, but will dissociate in the abomasum and duodenum. (Jones and Mangan, 1977). In this context, CT have been shown to exhibit a higher binding affinity to proteins, which is primarily due to their higher molecular weight (Mueller-Harvey, 2006). However, beyond these properties, HT offer notable advantages over CT, making them

a potentially valuable addition to the field of animal nutrition. For instance, HT has been demonstrated to reduce the digestibility of feed and nutrients to a lesser extent, exhibit higher acceptance rates, and demonstrate more effective methane formation mitigation (Deaville et al., 2010; Jayanegara et al., 2015). The latter can be attributed to their antimicrobial effects, which includes influencing cell walls and inhibiting oxidative phosphorylation (Huang et al., 2024).

In addition to their direct impact on feed intake and effects on digestion in the rumen and the postruminal gastrointestinal tract of ruminants, tannins also offer promising approaches as silage additives by reducing proteolysis during the ensiling of protein-rich forages. Research studies involving both naturally occurring tannins (typically CT present in legumes) and added tannin extracts have documented this mode of action (Deaville et al., 2010; Lorenz et al., 2010; Hymes-Fecht et al., 2013; Jayanegara et al., 2019). In addition to protecting proteins from hydrolysis through forming complexes, HT are also capable of reducing the activity of proteolytic enzymes in silages. According to Li et al. (2018), the three proteolytic plant enzymes responsible for proteolysis, carboxypeptidase, aminopeptidase and acid proteinase, are increasingly inactivated at higher concentrations of HT. This effect can be observed immediately after ensiling begins and continues over the following weeks, whereby the inactivation is explained by the binding affinity with the surfaces of the enzymes (Guo et al., 2007; Li et al., 2018). In addition, HT have antibacterial effects and influence the diversity and composition of the microbial community of ensiled forages. However, the available research findings differ from one another, making it difficult to identify a clear trend of influence (Wang et al., 2020; Ke et al., 2022; Chen et al., 2022). On the other hand, it is indisputable that increasing the application of HT reduces the accumulation of NPN compounds and end products of microbial degradation, such as ammonia nitrogen ($\text{NH}_3\text{-N}$), even under unfavourable ensiling conditions (Tabacco et al., 2006; Deaville et al., 2010; Li et al., 2018; Jayanegara et al., 2019; Wang et al., 2020; Ke et al., 2022; Taha et al., 2025). The influence of HT on the metabolism of individual AA and the formation of BA is currently insufficiently studied to allow valid interpretation.

Generally speaking, the literature on the factors having an impact on overall silage quality and particularly the protein quality compared to fresh forage is heterogeneous and often inconsistent. In an attempt to achieve a structured overview of protein quality of legume and grass forage crops that was expected to be instrumental in designing targeted ensiling experiments, a dataset was constructed from published scientific literature by pooling

laboratory and feeding trial studies based on ensiled forages with a detailed description of chemical composition as well as crude protein components.

The selected literature was obtained from databases of the search engines Google Scholar and Web of Science using “nitrogen fractions”, “silage”, “ensiled”, “grass”, “legume”, “proteolysis” and “protein breakdown” as keywords in various combinations. Hits were scrutinised and only hits were further considered that fulfilled the following inclusion criteria:

- The publication had to be written in English or German;
- C3 grasses and small seeded legumes from temperate and subtropical climate zones;
- Forage was grown as a pure stand and also ensiled individually;
- Each silage treatment had to be prepared at least in duplicate;
- The ensiling period under anaerobic conditions had to be no less than 30 days;
- Composition of CP, such as non-protein nitrogen and other CP fractions, were reported in the publication.

The publication hits were then reviewed for accordance with the inclusion criteria, resulting in a total number of 38 studies meeting the inclusion criteria, which were published between 1979 and 2019 (Table 1). Additionally, data from two at that time unpublished experiments of our group were also included in the database.

With regard to forage material, ensiling conditions and chemical analyses, distinct differences became obvious both between and within the studies. Pure forage stands were established in North America, Europe and Asia under various soil, climate and management conditions. Furthermore, variations occurred in stage of maturity and growth number. Anaerobic storage period and silo type covered a wide range from 30 to 150 days and from 0.1 L tubes to large steel silos. Due to the fact that additives or supplements, e.g., tannins, acids, molasses, and bacterial inoculants, as well as mechanical treatments could have an effect on silage fermentation characteristics and therefore also influence the extent of proteolysis and deamination, additive and mechanical treatments were included in the database in a simplified way (“control” or “treatment”). Additives with a supposedly negative effect on silage quality were sorted out in advance. Information about chopping length, storage temperature, fertilization and wilting time were also listed, but these were not taken into account. In addition to CP components (e.g., $\text{NH}_3\text{-N}$ and NPN), chemical composition and fermentation characteristics were also included in the dataset, in order to describe the large number of different silages in detail. Chemical composition included DM, ash, CP, neutral detergent fibre, acid detergent fibre, acid detergent lignin and WSC. Lactic acid, acetic acid, propionic acid,

butyric acid, ethanol and pH were included as fermentation characteristics. Arithmetical mean, range, number of observations and standard deviation of key chemical composition of silages are shown in Table 2.

Table 1. Studies included as data basis for the descriptive comparison of ensiled forage legumes and C3 grasses

Legumes				C3 Grasses							
Exp.	References	Species	Storage period (d)	Exp.	References	Species	Storage period (d)	Exp.	References	Species	Storage period (d)
1.	Contreras-Govea et al. 2011	Lucerne	60	23.	Mustafa et al. 2000	Lucerne	90	1.	Francisco et al. 2006	Winter wheat	100
2.	Coblentz & Grabber 2013	Birdsfoot trefoil	60	24.	Mustafa et al. 2002	Berseem clover	70	2.	Heron et al. 1988	Ryegrass	76
3.	Coblentz & Grabber 2013	Lucerne	60	25.	Nguyen et al. 2004	Lucerne	56	3.	Keles et al. 2014	Barley	45
4.	Dentinho et al. 2018	Lucerne	35	26.	Oshima et al. 1979	Lucerne	90	4.	Keles et al. 2014	Wheat	45
5.	Dong et al. 2019	Lucerne	30	27.	Owens et al. 2002	Red clover	35	5.	Keles et al. 2014	Rye	45
6.	Dong et al. 2019	Red clover	30	28.	Owens et al. 2002	Lucerne	35	6.	Keles et al. 2014	Triticale	45
7.	Dong et al. 2019	Lucerne	30	29.	Purwin et al. 2011	Red clover	90	7.	Keles et al. 2014	Oat	45
8.	Dong et al. 2019	Red clover	30	30.	Roscher 2017	Lucerne	90 / 180	8.	Mustafa et al. 2000	Barley	90
9.	Fairbairn et al. 1992	Lucerne	42 / 90	31.	Roscher 2017	Birdsfoot trefoil	90	9.	Nguyen et al. 2004	Orchardgrass	56
10.	Francisco et al. 2006	Kura clover	100	32.	Scherer et al. 2019	Lucerne	120	10.	Nguyen et al. 2005	Orchardgrass	120
11.	Grabber et al. 2009	Red clover	90	33.	Scherer et al. 2019	Red clover	120	11.	Niu et al. 2018	Smooth brome	56
12.	Grabber et al. 2009	Lucerne	90	34.	Seguin & Mustafa 2003	Kura clover	50	12.	Nsereko et al. 1998	Ryegrass	100
13.	Guo et al. 2008	Lucerne	35	35.	Tabacco et al. 2006	Lucerne	78 / 120	13.	Oshima et al. 1979	Ryegrass	90
14.	Guzatti et al. 2017	Red clover	100	36.	Tao et al. 2019	Lucerne	60	14.	Roscher 2017	Ryegrass	96
15.	Hartinger et al. 2019	Lucerne	120	37.	Wang et al. 2019	Lucerne	60	15.	Scherer et al. 2019	Ryegrass	120
16.	Li et al. 2018	Lucerne	35	38.	Wang et al. 2019	Stylo	60	16.	Tremblay 2005	Timothy	150
17.	Li et al. 2018	Lucerne	30	39.	Xu et al. 2011	Erect milkvetch	45	17.	Hilgers et al. 2025	Ryegrass	90
18.	Li et al. 2018	Red clover	30	40.	Yuan et al. 2017	Lucerne	60	18.	Scherer (unpublished)	Ryegrass	120
19.	Lorenz et al. 2010	Sainfoin	60	41.	Hilgers et al. 2025	Lucerne	115				
20.	Ke et al. 2017	Lucerne	64	42.	Hilgers et al. 2025	Red clover	120				
21.	Ke et al. 2018	Lucerne	60	43.	Scherer (unpublished)	Lucerne	120				
22.	Michalkova et al. 2009	Lucerne	84	44.	Scherer (unpublished)	Red clover	120				

Exp. = Experiment; d = days.

Table 2. Descriptive comparison of the chemical composition and fermentation characteristics of ensiled forage legumes and C3 grasses

Variables (g/kg DM)	Legumes				C3 Grasses			
	Mean	Range	SD	No.	Mean	Range	SD	No.
Dry matter (g/kg)	328	119-600	93	206	314	149-604	100	116
Ash	116	91.0-165	16.5	62	102	44.0-151	25.5	61
aNDFom	399	221-534	43	138	515	330-597	62.7	71
ADFom	309	180-435	37.1	140	314	200-395	42.3	71
ADL	70.8	34.0-102	17.5	66	50.8	9.60-110	20.3	60
WSC (prior ensiling)	77	35.1-153	27.2	36	94.9	19.3-236	51.6	36
WSC	26.9	0.80-237	39.3	95	43.4	1.0-181	36.1	114
Crude protein	194	126-294	34.7	200	141	63.0-242	36.8	117
NPN (g/kg CP) (prior ensiling)	251	49.9-462	110	52	276	137-375	57.8	13
NPN (g/kg CP)	542	167-831	158	207	560	186-843	133	118
NH ₃ -N (g/kg N)	92.6	11.0-292	55.1	175	82.8	17.0-141	30.5	101
pH	4.7	3.9-7.0	0.5	173	4.2	3.5-5.6	0.4	117
Lactic acid	61.4	3.70-132	28.1	144	67.8	6.00-188	44.9	93
Acetic acid	25.4	1.70-114	17.3	141	13.9	0.10-53.1	11.2	63
Propionic acid	1.3	0-23.7	3.6	73	1.4	0-13.5	2.6	36
Butyric acid	1.9	0-34.0	5.1	93	1.1	0-15.3	3.3	38
Ethanol	10.3	0.10-73.9	12.7	72	20.9	2.00-104	22.2	41

DM = dry matter; aNDFom = neutral detergent fibre assayed with heat-stable amylase and expressed exclusive residual ash; ADFom = acid detergent fibre expressed exclusive residual ash; ADL = acid detergent lignin; WSC = water-soluble carbohydrates; NPN = non-protein-nitrogen; NH₃-N = ammonia-nitrogen; SD = standard deviation; No. = number of values.

A closer inspection of the studies in the database (data not shown) revealed that DM concentration of the forage at ensiling can have a drastic effect on general silage and, in particular, protein quality. Further, tannins as chemical additives, although apparently effective in reducing CP degradation, have not been studied systematically. Therefore, the overall objective of the study reported in this thesis was to evaluate the interactions of different hydrolysable tannin (HT) concentrations and two DM concentrations at ensiling on selected grass and legume forage crop species with regard to:

1. Chemical composition, fermentation pattern, CP fractions and in vitro gas production. Special focus was directed on the fate of CP fractions caused by ensiling;
2. Short-term DMI and feed choice behaviour of goats;
3. The formation of free amino acids and biogenic amines.

The vital hypothesis was that an increase in DM concentration by wilting before ensiling and the addition of a HT commodity in defined amounts at ensiling would reduce the extent of proteolysis and amino acid degradation and, thus, improve the protein value of forages. An improved protein value in turn would lessen the need to supplement ruminant rations with protein feeds, thus contributing to an improved nitrogen use efficiency both at the animal and farm level.

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CHAPTER 2

Scope of the thesis

The research presented in this thesis is part of the “OptiKons-Project”, which was funded by the Federal Ministry of Food and Agriculture (BMEL) with the objective of examining approaches to reduce crude protein (CP) degradation in ensiled forages and thus to improve the efficiency of nitrogen (N) use by ruminants. Both, Chapter 3 and 4 contain two manuscripts that have been formatted in accordance to the guideline and structural layout of the respective scientific journal selected for publication, whereas Chapter 5 has been meticulously prepared for later submission to the same journal. In order to ensure a coherent appearance of the thesis, adjustments have been made to the font.

The central objective of this thesis was to ascertain the extent of proteolysis and the associated alterations in the composition of N fractions that occurred during the fermentation process, depending on forage species and pre-ensiling treatments. In addition to varying the dry matter (DM) concentration at ensiling, particular focus was given to the use of a hydrolysable tannin extract (HT) as silage additive. In this context, the intention was also to gain knowledge to which extent the different silage treatments affect the short-term feed intake and preference of goats in feed choice situations.

The initial manuscript (Chapter 3) focuses on the effects of DM concentration and HT level on the chemical composition, CP fractions and fermentation quality of ensiled grass and legume forages. In summary, lucerne, Italian ryegrass and red clover were subjected to wilting on the field at two distinct DM levels and treated with HT at three varying concentrations with the objective of achieving a broad range in terms of fermentation quality and CP degradation. Both, fresh forages and obtained silage treatments were systematically analysed using chemical laboratory methods, including proximate constituents, fibre fractions, fermentation products and CP fractions. Furthermore, *in vitro* gas production kinetic parameters were determined and energy value and organic matter digestibility were estimated.

The second manuscript (Chapter 4) deals with the effect of DM concentration and different HT levels on DM intake and the feed choice behaviour of goats. To achieve this objective, three independent preference trials were conducted, utilising the silage treatments that have been comprehensively described in Chapter 3. The goats were offered the silage treatments in two-choice situations, whereby both short-term DM intake and preference behaviour were

determined. Furthermore, possible correlations between silage characteristics and DM intake were investigated, with the intention of identifying influencing factors being indirectly or directly responsible for preference or avoidance of examined silage treatments.

In the third manuscript (Chapter 5), the focus shifts to the subject of N metabolism, with particular attention given to the changes in amino acid composition as well as the formation of biogenic amines resulting from proteolytic activities during the ensiling process. Furthermore, linear relationships between CP compounds and short-term DM intake were applied to ascertain the extent to which single or multiple CP compounds explain variance in silage consumption and thus serve as valid predictors in DM intake models.

Finally, Chapter 5 provides a general discussion of the aforementioned chapters 3, 4 and 5. A critical examination of the obtained results and substantial key points was conducted in great detail. The general discussion is completed by future prospects regarding the potential improvements in silage quality and N use efficiency as well as the implementation of HT extracts as silage additives under practical conditions on farm level.

CHAPTER 3

Effects of hydrolysable tannin extract and dry matter concentration on ensiled legume and grass forages – Chemical composition, crude protein fractions and *in vitro* gas production kinetics

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Published in **Animal Feed Science and Technology** (2025) 327:116430.

<https://doi.org/10.1016/j.anifeedsci.2025.116430>

Abstract

Pre-ensiling treatments can have a distinct impact on crude protein (CP) degradation and fermentation characteristics of ensiled high-protein forages. The objective of this study was to investigate the effects of different dosages of chestnut tannin (CT) and dry matter (DM) concentration on chemical composition, nitrogen (N) compounds as well as *in vitro* gas production (GP) in grass and legume silages. Pure stands of lucerne (*Medicago sativa* L.), Italian ryegrass (*Lolium multiflorum* L.) and red clover (*Trifolium pratense* L.) were harvested and wilted to DM contents of approximately 200 and 350 g/kg. The forages were then ensiled in 120-litre barrels with three levels of hydrolysable tannin (HT) supplementation (0, 20 and 40 g/kg DM). After ensiling, six silage treatments of each forage species were sampled for chemical analysis, particularly with regard to proximate constituents, fermentation products and other volatile compounds, CP fractions and 96-h *in vitro* gas production (GP) characteristics. In order to statistically evaluate the effects of HT and DM treatments, data were analysed using analysis of variance as well as orthogonal contrast test (linear and quadratic). In addition, the mean GP kinetics over time were estimated using nonlinear regressions. The stepwise increase of HT concentration linearly reduced the accumulation of non-protein-N (NPN) in all forages ($P < 0.01$) by at least 20% in low DM silages and by 10% in silages with a higher DM level. Both, HT and DM treatments resulted in a significant decrease ($P < 0.05$) of ammonia-N concentration. Among all forage species, the extent of proteolytic activities was highest in untreated low DM lucerne silages, as NPN and ammonia-N concentrations were 824 g/kg CP and 198 g/kg N, respectively, whereas the lowest CP degradation was generally determined in red clover silages. Maximum GP as well as organic matter digestibility (OMD) were negatively affected by increasing HT dosage in all forage species ($P < 0.05$), with the impact being more pronounced in moister silages. On the other hand, wilting to a higher DM content increased GP and OMD in red clover and Italian ryegrass silages. The present study revealed that HT markedly improved CP quality in silages due to higher stability of protein, whereas the DM treatment had more beneficial effects regarding fermentation quality, OMD and GP.

Abbreviations

A, non-protein N; ADFom, acid detergent fibre expressed exclusive residual ash; ADL, acid detergent fibre; aNDFom, neutral detergent fibre assayed with heat stable amylase and expressed exclusive residual ash; B1, rapidly degraded true protein; B2, intermediately degraded true protein; B3, slowly degraded true protein; c, gas production rate; C, undegradable true protein; CP, crude protein; DM, dry matter; DMI, DM intake; GP, gas production; GP2, gas production rate at 2 h of incubation; HI, silages with an approximate DM level of 350 g/kg; HT, hydrolysable tannin; IR, Italian ryegrass; LO, silages with an approximate DM level of 200 g/kg; LU, lucerne; ME, metabolizable energy N, nitrogen; NH₃-N, ammonia-N; NPN, non-protein-N; OMD, organic matter digestibility; RC, red clover; VFA, volatile fatty acids; WSC, water-soluble carbohydrates.

1. Introduction

Forage crops, e.g., Italian ryegrass (IR), lucerne (LU) and red clover (RC) with high nutritive value are major components of ruminant diets and main sources of home-grown energy as well as crude protein (CP) in grassland-based livestock production systems (Wilkins and Jones, 2000; Cavallarin et al., 2007; Lüscher et al., 2014). Feeding large quantities of forage is beneficial in several ways due to diminished competition with human food production, increased feed autonomy, environmental services and maintained optimal rumen function (Godfray et al., 2010; Eisler et al., 2014). To provide forage throughout the year, ensiling is the most common practice of preservation, especially in temperate regions (Südekum et al., 2016).

However, it is well established that during field wilting and ensiling of high-protein grass and legume forages, the nutritive value can be reduced by extensive proteolysis of plant CP (Guo et al., 2008; Edmunds et al., 2014). This degradation of CP to non-protein nitrogen (NPN) compounds is the result of proteolytic plant and microbial enzyme activity, whereby proportion of NPN increase up to 800 g/kg of CP (Pahlow et al., 2003; Guo et al., 2007; Ding et al., 2013). The extent of CP degradation is highly variable and is affected by several factors, such as temperature, DM content, rate of pH change and forage species (Kofahl, 2009; Hoedtke et al., 2010). In particular, legumes, such as LU, but also grasses, which have been intensively fertilized and harvested at early maturity stages, are highly susceptible to proteolysis during ensiling, since a high buffering capacity and low proportion of water-soluble carbohydrates (WSC) are key factors for an insufficient and slow pH drop (McDonald et al., 1991; Tremblay et al., 2005). A distinct accumulation of NPN compounds in silage, such as ammonia (NH₃) or biogenic amines, affects not only DM intake (DMI) negatively (Krizsan and Randby, 2007), but also reduces the nitrogen (N) use efficiency by microorganisms in the rumen (Givens and Rulquin, 2004; Broderick et al., 2007) and may negatively impact on DMI in feed choice situations (Scherer et al., 2019).

To impair the extent of CP degradation in ensiled forages and thus reduce negative impact on animals as well as the environment, multiple direct or indirect methods exist. In addition to ensiling technology (Slottnner and Bertilsson, 2006), treatments with commercially available bacterial inoculants (Winters et al., 2000) or chemical additives (Guo et al., 2008) are well established practices to maintain protein quality in silage. Meanwhile, it is known that various forage crops, e. g., Birdsfoot trefoil (*Lotus corniculatus* L.) (Hymes-Fecht et al., 2013), containing plant secondary compounds, such as tannins, that have the property to protect proteins against hydrolysis, both, during ensiling and in the rumen (Tabacco et al., 2006).

Tannins are classified as secondary plant metabolites, which have a high affinity to form reversible and irreversible hydrogen bonds with proteins, due to interaction between multiple phenolic hydroxyl groups of tannins and carboxyl groups of proteins. Based on their chemical structure and properties, tannins can be divided into two main groups: condensed and hydrolysable tannins (Makkar, 2003). In addition, tannins are able to reduce the activity of proteases (Li et al., 2018) and influence the microbial community composition in silage (Wang et al., 2020). Several studies depicted that the addition of both, condensed and hydrolysable tannins (HT) to forage crops before ensiling reduced accumulation of NPN compounds and $\text{NH}_3\text{-N}$ (Jayanegara et al., 2019a). Tabacco et al. (2006) investigated the effect of different HT concentrations on proteolysis in ensiled LU. Compared to the control, the addition of 60 g/kg DM of HT reduced NPN proportion about 17% ($P < 0.05$) and $\text{NH}_3\text{-N}$ by more than 30% ($P < 0.05$). In spite of the described beneficial effects, tannins are also associated with some negative properties, such as reduced palatability and digestibility of feedstuff that limit its use as an additive, depending on the type of tannin, tannin dosage and animal species (Aboagye and Beauchemin, 2019).

The objective of the present study was to evaluate the interactions of different HT concentrations, two varying DM concentrations at ensiling on various grass and legume forage crop species with chemical composition, fermentation pattern, CP fractions and *in vitro* gas production. Special focus was directed on the effect of varying HT and DM concentrations on fate of CP fractions caused by ensiling, because a reduction of the extent of proteolysis would improve the protein value of forages. An improved protein value in turn would lessen the need to supplement ruminant rations with protein feeds, thus contributing to an improved nitrogen use efficiency both at the animal and farm level.

2. Materials and methods

2.1. Preparation of silages

Silages of all forage species were prepared in 2018 and 2019 at the Campus Frankenforst of the Faculty of Agriculture, University of Bonn, Germany ($7^\circ 12' 22''$ E and $50^\circ 42' 49''$ N; annual precipitation, 650 - 850 mm).

In 2018, LU (*Medicago sativa* L., 2 hectares) and RC (*Trifolium pratense* L., 0.2 hectare) were cultivated in pure stands in the third and first year of use, respectively. No fertilizer was applied during the entire period. The first cut of LU was mowed at early-bud stage using a disc mower combination (DISCO 3100, CORTO 3150 F; CLAAS, Harsewinkel, Germany) with a stubble height of 7 cm. The RC was mowed with the same equipment at late-bud stage. In 2019, a pure stand of 1 hectare IR (*Lolium multiflorum* L.) was cultivated at the same site. In total, 130 kg N-fertilizer were applied on the field, which were divided into 50 kg N (swine slurry) before first cut and 80 kg N (calcium ammonium nitrate) before the second cut. The second cut of IR was mowed after six weeks of regrowth at late vegetative stage. Samples from mowed forages were taken from various locations on the fields and frozen (-18 °C) immediately for later analysis. After mowing, the herbage was spread out using a tedder to accelerate field wilting. During the complete field-wilting processes of LU and IR the weather conditions were sunny with maximum temperatures of 27 and 23°C, respectively, and no rainfall occurred. The weather conditions during RC harvest were changeable, temperatures up to 28°C but also clouds and precipitation occurred (≈ 0.5 l/m²). To produce silages with two different DM levels, one part of each forage species was picked up from the field and chopped to a theoretical length of chop of 45 mm after approximately 6-8 h wilting (low DM silages; LO ≈ 200 g/kg), whereas the second part was wilted until the next day for at least 26 h (high DM silages; HI ≈ 350 g/kg). The DM concentration of the forages during the wilting period was regularly determined using a hot air dryer (Fluid bed dryer TG 1, Retsch, Haan, Germany). The procedure was based on the method described by von Borstel (1981). The respective plant material was unloaded immediately in a shady place on 5 x 5 m plastic tarpaulin and samples from both DM levels were taken and frozen for later chemical analysis. For each of the three species, six silage treatments in a 2 x 3 factorial design were prepared in quadruplicate, differing in DM and HT concentration.

The HT used in this experiment were extracted from chestnut wood (*Castanea sativa* MILL.) and were applied as fine powder with pure tannin content about 770 g/kg DM (Silvafeed ENC powder, Silvateam, San Michele Mondovì CN, Italy) to LU and RC or as liquid with 460 g DM/kg and a total tannin content of minimum 750 g/kg DM (Silvafeed ENC liquid, Silvateam) to IR before ensiling. To achieve a consistent application, both HT products were dissolved in water and dispensed with a pressure sprayer on thin layers of the wilted forage (Prima 5, Gloria, Witten, Germany). The amount of water applied to all treatments was identical in order to obtain equal effects on DM, whereby the HT to water ratio increased from pure water (0 g HT/kg DM) in the control group to 1:4 (20 g HT/kg DM) to 1:2 (40 g HT/kg DM). The HT:water ratio of

1:2 represented the best possible compromise between product solubility and the lowest possible dosage of water.

Based on Tabacco et al. (2006), the following treatments were applied to each of the three forage crops (IR, LU and RC), such that a total of 18 treatments were prepared and investigated in this study:

- Three HT concentrations: 0 g/kg (0T), 20 g/kg (2T) and 40 g/kg (4T);
- Two DM levels: ~ 200 g/kg (low DM; LO) and ~ 350 g/kg (high DM; HI).

Before ensiling, the HI and LO DM plant material was compacted (HI: 212 kg DM/m³; LO: 190 kg DM/m³ for RC and LU and 177 kg DM/m³ for IR) following the recommendations of the Federal Working Group for Forage Preservation in Germany (Bundesarbeitskreis Futterkonservierung, 2011) using a hydraulic press, which was manufactured for this purpose by the Institute for Agricultural Engineering at the University of Bonn. Subsequently, the compacted forage treatments were sealed airtight in 120-litre barrels by covering the opening with an oxygen-impermeable plastic sheet and closing it with a lid and clamping ring.

All barrels were stored indoors at room temperature and after 115 d of ensiling, three barrels of each treatment were opened, thoroughly mixed and composite samples (1,000 g) of each barrel were taken and immediately frozen (-18°C) for chemical analyses. Furthermore, samples for analysis of fermentation products were collected and frozen (2 x 50.0 g for each barrel). Silages did not show any sign of visible spoilage, nevertheless the top 5 cm of each barrel were discarded.

2.2. General analyses

About 600 g of each replicate were taken, freeze-dried for 5 days (P18K-E-6, Dieter Piatkowski, Petershausen, Germany) in triplicate and ground stepwise using 3-mm and then 1-mm sieve aperture (SM100, Retsch, Haan, Germany). The DM of the silages was then estimated by oven-drying of duplicate samples at 105°C overnight. A correction of DM (DM_{cor}) was conducted according to (Weißbach and Strubelt, 2008) to take into account loss of volatiles during drying:

$$\text{DM}_{\text{cor}} = \text{DM} + (1.05 - 0.059 \times \text{pH}) * \text{total volatile fatty acids (VFA, C2-C6)} \times 0.08 \times \text{lactic acid} + 0.77 \times \text{1,2-propanediol} + 0.87 \times \text{2,3-butanediol} + 1 \times \text{total of other alcohols (C2-C4)}$$
 [g/kg].

Proximate analyses of silage samples were performed according to VDLUFA (2012), and method numbers are given. Crude lipids (CL) and ash were analysed by using methods 5.1.1 and 8.1. Crude protein was calculated by determining total N concentration with Kjeldahl method (4.1.1) using a Vapodest 50s carousel (Gerhardt, Königswinter, Germany) and multiplying N by 6.25. The proportions of neutral detergent fibre assayed with heat-stable amylase and expressed without residual ash (aNDFom), acid detergent fibre expressed without residual ash (ADFom), and acid detergent lignin (ADL) were analysed using an Ankom 2000 Fiber Analyser (Ankom Technology, Macedon, USA), according to the methods 6.5.1, 6.5.2, and 6.5.3, respectively. Because of higher pectin concentrations in LU and RC samples, ADFom analysis was performed sequentially on the ND insoluble residue.

Crude protein fractionation was done according to the Cornell Net Carbohydrate and Protein System (Sniffen et al., 1992), following standardizations and recommendations of Licitra et al. (1996).

2.4. Chemical analyses of fermentation products

Frozen herbage and silage subsamples (50 g) were used for analysis of fermentation products. The following fermentation variables were determined in duplicate: pH, lactic acid, volatile fatty acids (VFA; acetic acid, propionic acid and butyric acid), alcohols (methanol, ethanol, propanol, 1,2-propanediol, 2,3-butanediol, 1-butanol and 2-butanol), acetone, NH₃-N and WSC. In addition, samples were also analysed for ethyl lactate, ethyl acetate and propyl acetate. Cold water extracts were prepared by blending the frozen samples with 300 ml distilled water and 1 ml toluol and stored cool (4°C) overnight. The extracts were then filtered using filter paper (MN 615, Macherey-Nagel, Düren, Germany) and microfiltered subsequently (Minisart RC, 0.45 µm pore size, Sartorius, Göttingen, Germany).

After extraction the pH was determined potentiometrically by using a calibrated pH meter (827 pH lab, Metrohm, Herisau, Switzerland). Lactic acid was analysed by high-performance liquid chromatography with refractive index detection (LC-20 AB; Shimadzu Deutschland, Duisburg, Germany) according to Weiß and Kaiser (1995). Both, individual VFA and alcohols were determined by gas chromatography (GC) with flame ionization detection (detection limit: 0.01%) (GC-2010, Shimadzu, Duisburg, Germany), as described by Weiß (2001), whereas ethyl esters, acetone, propanol, 1,2-propanediol, methanol, 1-butanol, 2-butanol and 2,3-butanediol were analysed by GC as described by Weiß and Sommer (2012) (detection limit:

0.001%). The NH₃-N was analysed colourimetrically based on the Berthelot reaction using a continuous flow analyser (CFA) (Skalar Analytical, Breda, Netherland). The WSC concentration was determined by the anthrone method with the same CFA used for NH₃-N determination, according to Lengerken and Zimmermann (1991).

2.4. *In vitro* gas production

Freeze-dried samples (200 mg ± 10 mg) of each forage treatment were incubated in the Hohenheim gas test (HGT) (method 25.1; VDLUFA 2012,) system to measure *in vitro* GP and to estimate organic matter digestibility (OMD) (Menke and Steingass, 1988) and metabolizable energy (ME) concentrations (GfE, 2008). Rumen fluid was collected from six ruminally fistulated sheep fed with a mixed forage-concentrate diet consisting of perennial grass hay and a concentrate mix in a ratio of 2:1 twice daily (VDLUFA, 2012, method 25.1). Rumen fluid was always obtained before morning feeding and immediately used in the HGT. *In vitro* incubations were conducted in three sequential runs with a total of two replications for each silage repetition (n = 6) and four replications for fresh herbage. Following the procedure of Liu et al. (2002) the GP was recorded after 2, 4, 6, 8, 12, 24, 36, 48, 60, 72 and 96 h of incubation. The mean GP from two syringes per run (standardized to 200 mg DM) was used to estimate GP kinetics over time with a nonlinear regression equation (Ørskov and McDonald, 1979):

$$GP = a + b(1 - e^{-ct})$$

where *a* is the initial GP of soluble ingredients in the inoculum, *b* is potential GP of insoluble, fermentable ingredients, *c* is the rate of GP, and *t* the incubation time. Parameters *a* and *b* were assembled to (*a* + *b*), expressed as maximum GP, to improve the statistical validity. Short time GP was determined as cumulative GP at two hours of incubation (GP₂).

The GP after 24 h of incubation was taken to estimate ME of silage treatments using the following equations:

Grass silage (GfE, 2008):

$$ME(\text{MJ/kg DM}) = 7.81 + 0.07559 * GP - 0.00384 * \text{ash} + 0.00565 * \text{CP} + 0.01898 \text{ CL} - 0.00831 * \text{ADFom}$$

Legume silages (GfE, 2017):

$$\text{ME (MJ/kg OM)} = 12.49 - 0.0114 * \text{ADFom} + 0.00425 * \text{CP} + 0.02690 * \text{CL} + 0.01683 * \text{GP}$$

$$\text{ME (MJ/kg DM)} = \text{ME(MJ/kg OM)} * (1000 - \text{ash(g/kg DM)})/1000$$

OMD of silages (Menke and Steingass, 1988):

$$\text{OMD (g/kg)} = 15.38 + 0.8453 \text{ GP} + 0.0595 \text{ CP} + 0.0675 \text{ ash}$$

GP is expressed as ml/200 mg DM in 24 h and CP, Ash, ADFom and CL are presented in g/kg DM.

2.5. Statistical analysis

All statistical analyses were conducted using SAS 9.4 (SAS Institute Inc., Cary, North Carolina, USA). Residuals were initially analysed using the UNIVARIATE procedure to evaluate the normality of the response variables by the Shapiro-Wilk test. Next, a two-factorial analysis of variance (PROC GLM) was used to test the effect of DM treatment, HT treatment and the DM x HT interaction on proximate constituents, fermentation characteristics, GP and CP fractions for each forage species, while an orthogonal contrast test was performed (PROC GLM statement CONTRAST) to estimate linear and quadratic effects of different HT concentrations (0, 20 and 40 g/kg DM) within each DM level on chemical composition. Significance was defined at $P < 0.05$.

Similar to Liu et al. (2002), non-linear regression analysis with weighted least squares means (PROC NLIN) were used to estimate the GP parameters (a , b and c) which were described in section 2.4. The least squares means of treatment x time (t) from the mixed model analysis were used as time series measurements and the standard error of least squares means as weight. When the 95% confidence intervals of treatments did not overlap, GP parameters were considered to be significantly different between treatments. Besides, the effects of run and treatment and their interactions on GP per point in time were calculated by using a mixed model in SAS (PROC MIXED).

3. Results

3.1. General chemical composition and fermentation variables

The chemical composition and CP fractions of LU, IR and RC before ensiling ranged within expected values, considering the stage of maturity of the respective forage crops. The corresponding results are shown in Table 1. During wilting on the field, changes in DM, CP fractions and WSC were observed across species.

As shown in Table 2, the DM level had a strong impact on the CP content, and the HT addition also had an effect, namely CP concentrations were higher in LO silages without HT. Increasing the DM content led to a lower silage pH ($P < 0.001$) in LU silages but increased WSC concentration from less than 3 g/kg DM to more than 25 g/kg DM. The lactic acid concentration of the LU silages was decreased by HT treatment. Increasing the DM content effectively reduced concentrations of acetic acid, propionic acid and butyric acid. The HT treatment enhanced ethanol content in LO and HI silages, respectively, yet ethanol did not exceed 10 g/kg DM in any LU silage. More intensive wilting reduced the formation of $\text{NH}_3\text{-N}$ more than HT ($P < 0.001$).

The results of chemical analysis of IR silages are presented in Table 3. Both pre-ensiling treatments lowered CP and CL proportions, whereas the effects of HT were only significant in LO silages. The fermentation pattern was in many cases affected by HT dosage as well as DM content. Silage pH varied in a common range (4.1 – 4.7), considering the maturity stage and DM level. In LO HT treatment resulted in a pH decline ($P < 0.001$), in contrast the pH in HI showed almost no change ($P = 0.59$). Both, lactic acid and acetic acid were detected in LO silages in higher concentrations than in HI silages. Butyric acid was only found in negligible quantities in most silages and its concentration was not affected by pre-ensiling treatments. Similar to LU, the enhancement of DM content by wilting resulted in a rise of WSC concentrations in IR silages ($P < 0.001$). The ensiling process caused a considerable increase of $\text{NH}_3\text{-N}$, especially in the untreated, low DM IR silages. However, a higher DM content in combination with HT treatment diminished the concentration of $\text{NH}_3\text{-N}$ below 100 g/kg N.

The proximate nutrients and fermentation products of RC are given in Table 4. The content of CP was highest in the untreated, low DM RC silages and declined by DM and HT treatment.

Increasing the DM content decreased pH and concentrations of acetic acid, propionic acid and butyric acid, whereas lactic acid was elevated. The concentration of WSC was positively affected by DM content ($P < 0.001$). Likewise, the addition of HT to HI forages resulted in

higher concentrations of WSC ($P < 0.001$). Ammonia-N was highest in RC0TLO, but was significantly reduced by both DM and HT treatments. By gradually increasing HT application rate, $\text{NH}_3\text{-N}$ formation was linearly diminished in LO ($P < 0.001$) as well as HI silages ($P = 0.009$).

Table 1. Chemical composition and crude protein (CP) fractions of fresh and wilted lucerne (LU), Italian ryegrass (IR) and red clover (RC) before ensiling

Variables (g/kg DM)	Lucerne (LU)			Italian ryegrass (IR)			Red clover (RC)		
	Fresh	LO	HI	Fresh	LO	HI	Fresh	LO	HI
DM (g/kg)	162	251	367	132	193	384	116	169	282
Ash	112	121	118	109	131	112	128	150	150
CP	229	220	210	190	178	181	170	169	152
aNDFom	418	442	465	463	475	509	438	462	466
ADFom	347	298	343	273	275	284	338	349	349
ADL	144	128	131	21.2	24.5	26.7	73.6	82.9	84.8
WSC	110	94	104	95.5	172	148	153	67.2	112
ME (MJ/kg DM)	10.1	10.3	9.80	10.9	10.5	10.7	9.87	9.49	9.41
$\text{NH}_3\text{-N}$ (g/kg N)	8.20	14.2	14.9	0.00	21.1	17.3	25.7	40.8	49.4
<u>CP fractions (g/kg CP)</u>									
A	268	285	387	242	307	375	204	257	317
B1	195	170	79.9	177	91.1	97.4	165	47.9	29.2
B2	482	481	464	495	432	250	530	514	495
B3	0.00	20.9	9.50	59.9	127	220	39.8	84.1	78.1
C	56.0	42.6	59.6	26.0	42.5	57.5	61.0	77.1	76.9

DM = dry matter; LO = 200 g/kg; HI = 350 g/kg; aNDF = neutral detergent fibre assayed with heat-stable amylase and expressed exclusive residual ash; ADFom = acid detergent fibre expressed exclusive residual ash; ADL = acid detergent lignin; WSC = water-soluble carbohydrates; $\text{NH}_3\text{-N}$ = ammonia-nitrogen; ME = metabolizable energy; A = non-protein-nitrogen, calculated as CP-true protein (TP); B1 = buffer-soluble TP, quickly degradable; B2 = buffer-insoluble TP, moderately degradable; B3 = neutral detergent insoluble TP, slowly degradable; C = acid detergent insoluble TP, indigestible.

^aCrude protein fractionation according to the Cornell Net Carbohydrate and Protein system (Sniffen et al. 1992)

Table 2. Effect of different dosages of hydrolysable tannin (HT) and dry matter (DM) level on chemical composition and fermentation characteristics in lucerne (LU) silages

Variables (g/kg DM)	Treatments ^a						SEM	<i>P</i> -value						
	0TLO	2TLO	4TLO	0THI	2THI	4THI		DM	HT	DM x HT	Lin LO ^b	Quad LO ^b	Lin HI ^b	Quad HI ^b
Dry matter (g/kg)	230	228	248	366	365	370	2.10	<0.001	<0.001	0.009	<0.001	0.002	0.247	0.186
Ash	132	132	129	123	122	116	2.01	<0.001	0.055	0.669	0.265	0.599	0.037	0.301
Crude protein	245	237	230	224	218	220	2.80	<0.001	0.014	0.143	0.0026	0.962	0.332	0.230
Crude lipids	37.0	34.4	32.7	28.0	27.1	28.8	0.75	<0.001	0.059	0.016	0.002	0.622	0.466	0.173
aNDFom	391	395	404	392	391	381	11.1	0.357	0.994	0.535	0.423	0.857	0.484	0.773
ADFom	311	323	326	326	324	326	8.98	0.489	0.713	0.614	0.250	0.657	0.959	0.835
ADL	81.2	93.7	99.9	86.6	81.5	83.8	3.17	0.012	0.079	0.012	0.001	0.442	0.544	0.351
WSC	2.30	2.07	2.57	29.0	29.4	24.6	2.10	<0.001	0.518	0.403	0.929	0.888	0.161	0.317
ME (MJ/kg DM)	10.1	9.73	9.56	9.73	9.60	9.57	0.15	0.068	0.013	0.211	0.003	0.453	0.274	0.710
pH	5.12	5.55	5.59	4.97	4.97	5.01	0.10	<0.001	0.058	0.089	0.007	0.149	0.783	0.874
Lactic acid	90.5	51.7	41.6	70.2	67.2	56.0	0.37	0.314	<0.001	<0.001	<0.001	0.008	0.019	0.384
Acetic acid	42.6	38.3	30.0	13.9	18.0	15.5	0.19	<0.001	<0.001	<0.001	<0.001	0.143	0.285	0.022
Propionic acid	1.03	1.13	0.97	-	-	-	0.01	<0.001	0.794	0.794	0.707	0.391	-	-
Butyric acid	0.40	9.67	12.5	-	-	-	0.23	0.002	0.063	0.052	0.003	0.283	-	-
Methanol	1.70	3.07	2.83	2.03	2.70	2.10	0.38	0.431	0.062	0.399	0.059	0.115	0.904	0.203
Ethanol	6.73	10.3	9.20	5.40	6.43	7.80	0.80	0.004	0.013	0.201	0.043	0.026	0.048	0.863
Propanol	350	253	162	-	-	-	15.7	<0.001	<0.001	<0.001	<0.001	0.865	-	-
Butanol ^c	160	266	317	-	-	-	17.2	<0.001	0.002	<0.001	<0.001	0.219	-	-
Ethyl lactate ^c	20.3	-	-	12.3	35.6	35.0	9.93	0.026	0.991	0.081	-	-	0.133	0.370
Ethyl acetate ^c	63.7	53.0	18.7	16.0	38.0	57.3	20.3	0.638	0.929	0.143	0.145	0.643	0.176	0.958
Propyl acetate ^c	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NH ₃ -N (g/kg N)	198	200	172	121	111	100	8.26	<0.001	0.030	0.537	0.041	0.156	0.100	0.945

aNDF = neutral detergent fibre assayed with heat-stable amylase and expressed exclusive residual ash; ADFom = acid detergent fibre expressed exclusive residual ash; ADL = acid detergent lignin; WSC = water-soluble carbohydrates; ME = metabolizable energy; NH₃-N = ammonia-nitrogen; CP = crude protein; SEM = standard error of the mean; DM= effect of dry matter level; HT = effect of hydrolysable tannin; significance between treatments at P < 0.05.

^a treatments: 0T = 0 g/kg DM of HT; 2T = 20 g/kg DM of HT; 4T = 40 g/kg DM of HT; LO = 200 g/kg; HI = 350 g/kg

^b orthogonal contrasts: Linear (Lin) and quadratic (Quad) effects of different HT concentrations on the chemical composition of silages at the respective DM level

^c mg/kg DM

Table 3. Effect of different dosages of hydrolysable tannin (HT) and dry matter (DM) level on chemical composition and fermentation characteristics in Italian ryegrass (IR) silages

Variables (g/kg DM)	Treatments ^a						SEM	<i>P</i> -value						
	0TLO	2TLO	4TLO	0THI	2THI	4THI		DM	HT	DM x HT	Lin LO ^b	Quad LO ^b	Lin HI ^b	Quad HI ^b
Dry matter (g/kg)	197	204	203	359	367	371	4.03	<0.001	0.081	0.686	0.325	0.373	0.048	0.622
Ash	137	127	121	139	130	135	3.83	0.062	0.036	0.214	0.009	0.703	0.512	0.192
Crude protein	209	197	185	186	182	184	2.20	<0.001	<0.001	0.001	<0.001	0.904	0.533	0.240
Crude lipids	49.0	36.0	33.7	32.9	32.6	32.8	2.02	<0.001	0.004	0.004	<0.001	0.068	0.991	0.911
aNDFom	452	443	433	437	445	437	7.42	0.642	0.393	0.393	0.090	1	1	0.436
ADFom	308	280	276	280	283	274	5.12	0.046	0.010	0.027	0.001	0.087	0.423	0.384
ADL	39.5	44.2	44.9	26.6	28.9	26.9	6.56	0.014	0.855	0.928	0.571	0.808	0.975	0.801
WSC	3.41	4.16	6.81	43.2	38.5	41.5	6.06	<0.001	0.790	0.469	0.699	0.904	0.194	0.878
ME (MJ/kg DM)	10.4	10.2	9.98	10.4	10.3	10.3	0.08	0.033	0.019	0.023	0.004	0.875	0.289	0.754
pH	4.60	4.23	4.10	4.67	4.66	4.72	0.06	<0.001	0.005	0.002	<0.001	0.117	0.590	0.594
Lactic acid	75.0	120	117	63.4	63.6	53.0	16.5	<0.001	0.092	0.009	0.009	0.067	0.051	0.228
Acetic acid	49.9	21.7	14.4	15.1	15.5	15.1	2.07	<0.001	<0.001	<0.001	<0.001	0.001	0.135	0.306
Propionic acid	2.65	-	-	-	-	-	0.69	-	-	-	-	-	-	-
Butyric acid	-	1.73	0.71	0.18	0.09	0.02	0.58	0.178	0.390	0.325	0.402	0.077	0.965	0.929
Methanol	0.77	0.63	0.50	0.47	0.30	0.40	0.07	<0.001	0.053	0.208	0.015	1,000	0.490	0.128
Ethanol	8.22	12.9	14.1	5.71	7.35	6.36	0.89	<0.001	0.011	0.006	0.001	0.142	0.354	0.063
Propanol ^c	16,379	832	188	218	153	203	675	<0.001	<0.001	<0.001	<0.001	<0.001	0.987	0.945
Ethyl lactate ^c	51.3	163	178	48.3	50.3	47.0	11.5	<0.001	<0.001	<0.001	<0.001	0.005	0.936	0.853
Ethyl acetate ^c	29.3	60.0	-	55.3	65.3	47.3	13.9	0.039	0.048	0.350	0.134	0.788	0.691	0.426
NH ₃ -N (g/kg N)	141	127	98.3	106	103	97.9	4.46	<0.001	<0.001	0.055	<0.001	0.219	0.012	0.324

DM = dry matter; aNDF = neutral detergent fibre assayed with heat-stable amylase and expressed exclusive residual ash; ADFom = acid detergent fibre expressed exclusive residual ash; ADL = acid detergent lignin; WSC = water-soluble carbohydrates; ME = metabolizable energy; NH₃-N = ammonia-nitrogen; CP = crude protein; SEM = standard error of the mean; DM = effect of dry matter level; HT = effect of hydrolysable tannin; Significance between treatments at $P < 0.05$.

^a treatments: 0T = 0 g/kg DM of HT; 2T = 20 g/kg DM of HT; 4T = 40 g/kg DM of HT; LO = 200 g/kg; HI = 350 g/kg

^b orthogonal contrasts: Linear (Lin) and quadratic (Quad) effects of different HT concentrations on the chemical composition of silages at the respective DM level

^c mg/kg DM

Table 4. Effect of different dosages of hydrolysable tannin (HT) and dry matter (DM) level on chemical composition and fermentation characteristics in red clover (RC) silages

Variables (g/kg DM)	Treatments ^a						SEM	<i>P</i> -value						
	0TLO	2TLO	4TLO	0THI	2THI	4THI		DM	HT	DM x HT	Lin LO ^b	Quad LO ^b	Lin HI ^b	Quad HI ^b
Dry matter (g/kg)	163	174	167	293	323	327	4.89	<0.001	0.018	0.029	0.558	0.158	<0.001	0.044
Ash	165	161	147	162	150	148	3.40	0.135	0.002	0.201	0.003	0.198	0.011	0.238
Crude protein	184	178	172	164	165	159	1.97	<0.001	0.004	0.183	0.011	0.979	0.108	0.244
Crude lipids	35.5	33.2	30.0	27.2	25.7	23.2	0.84	<0.001	<0.001	0.713	0.001	0.682	0.005	0.615
aNDFom	406	462	458	447	426	430	18.6	0.663	0.518	0.102	0.058	0.196	0.538	0.588
ADFom	361	356	341	327	380	364	9.51	0.577	0.075	0.015	0.163	0.675	0.018	0.012
ADL	73.4	85.0	88.7	75.0	102	101	4.22	0.010	<0.001	0.206	0.025	0.461	<0.001	0.019
WSC	0.79	0.95	2.87	12.9	19.1	25.2	1.79	<0.001	0.006	0.043	0.428	0.696	<0.001	0.980
ME (MJ/kg DM)	8.96	8.81	8.86	9.22	8.78	8.70	0.08	0.724	0.003	0.052	0.403	0.293	<0.001	0.074
pH	4.93	5.07	4.70	4.40	4.41	4.43	0.06	<0.001	0.077	0.043	0.039	0.010	0.747	0.950
Lactic acid	79.6	55.1	87.7	113	99.5	91.2	7.36	<0.001	0.067	0.043	0.452	0.008	0.058	0.772
Acetic acid	48.6	41.2	42.4	26.4	23.3	21.5	2.44	<0.001	0.074	0.068	0.097	0.180	0.185	0.822
Propionic acid	4.52	5.28	2.53	0.01	-	-	0.43	<0.001	0.019	0.021	0.006	0.006	-	-
Butyric acid	20.4	34.0	13.5	0.99	0.13	-	3.85	<0.001	0.058	0.056	0.228	0.004	-	-
Methanol	4.50	2.97	2.94	2.90	2.87	1.97	0.49	0.045	0.070	0.337	0.042	0.233	0.201	0.482
Ethanol	5.58	2.77	3.95	1.77	2.25	1.48	0.59	0.005	0.334	0.134	0.176	0.066	0.799	0.538
Propanol ^c	3,588	2,333	2,159	301	60.0	122	433	<0.001	0.156	0.342	0.039	0.329	0.774	0.780
Butanol ^c	275	631	575	11.7	-	-	130	<0.001	0.392	0.342	0.039	0.329	-	-
Ethyl lactate ^c	-	-	-	25.3	19.0	11.7	4.92	<0.001	0.408	0.408	-	-	0.073	0.935
Ethyl acetate ^c	-	-	20.0	-	-	10.3	9.19	0.675	0.205	0.834	-	-	-	-
Propyl acetate ^c	47.3	58.0	70.7	-	-	-	22.7	-	0.877	-	0.481	0.972	-	-
NH ₃ -N (g/kg N)	156	131	91.5	113	92.2	88.6	9.40	<0.001	<0.001	0.005	<0.001	0.311	0.009	0.228

aNDF = neutral detergent fibre assayed with heat-stable amylase and expressed exclusive residual ash; ADFom = acid detergent fibre expressed exclusive residual ash; ADL = acid detergent lignin; WSC = water-soluble carbohydrates; ME = metabolizable energy; NH₃-N = ammonia-nitrogen; CP = crude protein; SEM = standard error of the mean; DM = effect of dry matter level; HT = effect of hydrolysable tannin; Significance between treatments at $P < 0.05$.

^a treatments: 0T = 0 g/kg DM of HT; 2T = 20 g/kg DM of HT; 4T = 40 g/kg DM of HT; LO = 200 g/kg; HI = 350 g/kg

^b orthogonal contrasts: Linear (Lin) and quadratic (Quad) effects of different tannin (HT) concentrations on the chemical composition of silages at the respective DM level

^c mg/kg DM

3.2. Crude protein fractions

The CP fractionation revealed various differences between fresh, wilted (Table 1) and ensiled plant material as well as between pre-ensiling treatments (Table 5).

During the ensiling process, NPN increased to the largest CP fraction in all LU silages. In all three forage species, non-protein-nitrogen proportion was highest in all but one (IR0TLO) silage without HT treatment, particularly in LU0TLO (824 g/kg CP). The addition of HT at 20 and 40 g/kg DM resulted in a stepwise decrease of NPN by 26% in LO silages ($P < 0.001$) and 22% in HI silages ($P < 0.001$), respectively. Fraction B2 declined during ensiling period but remained as second largest fraction and was almost 160 g/kg CP higher in LU4TLO than in LU0TLO ($P < 0.001$). An effect of HT ($P < 0.001$) in a similar range was also found in HI silages.

Similar to LU, NPN in IR increased during fermentation to become the largest CP fraction in all six IR silages. Concentrations ranged between 540 and 696 g/kg CP and largest differences were observed between IR0THI and IR4TLO. In accordance to the HT treatment in LU, 40 g/kg DM of HT linearly reduced the NPN formation in LO silages by 25% ($P < 0.001$), whereas an inferior decline by 11% was determined in HI silages ($P = 0.003$). Fraction B2, the second largest group, was affected by DM ($P < 0.001$) and HT treatment. However, only a tendency ($P = 0.078$) effect of HT was observed in HI silages. As with NPN, the largest discrepancy in fraction B2 was noted between IR0Thi and IR4Tlo.

Formation of NPN compounds in RC were lower than in LU and IR, as the NPN proportion did not exceed 500 g/kg CP in any silage treatment. The addition of HT resulted in a distinct effect in HI and LO silages ($P < 0.001$), with the most significant decline of NPN between RC0TLO and RC4TLO. Fraction B2 remained as the predominant CP fraction after ensiling with highest proportions in RC4TLO with over 500 g/kg CP. In addition, fraction B2 was positively influenced by DM as well as HT. Fraction C increased during ensiling and exceeded the concentrations determined in LU and IR, and was affected by DM ($P = 0.015$). Furthermore, a linear increment of fraction C in HI silages due to HT was observed ($P = 0.011$), whereas a comparable effect could not be detected in LO silages.

3.3. *In vitro* gas production characteristics

The GP parameters of LU, IR and RC are given in Table 6. The direct comparison between the three forage crops showed that GP2 as well as GP rate were highest in LU silages, whereas the highest maximal GP was determined in IR silages. With regard to LU silages, a significant dependence of the GP parameters on DM treatment was found. Wilting LU increased GP2 ($P = 0.006$), maximum GP ($P = 0.022$) and GP rate ($P = 0.031$), but tended to reduce the OMD ($P = 0.078$). A more pronounced decline in OMD of about 3% was observed as a result of the HT treatment. In addition, maximum GP ($P = 0.022$) as well as 24 h GP ($P < 0.001$) were also negatively affected by HT.

All *in vitro* GP parameters of IR silages were strongly affected by DM content. In general, an increase of DM, due to wilting, resulted in a rise of GP2, maximal GP, GP rate and 24 h GP ($P < 0.001$). Contrary to LU, the DM treatment had a positive on OMD of IR. The added HT extract linearly decreased OMD, 24 h GP and maximum GP, but improved the GP2 in LO silages ($P = 0.014$).

The two pre-ensiling treatments had similar effects on IR and RC. All listed parameters of RC silages were positively affected by DM ($P < 0.001$). In contrast, the gradual addition of HT up to 40 g/kg on DM basis resulted in a significant reduction of maximum GP, 24 h GP, OMD.

Table 5. Effect of different dosages of hydrolysable tannin (HT) and dry matter (DM) level on crude protein (CP) fractions in grass and legume silages

CP fractions ^c g/kg CP	Treatments ^a						SEM	<i>P</i> -value						
	0TLO	2TLO	4TLO	0THI	2THI	4THI		DM	HT	DM x HT	Lin LO ^b	Quad LO ^b	Lin HI ^b	Quad HI ^b
Lucerne (LU)														
A	824	715	656	778	713	640	11.5	0.050	<0.001	0.211	<0.001	0.103	<0.001	0.796
B1	11.9	18.9	11.1	12.1	20.9	23.0	5.72	0.331	0.402	0.566	0.929	0.313	0.203	0.636
B2	123	218	281	157	214	280	9.69	0.237	<0.001	0.136	<0.001	0.204	<0.001	0.684
B3	-	-	1.07	6.58	6.68	5.32	2.84	0.027	0.999	0.890	-	-	0.759	0.838
C	56.0	58.6	51.3	50.7	48.3	53.5	4.43	0.243	0.966	0.394	0.465	0.379	0.660	0.502
Italian ryegrass (IR)														
A	676	604	540	696	650	626	13.6	<0.001	<0.001	0.075	<0.001	0.176	0.003	0.512
B1	33.7	36.0	39.1	24.3	31.5	32.7	6.73	0.065	0.274	0.696	0.584	0.147	0.393	0.718
B2	251	301	359	199	212	230	11.2	<0.001	<0.001	0.015	<0.001	0.986	0.078	0.876
B3	2.01	4.34	10.0	22.3	50.5	53.1	8.06	<0.001	0.064	0.313	0.495	0.819	0.019	0.219
C	42.0	55.6	50.8	58.6	54.9	57.5	4.91	0.113	0.397	0.201	0.226	0.151	0.873	0.611
Red clover (RC)														
A	486	333	290	471	417	338	11.4	0.001	<0.001	0.003	<0.001	0.002	<0.001	0.395
B1	-	18.0	27.4	19.5	5.56	10.1	8.62	0.635	0.566	0.109	-	-	0.456	0.399
B2	381	459	506	353	406	453	10.2	<0.001	<0.001	0.375	<0.001	0.222	<0.001	0.818
B3	64.8	102	95.4	64.8	68.4	72.8	11.3	0.124	0.223	0.341	0.222	0.077	0.624	0.979
C	83.6	88.1	93.2	92.2	105	130	8.79	0.015	0.057	0.302	0.454	0.974	0.011	0.569

A = non-protein-nitrogen, calculated as CP-true protein (TP); B1 = buffer-soluble TP, quickly degradable; B2 = buffer-insoluble TP, moderately degradable; B3 = neutral detergent insoluble TP, slowly degradable; C = acid detergent insoluble TP, indigestible; TP = true protein; SEM = standard error of the mean; DM = effect of dry matter level; HT = effect of hydrolysable tannin; Significance between treatments at $P < 0.05$.

^a treatments: 0T = 0 g/kg DM of HT; 2T = 20 g/kg DM of HT; 4T = 40 g/kg DM of HT; LO = 200 g/kg; HI = 350 g/kg

^b orthogonal contrasts: Linear (Lin) and quadratic (Quad) effects of different HT concentrations on the chemical composition of silages at the respective DM level

^c Crude protein fractionation according to the Cornell Net Carbohydrate and Protein system (Sniffen et al. 1992)

Table 6. Effect of different dosages of hydrolysable tannin (HT) and dry matter (DM) level on cumulative gas production (GP) kinetics and organic matter digestibility (OMD) in grass and legume silages.

Variables	Treatments ^a						SEM	<i>P</i> -value						
	0TLO	2TLO	4TLO	0THI	2THI	4THI		DM	HT	DM x HT	Lin LO ^b	Quad LO ^b	Lin HI ^b	Quad HI ^b
	Lucerne (LU)													
GP2 ^c	10.0	9.48	8.84	11.1	11.8	10.4	0.62	0.006	0.223	0.610	0.204	0.944	0.434	0.185
(a + b) ^c	43.6	41.5	40.9	43.9	42.8	42.3	0.46	0.022	0.002	0.445	0.001	0.217	0.030	0.655
c (%/h)	12.7	13.1	12.6	14.1	14.5	13.2	0.01	0.031	0.335	0.674	0.948	0.572	0.267	0.242
24 h GP ^c	43.4	41.3	40.2	43.6	42.4	41.3	0.57	0.102	<0.001	0.708	0.022	0.538	0.014	0.957
OMD (g/kg)	756	734	718	739	725	713	6.39	0.078	<0.001	0.644	<0.001	0.709	0.012	0.885
	Italian ryegrass (IR)													
GP2 ^c	5.58	6.08	6.80	9.22	9.12	8.55	0.30	<0.001	0.650	0.024	0.014	0.756	0.142	0.537
(a + b) ^c	52.3	51.1	49.2	55.3	54.7	53.3	0.54	<0.001	0.002	0.638	0.002	0.616	0.020	0.570
c (%/h)	7.07	6.89	6.76	8.17	8.12	7.61	0.01	<0.001	0.198	0.295	0.520	0.375	0.062	0.361
24 h GP ^c	47.2	44.7	43.0	50.1	49.5	47.8	0.67	<0.001	<0.001	0.291	<0.001	0.665	0.033	0.518
OMD (g/kg)	770	735	709	782	769	759	7.08	<0.001	<0.001	0.057	<0.001	0.598	0.037	0.850
	Red clover (RC)													
GP2 ^c	7.04	7.15	6.88	9.60	9.53	9.90	0.40	<0.001	0.984	0.724	0.785	0.710	0.607	0.667
(a + b) ^c	42.8	40.6	40.2	45.3	45.2	42.7	0.41	<0.001	<0.001	0.036	<0.001	0.096	<0.001	0.034
c (%/h)	9.80	10.6	10.3	11.3	11.1	10.9	0.01	<0.001	0.340	0.066	0.123	0.058	0.169	0.941
24 h GP ^c	40.6	38.8	38.4	43.8	43.3	41.6	0.49	<0.001	0.003	0.335	0.010	0.289	0.008	0.302
OMD (g/kg)	717	697	680	731	719	699	4.99	<0.001	<0.001	0.673	<0.001	0.755	<0.001	0.536

GP2 = GP at 2 h of incubation; (a + b) = maximum GP of the immediately soluble and the insoluble, fermentable fraction; c = gas production rate; OMD = organic matter digestibility; SEM = standard error of the mean; DM = effect of dry matter level; HT = effect of hydrolysable tannin; Significance between treatments at $P < 0.05$

^a treatments: 0T = 0 g/kg DM of HT; 2T = 20 g/kg DM of HT; 4T = 40 g/kg DM of HT; LO = 200 g/kg; HI = 350 g/kg

^b orthogonal contrasts: Linear (Lin) and quadratic (Quad) effects of different HT concentrations on the chemical composition of silages at the respective DM level

^c ml / 200 mg DM

4. Discussion

4.1. General chemical composition and fermentation variables

Proximate constituents were in a typical range for Italian ryegrass (Broderick et al., 2002; Cooke et al., 2008) and legume silages (King et al., 2012; Hymes-Fecht et al., 2013; Hartinger et al., 2019), harvested at late vegetative and bud stage, respectively. The target of producing silages with two different DM levels was fulfilled in each experiment. Despite defined field wilting period, water losses varied between experiments due to plant characteristics and weather conditions. Significant differences in DM content within HI RC silages were attributable to technical issues with application of HT.

Crude protein concentrations were always highest in fresh herbage and declined stepwise with increasing wilting level. The reason for these shifts were probably leaf losses caused by harvesting procedure (Rotz and Abrams, 1988; Elawad et al., 2002). Besides DM, HT treatment also led to significant effects on CP proportions in silages. Most likely, addition of HT solution (water & chestnut tannin extract) resulted in a dilution of chemical constituents of forage crops. Similar effects were reported by Hashemzadeh-Cigari et al. (2011), who found that a stepwise molasses addition (50 and 100 g/kg DM) led to a significant decrease of CP in LU silage.

The analysis of fermentation variables in LU, IR and RC exposed large differences between both, forage species as well as pre-ensiling treatments. In general, a more intensive fermentation was determined in LO silages compared to HI silages, as the acetic acid concentration in LO silages ranged from 14 to 50 g/kg DM, while in HI silages a range between 14 and 26 g/kg DM was observed. The acetic acid concentration in several LO silages exceeded threshold values that have been considered detrimental in terms of DM intake due to reduced feed acceptance (Eisner et al., 2006; Gerlach et al., 2021). Furthermore, increased butyric acid concentrations (> 5 g/kg DM) in LO silages indicate a clostridia-related malfermentation, which results in a decreased fermentation quality and nutritional value of silages (Kung and Shaver, 2001). The generally more restrictive fermentation in HI silages was due to increasing water losses, which are negatively related with microbial activity and accordingly with the formation of fermentation acids (Huhtanen et al., 2002; Kung et al., 2018).

Although the effect of DM treatment led to similar results in all forage species, the effect of HT treatment on the formation of fermentation acids was much more inconsistent. The lactic acid concentration was reduced by HT in all HI and LO LU silages but in LO IR and RC silages an opposing trend was found. Decreased lactic acid concentrations in ensiled forages due to HT

treatment were reported for a grass-legume mixture, treated with 26 g/kg DM oak tannin extract (Herremans et al., 2020) and for ensiled LU, treated with 20 and 50 g/kg DM of tannic acids, resulting in a decline after 35 days by 31 to 25 g/kg DM due to an impairment of lactic acid bacteria activity (Li et al., 2018). On the other hand, Tabacco et al. (2006) and Cavallarin et al. (2006) observed no effect on lactic acid concentration, when chestnut tannin was added at different levels to LU silages. In the present study the acetic acid content was significantly reduced in LO silages of LU and IR and tended to decline in low DM RC silages but acetic acid concentration in HI was not affected. Likewise, published data are manifold regarding the effect of HT on acetic acid concentrations (Ding et al., 2013a; Li et al., 2018; Herremans et al., 2020). Concerning the effect on butyric acid, Salawu et al. (1999) concluded that condensed tannins are useful silage additives to inhibit butyric acid formation in low DM ryegrass silages. However, this finding does not agree with our results, since high amounts of butyric acid were determined in LU and RC silages. Besides, Jayanegara et al. (2019b) noted that the influence on butyric acid depends on tannin type. In accordance with our results, Li et al. (2018) reported an increasing butyric acid content within the first 35 days of ensiling in LU silages treated with 50 g/kg tannic acid (on DM basis) compared to the control treatment (3.7 vs. 1.8 g/kg DM). Contrary, Ding et al. (2013a) applied the same amount of tannic acid to LU silage and observed a significant reduction of butyric acid. Accordingly, no clear tendencies are recognisable, which suggests complex interactions between forage species, ensiling conditions and tannin type.

4.3. Crude protein fractions

It is well established that extensive changes in plant CP composition occur during the entire harvesting and ensiling process due to proteolysis and deamination (Hoedtke et al., 2010). In fresh forages, the majority of CP is present as protein, usually between 800 and 900 g/kg of CP (Fairbairn et al., 1988; Cavallarin et al., 2005; Guo et al., 2008; Ding et al., 2013b; Dong et al., 2019). In the present study, protein concentration was between 732 and 796 g/kg CP, thus tended to be lower than the average reported by others. Differences are attributable to factors such as forage species, maturity stage, N fertilization and sampling (Johnson et al., 2001; Yu et al., 2003). Moreover, Hartinger et al. (2019) mentioned that differences in NPN proportion might partly derive from different analytical methods.

Ensiling resulted in extensive quantitative changes of N fractions in each forage species. As reported by Guo et al. (2007), proteolysis of protein to NPN compounds takes place mainly during the first days of ensiling, whereby plant enzymes (e.g. carboxypeptidase,

aminopeptidase and acid proteinase) are predominantly responsible for the hydrolysis of peptide bonds, and further free amino acids are catabolised by microbial enzymes (Ding et al., 2013b; Dong et al., 2019). In our study, the magnitude of NPN accumulation depended in particular on forage species, and HT treatment, so that largest differences in terms of proteolysis were determined between LU0TLO (824 g/kg CP) and RC4TLO (290 g/kg CP). Pronounced variations in the extent of CP degradation in RC and LU silages have also been reported previously. Several authors observed that the formation of NPN in RC was up to 50% less than in LU silages (Broderick et al., 2007; Hymes-Fecht et al., 2013; Dong et al., 2019). Since both legume species have comparable buffer capacities and inherent proteolytic enzyme activities (Jones et al., 1995), the reduction of proteolysis may be attributed to polyphenol oxidase activity. Besides tannins, polyphenol oxidase also represents a natural protein-protection mechanism through formation of complexes with proteins, which are resistant to proteolysis (Winters et al., 2008).

With regard to the DM treatment, the effects on CP fractions were inconsistent between the forages. The HI LU silages contained significantly less NPN. However, opposing effects were found in RC silages, where the NPN concentrations were higher in HI silages. Contrary to NPN, increasing DM concentrations effectively diminished NH₃-N in all forage species, which is in consensus with the findings of Hristov and Sandev (1998) who showed that wilting LU from 265 to 469 g/kg DM reduced NPN by 6% and NH₃-N by 71%. These findings may implicate that the DM treatment affects the microbial deamination more than the hydrolysis of protein by plant proteases. According to Kofahl (2009), wilting plant material has less impact on hydrolysis than other factors, such as silage pH or temperature. Nevertheless, several authors studied the relation between moisture loss and proteolysis and concluded mostly a positive influence on protein quality. Hartinger et al. (2019) conducted an experiment with LU silages and DM concentrations similar to this study and reported that NPN and NH₃-N were lowered in high DM silages. In accordance, strong negative relationships between silage DM and NPN concentration ($r^2 = 0.90$) were obtained by Tabacco et al. (2006). In this context, it should be taken into account that the influence of DM content becomes more consistent with increasing water removal in terms of proteolysis, due to the decreasing relevance of silage pH (Edmunds et al., 2014). However, not only the extent of wilting but also the rate of moisture loss is decisive. A more rapid water loss within a specific time period results in a higher proportion of remaining protein (Cavallarin et al., 2005; Edmunds et al., 2014). Since the weather conditions during LU harvest were more favourable than for IR and especially RC, this could most likely be an explanatory approach for the inconsistent effect of DM treatment on CP fractions.

Hydrolysable tannins are characterized by their capability to form complexes with proteins due to multiple phenolic hydroxyl groups and restrict the activity of proteolytic enzymes, both in silage and in the rumen (Makkar, 2003; Ding et al., 2013a; Li et al., 2018). In the present study, the addition of 40 g/kg HT on DM basis to LU, IR and RC resulted in a significant decline of NPN compounds in LO silages by 20, 20 and 40% and in HI silages by 18, 10 and 28%, respectively. In addition, HT lowered the deamination of free amino acid to $\text{NH}_3\text{-N}$ in all silages, but the absolute effect was more pronounced in LO silages. Comparable effects on CP degradation were reported by Tabacco et al. (2006), as LU silages treated with 40 g/kg chestnut tannin on DM basis reduced the NPN and $\text{NH}_3\text{-N}$ content by 15 and 25%. Cavallarin et al. (2006) used the third regrowth of the same LU stand to investigate the effect of a *Lactobacillus plantarum* inoculant (10^6 cfu/g) and 40 g/kg DM chestnut tannin on overall silage and protein quality, respectively, and ascertained that chestnut tannin lowered the formation of NPN compounds more effectively than the inoculant (control: 612 g/kg CP; inoculant: 590 g/kg CP; tannin: 537 g/kg CP). Furthermore, the effects of chestnut and oak tannin on $\text{NH}_3\text{-N}$ formation in mixed legumes silages were examined by Herremans et al. (2019) with the conclusion that both types of tannins restricted proteolysis during ensiling but, also, a negative quadratic relationship between $\text{NH}_3\text{-N}$ and chestnut tannin was determined, as doses of more than 50 g/kg DM of chestnut tannin did not further reduce $\text{NH}_3\text{-N}$ concentrations.

The HT treatment was less effective in HI silages. A similar observation was reported by Martens et al. (2019), who found a more pronounced effect of tannins on proteolysis in silages with lower DM contents. Moreover, a second experiment of Tabacco et al. (2006) demonstrated that HT (40 g/kg of DM) reduced NPN and $\text{NH}_3\text{-N}$ concentrations in a silage with a DM of 400 g/kg by 75 and 43 g/kg N, whereas the same supplementation level of HT reduced NPN and $\text{NH}_3\text{-N}$ by only 50 and 3 g/kg N in silages with a higher DM level (500 g/kg). It can be assumed that the formation of tannin-protein-complexes is a time-consuming process, because no obvious inhibition of proteolysis occurs within the first hours of ensiling by HT. On the other hand, the increase of DM immediately restricts the accumulation of NPN compounds, thus masking the inhibitory effects of HT (Muck, 1987).

4.4. *In vitro* gas production characteristics

Based on close correlations between GP parameters and chemical composition of silages, the GP technique has proven to be a powerful indicator for both, silage quality and silage DMI (Huhtanen et al., 2002; Hetta et al., 2007). The present study exposed various factors relating

to GP kinetics. Overall, maximal GP and GP rate of IR was superior to legume forages, which is consistent with the findings of Navarro-Villa et al. (2011) and Zhong et al. (2016). These observations are due to a positive relation of total GP with WSC, NDF and digestibility of NDF, plus the negative association with CP, ADF and ADL concentration (Getachew et al., 1998; Brown et al., 2002). The HT treatment had obvious effects on maximal GP of grass and legume silages, whereby higher concentrations of HT resulted in linear reductions of GP of about 4 to 6%. Besides, a meta-analysis, comprising 107 observations, showed that in addition to methane, total *in vitro* GP decreased linearly with increasing dietary tannin level (Jayanegara et al., 2012). Our study supports findings of others, who have attributed these effects to the ability of tannins to form complexes with nutrients such as proteins and carbohydrates which are poorly soluble for the microorganisms in the rumen (Makkar, 2003; Kamalak et al., 2005; Colombini et al., 2009). Furthermore, tannins are capable to hinder microorganisms themselves (McMahon et al., 2000; Goel et al., 2005; Nascimento et al., 2017). Thereby, condensed tannins appear to decrease gas and methane production more through a lowered fibre digestion, whereas HT are more efficient in terms of inhibition of growth and activity of microorganisms (Jayanegara et al., 2015).

As previously mentioned, wilting forages resulted in higher rates for GP₂, maximal GP and GP rate, which corroborates results of previous work. Among others, Wan et al. (2021) ensiled sudangrass at two DM levels of about 190 g/kg and 280 g/kg for 60 days, respectively, and pointed out a slope in asymptotic gas production (51.2 vs. 55.2 ml/g DM) as well as GP rate (0.26 vs. 0.29 ml/h). Comparable results were also found for grass silages, ensiled with a DM of 191 or 307 g/kg, where total GP in high DM silage was 5% above silages with a lower DM content (Brown et al., 2002). This is most likely attributable to more intense fermentation in low DM silages leading to more extensive DM and energy losses and lower amounts of residual WSC (Kung et al., 2018).

4.5. Potential limitations in the use of hydrolysable tannins

In addition to the beneficial properties of HT in terms of protein and overall feeding value, possible limitations must also be pointed out for an objective evaluation of its practical use. One major limitation is the comparatively high application rate. To illustrate this, the dosage of tannin-containing products exceeds the application rate of a comparable chemical additive, such as formic acid, by a factor of around 10 to 20 (Muck et al., 2018). These quantities can result in logistic as well as economic constraints that make practical application challenging.

Furthermore, the increased viscosity of tannin-containing products must be taken into account, which entails increased demands on the dosing system.

5. Conclusions

Increasing the DM content due to prolonged wilting time significantly improved the fermentation quality in high DM silages by reducing the production of undesirable fermentation products such as butyric acid. The accumulation of $\text{NH}_3\text{-N}$ was limited, indicating a lower activity of proteolytic bacteria. Adding HT in a concentration of 40 g/kg DM to forages crops at ensiling was more effective than the DM treatment in decreasing the hydrolysis of protein. A linear decrease in gas production and organic matter digestibility was revealed by gradually increasing the HT extract dosage.

The results of the present study clearly demonstrate that external factors, such as HT, can effectively improve the protein quality of ensiled high-protein grass and legume forages, even under unfavourable, challenging ensiling conditions. More research is needed, especially *in vivo* studies, to assess at which concentrations and ensiling conditions the beneficial properties of HT overcome adverse effects in order to define more precise application recommendations.

Author Contributions

B. Hilgers: Methodology, Formal analysis, Investigation, Resources, Writing - Original Draft. **K. Gerlach:** Conceptualization, Methodology, Writing – Review & Editing, Supervision. **C. Böttger:** Formal analysis, Writing – Review & Editing. **K. Weiß:** Formal analysis, Writing – Review & Editing. **K.-H. Südekum:** Conceptualization, Methodology, Writing. Review & Editing, Supervision, Project administration, Funding acquisition.

Funding

This work was supported by the Federal Ministry of Food and Agriculture (BMEL) based on a decision of the Parliament of the Federal Republic of Germany via the Federal Office for

Agriculture and Food (BLE) under the innovation support program [grant number 2818300116].

Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Acknowledgements

The authors thank Petra Jacquemien for skilled technical support and chemical analysis. Furthermore, the authors acknowledge the support of the team at the Central Analytical Laboratory, Humboldt-University, Berlin, for fermentation pattern analysis. Special thanks to Thomas Hartinger, Helge Deitmers, Klara Schmitz, and Ziade Abraha Kahsay for their assistance with herbage harvest and silage preparation.

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CHAPTER 4

Effects of hydrolysable tannin extract and dry matter concentration on ensiled legume and grass forages – Short-term dry matter intake and feed choice behaviour of goats in relation to silage characteristics

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Abstract

The ensiling of high-protein forages often results in extensive proteolysis and poor fermentation quality, which may impair the voluntary feed intake of ruminants. Therefore, the present study evaluated the effects of different hydrolysable tannin (HT) extract and dry matter (DM) levels on preference and short-term dry matter intake (DMI) of grass and legume silages by goats. Accordingly, pure stands of lucerne (*Medicago sativa* L.), Italian ryegrass (*Lolium multiflorum* L.) and red clover (*Trifolium pratense* L.) were wilted to approximately 200 or 350 g/kg DM and ensiled with three HT levels (0, 20 and 40 g/kg on DM basis), resulting in six treatments per forage species. After opening, silages were analysed for chemical composition and fermentation characteristics. Forage preference and short-term DMI were examined in three independent trials using adult Saanen type wethers in a free-choice design. Each possible two-way combination of the six silage treatments and a lucerne hay was offered for 3 h daily. Increasing DM concentration consistently enhanced DMI and preference across all forage species. Supplementation with 40 g HT/kg DM generally improved DMI of low DM silages, but a comparable effect was not observed in high DM silages. The DMI was negatively correlated with most fermentation products, particularly acetic acid, propionic acid, butyric acid, propanol, butanol and ammonia-nitrogen. In contrast, residual water-soluble carbohydrates and in vitro gas production after two hours of incubation were positively related to DMI.

Abbreviations

ADFom, acid detergent fibre expressed exclusive residual ash; ADL, acid detergent fibre; aNDFom, neutral detergent fibre assayed with heat stable amylase and expressed exclusive residual ash; B1, rapidly degraded true protein; B2, intermediately degraded true protein; B3, slowly degraded true protein; BW, body weight; c, gas production rate; CP, crude protein; DM, dry matter; DMI, DM intake; GABA, gamma-aminobutyric acid; GP, gas production; GP2, gas production rate at 2 h of incubation; HI, silages with an approximate DM level of 350 g/kg; HT, hydrolysable tannin; IR, Italian ryegrass; LO, silages with an approximate DM level of 200 g/kg; LU, lucerne; N, nitrogen; NH₃-N, ammonia-N; NPN, non-protein-N; OM, organic matter; OMD, OM digestibility; RC, red clover; WSC, water-soluble carbohydrates.

1. Introduction

Ensiled grass and legume forages are common feeds in dairy and beef production, as they have the potential to provide ruminants with cost-effective and sustainably produced crude protein (CP), energy and fibre (Eisler et al., 2014; Lüscher et al., 2014). Accordingly, it is crucial to maximize the utilization of conserved high-quality forages, whereby voluntary feed intake represents the most meaningful influencing factor (Mertens, 1994). The regulation of voluntary feed intake involves multiple mechanisms that are related to dietary and animal factors, making it difficult to predict feed consumption (Huhtanen et al., 2011). Feeding behaviour and dry matter (DM) intake (DMI) are directly dependent on physical (e.g. DM content, particle size) and chemical (e.g. odour, taste, nutritive value, antinutritive factors) characteristics of the feed by linking sensory properties and a hedonic value to a post-ingestive feedback (Baumont, 1996).

Whilst DMI of fresh and dried forages is clearly related to forage type, organic matter (OM) digestibility (OMD), CP concentration and cell wall components (Kuoppala et al., 2008; Steinshamm, 2010; Riaz et al., 2014), the prediction of feed consumption of silages is more complex, as modification and degradation of carbohydrates and nitrogen (N) compounds occur during the ensiling process. Therefore, it has often been assumed that fermentation products such as volatile fatty acids (acetic acid, propionic acid, butyric acid, etc.), alcohols and ethyl esters are closely related to DMI of ruminants (Huhtanen et al., 2002; Gerlach et al., 2019; Gerlach et al., 2021). Furthermore, the accumulation of end-products of CP and amino acid degradation represent reliable indicators of feed acceptance and DMI. Krizsan and Randby (2007) reported that voluntary feed intake of different grass silages was impaired by increasing concentrations of ammonia-N ($\text{NH}_3\text{-N}$) and the total sum of biogenic amines, with histamine and tryptamine explaining up to 43% of the variation in feed intake. Close relationships between N compounds and DMI were also found by Hartinger et al. (2021), who conducted feed choice trials with goats offered lucerne (LU) silages with different fermentation qualities. The 3 h DMI was negatively correlated with concentrations of non-protein-N (NPN) ($r = -0.773$), gamma-aminobutyric acid (GABA) ($r = -0.866$) and $\text{NH}_3\text{-N}$ ($r = -0.806$).

The lower DMI of poorly fermented silages that have been subject to intensive proteolytic processes is due to the fact that these silages may contain a variety of biological and chemical agents, e.g. biogenic amines and ammonia, that are potentially hazardous to animal health if ingested at sufficiently high amounts leading to a negative post-ingestive feedback (Baumont, 1996; Driehuis et al., 2018). Moreover, fermentation products and NPN compounds influence

taste and odour of silages, which negatively affect the sensory input of animals and thus reduces palatability. This was confirmed as sheep without a sense of smell showed a significantly higher DMI of poorly preserved silages than unrestricted sheep (Arnold et al., 1980).

Accordingly, the improvement of the fermentation quality and limitation of protein degradation are decisive factors in order to promote DMI of ensiled forages and must be ensured by pre-ensiling treatments if the ensiling conditions are unfavourable. A simple but reliable method is to increase the DM level prior ensiling. Hartinger et al. (2021) pointed out that raising the DM of LU silages from 25% to 35% not only reduced the accumulation of NPN, GABA and $\text{NH}_3\text{-N}$, but also resulted in a significantly higher short-term DMI by goats.

Another well-studied method to improve silage and protein quality is the use of plant bioactive compounds, such as hydrolysable tannins (HT) (Niderkorn and Jayanegara, 2021). Hydrolysable tannins are water-soluble polyphenolic secondary plant compounds with the capacity to form complexes mainly with proteins due to their multiple phenolic hydroxyl groups (Patra and Saxena, 2011). When correctly used, many beneficial properties are associated with dietary tannins in relation to ruminant nutrition and metabolism. With regard to forage preservation, Hilgers et al. (2025) have shown recently that CP degradation in various grass and legume silages was reduced by adding HT up to 40 g/kg DM, even under unfavourable ensiling conditions. Furthermore, HT contributes to a reduction in proteolysis in the rumen, providing higher amounts of rumen-undegraded protein and resulting in less N excretion in urine. Extra attention is given to HT, as they have been found to restrict enteric methane emissions by acting as anti-methanogenic agents (Jayanegara et al., 2012). They also provide further beneficial aspects regarding animal health, such as anti-parasitic, anti-oxidative and anti-inflammatory properties (Niderkorn and Jayanegara, 2021). However, it must be considered that the potential effects are often inconsistent, because tannin source, type of tannin extract and level of supplementation determine whether the effect is beneficial or detrimental. Excessive concentrations in the diet (> 30-50 g/kg DM) may impair feed palatability and reduce digestibility of nutrients, with corresponding effects on DMI and animal performance (Addisu, 2016; Yanza et al., 2021). Accordingly, there is a need to clarify the application rate of HT to optimize the benefits and to avoid adverse effects. Therefore, the aim of the present study was to ascertain how the addition of HT extract in increasing concentrations affects the short-term DMI and preference behaviour of goats, considering different forage species and silage qualities.

2. Materials and methods

2.1. Preparation and general analysis of silages

The preparation and general analysis of the differently treated grass and legume forages were described in detail in Hilgers et al. (2025; Chapter 3).

In 2018, LU (*Medicago sativa* L.) and red clover (*Trifolium pratense* L.; RC) were cultivated in pure stands at the Campus Frankenforst of the University of Bonn, Germany, and were harvested as first regrowth at early and late bud stage, respectively. The following year, the first regrowth of a pure stand of Italian ryegrass (*Lolium multiflorum* L.; IR) was cut at the late vegetative stage at the same location. After cutting, the forage was spread out using a tedder to improve wilting conditions on the field. To produce silages with two different DM levels, one part of each forage species was collected from the field using a loading wagon and chopped to a theoretical cutting length of 45 mm after approximately 6-8 h of wilting (low DM silages; LO \approx 200 g/kg DM). The second part was wilted for at least 26 h until the next day (high DM silages; HI \approx 350 g/kg DM).

The HT extract used in this experiment was obtained from chestnut wood (*Castanea sativa* MILL.) and was applied as fine powder with pure HT content about 770 g/kg DM (Silvafeed ENC powder, Silvateam, San Michele Mondovì CN, Italy) to LU and RC after being dissolved in water. In the following year, the same extract was chosen for the treatment of IR, but in an already dissolved form to improve handling, with 460 g DM/kg and a HT content of about 750 g/kg DM (Silvafeed ENC liquid, Silvateam, Italy). To achieve a consistent application, both HT products were dissolved in water and dispensed with a pressure sprayer on the wilted forage (Prima 5, Gloria, Witten, Germany). The amount of water applied to all treatments was identical in order to obtain equal effects on DM, whereby the HT:water ratio increased from pure water in the control group to 1:4 (20 g HT/kg DM) to 1:2 (40 g HT/kg DM). The HT:water ratio of 1:2 represented the best possible compromise between product solubility and the lowest possible dosage of water.

Based on the work of Tabacco et al. (2006), the following treatments were involved in this study:

- Three HT concentrations: 0 g/kg (0T), 20 g/kg (2T) and 40 g/kg (4T)
- Two DM levels: \approx 200 g/kg (low DM; LO) and \approx 350 g/kg (high DM; HI).

With the further consideration of the three forage crops (IR, LU and RC), a total of 18 treatments were prepared and investigated.

Before ensiling, the HI and LO DM plant material was compacted (HI: 212 kg DM/m³; LO: 190 kg DM/m³ for RC and LU and 177 kg DM/m³ for IR) following the recommendations of the Federal Working Group for Forage Preservation in Germany (Bundesarbeitskreis Futterkonservierung, 2011) using a hydraulic press, which was manufactured for this purpose by the Institute of Agricultural Engineering at the University of Bonn. Subsequently, the compacted forage treatments were sealed airtight in 120-litre plastic barrels by covering the opening with an oxygen-impermeable plastic sheet and closing it with a lid and clamping ring. After 115 days of storage at room temperature, three barrels of each treatment were opened, thoroughly mixed and composite samples of each barrel were taken for analysis of proximate constituents, fibre fractions, fermentation products, CP fractions and gas production (GP) kinetics as presented in Hilgers et al. (2025). An overview of the most relevant silage characteristics is shown in Table 1. The fermentation process of all silage treatments was evaluated using the DLG scheme for silage quality (DLG, 2006), which classifies silages into five categories (very well fermented, well fermented, in need of improvement, poorly fermented, very poorly fermented) based on a point system and is positively or negatively influenced by the parameters pH value, DM content, as well as acetic and butyric acid concentrations. In particular, increased concentrations of the two fermentation acids mentioned above lead to a downgrading of the quality classification due to a significant score subtraction. For the following preference trials, silage material was spread out, mixed thoroughly on a clean tarpaulin and packed in portions into polyethylene vacuum bags (170 μ m, 400 mm x 600 mm; Frey Serviceverpackung, Dombühl, Germany). Small portions about 2.5 kg of HI and 3 kg of LO silages were filled in bags and stored at 8 °C until feeding.

Table 1. Effect of different dosages of hydrolysable tannin (HT) and dry matter (DM) level on the chemical composition and fermentation characteristics in lucerne (LU), Italian ryegrass (IR) and red clover (RC) silages

Variables (g/kg DM)	Treatments ^a						SEM	<i>P</i> -value						
	0TLO	2TLO	4TLO	0THI	2THI	4THI		DM	HT	DM x HT	Lin LO ^b	Quad LO ^b	Lin LO ^b	Quad HI ^b
Lucerne (LU)														
DM (g/kg)	230	228	248	366	365	370	0.21	<0.001	<0.001	0.009	<0.001	0.002	0.247	0.187
CP	245	237	230	224	218	220	2.80	<0.001	0.14	0.142	0.003	0.962	0.332	0.230
aNDFom	391	395	404	392	391	381	11.1	0.357	0.994	0.535	0.423	0.857	0.484	0.773
ADFom	311	323	326	326	324	326	8.98	0.489	0.713	0.614	0.250	0.657	0.959	0.835
ME (MJ/kg DM)	10.1	9.73	9.56	9.73	9.60	9.57	0.15	0.068	0.013	0.211	0.003	0.453	0.274	0.710
WSC	2.30	2.07	2.57	29.0	29.4	24.6	2.10	<0.001	0.518	0.403	0.929	0.888	0.161	0.317
OMD (g/kg)	756	734	718	739	725	713	6.39	0.078	0.001	0.644	0.001	0.709	0.012	0.885
pH	5.12	5.55	5.59	4.97	4.97	5.01	0.10	<0.001	0.058	0.089	0.007	0.149	0.783	0.874
Lactic acid	90.5	51.7	41.6	70.2	67.2	56.0	0.37	0.314	<0.001	<0.001	<0.001	0.008	0.019	0.384
Acetic acid	42.6	38.3	30.0	13.9	18.0	15.5	0.10	<0.001	<0.001	<0.001	<0.001	0.143	0.285	0.022
Butyric acid	0.40	9.67	12.5	-	-	-	0.23	<0.001	0.063	0.052	0.003	0.283	0.945	0.948
NH ₃ -N (g/kg N)	198	200	172	121	111	100	8.26	<0.001	0.030	0.573	0.042	0.156	0.100	0.945
NPN (g/kg CP)	824	715	656	778	713	640	11.5	0.050	<0.001	0.211	<0.001	0.103	<0.001	0.796
Italian ryegrass (IR)														
DM (g/kg)	197	204	203	359	367	371	4.03	<0.001	0.081	0.686	0.325	0.373	0.048	0.622
CP	209	197	185	186	182	184	2.20	<0.001	<0.001	0.001	<0.001	0.904	0.533	0.240
aNDFom	452	443	433	437	445	437	7.42	0.642	0.393	0.393	0.090	1.00	1.00	0.436
ADFom	308	280	276	280	283	274	5.12	0.046	0.010	0.027	0.001	0.087	0.423	0.384
ME	10.4	10.2	9.98	10.4	10.3	10.3	0.08	0.033	0.019	0.023	0.004	0.875	0.289	0.754
WSC	3.41	4.16	6.81	43.2	38.5	41.5	6.06	<0.001	0.790	0.469	0.699	0.904	0.194	0.878
OMD (g/kg)	770	735	709	782	769	759	7.08	<0.001	<0.001	0.057	<0.001	0.598	0.037	0.850

Table 1. (continued)

Variables (g/kg DM)	Treatments ^a						SEM	<i>P</i> -value						
	0TLO	2TLO	4TLO	0THI	2THI	4THI		DM	HT	DM x HT	Lin LO ^b	Quad LO ^b	Lin HI ^b	Quad HI ^b
Italian ryegrass (IR)														
pH	4.60	4.23	4.10	4.67	4.66	4.72	0.06	<0.001	0.005	0.002	<0.001	0.117	0.590	0.594
Lactic acid	75.0	120	117	63.4	63.6	53.0	16.5	<0.001	0.092	0.009	0.009	0.067	0.051	0.228
Acetic acid	49.9	21.7	14.4	15.1	15.5	15.1	2.07	<0.001	<0.001	<0.001	<0.001	0.001	0.135	0.306
Butyric acid	-	1.73	0.71	0.18	0.09	0.02	0.58	0.178	0.390	0.325	0.402	0.077	0.965	0.929
NH ₃ -N (g/kg N)	141	127	98.3	106	103	97.9	4.46	<0.001	<0.001	0.055	<0.001	0.219	0.012	0.324
NPN (g/kg CP)	676	604	540	696	650	626	13.6	<0.001	<0.001	0.075	<0.001	0.176	0.003	0.512
Red clover (RC)														
DM (g/kg)	163	174	167	293	323	327	4.89	<0.001	0.018	0.030	0.558	0.158	<0.001	0.044
CP	184	178	172	164	165	159	1.97	<0.001	0.004	0.183	0.011	0.979	0.108	0.244
aNDFom	406	462	458	447	426	430	18.6	0.663	0.518	0.102	0.058	0.196	0.538	0.588
ADFom	361	356	341	327	380	364	9.51	0.577	0.075	0.015	0.163	0.675	0.018	0.012
ME (MJ/kg DM)	8.96	8.81	8.86	9.22	8.78	8.70	0.08	0.724	0.003	0.052	0.403	0.293	<0.001	0.074
WSC	0.79	0.95	2.87	12.9	19.1	25.2	1.79	<0.001	0.006	0.043	0.428	0.696	<0.001	0.980
OMD (g/kg)	717	697	680	731	719	699	4.99	<0.001	<0.001	0.673	<0.001	0.755	<0.001	0.536
pH	4.93	5.07	4.70	4.40	4.41	4.43	0.06	<0.001	0.077	0.043	0.039	0.010	0.747	0.950
Lactic acid	79.6	55.1	87.7	113	99.5	91.2	7.36	<0.001	0.067	0.044	0.452	0.008	0.058	0.772
Acetic acid	48.6	41.2	42.4	26.4	23.3	21.5	2.44	<0.001	0.074	0.682	0.097	0.180	0.185	0.822
Butyric acid	20.4	34.0	13.5	0.99	0.13	-	3.85	<0.001	0.058	0.005	0.228	0.004	0.858	0.934
NH ₃ -N (g/kg N)	156	131	91.5	113	92.2	88.6	9.40	<0.001	<0.001	<0.001	<0.001	0.312	0.056	0.228
NPN (g/kg CP)	486	333	290	471	417	338	11.4	0.001	<0.001	0.003	<0.001	0.002	<0.001	0.395

CP = crude protein; aNDF = neutral detergent fibre assayed with heat-stable amylase and expressed exclusive residual ash; ADFom = acid detergent fibre expressed exclusive residual ash; ME = metabolizable energy; WSC = water-soluble carbohydrates; OMD = organic matter digestibility; NH₃-N = ammonia-nitrogen; NPN = non-protein-nitrogen; SEM = standard error of the mean; Significance between treatments at *P* < 0.05.

^a treatments: 0T = 0 g/kg DM of HT; 2T = 20 g/kg DM of HT; 4T = 40 g/kg DM of HT; LO = 200 g/kg; HI = 350 g/kg

^b orthogonal contrasts: Linear (Lin) and quadratic (Quad) effects of different HT concentrations on the chemical composition of silages at the respective DM level

2.2. Preference trials

Three forage preference trials were performed in 2018 and 2019, in order to determine and evaluate short-time DMI and feed choice behaviour by goats of the silage treatments according to Burns et al. (2001). All trials were conducted with adult Saanen-type wethers (German Improved White Goat breed; LU trial: n = 6, body weight (BW) 91.3 kg [\pm 12.3 kg]; IR trial: n = 6, BW 73.2 kg [\pm 5.1 kg]; RC trial: n = 5, BW 95 kg [\pm 8.5 kg]). In each trial, two goats shared an indoor pen of approximately 2 x 3 m bedded with straw. To measure individual feed intake, goats were tied up for feeding during the experimental period, with the possibility of lying down and unrestricted access to water and salt-licks.

Similar to previous studies (Gerlach et al., 2014; Gerlach et al., 2017; Scherer et al., 2019b) all trials started with an adaptation period of 7 days, where single meals of each silage treatment and LU hay were offered once to allow the goats to associate the silage with individual smell, taste and post-ingestive metabolic response. Every morning, forages were offered in a randomized order for 3 h and LU hay was used as a standard to enable quantitative comparisons between trials. During the consecutive experimental phase (21 days), every possible 2-way combination of six silage treatments per forage species and the hay standard (n = 21 combinations) was presented side by side to each goat in plastic boxes (400 x 340 x 250 mm) with free access to both feeding boxes to ensure a free choice. To avoid a habit reflex of the goats, the left-right positions of the forage treatments were randomized for all combinations. Each day, the forages were weighed and offered for 3 h, starting at 07:30 h. Boxes were weighed every 30 min over a period of 3 h to determine the amount of forage ingested. During the weighing procedures feeding time was paused. To guarantee *ad libitum* feed intake, forage was added as soon as less than 300 g fresh matter of a meal remained in a box. Silage vacuum bags were stored in a cooling chamber during the trials and were removed one day before feeding to offer forages at room temperature. Grass hay was offered in plastic boxes for *ad libitum* intake at 15:30 h for 2 h throughout all trials. In addition, feed intake in the afternoon was determined for each goat during the experimental phase to calculate total daily DMI. Water was offered to the goats for voluntary consumption halfway through each of the two daily feeding times.

Baled LU and grass hays, used for feeding in the morning and afternoon, respectively, were obtained from regional producers. Before feeding, bales were surveyed for spoilage and homogeneity. Samples of each hay were taken every second day in the morning throughout the feeding trials by hand-plucking from multiple locations. Composite samples for both hays were prepared and used for subsequent chemical analysis, with the following concentrations of DM,

ash, CP, neutral detergent fibre assayed with heat-stable amylase and expressed exclusive residual ash (aNDFom), acid detergent fibre expressed exclusive residual ash (ADFom), and acid detergent lignin (ADL) for LU (grass) hay: 906 (908) g/kg, 103 (111), 173 (122), 503 (530), 368 (320) and 104 (72) g/kg DM.

2.3. Statistical analysis

All statistical analyses were conducted using SAS 9.4 (SAS Institute Inc., Cary, North Carolina, USA). Residuals were initially analysed using the UNIVARIATE procedure to evaluate the normality of the response variables by the Shapiro-Wilk test. Next, a two-factorial analysis of variance (PROC GLM) was used to test the effects of DM and HT treatments and the DM x HT interaction on proximate constituents, fermentation characteristics, OMD and N compounds in each forage species, while an orthogonal contrast test was performed (PROC GLM statement CONTRAST) to estimate linear and quadratic effects of different HT concentrations (0, 20 and 40 g/kg on DM basis) within each DM level on chemical composition. Significance was defined at $P < 0.05$.

To obtain individual preferences for offered forage treatments, the statistical procedure of multidimensional scaling (MSD) was used for each feeding trial, according to Buntinx et al. (1997) and Burns et al. (2001). Therefore, an observation of the magnitude of perceived differences, or dissimilarity (δ), between each pair of a set of forage combination is required. The MDS is applied to estimate the coordinates in a two-dimensional space, for each forage treatment, so that the distances between the respective positions will correspond closely to their experimentally measured dissimilarities (Schiffman et al., 1981). Magnitude of preference or rejection between two silages was expressed numerically as a relative distance (δ) and was calculated from measured intake data obtained in each preference trial by subtracting the amount of the least preferred forage from the most preferred forage and dividing the difference by the sum of both intakes. Thus, δ ranged from 0 to 1 for each pair combination. If a goat consumed equal quantities in one set and showed no preference for one forage over the other, δ was close or equal to zero. If δ was close or equal to one the animal revealed a strong preference for one forage and the combination was judged to be very different (Buntinx et al., 1997). PROC MDS is an iterative fitting procedure for data with the aim to minimize residual sum of squares by arranging the forages by estimating coordinates in a limited number of orthogonal dimensions to model the observed differences, as described by Burns et al. (2001). Forages with coordinates that are similar in the dimensional space are modelled as similar in

preference and, conversely, coordinates far apart in the dimensional space indicate forages that differ in preference (Buntinx et al., 1997). For each trial two sets of δ matrices with forage stimuli coordinates and dimension weights by included animals were obtained, one for 30-min DMI and a second for 3-h DMI. In order of fit is dimension one first, which will generally include the most important variables, followed by dimension two. The forage with the highest DMI in each run was used as a positive control by assigning it positive coordinates (Burns et al., 2001).

Furthermore, data from each forage preference trial were analysed by two-way ANOVA (proc GLM) for average DMI with animal and forage as independent variables. When the calculated values were significant ($P < 0.05$), the Waller-Duncan-k-ratio t-test was performed to outline significant differences between treatments by separating the means based on the minimum significant difference (MSD). In addition, correlation coefficients (Pearson coefficient) between DMI and silage composition were calculated using the CORR procedure. The significance level was set at $P < 0.05$, and a trend towards a significant effect was noted when $0.05 \leq P \leq 0.10$.

3. Results

3.1. Effects of silage treatments on animal preference and short-time dry matter intake

Table 1. provides a brief overview of the effects of pre-ensiling treatments on the chemical composition of silage. For a detailed description and classification of the results, as well as data on further variables not reported here, see Hilgers et al. (2025).

All animals were apparently healthy throughout all preference trials and showed no visible signs of physical impairments or disorders that might have affected the outcome of the experiments. At opening, no silage showed visible signs of moulds or other anomalies. In general, LO silages had a more pronounced odour compared to HI silages. Regarding sensory aspects, stepwise enhancement of HT supplementation led to a brownish discoloration of silages. Results of the respective preference trials, expressed as DMI (g/30 min and g/3 h) are given in Table 2. In the LU trial, the 30 min and 3 h DMI in response to HI silages was higher than for LO silages, with the highest DMI measured for LU0THI and lowest for LU0TLO and LU2TLO, respectively ($p < 0.05$). In HI silages, the application of HT up to 40 g/kg DM

affected the DMI adversely. Among LO silages, LU4TLO was preferably consumed, with the difference to LU0TLO and LU2TLO being significant after 3 h of intake. Considering all three trials, the gap between the most consumed and the least preferred silage was most distinct in the LU trial. In the IR preference trial, no differences were observed for 3 h DMI between IR2TLO, IR4TLO, IR0THI, IR2THI and IR4THI, only LU hay and IR0TLO were less consumed by goats. The results of the RC trial revealed that both for 30 min and 3 h, the DMI of the HI treatments was higher than that of LO silages. As with the IR trial, the HT treatment did not affect the DMI of HI silages. In RCLO silages, the highest DMI was measured for RC4TLO, followed by RC0TLO.

The overall results of all trials showed a major impact of DM level on initial 30 min DMI and 3 h DMI. In addition, DMI was affected by different amounts of HT, although the effect on DMI was comparatively small and inconsistent. Whilst the addition of HT at 40 g/kg DM in LO silages resulted in improved DMI, a beneficial effect of 20 g/kg DM of HT addition was not observed. In HI silages, 3 h DMI was not different when 20 or 40 g/kg DM of HT were added. It is conspicuous that, as feeding time progressed, the acceptance of HT treated silages increased, compared with the initial 30 min DMI.

Table 2. Effect of different dosages of hydrolysable tannin (HT) and dry matter (DM) level on short-term dry matter intake (DMI) of lucerne (LU), Italian ryegrass (IR) and red clover (RC) silages and lucerne hay by goats

Treatments ¹	Lucerne (LU)		Italian ryegrass (IR)		Red clover (RC)		All forage species	
	DMI		DMI		DMI		DMI	
	(g/30 min)	(g/3 h)	(g/30 min)	(g/3 h)	(g/30 min)	(g/3 h)	(g/30 min)	(g/3 h)
0TLO	84.1 ^d	210 ^d	131 ^c	345 ^b	97.9 ^{bc}	294 ^{bc}	103 ^{de}	275 ^c
2TLO	71.2 ^d	211 ^d	127 ^c	479 ^a	76.1 ^c	236 ^c	92.5 ^e	312 ^c
4TLO	87.6 ^d	286 ^c	170 ^{bc}	482 ^a	124 ^b	333 ^b	131 ^d	373 ^b
0THI	338 ^a	683 ^a	223 ^a	528 ^a	184 ^a	441 ^a	250 ^a	549 ^a
2THI	243 ^b	537 ^b	209 ^{ab}	556 ^a	191 ^a	471 ^a	218 ^b	529 ^a
4THI	222 ^{bc}	533 ^b	216 ^a	554 ^a	181 ^a	464 ^a	209 ^b	523 ^a
Lucerne hay	198 ^c	474 ^b	148 ^c	383 ^b	123 ^b	245 ^c	161 ^c	380 ^b
Mean	178	419	175	475	134	355	166	418
MSD	40.1	70.3	44.7	79.3	40.8	80.5	28.2	52.4
	<i>P-value</i>							
Treatment	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Animal	<0.001	<0.001	0.001	0.002	<0.001	<0.001	<0.001	<0.001
Treatment x Animal	0.003	0.007	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

MSD = minimum significant difference (Waller-Duncan *k-ratio t-test*); Significance between treatments at $P < 0.05$.

¹ Treatments: 0T = 0 g/kg DM of HT; 2T = 20 g/kg DM of HT; 4T = 40 g/kg DM of HT; LO = 200 g/kg; HI = 350 g/kg.

^{a-d} Means within a row not sharing the same superscript differ significantly ($P < 0.05$).

The graphical solutions of the MDS analysis for preferences in each trial, consisting of 30 min and 3 h data are shown in Figure 1. Multidimensional scaling pointed out apparent differences between forage species, pre-ensiling treatments as well as duration of feed intake. In contrast to measured DMI of the LU trial, the MDS indicated that LU2THI was highly preferred, having two positive coordinates. Nevertheless, a higher preference for all HI silages was noticeable, due to at least one positive coordinate (≥ 1) for LU0THI and LU4THI, while LU0TLO, followed by LU4TLO were most-avoided when goats had the possibility to choose, owing to negative coordinates in dimensional space and largest distance to silage treatments with highest acceptance. The MDS analysis of the IR trial revealed several differences to the MDS of the LU trials. A majority of silages had one negative and one positive coordinate and were generally of intermediate preference, which coincides with the results of the analysis of variance, because no differences were detected regarding the 3 h DMI among IR2TLO, IR4TLO, IR0THI, IR2THI and IR4THI. Among all IR silages, IR2THI and IR4THI ranked positive in both dimensions within the first 30 min of DMI, whereas the goats appeared to have a preference for the IR0TLO treatment after 3 h feeding time. In the RC trial, RC0THI, RC2THI, RC4THI and LU hay were strongly preferred, however, a shift of preference was observed between 30 min and 3 h DMI, especially for LU hay and RC0THI. Looking at all trials, the MDS highlighted the strong preference for HI silages six times, while LO silages were preferred only once. Furthermore, across all trials two positive coordinates were assigned only once to a silage without HT treatment (0T), four times to silages with HT at 20 g/kg DM (2T) and twice to silages at 40 g/kg DM (4T).

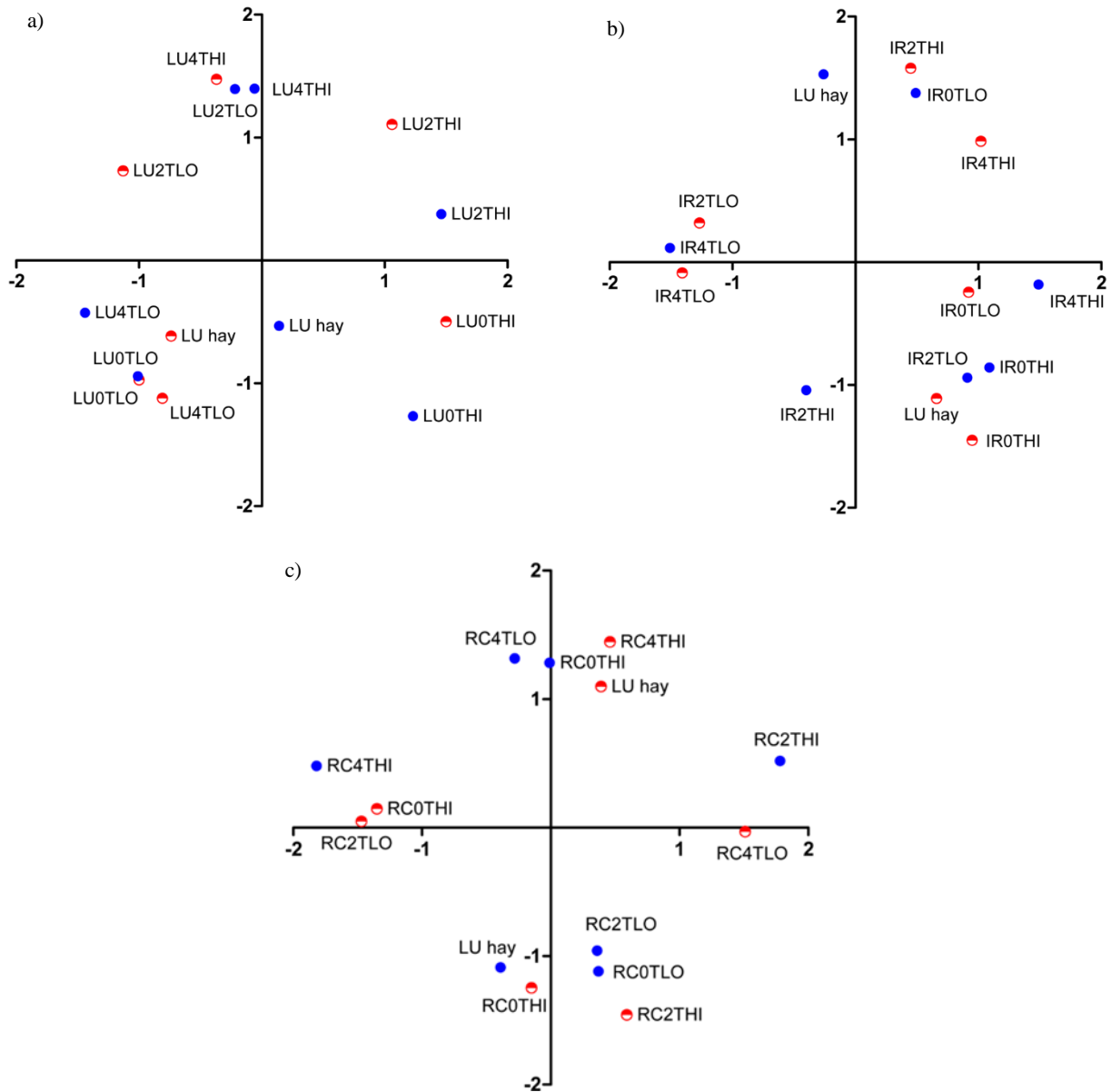


Figure 1. Multidimensional scaling of feed choice behaviour of goats (30 min dry matter intake (DMI) \ominus and 3 h DMI \bullet). a) Two-dimensional stimulus coordinates of preference for different lucerne (LU) silage treatments and a LU hay among six goats; b) Two-dimensional stimulus coordinates of preference for different Italian ryegrass (IR) silage treatments and a LU hay among six goats; c) Two-dimensional stimulus coordinates of preference for different red clover (RC) silage treatments and a LU hay among 5 goats. 0T = 0 g hydrolysable tannin per kg of DM; 2T = 20 g hydrolysable tannin per kg of DM; 4T = 40 g hydrolysable tannin per kg of DM; LO = DM at 200 g/kg; HI = DM at 350 g/kg

3.2. Silage characteristics influencing short-time dry matter intake

The results of correlation analysis between silage characteristics and 3 h DMI are presented in Table 3. Regarding fermentation acids, a highly significant negative relationship was observed between DMI and both acetic acid and propionic acid in all three ensiled forage crops ($r < -0.89$; $p < 0.05$). In RC silages, a close relationship between butyric acid and reduced feed intake in goats was also observed ($r = -0.94$; $p < 0.01$). The alcohol propanol was also associated with a negative impact on feed intake in all forage species ($r < -0.85$; $p < 0.05$). A close relationship between NH_3 accumulation and 3 h DMI was found in all three trials ($r < 0.85$; $p < 0.05$) but, comparable positive correlations for the CP fractions with 3 h DMI were only found for ruminally slowly degradable CP (fraction B3) in LU and IR silages, whereas there was no correlation for the remaining four CP fractions. Across all preference trials, it was found that the concentration of WSC was also closely related to the goats' feed intake ($r > 0.82$; $p < 0.05$). With regard to the in vitro gas production parameters, GP after 2 h of incubation (GP2) was positively correlated with 3 h DMI in all experiments ($r > 0.78$; $p < 0.1$), whereas only individual correlations were identified for the other parameters, all of which were also positively associated to 3 h DMI.

Table 3. Correlation (Pearson coefficient) between lucerne (LU), Italian ryegrass (IR) and red clover (RC) silage characteristics and 3 h DM intake (DMI) of goats

Variables	3 h DMI of goats		
	Lucerne (LU)	Italian ryegrass (IR)	Red clover (RC)
DM	0.96**	0.81*	0.83*
DMI (30 min)	0.99***	0.86*	0.98***
Crude ash	-0.82*	0.01	-0.74Ψ
Crude protein	-0.83*	-0.92**	-0.89*
Crude lipids	-0.92**	-0.95*	-0.92**
aNDFom	-0.44	-0.66	-0.10
ADFom	0.56	-0.85*	0.14
ADL	-0.40	-0.65	0.67
ME	-0.43	-0.04	-0.15
pH	-0.75Ψ	0.26	-0.95**
Lactic acid	0.07	-0.34	0.79Ψ
Acetic acid	-0.95**	-0.92**	-0.94**
Propionic acid	-0.96**	-0.89*	-0.95**
Butyric acid	-0.62	-0.08	-0.94**
Ethanol	-0.71	-0.33	-0.61
Methanol	-0.27	-0.90*	-0.64
Propanol	-0.92**	-0.90*	-0.85*
Butanol	-0.86*	-	-0.70
Ethyl lactate	0.51	-0.16	0.69
Ethyl acetate	-0.46	0.44	0.32
Propyl acetate	-	-	-0.69
NH ₃ -N	-0.91*	-0.85*	-0.87*
WSC	0.96**	0.82*	0.85*
OMD	-0.40	0.39	0.62
<u>Crude protein fractions</u>			
A	-0.08	-0.07	-0.09
B1	0.24	-0.34	0.04
B2	0.01	0.74	-0.01
B3	0.97***	0.94**	-0.41
C	-0.71	0.45	0.72
<u>Gas production</u>			
GP2	0.78Ψ	0.89*	0.78Ψ
(a + b)	0.51	0.48	-0.01
c	0.80Ψ	0.67	0.65
24 h GP	0.38	0.40	0.60

ME = metabolizable energy; aNDF = neutral detergent fibre assayed with heat-stable amylase and expressed exclusive residual ash; ADFom = acid detergent fibre expressed exclusive residual ash; ADL = acid detergent lignin; WSC = water-soluble carbohydrates; ME = metabolizable energy; NH₃-N = ammonia-nitrogen A = non-protein-nitrogen, calculated as CP-true protein (TP); B1 = buffer-soluble TP, quickly degradable; B2 = buffer-insoluble TP, moderately degradable; B3 = neutral detergent insoluble TP, slowly degradable; C = acid detergent insoluble TP, indigestible; GP = gas production; GP2 = GP at 2 h of incubation; (a + b) = maximum GP of the immediately soluble and the insoluble, fermentable fraction; c = gas production rate; OMD = organic matter digestibility; Ψ = P < 0.1; * = P < 0.05; ** = P < 0.01; *** = P < 0.001.

4. Discussion

4.1. Effects of dry matter level and silage characteristics on short-time dry matter intake and preference behaviour

The preference trials revealed considerable variations in preference and short-time DMI of the forage treatments by goats. In a direct comparison between ensiled forage species, mean 3 h DMI was greatest for IR, followed by LU and lowest for RC silages. However, it must be considered that goats offered LU0THI silage had by far the greatest DMI. Several authors reported that well fermented legume silages were ingested by dairy cows in greater amounts than grass silages (Hoffman et al., 1998; Broderick et al., 2002; Dewhurst et al., 2003b). These findings are in line with the fact that legumes are characterized by lower fibre concentrations (Albrecht and Beauchemin, 2003) as well as higher passage rates of particles from the rumen, when harvested at an appropriate maturity stage (Dewhurst et al., 2003a). The lower DMI in the present study for RC in relation to IR and LU is substantially attributed to differences in OMD, DM content and fermentation quality. The RC contained larger amounts of ash and was harvested at a slightly more advanced stage of maturity compared to the other forages, causing a decrease in both OMD and energy content. Ruminants prefer energy-rich and highly digestible feeds due to a positive post-ingestive feedback, but this is more relevant for high-yielding animals where rumen fill is a limiting factor for DMI (Baumont, 1996; Kornfelt et al., 2013; Álvarez et al., 2022), which accordingly did not apply to the animals in the present preference trials. However, by far the most decisive point for the lower mean DMI in the RC trial was the comparatively low DM content of the silages and the associated inferior silage quality, resulting in an unpleasant alteration of texture and odour, which was observed after silo opening.

The key factor among silage treatments affecting fermentation quality turned out to be the DM treatment, because all HI silages were rated as “well fermented”, while 7 of the 9 LO silages were assessed as “moderate to poorly fermented” according to the DLG classification (DLG, 2006). Of the LO silages, only two (IR2TLO and IR4TLO) achieved good fermentation quality, resulting in a higher DMI compared to the others.. Huhtanen et al. (2002) validated that silage quality is closely related to DMI and concluded that a restrictive fermentation with concentrations of 80 g/kg DM total fermentation acids and less than 50 g NH₃-N per kg N should be aimed for. Furthermore, lactic acid should constitute the largest proportion of total fermentation acids and account for at least 650 to 700 g/kg of total fermentation acids (Kung and Shaver, 2001). A too intensive accumulation of fermentation acids not only impairs sensory acceptance, which is particularly important in preference trials (Scherer et al., 2019a), but also

increases the osmotic pressure and lowers the pH in the rumen, resulting in less feed consumption per meal (Baumont et al., 2000).

An effective approach to restrict the fermentation process and thus increasing DMI is to increase the DM content of plant material prior to ensiling through field wilting, resulting in a decreased water availability for microbial metabolism and growth (Whiter and Kung, 2001; Santos and Kung, 2016). In this context, Hartinger et al. (2021), in a preference trial similar to our study, observed that, under good wilting conditions, the increase in DM content of LU prior ensiling from 250 to 350 g/kg improved the 3 h DMI from 38 to 158 g. A similar conclusion was drawn by Scherer et al. (2019b), who measured a significant increase in feed consumption for LU, IR and RC silages when plant material was ensiled with higher DM concentrations. Furthermore, a meta-analysis estimated an elevated DMI of 11 g when silage DM content was increased by 1 g/kg (Eisner, 2007). However, the relationship between DMI and silage DM content is not based on a linear but rather a quadratic function, with the maximum feed intake expected at 350 to 400 g/kg DM (Huhtanen et al., 2013). All authors agreed that the positive relationship between DMI and silage DM concentration did not primarily depend on the DM per se, but rather improved silage quality due to the suppression of certain fermentation products such as acetic acid, propionic acid, butyric acid and various alcohols and esters (Steen et al., 1998; Huhtanen et al., 2002; Eisner, 2007). In the present study, LO silages contained higher amounts of acetic acid than HI silages. Except IR2TLO and IR4TLO, all LO silages exceeded the upper threshold of 30 g/kg DM defined by Kung and Shaver (2001). According to Eisner et al. (2006), acetic acid proved to be the most valid single predictor for DMI in wilted grass silages using a logarithmic equation. A current data analysis ascertained a distinct reduction of DMI by dairy cows already when the acetic acid concentration in silages exceeded 17 g/kg DM (Gerlach et al., 2021).

The present correlation analysis also confirmed that in addition to acetic acid, propionic acid and also butyric acid were negatively related to DMI. Both, Huhtanen et al. (2007) and Krizsan and Randby (2007), mentioned that propionic acid was the best single predictor for DMI among all fermentation acids, whereby a rise of propionic acid by 1 g/kg DM impaired the DMI of cattle by 89 g/day. The occurrence of butyric acid in significant concentrations is often associated with reduced DMI and feed acceptance, as butyric acid on the one hand has an unpleasant as well as intensive foul, rancid-butter smell which affects sensory characteristics, on the other hand butyric acid may cause ketosis, resulting in a negative post-ingestive feedback (Vicente et al., 2014; Kung et al., 2018). Nevertheless, it must be taken into account that butyric acid does not only influence DMI directly, but is rather considered as an indicator for mal-

fermentation, which can be attributed to activity of clostridia (Driehuis et al., 2018; Kung et al., 2018). Besides butyric acid, clostridia may also produce other undesirable compounds such as $\text{NH}_3\text{-N}$ and biogenic amines that may also contribute to a drop if not refusal of silage intake. In line with the present study, several other authors concluded that $\text{NH}_3\text{-N}$ is closely associated with a diminished feed consumption of poorly fermented silages, albeit it is disputable whether the decline is directly or indirectly – i.e. through its close relation to other fermentation and proteolysis end-products – caused by $\text{NH}_3\text{-N}$ (Steen et al., 1998; Stedilová and Kalac, 2004; Krizsan and Randby, 2007). In addition to $\text{NH}_3\text{-N}$, extensive microbial degradation of CP also involves the formation of biogenic amines, which can lead to metabolic disorders or even health risks (Scherer et al., 2015; Özogul and Özogul, 2020). Data of our study (Hilgers, et al., unpublished; Chapter 5) show that the concentration of total biogenic amines was the best single predictor among NPN compounds for the 3 h DMI, explaining more than 50% of variation in the feed choice trials. In this context, a strong dependency on silage DM level also emerged, as in HI silages the concentration of total biogenic amines was on average 90% lower than in LO silages. Krizsan and Randby (2007) reported a close linkage between individual biogenic amines (tryptamine, histamine and cadaverine) and DMI by growing cattle, but the linear relationship to total biogenic amine concentration turned out to be inferior, which can most likely be explained by the higher sensitivity in feed choice trials than in single feed trials without choice.

4.2. Effect of hydrolysable tannins on short-time dry matter intake and preference behaviour

A variety of studies has investigated the effect of different sources and types of tannins on DMI, rumen fermentation, nutrient digestibility, metabolism and animal performance and have been reviewed, e.g., by Mueller-Harvey (2006). Tannins are known to be a very heterogeneous group of polyphenolic polymers, varying in molecular weight, structure and composition (Frutos et al., 2004a; Piluzza et al., 2013). Likewise, the effect of tannins on DMI is influenced by the applied dose, forage preservation method, timing of application (before or after ensiling), and individual interactions with plant and animal species (Santos et al., 2000; Getachew et al., 2008; Deaville et al., 2010; Aguerre et al., 2016; Min and Solaiman, 2018). Hence, a general consideration of separate experiments is complicated and often several influencing factors determine whether the usage of dietary tannin has positive or disadvantageous characteristics.

The preference and short-time DMI of goats was clearly affected by different concentrations of HT. However, the influence was not consistent, since both, forage species as well as DM

level, led to varying results. Hydrolysable tannins increased the DMI in all LO silages, which is most likely attributable to the decreased extent of proteolysis. The application of HT not only reduced the hydrolysis of protein by plant-derived proteases, but also the deamination and decarboxylation of free amino acids. This resulted in a significant lower accumulation of the amino acid degradation end-products $\text{NH}_3\text{-N}$ and total biogenic amines, which were strongly and negatively correlated with 3 h DMI in all preference trials ($\text{NH}_3\text{-N}$: $r > -0.85$; $p < 0.05$; total biogenic amines $r > -0.86$; $p < 0.05$; Hilgers et al., unpublished data; Chapter 5). It is well known that tannins limit proteolytic activities both in the silo and in the rumen due to several different mechanisms. In general, tannins are able to form complexes with plant protein due to hydroxyl groups (Patra and Saxena, 2011). Furthermore, tannins influence the composition of the microbial community and reduce the activity of various enzymes (Chen et al., 2021; Ke et al., 2022).

A comparable inhibitory effect on proteolysis was also observed in HI silages of all forage species, however, other than in LO silages, the HT treatment did not lead to a comparable rise in DMI. In contrast, a detrimental effect in LUHI silages was determined when HT level rose up to 40 g/kg DM.

Accordingly, it can be assumed that the beneficial effect of the reduced protein degradation caused by the HT treatment increased the acceptance of poorly fermented silages, but this effect became less important in forage selection due to a superior influence of the DM concentration. The higher DM concentration not only lowered the accumulation of $\text{NH}_3\text{-N}$ and biogenic amines, but also restricted the production of undesirable volatile acids and alcohols, which is crucial for an improved silage DMI (Huhtanen et al., 2002; Krizsan and Randby, 2007). The more pronounced effect of the increase of DM on DMI is most likely less explained by post-ingestive signals, but more by a change in sensory properties, especially olfactory properties, as unpleasant odours, such as from acetic and butyric acid, decrease at higher DM concentrations. In this context, it must be pointed out that in two-choice experiments the sensory properties of silages have stronger influence on animal preference and feed intake than when no choice is offered (Baumont, 1996). In similarly designed preference trials it was shown that 90% of variation in 3 h DMI were explained by the first 3 min of initial feed intake (Scherer et al., 2019a). In previous experiments goats were also able to develop preferences already after a single meal of each feed in the adaptation phase, which were then reflected in the actual experimental period (Hartinger et al., 2021). The fact that smell is of particular relevance in terms of silage intake became clear in an experiment with anosmic sheep, as these sheep

consumed 33% more of a poorly fermented silage than sheep with an intact sense of smell (Arnold et al., 1980).

Besides the favourable ability to bind proteins and thus reducing CP degradation both in the silo (Tabacco et al., 2006) and the rumen (Aboagye et al., 2019), the use of HT is also accompanied by detrimental properties that may affect feed intake. Tannins, especially condensed tannins, are known to reduce the OMD and impair the palatability of feedstuff due to an astringent flavour (Waghorn, 2008). Others have linked reduced DMI with a slower passage rate through the gastrointestinal tract due to lessened DM digestibility and OMD, respectively (Deaville et al., 2010; Aguerre et al., 2016). Thereby, the reduced digestibility is caused by inhibition of bacteria or their cellulolytic enzymes, or both (Jayanegara et al., 2015) as well as formation of complexes with feed components such as protein and carbohydrates (Aguerre et al., 2016). Furthermore, the microbial population and *in vitro* GP were highly affected by tannin dose. Close relationships between condensed tannin and HT extract dose and digestibility coefficients were reported several times (Tabacco et al., 2006; Getachew et al., 2008; Aguerre et al., 2016; Henke et al., 2017, 2017; Gerlach et al., 2018). This is consistent with findings of our study reported previously (Hilgers et al., 2025), as 72 h GP kinetic parameters, 24 h GP and OMD were linearly reduced by increasing concentrations of HT. However, it has to be mentioned that for the estimation of these parameters in our study, rumen fluid of sheep was used and not rumen fluid of goats. Due to their proline-rich saliva, goats are more tolerant than sheep toward a higher consumption of tannin-containing forages, as tannins are bound by their salivary proteins and thus have less effect on the rumen metabolism and on feed intake (Zimmer and Cordesse, 1996; Min and Solaiman, 2018). With regard to the DMI in the preference trials, it should also be noted that the post-ingestive feedback as response to silage digestibility is more important in producing animals, where rumen fill is a limiting factor, than in a two-choice experiment, where the sensory characteristics are more decisive.

Moreover, the use of HT as dietary additive has long been considered critical because of their potential toxic effects if animals consume sufficiently large amounts, leading to anorexia, depression, rumen atony, and kidney and liver necrosis (Zhu et al., 1992; Plumlee et al., 1998; Clifford and Scalbert, 2000). While oak tannin, tannic acid and some other HT are classified as harmful in higher amounts, the consumption of chestnut tannin by ruminants is comparatively harmless. Frutos et al. (2004b) included 20.8 g/kg DM of chestnut tannin in the diet of finishing lambs and did not find either a sign of intoxication or residues of HT metabolites in the carcasses. Even when the diet contained a higher proportion of chestnut tannin (80 g/kg DM), no health impairment or loss of BW were reported (Zimmer and Cordesse, 1996). As a

consequence, no health risks could be expected in the present study that may result in an aversion of feedstuff, since the maximum concentrations was about 40 g/kg silage DM.

In direct relation to feed intake, HT, especially chestnut tannin extracts, do not appear to have significant negative effects and are often better accepted than condensed tannins. Deaville et al. (2010) prepared ryegrass silages with 75 g/kg of HT or condensed tannin extracts (55 g/kg DM actual tannin concentration in silages) and observed distinct variations in DMI when silages were fed to sheep. The daily DMI of the silage treated with condensed tannins was significantly lower (591 g/day) than the control (704 g/day) and HT treated silage (746 g/day). According to Deaville et al. (2010), these differences were mainly attributable to the influence of condensed tannins on digestibility of DM, OM and neutral detergent fibre. Also, when feeding a protein-rich diet containing LU silage, no effect on DMI was observed when various HT extracts (tannic acid, gallic acid and chestnut tannin) were added at a concentration of 15 g/kg DM. With the exception of CP, the addition also resulted in no decrease in OMD and digestibility of individual nutrients (Aboagye et al., 2019). These findings are in agreement with other results of feeding trials, as no significant effects on DMI were reported, when chestnut tannin (Frutos et al., 2004b; Colombini et al., 2009; Liu et al., 2013; Taha et al., 2022) and other HT sources, such as oak tannin (Focant et al., 2019; Herremans et al., 2020), were added to forages before ensiling or directly to the diet of ruminants at different concentrations (10-30 g/kg DM). Yanza et al. (2021) concluded that HT are more favourable than condensed tannins to avoid detrimental effects and to maintain DMI, digestibility and animal performance, with dietary concentrations in a moderate range of up to 30 g/kg DM.

5. Conclusions

The present study showed that the DM concentration of silages was the primary factor influencing DMI and feed choice behaviour of goats, regardless of the forage species. The preference for silages with higher DM concentration was attributed to a substantially improved fermentation quality. The higher DM content was negatively related to most fermentation products, which were also associated with decreased DMI, such as acetic acid, propionic acid, butyric acid, propanol, butanol and NH₃-N. With regard to the use of HT extract, an enhanced DMI by goats was determined for LO silages, which most likely can be explained by reduced CP degradation in HT treated silages. However, in silage with a higher DM level, this beneficial

effect of HT could no longer be observed to the same extent. Accordingly, it was concluded that at a certain point the negative properties of HT, i.e. the astringent taste and the impairment of nutrient digestibility, become more relevant. In this context, it is evident that HT can positively influence not only silage quality but also feed intake. Nevertheless, more research is needed to clearly identify under which environmental conditions and application rates the maximum benefit of HT is achieved. Furthermore, the influence of the animal species needs to be further clarified.

Author Contributions

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K. Gerlach: Conceptualization, Methodology, Writing – Review & Editing, Supervision.

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Funding

This work was supported by the at that time Federal Ministry of Food and Agriculture (BMEL) based on a decision of the Parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support program [grant number 2818300116].

Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Acknowledgments

The authors thank Petra Jacquemien and their team for technical support and chemical analysis. Furthermore, the authors acknowledge the support of the team at the Central Analytical

Laboratory, Humboldt-University, Berlin, for fermentation pattern analysis. Special thanks to Thomas Hartinger, Helge Deitmers, Klara Schmitz, and Ziade Abraha Kahsay for their assistance with the silage preparation.

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CHAPTER 5

Effects of hydrolysable tannin extract and dry matter concentration on ensiled legume and grass forages – Amino acid composition and formation of biogenic amines

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In preperation

Abstract

Successful ensiling of high-protein forages is often limited by extensive proteolysis during the process. In addition to increasing the dry matter (DM) content, hydrolysable tannins (HT) have been shown to be an effective approach to lowering proteolysis. The objective of the present study was to examine the effect of DM level and different dosages of a HT extract on crude protein (CP) fractions, amino acid (AA) concentrations and the formation of end-products of AA degradation in three forage species. Lucerne (*Medicago sativa* L.), Italian ryegrass (*Lolium multiflorum* L.) and red clover (*Trifolium pratense* L.) were wilted to DM contents of 200 and 350 g/kg, respectively, and treated with three HT levels (0, 20 and 40 g/kg DM) before ensiling in 120-L barrels in quadruplicate. Silage samples were analysed to determine their chemical composition, with particular attention on the proportions of CP fractions, total and free AA and biogenic amines (BA). Furthermore, relationships were estimated between N compounds and feed choice behaviour of goats. The ensiling process resulted in a considerable increase of non-protein-nitrogen (NPN), free AA, ammonia-N ($\text{NH}_3\text{-N}$) and total BA concentrations in all silages, with the highest proportion in untreated low DM lucerne silage. Improved fermentation quality of silages of all forage species was shown at the higher DM level, along with a reduction of NPN ($P \leq 0.05$), $\text{NH}_3\text{-N}$ ($P < 0.001$) as well as total BA ($P < 0.001$). Likewise, the results indicate that the concentrations of NPN ($P < 0.001$), free AA ($P < 0.01$) and BA ($P < 0.001$) were lowered by HT and the influence of HT was more pronounced in low compared to high DM silages. Moreover, regression analysis demonstrated that ammonia-N and total BA were reliable indicators of feed choice behaviour and preference in a small ruminant species, thus providing insights into silage quality.

Abbreviations:

AA, amino acids; BA, biogenic amines; CP, crude protein; DM, dry matter; DMI, dry matter intake, FM, fresh matter; GABA, gamma-aminobutyric acid; HI, silages with an approximate DM level of 350 g/kg; HT, hydrolysable tannin; IR, Italian ryegrass; LO, silages with an approximate DM level of 200 g/kg; LU, lucerne; N, nitrogen; NH₃-N, ammonia-N; NPN, non-protein-N; RC, red clover; WSC, water-soluble carbohydrates

1. Introduction

Over the last decades, ensiling has become the most common practice for preserving forages in intensive livestock production systems, especially in temperate regions due to several advantages compared to other methods, e.g. hay making (Wilkinson and Rinne, 2018; Bernardes et al., 2018). The objective is to provide feed for ruminants year-round and to maintain the feeding value of fresh plant material to the greatest possible extent (McDonald et al., 1991). However, undesirable, but unavoidable processes occur during the fermentation procedure, resulting not only in dry matter (DM) and energy losses but also in degradation of plant crude protein (CP) (Borreani et al., 2018; Dong et al., 2019). The loss of protein and the subsequent increase in the concentration of non-protein nitrogen (NPN) compounds during the processes of wilting and ensiling can be attributed to two pathways, which are the result of plant and microbial proteolytic enzymes. Firstly, proteolytic enzymatic activity results in the hydrolysis of peptide bonds and the accumulation of peptides and free amino acids (AA) (Guo et al., 2007; Ding et al., 2013). Secondly, the further degradation of AA to ammonia-N ($\text{NH}_3\text{-N}$), organic acids, CO_2 and biogenic amines (BA) by deamination and decarboxylation is mainly attributable to proteolytic microorganisms such as species of the genera *Clostridia* and *Enterobacteria* (Pahlow et al., 2003; Hoedtke et al., 2010; Li et al., 2022). An extensive formation of NPN compounds, which emerges particularly in poorly-fermented silages, e.g. silages with high proportions of moisture and CP (Hartinger et al., 2019), has to be evaluated critically. Besides an inefficient microbial CP synthesis in the rumen, leading to increased environmental pollution and necessitating the purchase of supplemental protein (Givens and Rulquin, 2004), excessive ingestion of $\text{NH}_3\text{-N}$ and BA may pose a health risk to ruminants (Driehuis et al., 2018). According to Scherer et al. (2015) and Özogul, Y. and Özogul, F. (2019), elevated intake of BA may cause detrimental physiological effects, such as a reduced protein degradation in rumen and damage to the ruminal microflora and mucosa. Furthermore, several studies have attributed $\text{NH}_3\text{-N}$ and BA to an inverse relationship with DM intake, but the reasons for this remain unclear due to inconsistent results (Huhtanen et al., 2002; Krizsan and Randby, 2007).

However, various factors are able to restrict the rate and extent of proteolysis and thus also the formation of BA during ensiling (Fijalkowska et al., 2015). One of the most common methods is to increase the DM content of forages before ensiling by field wilting. The rate of moisture loss is therefore crucial (Edmunds et al., 2014). Similarly, the use of chemical additives (e.g. formic acid) or microbial inoculants (e.g. homofermentative lactic acid

bacteria) are well-established practices for improving silage and, although not consistently, protein quality by achieving a rapid and extensive pH decline (Muck et al., 2018). One other option to prevent the degradation of CP in silage is the addition of hydrolysable tannins (HT) (Jayanegara et al., 2019). In general, HT are classified as secondary plant metabolites with multiple phenolic hydroxyl groups, which have the capacity to form complexes primarily with proteins (Makkar, 2003). Furthermore, HT have been shown to affect both the microbial population and the activity of proteolytic enzymes in silage and the rumen (Li et al., 2018a; Chen et al., 2021). As reported by Tabacco et al. (2006), addition of 40 g HT/kg DM to lucerne (LU) prior to ensiling resulted in a decline of NPN and $\text{NH}_3\text{-N}$ by 15% and 20%, respectively. In LU silage with 50 g/kg DM chestnut tannin, $\text{NH}_3\text{-N}$ was significantly lowered from 120 to 78 g/kg N (Chen et al., 2021). As both decarboxylation and deamination are likely concomitant processes in AA catabolism and therefore the accumulation of BA and NH_3 is also closely correlated (Jia and Yu, 2022), it can be assumed that HT may restrict BA formation, too.

However, to the best of our knowledge, there is currently a lack of systematic data available on the effect of HT on AA degradation and the accumulation of BA in several grass and legume silages. The objective of this study was to investigate the extent to which HT influences the formation of free AA and BA under various ensiling conditions in different forage species. Therefore, different concentrations of a commercial chestnut tannin extract were added to LU, Italian ryegrass (IR) and red clover (RC) ensiled at two DM levels to achieve significant variations in the extent of proteolysis. Furthermore, short-term DM intake data from three previously presented forage preference trials (Hilgers et al., under review; Chapter 4), in which the identical silages were fed to goats, were used to examine the relationship between feed choice behaviour and N compounds.

2. Material & Methods

2.1. Silage preparation

The present study primarily focuses on the AA and BA concentrations of fresh and ensiled LU, IR and RC. Detailed information regarding silage preparation, general analysis, chemical

composition and preference for silages when offered to goats in choice situations (preference trials) are described in detail in Chapter 3 and Chapter 4 of this thesis.

In summary, pure stands of lucerne (*Medicago ativa* L.; first cut), red clover (*Trifolium pratense* L.; first cut) and Italian ryegrass (*Lolium multiflorum* L.; second cut) were cultivated at the Campus Frankenforst of the Faculty of Agricultural, Nutritional and Engineering Sciences, University of Bonn, Germany (50°43'14''N and 7°12'22''E). Lucerne (first cut) was harvested at the early bud stage, whereas the first cut of RC was harvested at the late bud stage. Italian ryegrass was fertilized with 130 kg N (50 kg N/ ha first cut, 80 kg N/ha second cut) and mown at the late vegetative development stage. In order to produce silages with two different DM levels, one part of each forage species was wilted for 6-8 hours (approximately 200 g/kg DM; low DM, LO), whereas the second part was subjected to wilting for a period of no less than 26 hours to reach a target DM concentration of 350 g/kg (high DM, HI).

Hydrolysable tannins utilized in this experiment were extracted from chestnut wood (*Castanea sativa* (MILL.)) and are commercially available as an extract with a total tannin content of about 750 g/kg DM as stated by the manufacturer (Silvafeed ENC, Silvateam, San Michele Mondovì CN, Italy). In order to ascertain the actual HT content of the chestnut extracts as the difference between total tannins and condensed tannins, the proportions of condensed tannins were also determined according to the acid butanol assay (Porter et al., 1986) and Folin-Ciocalteu reagent (Singleton, 1999). To achieve a consistent application, HT extract was dissolved in water and dispensed with a pressure sprayer onto the wilted forage (Prima 5, Gloria, Witten, Germany). According to (Tabacco et al., 2006), the following HT concentrations (DM basis) were studied: 0 g/kg (0T), 20 g/kg (2T) and 40 g/kg (4T).

Consequently, in combination with the two DM levels (LO: ~ 200 g/kg and HI: ~ 350 g/kg), six silage treatments were prepared for each forage species (LU, IR and RC) in quadruplicate. This resulted in the following acronyms: 0TLO, 2TLO, 4TLO, 0THI, 2THI, 4THI.

Following the preparation of the differently treated forages, the chopped plant material was compacted and sealed airtight in 120-L plastic barrels. All barrels were stored indoors at ambient temperature for at least 90 days.

2.2. General analysis

After the anaerobic storage period, three barrels from each treatment were opened and examined for the presence of mould or other indications of spoilage. In order to obtain

composite samples, the contents of the barrels were thoroughly mixed and about 1500 g fresh matter (FM) of each replication was taken from different spots. All samples were freeze-dried in triplicate for a period of 5 days (P18K-E-6, Dieter Piatkowski, Petershausen, Germany) and then ground successively using 3-mm and then 1-mm sieves (SM100, Retsch, Haan, Germany), with the exception of the silage samples which were analysed for AA and BA. The DM of the silages was subsequently estimated by oven-drying duplicate samples at 105°C. According to Weißbach, F. and Strubelt, C. (2008), correction of DM (DM_{cor}) was conducted to account for the loss of volatile compounds during the drying process.

Proximate analyses of fresh and ensiled forages were conducted in accordance with the guidelines outlined in VDLUFA (2012). Crude protein was calculated by determining total N concentration with Kjeldahl method (4.1.1) using a Vapodest 50s carousel (Gerhardt, Königswinter, Germany) and multiplying N by 6.25. Furthermore, CP fractionation was conducted according to the Cornell Net Carbohydrate and Protein System (Sniffen et al., 1992), following standardisations and recommendations of Licitra et al. (1996).

2.3. Chemical analyses of fermentation variables

Subsamples (50 g FM) of frozen forage and silage were analysed of fermentation products. A comprehensive analysis of the fermentation variables, procedures as well as detection limits are provided in detail in Hilgers et al. (2025; Chapter 3). Cold water extracts were prepared by blending the frozen samples with 300 ml deionized water and 1 ml toluol and refrigerating the mixture overnight at 4°C. The extracts were then filtered using filter paper (MN 615, Macherey-Nagel, Düren, Germany) and subsequently microfiltrated (Minisart RC, 0.45 µm pore size, Sartorius, Göttingen, Germany). The pH was determined potentiometrically by using a calibrated pH meter (827 pH lab, Metrohm, Herisau, Switzerland). Lactic acid was analysed by high-performance liquid chromatography (HPLC) with refractive index (RI) detection (LC-20 AB; Shimadzu Deutschland, Duisburg, Germany) in accordance with the methodology established by Weiß and Kaiser (1995). Acetic acid and butyric acid were determined by gas chromatography (GC) with flame ionization detection (detection limit: 0.01%) (GC-2010, Shimadzu, Duisburg, Germany), as described by Weiß (2001). The concentration of NH_3 -N was analysed colourimetrically, based on the Berthelot reaction, using a continuous flow analyser (Skalar Analytical, Breda, Netherlands) (Hinds, A. A. and Lowe, L. E., 1980). The determination of water-soluble carbohydrates (WSC) was conducted using the anthrone method (von Lengerken and Zimmermann, 1991).

2.4. Analysis of amino acids and biogenic amines

The concentrations of gamma-aminobutyric acid (GABA) and total AA (sum of peptide-bound and free AA) were assayed using ion-exchange chromatography following the guidelines of the European Commission (2009). The method does not differentiate between salts of AA or between D- and L-forms of AA. Moreover, acid hydrolysis has been shown to destroy tryptophan, thus rendering this method unsuitable for the determination of this amino acid. Prior to AA analysis, freeze-dried samples were ground to pass through a 0.25-mm sieve aperture. Prior to hydrolysis, performic acid oxidation was performed at 0 °C, employing a mixture of hydrogen peroxide and formic acid-phenol solution to oxidise cysteine and methionine, respectively, to yield cysteic acid and methionine sulfone. The excess oxidation reagent was decomposed with sodium disulphite. The oxidised or unoxidised samples were hydrolysed with hydrochloric acid for a period of 23 h in order to liberate AA from proteins. Hydrolysates were diluted with sodium citrate buffer or neutralised, and pH values were adjusted to 2.2. The individual AA were separated by ion-exchange chromatography and determined by reaction with ninhydrin using photometric detection at 570 nm (440 nm for proline).

Concentrations of free AA were analysed following the analytical method-guideline of Evonik Operations GmbH (2020). The extraction of the finely ground feed sample was conducted using diluted hydrochloric acid at room temperature, with continuous stirring. Norleucine was incorporated as an internal standard during this process. The chromatographic separation of the extract, which had been diluted with citrate buffer (pH adjusted to 2.2), was carried out on an AA analyser (cation exchange resin, sulfonated polystyrene). Subsequently, the separated individual AA were mixed in the unit with ninhydrin, to achieve a heated reaction loop in order to give a specific, purple colour (post-column derivatization). Photometric detection was then conducted at a wavelength of 570 nm.

The analysis of BA was performed using a liquid chromatography method (Smela et al., 2003). A sample was added to an internal standard (1,7-diaminoheptane) and subsequently extracted with trichloroacetic acid. The suspension was centrifuged and the resulting supernatant was filtered through filter paper. The solid residue extract was made up with deionised water and filtered through a disposable nylon filter. The obtained extract was mixed with saturated Na₂CO₃, followed by the addition dansyl chloride. The derivatisation process was then conducted for a duration of one hour. Subsequent to the removal of dansyl chloride using ammonia, the amine derivatives were extracted with diethyl ether, resulting in the

retention of AA derivatives within the water phase. The organic phase was evaporated to dryness and the solid residue was dissolved in acetonitrile, filtered through a nylon membrane filter and injected onto a chromatographic column. The identification of separated amines was achieved through a comparison of their respective retention times with those of the standards. The quantification process was conducted in accordance with the following calculation method:

$$c_x = RF_x * (C_{IS} * A_x) / A_{IS}$$

Where c_x is biogenic amine in the sample, RF_x refers to response factor of the amine, A_{IS} is peak of the internal standard, A_x refers to peak area of biogenic amine, and C_{IS} is the concentration of the internal standard.

2.5. Preference trials

A comprehensive account of the preference trials is provided in Hilgers et al. (under review; Chapter 4). In summary, three consecutive trials were conducted, with each trial focusing on a distinct forage species. These trials were designed to assess the impact of DM level and HT concentration on preference behaviour and short-term DM intake of Saanen-type wethers (German Improved White Goat breed). Six wethers were used to test the IR and LU silages and five wethers were used in the examination of RC silages.

Single meals of each forage treatment were offered individually during adaptation periods prior to all experimental phases. This was done to allow the animals to associate the silages with sensory characteristics, e.g., taste and smell, and with post-ingestive metabolic feedback. Each morning, silages were offered in a randomized order for 3 h. In order to facilitate quantitative comparisons between the three trials, lucerne hay was used as a standard. During the subsequent experimental period (21 days), every possible 2-way combination of six silage treatments per forage species and the LU hay ($n = 21$ combinations) was presented side by side to each goat in plastic boxes with free access to both feeding boxes to ensure a free choice. On a daily basis, the forage was weighed and offered for a period of 3 h, starting at 07:30 h in the morning. Subsequent to the commencement of the experiment, the boxes were weighed at 30-minute intervals for a duration of 3 h, with the objective of ascertaining the quantity of ingested silages. To ensure *ad libitum* feed intake, the respective forage was added as soon as less than 300 g FM of a meal remained in a box. In the afternoon, grass hay was offered to the goats for *ad libitum* intake at 15:30 for 2 h throughout all trials.

2.6. *Statistical analysis*

All statistical analyses were conducted using SAS 9.4 (SAS Institute Inc., Cary, North Carolina, USA). Residuals were initially analysed using the UNIVARIATE procedure to evaluate the normality of the response variables by the Shapiro-Wilk test. Subsequently, an analysis of variance (PROC GLM) was implemented to assess the impact of DM treatment, HT treatment, and the interaction between DM x HT on fermentation characteristics and N compounds in each forage species. Moreover, an orthogonal contrast test was executed (PROC GLM statement CONTRAST) to ascertain linear and quadratic effects of varying HT concentrations within each DM level on fermentation characteristics and N compounds. The significance of the results was established at $P < 0.05$.

The relationship between $\text{NH}_3\text{-N}$ and total BA, as well as NPN and free AA, was examined using simple linear regression (PROC REG). Stepwise multiple regression analysis was employed to establish a correlation between N compounds and short-term DM intake.

3. Results

3.1. *General chemical composition and crude protein fractions*

The chemical composition of LU, IR and RC before ensiling, including free AA and BA, is presented in Table 1. The effects of DM level and HT treatments on fermentation characteristics and chemical composition of silages are shown in Table 2. A more detailed description of the chemical analysis of silages, including proximate constituents, fermentation characteristics and gas production kinetics, is presented in Chapter 3.

Table 1. Chemical composition and nitrogen (N) compounds in fresh and wilted lucerne (LU), Italian ryegrass (IR) and red clover (RC) before ensiling

Variables (g/kg DM)	Lucerne (LU)			Italian ryegrass (IR)			Red clover (RC)		
	Fresh	LO	HI	Fresh	LO	HI	Fresh	LO	HI
DM (g/kg)	162	251	367	132	193	384	116	169	282
WSC	110	94	104	95.5	172	148	153	67.2	112
CP	229	220	210	190	178	181	170	169	152
NPN (g/kg CP)	268	285	387	242	307	375	204	257	317
Total AA	191	162	142	149	139	128	133	135	123
Free AA	4.91	9.99	17.3	2.24	4.65	14.2	3.01	5.12	7.84
Total BA (mg/kg DM)	71.0	157	130	49.0	44.0	107	14.3	15.4	15.5
NH ₃ -N (g/kg N)	8.20	14.2	14.9	-	21.1	17.3	25.7	40.8	49.4

LO = 200 g/kg; HI = 350 g/kg; DM = dry matter; WSC = water-soluble carbohydrates; CP = crude protein; NPN = non-protein-nitrogen; AA = amino acids; BA = biogenic amines; NH₃-N = ammonia-nitrogen.

In all forage species, silage pH was found to be affected by DM level ($P < 0.001$), with pH values decreasing in LU and RC but increasing in IR with increasing DM content. As a consequence of the elevation of the DM content prior to ensiling, a significant decline in both acetic and butyric acid was observed. Furthermore, the HT treatment led to a linear reduction of acetic acid in low-DM LU and IR silages ($P < 0.001$). With regard to the CP composition, ensiling of forages resulted in a distinct rise of NPN in all silages and the highest concentrations of NPN were found in LU silages, while CP breakdown was least intense in RC silages. Except for RC silages, NPN was identified as the predominant CP constituent in all of the examined silages. Both, DM and HT treatments led to a significant decrease in the proportion of NPN in ensiled grass and legumes. However, the addition of HT (40 g/kg DM) was most effective in inhibiting the NPN accumulation, especially in low DM silages. In low DM silages of LU, IR and RC, NPN was reduced by 20, 20 and 40%, respectively, whereas in high DM silages the decrease was only 18, 10 and 28%.

In addition to NPN, NH₃-N accumulation was reduced by higher DM ($P < 0.001$) and addition of HT ($P < 0.05$). In a similar manner to the linear effect of HT on NPN concentration, a more pronounced inhibition of NH₃-N formation was observed in low DM silages.

Table 2. Effect of hydrolysable tannin (HT) and dry matter (DM) level on chemical composition and fermentation characteristics in lucerne (LU), Italian ryegrass (IR) and red clover (RC) silages

Variables (g/kg DM)	Treatments ^a						SEM	<i>P</i> -value						
	0TLO	2TLO	4TLO	0THI	2THI	4THI		DM	HT	DM x HT	Lin LO ^b	Quad LO ^b	Lin HI ^b	Quad HI ^b
Lucerne (LU)														
pH	5.12	5.55	5.59	4.97	4.97	5.01	0.10	<0.001	0.058	0.089	0.007	0.149	0.783	0.874
Lactic acid	90.5	51.7	41.6	70.2	67.2	56.0	0.37	0.314	<0.001	<0.001	<0.001	0.008	0.019	0.384
Acetic acid	42.6	38.3	30.0	13.9	18.0	15.5	0.10	<0.001	<0.001	<0.001	<0.001	0.143	0.285	0.022
Butyric acid	0.4	9.67	12.5	-	-	-	0.23	-	0.063	0.052	0.003	0.283	-	-
NH ₃ -N (g/kg N)	198	200	172	121	111	100	8.26	<0.001	0.030	0.54	0.04	0.156	0.100	0.945
NPN (g/kg CP)	824	715	656	778	713	640	11.5	0.050	<0.001	0.211	<0.001	0.1033	<0.001	0.796
Italian ryegrass (IR)														
pH	4.60	4.23	4.10	4.67	4.66	4.72	0.06	<0.001	0.005	0.002	<0.001	0.117	0.590	0.594
Lactic acid	75.0	120	117	63.4	63.6	53.0	16.5	<0.001	0.092	0.009	0.009	0.067	0.051	0.228
Acetic acid	49.9	21.7	14.4	15.1	15.5	15.1	2.07	<0.001	<0.001	<0.001	<0.001	0.001	0.135	0.306
Butyric acid	-	1.73	0.71	0.18	0.09	0.022	0.58	0.178	0.390	0.325	0.402	0.077	0.965	0.929
NH ₃ -N (g/kg N)	141	127	98.3	106	103	97.9	4.46	<0.001	<0.001	0.055	<0.001	0.219	0.012	0.324
NPN (g/kg CP)	676	604	540	696	650	626	13.6	<0.001	<0.001	0.075	<0.001	0.176	0.003	0.512
Red clover (RC)														
pH	4.93	5.07	4.70	4.40	4.41	4.43	0.06	<0.001	0.077	0.043	0.039	0.010	0.747	0.950
Lactic acid	79.6	55.1	87.7	113	99.5	91.2	7.36	<0.001	0.067	0.044	0.452	0.008	0.058	0.772
Acetic acid	48.6	41.2	42.4	26.4	23.3	21.5	2.44	<0.001	0.074	0.682	0.097	0.180	0.185	0.822
Butyric acid	20.4	34.0	13.5	0.99	0.13	-	3.85	<0.001	0.058	0.005	0.228	0.004	0.858	0.934
NH ₃ -N (g/kg N)	156	131	91.5	113	92.2	88.6	9.40	<0.001	<0.001	<0.001	<0.001	0.312	0.056	0.228
NPN (g/kg CP)	486	333	290	471	417	338	11.4	0.001	<0.001	0.003	<0.001	0.002	<0.001	0.395

NH₃-N = ammonia-nitrogen; N = nitrogen; NPN = non-protein-nitrogen CP = crude protein; SEM = standard error of the mean; Significance between treatments at P < 0.05.

^a treatments: 0T = 0 g/kg DM of HT; 2T = 20 g/kg DM of HT; 4T = 40 g/kg DM of HT; LO = 200 g/kg; HI = 350 g/kg.

^b orthogonal contrasts: Linear (Lin) and quadratic (Quad) effects of different HT concentrations on the chemical composition of silages at the respective DM level.

3.2. Total and free amino acids

The concentrations of total and free AA in fresh and wilted forages are presented in Table 1. The process of overnight wilting of mowed forage crops resulted in a decrease in total AA concentration, with the most distinctive decline in LU (25%) and least in RC (7%).

The effects of DM and HT treatments on individual and total AA (sum of peptide-bound and free AA) in LU, IR and RC silages are presented in Tables 3, 4 and 5, respectively. The total concentrations of AA in silages ranged from 147 to 172 g/kg DM in LU, from 114 to 131 g/kg DM in RC, and from 139 to 154 g/kg DM in IR. Both DM level and HT treatments affected several individual AA, but the effects were variable and also depended on forage species. In LU silages, the the higher DM level increased the concentrations of Cys, Thr, Trp, Ser, Pro, Asp and Glu ($P < 0.01$), whilst simultaneously diminishing those of Met, Ile, Leu, Val, Gly and Ala ($P < 0.01$). With regard to HT treatment, more linear effects were observed in low DM silages (Met, Lys, Thr, Trp, Ile, Val, Gly, Ser, Glu, GABA) than in high DM silages (Met, Ile Leu, Val, Phe, Ser), showing that the impact of HT addition was inconsistent throughout. Quadratic effects were not observed.

Table 3. Effect of hydrolysable tannin (HT) and dry matter (DM) level on concentrations of total amino acids (AA; sum of peptide-bound and free AA) and gamma-aminobutyric acid (GABA) in lucerne (LU) silages

Variables (g/kg DM)	Treatments ^a						SEM	<i>P</i> -value						
	0TLO	2TLO	4TLO	0THI	2THI	4THI		DM	HT	DM x HT	Lin LO ^b	Quad LO ^b	Lin HI ^b	Quad HI ^b
MET	3.29	2.84	3.03	2.82	2.62	2.57	0.07	0.003	0.053	0.518	0.035	0.567	0.019	0.448
CYS	1.16	1.30	1.44	1.60	1.51	1.42	0.07	0.005	0.806	0.034	0.264	1.00	0.140	0.858
LYS	5.86	9.13	9.16	9.93	9.64	9.47	0.96	0.060	0.255	0.131	0.031	0.193	0.755	0.967
THR	3.01	3.99	4.90	8.35	7.85	7.59	0.34	<0.001	0.285	0.007	0.002	0.907	0.156	0.756
TRP	1.45	1.93	2.51	2.66	2.49	2.52	0.21	0.004	0.114	0.041	0.004	0.949	0.660	0.703
ARG	1.64	2.70	3.49	2.56	2.89	3.37	0.24	0.076	<0.001	0.068	<0.001	0.752	0.016	0.752
ILE	10.4	9.58	9.32	9.09	8.41	8.17	0.21	<0.001	0.001	0.926	0.003	0.259	0.012	0.375
LEU	14.0	13.9	14.3	14.0	12.9	12.6	0.32	0.005	0.126	0.073	0.670	0.568	0.010	0.296
VAL	13.2	12.3	11.9	11.4	10.6	10.2	0.23	<0.001	<0.001	0.966	0.002	0.397	0.003	0.367
HIS	2.31	2.95	3.33	3.67	3.46	3.45	0.46	0.098	0.687	0.404	0.137	0.839	0.763	0.816
PHE	8.41	8.31	8.53	8.78	8.11	8.00	0.21	0.521	0.190	0.156	0.668	0.621	0.022	0.275
GLY	10.5	9.06	9.13	8.79	8.01	7.81	0.36	<0.001	0.012	0.707	0.024	0.111	0.079	0.528
SER	2.30	3.04	3.65	8.14	8.19	7.13	0.23	<0.001	0.259	<0.001	0.002	0.774	0.008	0.077
PRO	10.7	9.36	10.0	14.1	14.0	13.6	0.51	<0.001	0.360	0.396	0.351	0.119	0.502	0.735
ALA	22.3	19.5	18.8	11.5	10.7	10.4	1.22	<0.001	0.185	0.581	0.064	0.517	0.549	0.879
ASP	25.6	24.9	21.8	32.1	30.7	30.1	1.69	<0.001	0.268	0.737	0.139	0.574	0.420	0.850
GLU	6.48	8.12	9.61	12.0	11.6	12.1	0.30	<0.001	<0.001	0.001	<0.001	0.793	0.879	0.267
Total AA	143	143	145	161	154	151	4.44	<0.001	0.608	0.271	0.591	0.862	0.090	0.656
GABA	14.2	12.7	11.5	6.59	6.20	5.84	0.26	<0.001	<0.001	0.005	<0.001	0.720	0.149	0.798

SEM = standard error of the mean; Significance between treatments at $P < 0.05$.

^a treatments: 0T = 0 g/kg DM of HT; 2T = 20 g/kg DM of HT; 4T = 40 g/kg DM of HT; LO = 200 g/kg; HI = 350 g/kg.

^b orthogonal contrasts: Linear (Lin) and quadratic (Quad) effects of different HT concentrations on the chemical composition of silages at the respective DM level.

Table 4. Effect of hydrolysable tannin (HT) and dry matter (DM) level on concentrations of total amino acids (AA; sum of peptide-bound and free AA) and gamma-aminobutyric acid (GABA) in Italian ryegrass (IR) silages

Variables (g/kg DM)	Treatments ^a							<i>P</i> -value						
	0TLO	2TLO	4TLO	0THI	2THI	4THI	SEM	DM	HT	DM x HT	Lin LO ^b	Quad LO ^b	Lin HI ^b	Quad HI ^b
MET	3.29	3.48	3.11	2.66	2.55	2.56	0.07	<0.001	0.043	0.050	0.060	0.008	0.319	0.560
CYS	1.19	1.34	1.27	1.30	1.20	1.25	0.08	1.00	0.970	0.340	0.768	0.246	0.768	0.401
LYS	5.37	10.4	9.40	7.57	7.55	7.48	0.31	0.005	<0.001	<0.001	<0.001	<0.001	0.880	0.894
THR	7.07	7.95	7.17	7.05	6.65	6.83	0.23	0.011	0.370	0.047	0.843	0.013	0.433	0.368
TRP	2.22	2.94	2.61	2.52	2.49	2.49	0.14	0.351	0.0810	0.078	0.066	0.013	0.869	0.924
ARG	2.98	4.49	5.02	4.37	6.04	6.09	0.18	0.002	0.0014	0.820	0.004	0.352	0.014	0.135
ILE	8.28	8.64	7.59	7.40	6.93	7.13	0.16	<0.001	0.0360	0.008	0.015	0.005	0.339	0.110
LEU	14.4	15.1	13.4	12.4	11.7	11.9	0.31	<0.001	0.0540	0.022	0.033	0.009	0.347	0.231
VAL	10.9	11.1	9.80	9.73	9.07	9.42	0.22	<0.001	0.0160	0.009	0.003	0.017	0.299	0.085
HIS	1.77	2.92	2.75	2.76	2.65	2.64	0.24	0.350	0.1140	0.044	0.014	0.045	0.771	0.780
PHE	8.97	9.80	8.69	7.91	7.59	7.67	0.24	<0.001	0.1340	0.048	0.441	0.006	0.499	0.539
GLY	9.55	9.81	8.75	8.20	7.68	7.88	0.19	<0.001	0.0344	0.018	0.014	0.012	0.283	0.155
SER	6.06	4.63	4.71	6.81	6.43	6.58	0.35	<0.001	0.0464	0.252	0.020	0.099	0.646	0.521
PRO	8.97	9.15	8.24	12.0	11.1	11.8	0.17	<0.001	0.0591	0.002	0.010	0.026	0.592	0.002
ALA	15.6	14.6	12.6	12.0	11.1	11.6	0.45	<0.001	0.0089	0.025	0.001	0.356	0.512	0.288
ASP	18.0	18.4	17.1	20.2	19.0	19.7	0.58	0.002	0.4920	0.223	0.315	0.232	0.506	0.209
GLU	11.2	14.8	14.5	14.3	14.1	14.3	0.73	0.261	0.0590	0.046	0.007	0.046	0.912	0.855
Total AA	136	150	137	139	134	137	3.70	0.108	0.644	0.094	0.624	0.008	0.718	0.354
GABA	11.2	8.75	7.49	6.34	5.81	5.94	0.62	<0.001	0.0161	0.057	0.001	0.421	0.656	0.700

SEM = standard error of the mean; Significance between treatments at $P < 0.05$.

^a treatments: 0T = 0 g/kg DM of HT; 2T = 20 g/kg DM of HT; 4T = 40 g/kg DM of HT; LO = 200 g/kg; HI = 350 g/kg.

^b orthogonal contracts: Linear (Lin) and quadratic (Quad) effects of different HT concentrations on the chemical composition of silages at the respective DM level

Table 5. Effect of hydrolysable tannin (HT) and dry matter (DM) level on concentrations of total amino acids (AA; sum of peptide-bound and free AA) and gamma-aminobutyric acid (GABA) in red clover (RC) silages

Variables (g/kg DM)	Treatments ^a						SEM	<i>P</i> -value						
	0TLO	2TLO	4TLO	0THI	2THI	4THI		DM	HT	DM x HT	Lin LO ^b	Quad LO ^b	Lin HI ^b	Quad HI ^b
MET	2.19	2.42	2.35	1.99	1.82	2.04	0.13	0.004	0.689	0.308	0.374	0.366	0.786	0.236
CYS	1.10	1.10	1.08	0.98	0.90	0.98	0.03	<0.001	0.5007	0.332	0.692	0.819	0.947	0.080
LYS	6.14	7.54	8.07	7.35	6.83	7.27	0.30	0.703	0.031	0.009	0.001	0.261	0.853	0.212
THR	4.79	5.75	6.39	6.40	5.86	6.39	0.29	0.031	0.047	0.029	0.002	0.660	0.975	0.158
TRP	2.36	2.65	2.76	2.05	1.94	2.36	0.13	<0.001	0.061	0.284	0.045	0.560	0.147	0.123
ARG	5.08	6.37	6.48	4.26	4.46	5.65	0.28	<0.001	0.0012	0.122	0.004	0.110	0.004	0.173
ILE	7.61	7.39	7.12	6.72	6.05	6.47	0.31	0.003	0.347	0.556	0.290	0.963	0.593	0.181
LEU	13.1	13.0	12.4	11.0	9.98	11.0	0.53	<0.001	0.5925	0.369	0.406	0.749	0.972	0.146
VAL	9.87	9.50	9.14	8.73	7.92	8.44	0.39	0.004	0.288	0.548	0.209	0.984	0.598	0.186
HIS	2.39	2.86	2.91	2.55	2.40	2.67	0.10	0.061	0.031	0.032	0.004	0.121	0.433	0.116
PHE	8.29	8.37	8.04	7.13	6.52	7.27	0.34	<0.001	0.730	0.320	0.616	0.634	0.777	0.135
GLY	7.44	8.00	7.87	7.06	6.59	7.31	0.34	0.015	0.566	0.297	0.378	0.416	0.619	0.176
SER	4.76	5.65	5.73	6.29	6.04	6.49	0.19	<0.001	0.034	0.034	0.004	0.113	0.495	0.163
PRO	5.37	6.89	6.65	9.12	8.43	8.65	0.23	<0.001	0.1747	0.001	0.002	0.010	0.174	0.142
ALA	14.8	13.8	11.9	9.37	8.19	8.77	1.47	0.002	0.517	0.659	0.197	0.801	0.778	0.632
ASP	9.72	11.8	14.9	19.0	18.1	18.9	1.03	<0.001	0.0725	0.071	0.004	0.674	0.929	0.510
GLU	10.5	12.8	13.0	11.8	11.3	12.9	0.45	0.743	0.006	0.031	0.002	0.085	0.118	0.099
Total AA	115	126	127	122	113	123	5.08	0.122	0.461	0.199	0.133	0.447	0.939	0.169
GABA	6.90	4.39	3.82	3.03	2.29	1.88	0.16	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.412

SEM = standard error of the mean; Significance between treatments at $P < 0.05$.

^a treatments: 0T = 0 g/kg DM of HT; 2T = 20 g/kg DM of HT; 4T = 40 g/kg DM of HT; LO = 200 g/kg; HI = 350 g/kg.

^b orthogonal contrasts: Linear (Lin) and quadratic (Quad) effects of different HT concentrations on the chemical composition of silages at the respective DM level.

With the exception of Lys, His and Glu, the AA in RC silages were affected ($P < 0.05$) by DM level; wilting to a higher DM content enhanced Thr, Ser, Pro and Asp ($P < 0.05$), but lowered Met, Cys, Trp, Arg, Ile, Leu, Val, Phe, Gly and Ala concentrations ($P < 0.05$). In a comparable manner to LU silages, HT treatment exerted a linear effect on several AA. However, a significant effect of gradual HT addition was found exclusively in low DM silages, with the exception of GABA in high DM silages ($P < 0.001$). As with LU and RC, IR silages were more affected by DM level than HT treatment. Increasing DM level was found to decrease the concentrations of Met, Lys, Thr, Ile, Leu, Val, Phe, Gly, Ala ($P < 0.05$) and increase Arg, Ser, Pro, Asp ($P < 0.05$). Apart from a linear effect on Arg and a quadratic effect on Pro, the HT treatment exerted no effect on AA composition in high DM silages. Unlike LU and RC, quadratic effects ($p < 0.05$) were found in two-thirds of analysed AA in low-DM IR silages (Met, Lys, Thr, Trp, Ile, Leu, Val, His, Phe, Gly, Ser, Pro, Ala and Glu).

The ensiling of forage crops resulted in a considerable increase in free AA content, ranging from three to 16 times the initial level, depending on the pre-ensiling treatments and forage species. The sum of free AA exhibited significant variation, ranging from 68.7 to 94.2 g/kg DM in LU (Table 6), to 48.1 to 71.4 g/kg DM in IR silages (Table 7), and 16.8 to 37.6 g/kg DM in RC (Table 8). In the case of both LU and IR silages, the highest concentrations of free AA were detected in low DM silages (LUT0LO and IRT0LO). In RC silages, the highest concentrations were found in untreated high DM silage (RC0THI). Across all three forage species, Ala and Asp were the predominant free AA, followed by Pro, Val and Leu. Both DM level and HT treatment resulted in substantial alterations to the free AA profile of silages. The effects of the DM level in LU silages were significant, with the majority of free AA showing alterations in concentration. Wilting to a higher DM concentration decreased the levels of Met, Val, Ala, Ile, Gly and Leu and concurrently increased the concentrations of Thr, Glu and Pro. In IR and RC silages, the higher DM concentration resulted in a decline of Met and Ala ($P < 0.05$), as well as in an enhancement of Asp, Glu and Pro ($P < 0.05$). Regarding the addition of HT to silages, accumulation of free AA was reduced as the proportion of HT increased ($P < 0.01$). In LU silages, the HT treatment ($P = 0.003$) but not the DM level ($P = 0.161$) had a pronounced effect on free AA than. Linear declines of 23% ($P = 0.002$) and 20% ($P = 0.033$) were observed in both low and high DM silages, respectively. The HT treatment in high DM RC silages decreased the concentration of 11 free AA ($P < 0.05$) and total free AA by 40% ($P < 0.003$). In low DM, silages Met, Val, Ile, Gly, Leu and Phe were linearly affected by adding HT. The DM treatment led to an accumulation of total free AA in high DM IR silages compared to low DM RC silages ($P = 0.01$). In contrast to RC silages, the HT treatment had a greater effect on total

free amino acids in low DM silages ($P < 0.001$) than in high DM silages ($P = 0.043$). Free Met, Lys, Thr, Val, Ala, Asp, Ile, Gly, Leu, Phe, Pro were linearly decreased in low DM silages by the HT treatment. With regard to the DM, only a tendency effect on total free AA could be detected.

Table 6. Effect of hydrolysable tannin (HT) and dry matter (DM) level on concentrations of free amino acids (AA) in lucerne (LU) silages

Variables (g/kg DM)	Treatments ^a						SEM	<i>P</i> -value						
	0TLO	2TLO	4TLO	0THI	2THI	4THI		DM	HT	DM x HT	Lin LO ^b	Quad LO ^b	Lin HI ^b	Quad HI ^b
MET	2.22	1.65	1.40	1.14	1.04	0.85	0.08	<0.001	<0.001	0.008	<0.001	0.154	0.022	0.621
LYS	3.51	5.43	4.56	6.33	5.86	5.08	1.63	0.129	0.647	0.385	0.469	0.250	0.342	0.888
THR	1.14	1.32	1.54	5.56	5.11	4.36	0.34	<0.001	0.501	0.096	0.417	0.937	0.027	0.693
VAL	10.3	8.37	7.19	7.01	6.34	5.47	0.19	<0.001	<0.001	0.004	<0.001	0.115	<0.001	0.630
ALA	19.4	15.7	14.2	8.20	7.57	6.63	1.60	<0.001	0.045	0.358	0.039	0.597	0.617	0.455
ARG	-	-	-	0.24	0.70	0.16	0.16	-	-	-	-	-	0.556	0.142
ASP	15.5	15.2	11.2	14.0	12.5	11.8	1.69	0.389	0.173	0.632	0.093	0.381	0.362	0.863
ILE	8.11	6.49	5.57	5.78	5.17	4.38	0.18	<0.001	<0.001	0.016	<0.001	0.105	<0.001	0.603
GLU	1.19	0.99	1.1	4.92	4.25	3.51	0.14	<0.001	<0.001	0.001	0.616	0.298	<0.001	0.772
GLY	7.43	5.27	4.75	4.71	4.10	3.42	0.31	<0.001	<0.001	0.051	<0.001	0.059	0.012	0.898
HIS	1.45	1.72	2.04	2.88	2.61	2.18	0.55	0.095	0.994	0.530	0.482	0.942	0.410	0.923
LEU	10.6	8.95	8.20	9.55	8.49	7.22	0.29	0.004	<0.001	0.548	<0.001	0.218	<0.001	0.751
PHE	5.46	4.56	4.2	5.17	4.66	3.94	0.17	0.312	<0.001	0.498	<0.001	0.294	0.000	0.594
PRO	7.78	6.44	6.82	10.7	10.7	9.79	1.03	<0.001	0.179	0.343	0.047	0.806	0.549	0.747
Total free AA	94.2	82.1	72.7	86.2	79.1	68.7	4.31	0.161	0.003	0.556	0.002	0.968	0.033	0.610

SEM = standard error of the mean; Significance between treatments at $P < 0.05$.

^a treatments: 0T = 0 g/kg DM of HT; 2T = 20 g/kg DM of HT; 4T = 40 g/kg DM of HT; LO = 200 g/kg; HI = 350 g/kg.

^b orthogonal contracts: Linear (Lin) and quadratic (Quad) effects of different HT concentrations on the chemical composition of silages at the respective DM level.

Table 7. Effect of hydrolysable tannin (HT) and dry matter (DM) level on concentrations of free amino acids (AA) in Italian ryegrass (IR) silages

Variables (g/kg DM)	Treatments ^a						SEM	<i>P</i> -value						
	IR0TLO	IR2TLO	IR4TLO	IR0THI	IR2THI	IR4THI		DM	HT	DM x HT	Lin LO ^b	Quad LO ^b	Lin HI ^b	Quad HI ^b
MET	1.91	1.60	1.21	1.23	1.08	1.02	0.10	<0.001	0.001	0.044	<0.001	0.780	0.163	0.578
LYS	1.84	5.32	4.06	4.17	3.90	3.82	0.16	0.068	<0.001	<0.001	<0.001	<0.001	0.156	0.733
THR	4.49	4.23	3.25	4.09	3.65	3.65	0.24	0.322	0.011	0.125	0.003	0.231	0.190	0.438
VAL	6.97	5.58	4.14	5.43	4.74	4.86	0.03	0.031	<0.001	0.004	<0.001	0.701	0.169	0.275
ALA	11.3	8.84	6.69	7.76	6.78	6.88	0.53	0.001	<0.001	0.012	<0.001	0.803	0.290	0.444
ARG	-	0.17	0.58	1.46	2.71	2.64	0.18	<0.001	0.003	0.022	0.183	0.612	<0.001	0.013
ASP	12.0	6.95	5.54	9.91	8.74	8.99	0.43	0.011	<0.001	<0.001	<0.001	0.005	0.148	0.205
ILE	4.93	4.01	2.91	4.14	3.56	3.63	0.19	0.242	<0.001	0.005	<0.001	0.777	0.085	0.155
GLU	2.97	3.64	3.00	5.72	4.54	4.48	0.51	0.001	0.494	0.209	0.964	0.315	0.102	0.378
GLY	5.18	4.16	3.02	3.48	2.93	2.80	0.22	<0.001	<0.001	0.015	<0.001	0.808	0.044	0.436
HIS	0.66	1.38	0.99	1.87	1.67	1.57	0.21	0.002	0.418	0.125	0.288	0.050	0.392	0.802
LEU	9.14	7.78	5.97	7.19	6.20	6.21	0.32	0.001	<0.001	0.011	<0.001	0.586	0.045	0.221
PHE	4.97	4.48	3.47	4.17	3.69	3.70	0.18	0.009	<0.001	0.014	<0.001	0.307	0.139	0.307
PRO	5.07	3.89	3.26	7.60	6.80	7.24	0.17	<0.001	<0.001	0.003	<0.001	0.231	0.197	0.020
Total free AA	71.4	62.0	48.1	68.2	61.0	61.5	2.11	0.094	<0.001	0.004	<0.001	0.394	0.043	0.160

SEM = standard error of the mean; Significance between treatments at $P < 0.05$.

^a treatments: 0T = 0 g/kg DM of HT; 2T = 20 g/kg DM of HT; 4T = 40 g/kg DM of HT; LO = 200 g/kg; HI = 350 g/kg.

^b orthogonal contracts: Linear (Lin) and quadratic (Quad) effects of different HT concentrations on the chemical composition of silages at the respective DM level.

Table 8. Effect of hydrolysable tannin (HT) and dry matter (DM) level on concentrations of free amino acids (AA) in red clover (RC) silages

Variables (g/kg DM)	Treatments ^a						SEM	<i>P</i> -value						
	RC0TLO	RC2TLO	RC4TLO	RC0THI	RC2THI	RC4THI		DM	HT	DM x HT	Lin LO ^b	Quad LO ^b	Lin HI ^b	Quad HI ^b
MET	0.73	0.31	0.07	0.24	0.07	-	0.07	<0.001	<0.001	0.019	<0.001	0.360	0.022	0.514
LYS	0.07	0.12	1.11	1.89	1.28	0.81	0.16	<0.001	0.067	0.003	0.200	0.480	0.000	0.716
THR	0.16	0.07	0.62	2.11	1.41	0.98	0.20	<0.001	0.141	0.006	0.125	0.219	0.002	0.578
VAL	3.39	2.04	1.49	2.88	2.06	1.57	0.32	0.598	0.001	0.607	0.001	0.330	0.014	0.698
ALA	8.55	6.67	4.64	4.17	3.01	2.38	1.44	0.013	0.184	0.760	0.079	0.965	0.398	0.882
ARG	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ASP	0.38	0.32	3.02	10.1	8.81	7.69	0.95	<0.001	0.066	0.039	0.051	0.262	0.092	0.933
ILE	2.53	1.47	1.06	1.98	1.37	0.98	0.23	0.215	<0.001	0.529	<0.001	0.276	0.009	0.629
GLU	0.26	0.29	0.29	2.67	1.95	1.41	0.06	<0.001	<0.001	<0.001	0.774	0.868	<0.001	0.223
GLY	1.46	1.01	0.8	1.47	0.98	0.64	0.18	0.701	0.005	0.869	0.025	0.576	0.007	0.756
HIS	-	-	-	0.44	0.28	0.19	0.02	-	-	-	-	-	<0.001	0.109
LEU	4.11	2.39	1.64	3.43	2.26	1.49	0.32	0.235	<0.001	0.633	<0.001	0.233	<0.001	0.620
PHE	1.93	1.15	0.87	1.87	1.25	0.90	0.16	0.837	<0.001	0.877	<0.001	0.208	<0.001	0.482
PRO	0.66	1.38	1.19	4.29	3.84	3.24	0.19	<0.001	0.137	0.003	0.071	0.068	0.002	0.743
Total free AA	24.2	17.2	16.8	37.6	28.6	22.3	2.96	0.001	0.006	0.462	0.082	0.435	0.003	0.715

SEM = standard error of the mean; Significance between treatments at $P < 0.05$.

^a treatments: 0T = 0 g/kg DM of HT; 2T = 20 g/kg DM of HT; 4T = 40 g/kg DM of HT; LO = 200 g/kg; HI = 350 g/kg.

^b orthogonal contracts: Linear (Lin) and quadratic (Quad) effects of different HT concentrations on the chemical composition of silages at the respective DM level.

Figure 1 illustrates the coherence between the concentrations of NPN and free AA across treatments of all forage species before and after ensiling. Simple regression analysis revealed a strong positive correlation between the concentration of NPN and free AA in all silages, with a high coefficient of determination ($R^2 = 0.924$; $p < 0.001$).

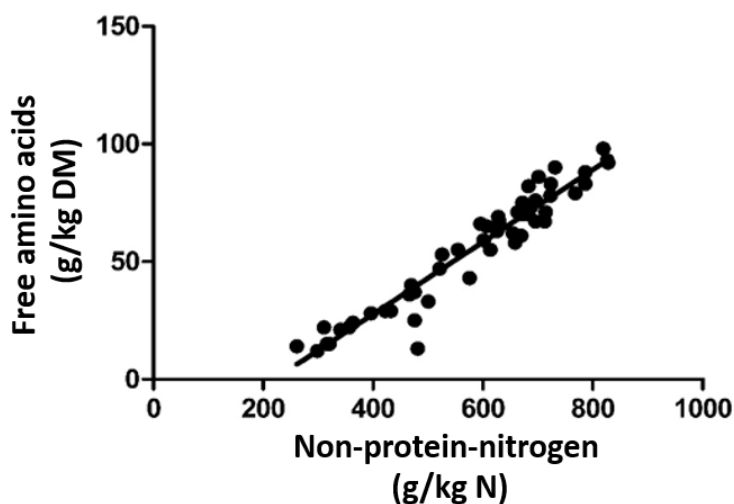


Figure 1. Relationship between concentration of non-protein-nitrogen and free amino acids in silage treatments and forage species; $n = 54$; $y = 219.4 + 6.512x$; $R^2 = 0.924$; $P < 0.001$.

3.3. Biogenic amines

In ensiled forages, total BA were detected within a range of 185 and 17,256 mg/kg DM. The concentrations in fresh and wilted plant material prior to ensiling were considerably lower and did not exceed 157 mg/kg DM. Lucerne silages had the highest average total BA concentrations of all forages (Table 9), followed by IR (Table 10) and RC silages (Table 11). Regarding the pre-ensiling treatments, the greatest proportions of total BA were found in OTLO treatments, regardless of the forage crop. In general, the content of BA in silages differed greatly; however, cadaverine, tyramine and putrescine were predominant in grass and legume silages, accounting for around 80% of total BA. Except for spermine (not significant in IR; $P = 0.952$), the accumulation of all BA was strongly affected by the DM level, leading to a distinct decline in high DM silages. Higher DM concentrations due to wilting restricted the formation of total BA by 89, 86 and 91% in LU, IR and RC silages, respectively ($P = 0.001$). The stepwise addition of HT to low DM silages caused a linear decrease in the level of most individual BA,

as well as in total BA in all grass and legume silages. However, the effect of HT treatment on high DM silages was negligible for the most part; only a few differences were observed. Only tyramine and phenylethylamine in IR ($P < 0.05$), spermidine, tyramine and spermine in LU ($P < 0.05$) and spermidine and spermine in RC ($P < 0.05$) were linearly affected by adding the HT extract at concentrations up to 40 g/kg DM in high DM silages.

Table 9. Effect of hydrolysable tannin (HT) and dry matter (DM) level on concentrations of biogenic amines (BA) in lucerne (LU) silages

Variables (mg/kg DM)	Treatments ^a						SEM	<i>P</i> -value						
	LU0TLO	LU2TLO	LU4TLO	LU0THI	LU2THI	LU4THI		DM	HT	DM x HT	Lin LO ^b	Quad LO ^b	Lin HI ^b	Quad HI ^b
Putrescine	3,742	2,700	2,005	127	72.1	53.3	106	<0.001	<0.001	<0.001	<0.001	0.208	0.631	0.891
Histamine	1,938	1,147	641	71.4	62.3	56.2	313	<0.001	0.150	0.161	0.013	0.717	0.973	0.997
Cadaverine	4,907	2,294	1,917	255	124	83.7	480	<0.001	0.013	0.0243	<0.001	0.081	0.805	0.940
Octopamine	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Spermidine	91.6	73.5	62.4	47.1	39.0	38.4	2.22	<0.001	<0.001	0.0031	<0.001	0.291	0.017	0.196
Tyramine	4,633	3,693	3,194	1,035	887	739	86.6	<0.001	<0.001	<0.001	<0.001	0.059	0.033	0.997
Spermine	42.5	32.1	28.3	33.1	29.2	27.5	1.18	<0.001	<0.001	0.008	<0.001	0.044	0.006	0.449
Phenethylamine	557	558	436	78.4	71.4	45.3	29.5	<0.001	0.037	0.235	0.013	0.111	0.443	0.795
Serotonine	-	-	-	6.17	9.00	9.00	0.83	-	-	-	-	-	0.055	0.146
Tryptamine	1,346	853	422	3.70	4.00	2.50	115	<0.001	0.006	0.006	<0.001	0.832	0.994	0.995
Total BA	17,256	11,351	8,706	1,658	1,299	1,055	1588	<0.001	0.001	0.003	<0.001	0.172	0.649	0.959

SEM = standard error of the mean; Significance between treatments at $P < 0.05$.

^a treatments: 0T = 0 g/kg DM of HT; 2T = 20 g/kg DM of HT; 4T = 40 g/kg DM of HT; LO = 200 g/kg; HI = 350 g/kg.

^b orthogonal contracts: Linear (Lin) and quadratic (Quad) effects of different HT concentrations on the chemical composition of silages at the respective DM level.

Table 10. Effect of hydrolysable tannin (HT) and dry matter (DM) level on concentrations of biogenic amines (BA) in Italian ryegrass (IR) silages

Variables (mg/kg DM)	Treatments ^a						SEM	<i>P</i> -value						
	IR0TLO	IR2TLO	IR4TLO	IR0THI	IR2THI	IR4THI		DM	HT	DM x HT	Lin LO ^b	Quad LO ^b	Lin HI ^b	Quad HI ^b
Putrescine	2,483	1,521	970	107	51.5	48.2	197	<0.001	0.006	0.010	<0.001	0.411	0.837	0.916
Histamine	1,418	661	435	27.1	16.4	11.0	168	<0.001	0.028	0.034	0.001	0.221	0.947	0.990
Cadaverine	3,730	735	584	271	74.8	91.9	185	<0.001	<0.001	<0.001	<0.001	<0.001	0.509	0.648
Octopamine	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Spermidine	35.6	17.3	37	3.23	1.00	1.00	7.4	<0.001	0.378	0.451	0.893	0.077	0.837	0.905
Tyramine	3,150	2,592	1,738	1,260	347	423	163	<0.001	<0.001	0.043	<0.001	0.474	0.003	0.029
Spermine	4.36	3.67	7.55	3.66	6.21	6.25	1.6	0.952	0.231	0.517	0.185	0.332	0.276	0.535
Phenethylamine	176	197	122	102	6.21	23.4	15.5	<0.001	0.004	0.007	0.030	0.029	0.004	0.012
Serotonine	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tryptamine	338	70.1	33.9	11.3	8.40	6.62	31.4	<0.001	<0.001	<0.001	<0.001	0.011	0.917	0.988
Total BA	11,335	5,798	3,929	1,785	511	611	648	<0.001	<0.001	0.001	<0.001	0.040	0.225	0.404

SEM = standard error of the mean; Significance between treatments at $P < 0.05$.

^a treatments: 0T = 0 g/kg DM of HT; 2T = 20 g/kg DM of HT; 4T = 40 g/kg DM of HT; LO = 200 g/kg; HI = 350 g/kg.

^b orthogonal contracts: Linear (Lin) and quadratic (Quad) effects of different HT concentrations on the chemical composition of silages at the respective DM level.

Table 11. Effect of hydrolysable tannin (HT) and dry matter (DM) level on concentrations of biogenic amines (BA) in red clover (RC) silages

Variables (mg/kg DM)	Treatments ^a						SEM	<i>P-value</i>						
	RC0TLO	RC2TLO	RC4TLO	RC0THI	RC2THI	RC4THI		DM	HT	DM x HT	Lin LO ^b	Quad LO ^b	Lin HI ^b	Quad HI ^b
Putrescine	1,563	779	513	160	55.6	20.3	52.8	<0.001	<0.001	<0.001	<0.001	0.002	0.087	0.574
Histamine	865	367	284	57.9	30.8	22.5	46.8	<0.001	<0.001	<0.001	<0.001	0.004	0.603	0.872
Cadaverine	2,924	1,502	819	340	142	99	101	<0.001	<0.001	<0.001	<0.001	0.011	0.117	0.544
Octopamine	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Spermidine	50.9	38.1	36.5	18.9	12.2	8.5	2.44	<0.001	<0.001	0.470	0.001	0.086	0.011	0.625
Tyramine	1,836	974	602	149	35.2	18.8	78.5	<0.001	<0.001	<0.001	<0.001	0.026	0.262	0.620
Spermine	12.1	9.05	9.68	10.4	8.16	6.65	0.80	0.014	0.005	0.446	0.051	0.087	0.007	0.716
Phenethylamine	56.3	49.3	33.5	12.6	8.15	6.28	8.51	<0.001	0.265	0.594	0.083	0.676	0.609	0.903
Serotonine	-	-	-	-	2,667	-	-	-	-	-	-	-	-	-
Tryptamine	221	107	27.9	2.97	3.71	2.59	20.3	<0.001	0.002	0.002	<0.001	0.501	0.990	0.970
Total BA	7,530	3,826	2,324	752	296	185	275	<0.001	<0.001	<0.001	<0.001	0.007	0.171	0.613

SEM = standard error of the mean; Significance between treatments at $P < 0.05$.

^a treatments: 0T = 0 g/kg DM of HT; 2T = 20 g/kg DM of HT; 4T = 40 g/kg DM of HT; LO = 200 g/kg; HI = 350 g/kg.

^b orthogonal contracts: Linear (Lin) and quadratic (Quad) effects of different HT concentrations on the chemical composition of silages at the respective DM level.

The relationship between $\text{NH}_3\text{-N}$ and total BA, both end products of amino acid catabolism, is presented in Figure 2. The simple linear regression analysis revealed a correlation between total BA and $\text{NH}_3\text{-N}$ concentrations ($R^2 = 0.755$; $P < 0.001$) in silages across forage species.

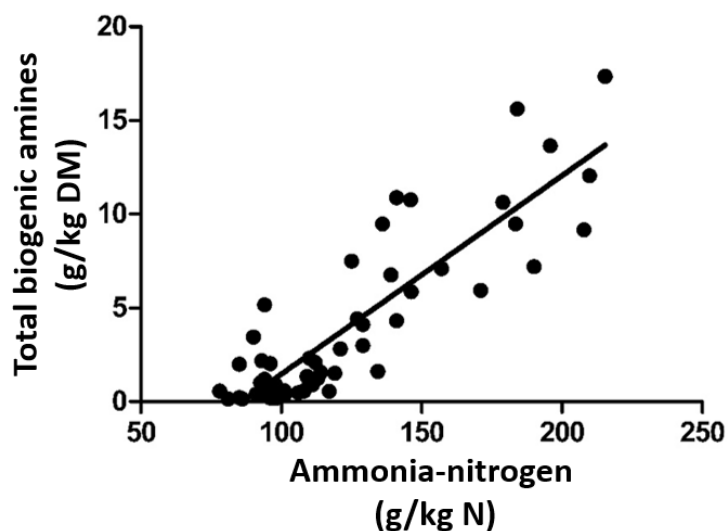


Figure 2. Relationship between concentrations of total biogenic amines and ammonia-nitrogen in silage treatments and forage species; $n = 54$; $y = 86.12 + 9.445 x$; $R^2 = 0.755$; $P < 0.001$.

3.4. Relationship between short-time dry matter intake and N compounds

Table 12 shows the relationships between the concentrations of selected nitrogen N compounds (NPN, free AA, $\text{NH}_3\text{-N}$ and total BA) in silages and the short-time dry matter (DM) intake of goats. The R^2_{adj} values for simple regression analysis were very low for the concentrations of NPN ($R^2_{\text{adj}} = 0.012$) and free AA ($R^2_{\text{adj}} = 0.002$), and coefficients were not significant ($P = 0.203$; $P = 0.297$, respectively). In contrast, the concentrations of both total BA and $\text{NH}_3\text{-N}$ were closely related to feed choice behaviour; the R^2_{adj} values explained 51% and 48% of the total variation in short-time DM intake, respectively. Including all four N compounds as regressors in a stepwise multiple regression analysis provided the best explanation of short-time DM intake ($R^2 = 0.869$). However, the effect of free AA was negligible and did not improve the adjustment of the model further.

Table 12. Linear and multiple regression analysis of crude protein compounds and short-time dry matter intake of silage treatments and forage species by goats

Variables	Intercept	Coefficient	P-value	R ² _{adj}
Simple regressions				
NPN	336.54	0.15195	0.203	0.012
Total free AA	381.37	0.78170	0.297	0.002
Total BA	513.81	-0.02221	<0.001	0.515
NH ₃ -N	749.34	-2.61449	<0.001	0.483
Multiple regression				
	376.48			0.869
NPN		0.76431	<0.001	
Total free AA		-1.34691	0.179	
Total BA		-2.0753	<0.001	
NH ₃ -N		-0.01553	<0.001	

NPN = non-protein-nitrogen; AA = amino acids; BA = Biogenic amines; NH₃-N = ammonia-nitrogen; R²_{adj} = adjusted coefficient of determination.

4. Discussion

4.1. General chemical composition and crude protein fractions

The DM level had a marked effect on silage quality due to a reduction in acetic acid and butyric acid, thereby confirming the findings of Santos, M. C. and Kung, L. (2016) and Kung et al. (2018). Concerning the effect of tannins on the production of fermentation acids, available conclusions are inconsistent. On the one hand, Tabacco et al. (2006) and Herremans et al. (2020) reported no significant influence on the quantity and composition of fermentation acids. On the other hand, Salawu et al. (1999) and He et al. (2020) revealed inhibiting effects of various condensed and hydrolysable tannins on fermentation products. The decline in the formation of fermentation acids caused by HT can be explained by direct modification of the microbial community, as well as by a restricted enzyme activity and the formation of complexes with carbohydrates (Chen et al., 2021; Ke et al., 2022). However, these results are not entirely consistent, as a study by Wang et al. (2020) demonstrated that the concentrations of lactic and acetic acid in high-moisture mulberry leaf silage were increased by the addition of tannic acid (20 g/kg DM). In this context, tannic acid also enhanced the abundance of *Weissella* and *Acinetobacter*. This positive effect was also found repeatedly in a subsequent publication with whole plant soybean silage (Wang et al., 2021). However, when interpreting the findings of the aforementioned studies, it is imperative to consider the substantial disparities between the

conserved fodder plants and the ensiled forages examined in this study. In this respect, the results can only be compared with caution.

The effect of the DM treatment on NPN concentrations was inconsistent and depended on the forage species. In LU silages, an increase in the DM level and thus, reducing the water activity, has resulted in a decline of NPN compounds ($P = 0.05$). These findings align with those reported in previous studies (Edmunds et al., 2014; Hartinger et al., 2019), and can most likely be attributed to a lower activity of proteolytic plant enzymes (Kofahl, 2009) and a suppressed microbial metabolism (Pahlow et al., 2003). In contrast to the effect on LU silages, the DM treatment in IR and RC silages led to a rise in the proportion of NPN in high DM silages. This unanticipated effect can be ascribed to unfavourable weather conditions during the field wilting period, causing plant-derived proteases remaining active before their inhibition during the ensiling process with the associated pH decline (Edmunds et al., 2014).

A remarkable impact on NPN concentrations was found when HT was applied to forages prior to ensiling. The reduced protein degradation during ensiling reflects the ability of tannins to form stable complexes with proteins in a pH range of 3.5 to 7.5 (Mueller-Harvey, 2006) and simultaneously by inhibition of proteases (Li et al., 2018a; Ke et al., 2022). Another factor is also the influence of HT on the microbial community, inhibiting bacteria whose abundance correlates with an increase in NPN compounds (Wang et al., 2020; Ke et al., 2022). As described by Ke et al. (2022), the interplay of these HT actions induced a remarkably lower NPN level (731 vs. 541 g/kg CP) when LU was treated with 40 g/kg HT before ensiling. Moreover, the results of this study corroborated those of Tabacco et al. (2006), who applied 0, 20, 40 and 60 g/kg HT to LU before ensiling. The stepwise enhancement of chestnut tannin proportion resulted in a continuous decrease of NPN in silages from 759 to 646 g/kg CP.

Jayanegara et al. (2019) summarised the effect of various tannin extracts and dosages on ensiled grass and legume forages and corroborated that the extent of proteolysis was significantly affected by the quantity of applied tannins, but also by the type of tannin extract. Whilst the inhibitory effect on the accumulation of NPN increased with higher application rates, it must be considered that nutritional characteristics such as *in vitro* gas production, as well as digestibility of DM, CP and neutral detergent fibre, were progressively lowered. This is exemplified by the findings of Chen et al. (2022). Increasing dosages of gallnut tannin (20 and 50 g/kg DM) on the one hand decreased the NPN in LU silages by more than 50 g/kg CP ($P < 0.001$), but on the other hand also decreased the ruminal digestibility of DM (636 vs. 617 vs. 586 g/kg) and neutral detergent fibre (466 vs. 444 vs. 422 g/kg).

4.2. Total and free amino acids

In the present study, concentrations of total AA in fresh forage ranged from 133 to 191 g/kg DM (784 to 834 g/kg CP). Comparable concentrations were reported for LU (180 g/kg DM) (Guo et al., 2008) and RC (141 g/kg DM) (Purwin et al., 2014). Regarding IR, no study has thus far observed CP proportions similar to those previously mentioned, but when total AA are expressed as g/kg CP, a closely related concentration of 786 g/kg CP was reported by Winters et al. (2000).

The substantial degradation of protein, which was accompanied by considerable alteration in concentrations of total and individual AA, is consistent with the findings of Winters et al. (2000), Guo et al. (2008) and Purwin et al. (2015). Furthermore, the effects of DM and HT treatments on the AA composition of preserved forages were clearly evident. Hartinger et al. (2019) reported that increasing the DM of LU forage from 250 to 350 g/kg reduced the amounts of Ile, Leu, Val, Ala and GABA ($p < 0.01$), whereas concentrations of Cys, Lys, Thr, Arg, His, Ser, Pro, Asp, and Glu ($p < 0.05$) increased. The findings of the present study are largely consistent with the results of the aforementioned studies, particularly with regard to the AA, which were lower at higher concentrations of DM. It can be hypothesised that higher concentrations of Leu, Gly, Lys, Thr, Glu, Arg, His, Lys and Phe are most likely an indicator for an reduced AA catabolism, and thus for a diminished formation of $\text{NH}_3\text{-N}$ and BA (Hoedtke et al., 2010; Scherer et al., 2015). Apart from that, higher concentrations of Ala and GABA are generally associated with poorly-fermented silages (Winters et al., 2000; Winters et al., 2001; Hartinger et al., 2019). Moreover, an increased concentration of Ala in low DM silages is attributed to the decarboxylation of Asp by microorganisms, while the formation of GABA is mainly related to the decarboxylation of Glu by plant enzymes in the early stage of fermentation (Oshima et al., 1979; Winters et al., 2000).

Gamma-aminobutyric acid is a four-carbon non-proteinogenic amino acid and is one of the most abundant inhibitory neurotransmitters. The synthesis of GABA via decarboxylation of glutamic acid has been confirmed not only in the central nervous system of mammals but also in plants and several bacterial species. Despite some beneficial aspects, such as promotion of growth performance and health, high concentrations of GABA in silages are considered as detrimental, as the intake can lead to reduction of DM intake and rumen digestibility of DM (Scherer et al., 2015; Wang et al., 2023).

The addition of HT to forages prior to ensiling did not result in a consistent effect on the concentration of total AA. In contrast, Guo et al. (2008) determined a tendency towards an

increase in total AA from 156 to 167 g/kg DM after adding tannic acid (15 g/kg FM) to LU before ensiling. However, it should be noted that the application rate used by Guo et al. (2008) was significantly lower than in our study. Furthermore, an increase in total AA was found in LU silages treated with 40 g/kg DM chestnut tannin by Tabacco et al. (2006), but the total AA concentration was more affected by an elevation in DM content from 400 to 500 g/kg. In general, the effect of HT treatment was more distinct in low than in high DM silages. In all low DM silages, HT induced a linear decrease of GABA by 20-45%, whereas the level of the corresponding AA Glu was 24-48% higher in silages treated with HT (40 g/kg DM) than in the untreated control. This finding suggests that decarboxylation in low DM silages was effectively restricted by HT, whereas in high DM silages the reduction of water activity became more important to limit microbial proteolysis. Accordingly, it can be concluded that the catabolism of AA is more sensitive to the effect of increasing DM than to a HT concentration of up to 40 g/kg DM. A constant effect of HT on AA concentrations in both low- and high-DM silages was only found for Arg, suggesting that the corresponding pathway of degradation has a considerably higher tolerance to reduced water activity.

Besides NPN, free AA have been identified as a direct indicator of CP hydrolysis (Guo et al., 2011). Prior to ensiling, proteolytic enzymes in mowed plant material are highly active, thereby enhancing the proportion of free AA of total AA (Cavallarin et al., 2005; Guo et al., 2007). In fresh grass and legume forages, free AA are only present in low concentrations, ranging from 7 to 16 g/kg DM, with the majority of this variation attributable to variations in forage species, CP content, and duration of field wilting time (Oshima et al., 1979; Guo et al., 2008; Dong et al., 2019).

As a consequence of protein hydrolysis, a remarkable increase in free AA can be expected during the initial days of ensiling, caused predominantly by the action plant endo- and exopeptidases. Guo et al. (2011) and (Tao et al., 2011) reported that the inhibition of these plant peptidases lowered the formation of free AA in the first weeks of ensiling about 70% each, when tested independently. The activity of proteolytic plant enzymes is susceptible to external factors such as acidification of silage by homofermentative inoculants or formic acid, but also by protein-binding agents, temperature and osmolarity (Winters et al., 2001; Guo et al., 2007; Tao et al., 2011). The increase of free AA during the ensiling process was also occurred in this study, although considerable differences in the extent of free AA accumulation were found between forage species. In RC silages, the concentrations of free AA were considerably lower than in IR and LU, which can be attributed to the activity of polyphenol oxidase in RC (Li et al., 2018b; Dong et al., 2019).

Hristov and Sandev (1998) found that enhancing the DM content from 260 to 460 g/kg resulted in a decline of total free AA by 11%. In this context, the intensity of moisture loss per time unit is more relevant than the actual wilting time (Cavallarín et al., 2005). Accordingly, a slow rate of wilting leads to a more extensive formation of free AA, such as Pro (Edmunds et al., 2014). Hence, the unfavourable weather conditions during the field-wilting period of RC may provide a rationale for the significant increase in total free AA in high DM RC silages, whereas in IR and LU, which were wilted under more favourable conditions, no corresponding change was detected. In contrast, (Hartinger et al., 2019) indicated that the concentrations of free AA in LU silages were less affected by wilting intensity than by the addition of sucrose prior to ensiling or by elevating the DM from 250 to 350 g/kg. As with NPN, the effect of HT on the production of free AA was more consistent and reduced the concentration of free AA more efficiently than an increase in the DM concentration, indicating the effectiveness of HT against the activity of plant proteases.

Guo et al. (2007) and Li et al. (2018a) both ensiled LU for 35 days and added 20 and 50 g/kg DM of tannic acid and gallnut tannin extract respectively, before sealing, and both confirmed a reduction of free AA content with increasing tannin dosage. With regard to the 20 g/kg DM treatments, the decrease ranged from 10 to 20%, which is in approximate agreement with the 20 g/kg DM HT treatment of this study. The findings of a recent study revealed that the application of 40 g/kg DM of chestnut tannin into LU silage prior to ensiling led to a suppression in the formation of both NPN and $\text{NH}_3\text{-N}$, surpassing the efficiency of increasing the silage DM, but did not exert an influence on free AA (Tabacco et al., 2006). One possible explanation might be the activity of certain plant and microbial enzymes. These are essential for the formation of free AA and are more sensitive to osmolarity than to tannins. Moreover, the variance could be based on additional factors, including type of tannin and forage species. Concentrations of free AA in LU silages are adversely affected by both hydrolysable and condensed tannins, whereas condensed tannins more strongly limit CP degradation (Ke et al., 2022).

4.3. Biogenic amines

Biogenic amines, which are classified as low-molecular-weight N-containing organic bases, are naturally present in both plant and animal organisms. It has been established that these substances fulfil numerous functions in the processes of RNA, DNA and protein synthesis. Consequently, BA in physiological concentrations are considered essential for normal growth

and differentiation of cells (Fusi et al., 2004). The presence of BA in silage is generally undesirable, as higher concentrations have been associated with reduced DM intake and increased health risks (van Os et al., 1995; Ladero et al., 2010). The paucity of available data on BA in plant material prior to ensiling is primarily attributable to the markedly low concentrations of BA. van Os et al. (1996b) determined concentrations of total BA in wilted perennial ryegrass between 117 and 241 mg/kg DM. Even lower concentrations (39.3 mg/kg DM of total BA) were measured in wilted and chopped oat (*Avena sativa* L.), with putrescine (36 mg/kg DM) as the most prevalent BA (Jia and Yu, 2022). In the present study, the total BA concentration ranged between 14 mg/kg DM in RC and 71 mg/kg DM in LU, whereas wilting increased the concentration in both LU and IR up to 157 mg/kg DM.

The ensiling of forages resulted in a considerable increase in total BA, most notable in OTLO silages. These alterations are mainly attributable to the enzymatic decarboxylation of the corresponding free AA by proteolytic bacteria, such as *Clostridia*, but also various strains of Enterobacteriaceae and lactic acid bacteria (Rooke and Hatfield, 2003; Nishino et al., 2007). Recent findings using metagenome sequencing demonstrate that the family of Enterobacteriaceae, comprising genera such as *Enterobacter*, *Klebsiella* and *Escherichia*, are predominantly responsible for the production of NH₃-N, cadaverine, putrescine and tyramine in poorly fermented LU silages. In addition, a strong positive correlation was detected between high levels of acetic acid and the relative abundance of major members of the family Enterobacteriaceae (Li et al., 2022). This correlation between BA-forming Enterobacteriaceae and high concentrations of acetic acid in poorly fermented silages could be an approach to explain the high concentrations of total BA in LU0TLO and IR0TLO treatments, although the silages did not contain large proportions of butyric acid, which may indicate low activity of *Clostridia*. However, the proliferation of most *Clostridia* is selectively inhibited by the accumulation of nitrite and nitric oxide, which is associated with the nitrate-reducing metabolism of Enterobacteriaceae. Consequently, there is potential for substantial formation of NH₃-N and BA, despite low levels of butyric acid and *Clostridia*, respectively (Li et al., 2022).

As with the hydrolysis of protein, the extent of BA formation is highly variable and depends on a number of factors, including ensilability of the forage, especially with regard to a rapid and sufficient pH drop, DM content, storage duration and temperature. Correspondingly large differences can be found for individual and total BA concentrations in silages (Scherer et al., 2019; Jia and Yu, 2022). In the research conducted by Jia and Yu (2022), the silage temperature had a significant impact on the accumulation of BA in oat silage, as a rise from 10 to 37°C led to a threefold increase in total BA. The temperature rise was also associated with a pronounced

increase of $\text{NH}_3\text{-N}$ and acetic acid, leading to the conclusion that an increased ensiling temperature negatively affects CP degradation and fermentation quality.

Furthermore, the current data demonstrated significant variations among different plant species and treatments. In addition to the highest concentration of NPN, the highest concentrations of total BA were also found in LU silages, followed by IR and RC silages. It is evident that in RC silages, the formation of BA was comparatively less pronounced in comparison to that of $\text{NH}_3\text{-N}$. Whereas the $\text{NH}_3\text{-N}$ content in RC was similarly high as in IR treatments, proportion of BA was about 50% lower in RC than in IR silages. It can be assumed that the respective precursor AA of the individual BA were more sensitive to the activity of polyphenol oxidase, and thus less AA were available for decarboxylation than for deamination. Differences between ensiled forage were also reported by Scherer et al. (2019), but the variations between forage species were comparatively less and the concentrations of BA generally lower. Weisbjerg et al. (2012) examined the effect of forage species on amine-N as proportion of total N in ensiled grasses and legumes and showed considerable differences between LU and the other forages ($P < 0.001$). Lucerne had the highest amine-N concentration (35.4 g/kg total N), whereas no significant differences were found between perennial ryegrass (20.5 g/kg total N) and RC (15.3 g/kg total N).

Despite the fact that the influence of the forage species was largely in accordance with published data, the decarboxylation of free AA in our study clearly exceeded the concentrations determined by other authors. As Scherer et al. (2015) summarised, data from 10 studies demonstrated an average level of 8 BA (histamine, tyramine, putrescine, cadaverine, tryptamine, phenylethylamine, spermidine and spermine) in untreated silages of 2,318 mg/kg DM. It is noteworthy that only a limited number of authors have reported concentrations that are comparable to, or even greater than those observed in the present study. Selwet et al. (2013) examined wilted LU silages and found a mean content of total BA of 7,091 mg/kg DM (including histamine, putrescine, cadaverine and tyramine), with cadaverine accounting for 6,021 mg/kg DM. In the present study, cadaverine was also identified as the predominant BA in all OTLO silages, but concentrations did not exceed 4,907 mg/kg DM. In an ensiled legume-grass mixture with a low DM content (140 g/kg), Olt et al. (2005) found 10,420 mg/kg DM total BA, which is in agreement with the findings of Krizsan et al. (2007), who determined a maximum concentration of 13,800 mg/kg DM in 24 silages. In both studies, the high BA levels were accompanied by high concentrations of butyric acid, acetic acid as well as $\text{NH}_3\text{-N}$. In this context, it has also been shown that the formation of BA is strongly dependent on external factors such as chemical silage additives (Olt et al., 2005). A considerably higher sensitivity to

silage treatments compared to $\text{NH}_3\text{-N}$ was also evident in our findings, despite the close linkage of the two degradation pathways of free AA, as shown in Figure 2. In terms of the quantitative comparison of BA in silages with literature data, it is also important to note that the number of examined BA frequently varies, which can influence the total BA content. Furthermore, differences in BA concentrations can be explained by ensiling duration, as an increase can still be detected after several weeks, especially under unfavourable ensiling conditions (van Os et al., 1996b; Jia and Yu, 2022). Accordingly, a direct comparison of numerical data with publications reporting shorter ensiling durations is only possible to a limited extent.

In the present study, the increase of DM concentration resulted in a tremendous decrease of BA in all forage species by approximately 90%. A distinct diminishing effect on BA was expected, as previous authors had made similar observations. van Os et al. (1996b) concluded that the formation of BA in grass silages was significantly reduced not only by improving the acidification rate due to formic acid or homofermentative lactic acid bacteria, but also by enhancing the DM during field wilting up to 550 g/kg. Other researchers found that a decline of $\text{NH}_3\text{-N}$ by 66% was accompanied by an increased in the silage DM content of a red clover-timothy mixture from 140 g/kg to 256 g/kg. This, moreover, resulted in a drastic and almost completely reduced accumulation of BA by 99% (Olt et al., 2005). An experiment conducted by (Weisbjerg et al., 2012) revealed that wilting different grass and legume forages before ensiling to a DM content of 310 g/kg instead of 250 g/kg, reduced amine-N and $\text{NH}_3\text{-N}$ by 44% and 7%, respectively, which in turn supports the assertion that the pathway of free AA decarboxylation is more susceptible to pre-ensiling treatments than free AA deamination. In this context, Weisbjerg et al. (2012) also observed that the most significant differences in BA concentrations among the five examined forage species were between RC and LU, which is consistent with our findings. The suppression of BA formation through field wilting prior to ensiling is based on the reduction of water activity. As a result, the proliferation and activity of low osmo-tolerant microorganisms, such as Clostridia and Enterobacteria, are constrained. This, in turn, limits the extent of decarboxylation and deamination (Pahlow et al., 2003). Furthermore, a prolonged wilting period before ensiling minimises the hydrolysis of protein during the anaerobic storage period, thus decarboxylation is also indirectly suppressed, since fewer free AA are available for metabolism (Edmunds et al., 2014).

Besides to the effects of DM treatment, the stepwise addition of HT also negatively affected the enrichment of BA in LO silages. However, to the authors' knowledge, no information about the influence of tannins on BA accumulation in ensiled forages is available. The findings of the present study demonstrated that HT treatment exerted a substantial impact on the concentration

of total BA in LU4TLO, IR4TLO and RC4TLO silages, with a reduction of 50, 65 and 70%, respectively. Two explanatory attempts have been made for these findings, which most likely also interact. As previously stated, an increase in HT proportion limits the formation of NPN and free AA in silages by forming complexes with proteins and, sequentially, also inhibit the activity of plant proteases (Guo et al., 2007; Ke et al., 2022). Consequently, a lower amount of free AA is available for the synthesis of BA. Furthermore, several studies have revealed that tannins possess selective antimicrobial properties against several species of Clostridia and Enterobacteria that are associated with the decarboxylation and deamination of free AA (He et al., 2020).

Despite the absence of literature directly comparing the effects of tannins on the formation of BA in silages, the potential influence can be inferred indirectly through the observed correlation between the two end-products of AA degradation, NH₃-N and BA (Nishino et al., 2007; Jovaišienė et al., 2017; Jia and Yu, 2022). It can be hypothesised that a negative effect on the formation of BA may be evident, because a number of researchers have reported that the addition of various tannin extracts as a pre-ensiling treatment resulted in a significant reduction of NH₃-N in silages, as observed in high DM silages. Merely scattered significant linear effects were detectable due to a large variation, although means were pronounced (LU: 36%; IR: 66%; RC: 75%). A possible explanation for the subordinate effect of HT in high DM silages could be attributed to the delayed effect on CP degradation, when HT extract was added before ensiling, compared to the DM treatment. Guo et al. (2007) concluded that an effective inhibition of proteolysis due to tannic acid (50 g/kg DM) was hardly detectable within the first hours after ensiling but more so in the following days, as the cross-linking process between tannic acid and N compounds was relatively time-consuming. As a consequence, an insufficient acidification immediately at the onset of the ensiling process may be reflected in a rapid increase of NPN compounds, without being noticeably affected by the added tannin extract. In contrast, an elevated DM content of ensiled forages by wilting decreases water activity directly and thus reduces the decarboxylation rate by proteolytic bacteria (Wieringa, 1958; Muck, 1987), which may mask the later onset of the HT effect.

4.4. Relationships between nitrogen compounds and short-term dry matter intake

Voluntary DM intake is generally closely related to the variations in the performance of ruminant livestock. However, silage DM intake by ruminants in detail is still poorly understood because of complex regulation mechanisms, related to dietary and animal factors (Huhtanen et al., 2002; Huhtanen et al., 2007; Grant and Ferraretto, 2018). It has been hypothesised that the presence of certain fermentation and CP degradation end-products in silages may contribute to a reduction in DM intake, particularly in cases of insufficient fermentation (Krizsan and Randby, 2007). However, the findings from studies conducted thus far have been inconsistent.

The present study therefore aimed to examine the potential influence of certain N compounds on short-term DM intake in a choice feeding design. As anticipated, silage DM intake by goats was diminished by elevated concentrations of total BA and $\text{NH}_3\text{-N}$, respectively, while NPN and free AA did not alter the preference or avoidance. With regard to NPN compounds, Krizsan and Randby (2007) also failed to observe any linear ($R^2 = 0.019$; $P = 0.24$) or quadratic ($R^2 = 0.03$; $P = 0.28$) relationships with DM intake of 24 different grass silages. This is consistent with the findings by Steen et al. (1998) and Scherer et al. (2019), who did not identify considerable correlations between DM intake and NPN or soluble N. Based on the close relationship between NPN and free AA concentration in the present study ($R^2 = 0.9241$; $p < 0.001$), the lack of an effect of free AA on DM intake appears plausible. The linear regression analysis indicated that an increase in ammonia nitrogen concentrations resulted in a decrease in silage dry matter intake by goats ($R^2 = 0.4828$; $P < 0.001$). Huhtanen et al. (2007) examined 240 relationships between $\text{NH}_3\text{-N}$ and silage DM intake, reporting a close negative regression ($R^2 = 0.89$; $P < 0.001$). In addition, a previous publication demonstrated a linear relationship between $\text{NH}_3\text{-N}$ and silage intake, although the equation could only account for 29% of the variation in DM intake (Huhtanen et al., 2002). Meanwhile, Steen et al. (1998) and Krizsan and Randby (2007) were only able to explain approximately 10% of the variation in silage intake. The comparatively higher dependence of DM intake on $\text{NH}_3\text{-N}$ proportion in the present study may be due to two factors. Firstly, very large differences in $\text{NH}_3\text{-N}$ concentrations occurred between silages. Secondly, a higher sensitivity to aversions can be assumed in feed choice behaviour experiments than in usual feeding experiments.

Several authors have emphasised that $\text{NH}_3\text{-N}$ is not a direct trigger for a lowered DM intake *per se* but rather an indirect indicator due to strong positive correlations to BA and other fermentation end-products (van Os et al., 1996a; Steen et al., 1998; Huhtanen et al., 2002). With regard to the impact of BA formation on silage DM intake, only few data are currently available.

In the present study, the total concentration of total BA in silages was found to be a suitable predictor of feed preference ($R^2 = 0.5146$; $p < 0.001$), as opposed to Krizsan and Randby (2007) who reported that linear or quadratic relationships between DM intake and total BA were not significant. However, the researchers concluded that histamine was a reliable indicator for DM intake, since the linear regression model was able to explain 43% of the variation in DM intake. However, Phuntsok et al. (1998) outlined that DM intake was most negatively correlated with the concentration of cadaverine, when LU silage was fed to steers ($r = -0.69$; $P = 0.01$). In general, a lowered palatability can be expected in poorly fermented silages containing high amounts of BA. Nevertheless, the addition of $\text{NH}_3\text{-N}$ or BA to well-fermented silages did not affect daily DM intake, indicating further interactions with other metabolites (van Os et al., 1995).

It is hypothesised that DM intake is also most likely diminished through negative effects of BA on chemostatic intake regulation, particularly when ruminants are not adapted to elevated BA concentrations in the diet (van Os et al., 1997). Besides the adverse impact on DM intake, BA has the potential to cause detrimental effects on animal health, when the amount consumed exceeds the amine-degrading capacity of rumen microbiota as well as amine oxidising enzymes in the intestinal wall. Consequently, BA are absorbed in the small intestine (van Os et al., 1997; Fusi et al., 2004). The ingestion of high amounts of BA have induced adverse effects, such as inflammation, on the histological characteristics of the rumen, the abomasum, the intestine, and the liver (Fusi et al., 2004). Furthermore, BA have been shown to induce neurological and cardiovascular disorders (Ladero et al., 2010). Nevertheless, ruminants can adapt to BA in the diet, with the duration of the experiment and the amount of ingested BA with silage may have an effect on the relation between DM intake and BA concentration (van Os et al., 1996a; van Os et al., 1997).

In the preference trials conducted by Hilgers et al. (under review; Chapter 4), the goats were permitted to select daily between two randomly offered silages. Moreover, the silages were fed only in the morning and a hay was fed in the afternoon. Consequently, a rapid adaptation to silages containing high amounts of BA appears improbable, which may provide an explanation for the strong correlation between total BA concentration and DM intake.

Multiple regressions yielded a marked enhancement in the prediction of DM intake in comparison with simple linear regressions. This finding suggests that individual components do not directly influence feed choice behaviour; rather, they serve as indicators for other fermentation end-products and metabolites. This assumption has been expressed by other

authors, who have noted that the inclusion of multiple parameters into regression equations results in more precise clarification of variations in DM intake (Huhtanen et al., 2002; Steen et al., 1998; Krizsan and Randby, 2007; Huhtanen et al., 2007). However, it must be underlined that the multiple regression shown in this study is comparatively accurate. Possible justifications for the accuracy could be the vast differences in terms of quality between the tested silages and thereby the aggravated adaptation to high concentrations of BA due to the variations in the offered feeds. Furthermore, the uniform environment and homogeneous group of animals most likely contribute to the accuracy of the regressions.

5. Conclusion

Extensive proteolysis occurred in almost all preserved forages. In addition to NPN, considerable increases in the concentrations of free AA, NH₃-N and BA were found in all silage treatments. Wilting plant material to a DM content of more than 300 g/kg, as well as applying HT at concentrations of up to 40 g/kg DM before ensiling, effectively decreased protein hydrolysis and thus maintained the protein value of the forages. However, the aforementioned methods employ diverse strategies to limit protein degradation. Increasing the DM concentration effectively reduced the catabolism of free AA and the resulting accumulation of BA and NH₃-N, whereas the influence on NPN or free AA content was not as pronounced than with HT addition. It was proven that the HT extract restricted not only the hydrolysis of protein to free AA and other NPN compounds, but also decreased deamination and decarboxylation as well as the associated end products: NH₃-N and BA. The effect of HT on protein degradation was generally more pronounced in low DM silages than in high DM silages. Furthermore, regression analysis indicated that N compounds resulting from protein degradation may be suitable indicators for predicting the preference or avoidance of ensiled high-protein forages by goats.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The author would like to express his sincere thanks to Claudia Parys and her colleagues at Evonik for their important contribution to this manuscript through their analyses of the biogenic amines and amino acids in all silages.

Fundings

This work was supported by the “Deutsche Forschungsgemeinschaft” (DFG, German Research Foundation, SU124/33–1).

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CHAPTER 6

General conclusions

The essential hypothesis of this thesis was that an increase in dry matter (DM) concentration by wilting before ensiling and the addition of a commercially available chestnut hydrolysable tannin (HT) extract, in defined amounts at ensiling would reduce the extent of proteolysis and amino acid degradation and, thus, improve the protein value of forages. An improved protein value in turn would lessen the need to supplement ruminant rations with protein feeds, thus contributing to an improved nitrogen (N) use efficiency (NUE) both at the animal and farm level, likely resulting in economic and environmental benefits.

The hypothesis was tested with the overall objective to evaluate the effects of different HT concentrations at two DM levels at ensiling on selected grass (Italian rygrass (*Lolium multiflorum* L.)) and legume forage crop species ((lucerne (*Medicago sativa* L.), red clover (*Trifolium pratense* L.)) with regard to the chapters:

- (1) Chemical composition, fermentation pattern, crude protein (CP) fractions and *in vitro* gas production. Special focus was directed on the fate of CP fractions caused by ensiling;
- (2) Short-term DM intake (DMI) and feed choice behaviour of goats in correlation with individual silage characteristics;
- (3) The formation of free amino acids (AA) and biogenic amines (BA) during the ensiling process.

From the outcome of the experiments the following general, summarizing conclusions and suggestions for further, targeted studies can be drawn:

(1) Increasing the DM content due to prolonged wilting time significantly improved the fermentation quality in high DM silages by reducing the production of undesirable fermentation products such as butyric acid. Furthermore, the accumulation of ammonia-N ($\text{NH}_3\text{-N}$) was limited, indicating a lower activity of proteolytic bacteria in high DM silages. Adding HT in a concentration of 40 g/kg DM to forage crops at ensiling was more effective than the DM treatment in decreasing the hydrolysis of protein. A linear decrease in gas production and organic matter digestibility was revealed by gradually increasing the HT extract dosage.

The results of the present study clearly demonstrate that external factors, such as HT, can effectively improve the protein quality of ensiled high-protein grass and legume forages, even

under unfavourable, challenging ensiling conditions. More research is needed, especially *in vivo* studies, to assess at which concentrations and ensiling conditions the beneficial properties of HT overcome adverse effects in order to define more precise application recommendations.

(2) The DM concentration of silages was the primary factor influencing DMI and feed choice behaviour of goats, regardless of the forage species. The higher acceptance of silages, due to an increase in DM concentration, was attributed to a substantially improved fermentation quality. The higher DM content was negatively related to most fermentation products, which were also associated with decreased DMI, such as acetic acid, propionic acid, butyric acid, propanol, butanol and $\text{NH}_3\text{-N}$. With regard to the use of HT extract, an enhanced DMI by goats was determined for low DM silages, which most likely can be justified on the basis of reduced CP degradation in HT treated silages. However, in silage with a higher DM level, this beneficial effect of HT could no longer be observed to the same extent. It appears that, at a certain point the negative properties of HT, i.e., the astringent taste and the impairment of nutrient digestibility, become more relevant. In this context, it is evident that HT can positively influence not only silage quality but also feed intake, at least in low DM silages.

Nevertheless, more research is needed to clearly identify under which environmental conditions and application rates the maximum benefit of HT is achieved. It must also be clarified to what extent the timing of HT application, i.e., before ensiling or before feeding, affects animal acceptance. According to the literature, tannins and their extracts also differ in terms of their perceptible astringency. An investigation of condensed tannins and HT with regard to their sensory acceptance could be one approach to overcome this adverse effect. Furthermore, the influence of the animal species needs to be further clarified, with a primary focus on dairy cows and beef cattle. With a more generalized scope, it is obvious that the number of fermentation products and other silage characteristics that can be analysed and detected will likely continue to increase in the future; on the other hand, the animal which perceives a multiple of these compounds by sensory impression and its feedback (shown by increased or decreased preference and intake) should not be neglected. Sensory characteristics of feedstuffs have a more important role than generally considered in the feeding behaviour of domestic ruminants. It is worth putting the animal itself and its preference behaviour in focus in further investigations, as it is the only possibility of covering all silage characteristics (sensory and post-ingestive feedback) the animals perceive.

In addition to focusing on preference behaviour, future work should also examine and evaluate the effects of silage treatments on metabolism, NUE and environmental impact in a more coherent manner in order to assess their actual potential.

In two unpublished feeding trials of our group conducted with dairy goats, a shift in N excretion was observed when the animals were fed ryegrass and lucerne silages with a HT addition of 40/kg DM. In general, a greater quantity of N was excreted via faeces, while a reduced amount of excreted N was observed in the urine and in form of milk urea. However, no substantial enhancement in NUE was observed in either trial. However, it should be noted that the rumen metabolism and the potential influence on methane formation as well as the environmental impact of excreted N were not examined in the present experiments. Consequently, no conclusions can be drawn from this work on these open questions, which requires further investigation if these knowledge gaps should be closed.

(3) Extensive proteolysis occurred in almost all preserved forages. In addition to NPN, considerable increases in the concentrations of free AA, $\text{NH}_3\text{-N}$ and BA were found in all silage treatments. Wilting plant material to a DM content of more than 300 g/kg, as well as applying HT at concentrations of up to 40 g/kg DM before ensiling, effectively decreased protein hydrolysis and thus maintained the protein value of the forages. However, the aforementioned methods employ diverse strategies to limit protein degradation. Increasing the DM concentration effectively reduced the catabolism of free AA and the resulting accumulation of BA and $\text{NH}_3\text{-N}$, whereas the influence on non-protein-N (NPN) compounds or free AA content was not as pronounced as with HT addition. It was proven that the HT extract restricted not only the hydrolysis of protein to free AA and other NPN compounds, but also decreased deamination and decarboxylation as well as the associated end products: NH_3 and BA. The effect of HT on protein degradation was generally more pronounced in low DM silages than in high DM silages. Furthermore, regression analysis indicated that N compounds resulting from protein degradation may be suitable indicators for predicting the preference or avoidance of ensiled high-protein forages by goats.

In this context, however, it remains to be clarified whether these indicators from ensiled high-protein forages can be transferred to other feedstuffs, ensiling conditions and animal species. Future studies should promote in-depth investigations on nitrogenous and non-nitrogenous volatile organic compounds (VOC), of which a wide variety are formed during different stages of ensiling, with concentrations ranging from 1 to 200 g/kg DM. To elucidate

currently inconsistent results regarding the role of VOC in their role in DMI, they should be added to a non-fermented diet just before feeding, so that there are negligible losses of VOC to the atmosphere. An in-depth quantification of metabolites formed in silage, in addition to routine chemical analysis and evaluation in preference and DMI trials, might provide a better picture of the role of silage fermentation compounds in preference and DMI. This also justifies further studies involving several forage crop species and treatments of harvested material before ensiling.

In order to gain a deeper understanding of the breakdown of free amino acids and the formation of ammonia, BA and other undesirable microbial metabolites, it is essential to take a closer look at the microbiota composition. The inclusion of the microbial level into the research, such as the taxonomic profile and the relative abundance of the bacterial microbiota at different time points throughout the ensiling period, could also offer promising approaches to better understand the interaction between HT, ensiling conditions and proteolytic bacteria. A detailed examination of the microbial and chemical changes that occur in the first few days after ensiling begins could also provide insights into the observation that the effect of HT on proteolytic activities becomes increasingly less significant at higher DM concentrations. Taking into account the various sources of tannins, it is therefore possible to gain a better understanding of the exact mode of action of tannins in general, particularly with regard to their protein-binding capacity, antibacterial effects or inhibition of plant and microbial enzymes.

(4) In addition to the beneficial properties of HT in terms of protein and overall feeding value of silages, potential limitations in the use of HT must also be considered for an objective evaluation of its practical use. One major limitation is the comparatively high application rate. For example, the dosage of tannin-containing products exceeds the application rate of a comparable chemical additive, such as formic acid, by a factor of around 10 to 20. These quantities can result in logistical as well as economic constraints that make practical application challenging.

Case scenario:

For lucerne silage with a DM concentration of 250 g/kg, an approximate application rate of 4 l/t FM formic acid (75%) is recommended. Based on a current price of 1.5 €/kg (September 2025), this results in a price of 6 € per t FM. With reference to the HT application rate of 40 g/kg DM (dissolved in water) to the identical plant material, the dosage would result in an

application rate for the mixture of 37.5 l/t FM at a price of 18 €/t. When considering bacterial inoculants, which are applied in quantities of less than one l/t FM and traded at a price of around 1 €/t FM, the difference becomes even more apparent.

Furthermore, the increased viscosity and sticky properties of tannin-containing products must be taken into account, as this puts greater demands on the dosing system. The low solubility of the powdered extract could also turn out to be a challenge in practice, as clumping is more likely to occur in cold water. For this reason, the liquid product proved to be the more practical option in this study, despite this compromising its transportability.

Although the practical application of tannins faces considerable challenges, this scientific work clearly demonstrates the enormous potential of secondary plant compounds for feed preservation. This provides motivation to seek solutions that are practicable also on a larger scale. Possible approaches could be found in the field of plant cultivation. Both, forage (e.g., birdsfoot trefoil (*Lotus corniculatus* L.)) and shrub legumes (e.g., Stylo (*Stylosanthes guianensis* L.)), contain significant amounts of condensed tannins, obviating the need for extra additions. However, these plants often tend to have a lower feeding value and yield potential than traditional forage plants such as ryegrass or lucerne, which still needs to be addressed from a plant breeding perspective. To exploit synergistic effects, more attention should be paid to integrating plants with secondary metabolites into grassland. In addition to the group of tannins, other secondary metabolites should also be taken into consideration, such as those identified in red clover, chicory (*Cichorium intybus* L.) and ribwort plantain (*Plantago lanceolata* L.). For example, in the studies presented ensiled red clover had around 40% less protein hydrolysis than ensiled lucerne probably due to its polyphenol oxidase activity. Clearly, the influence on proteolytic activities cannot be assured, even with the use of chemical additives. This highlights the potential that must be exploited if higher NUE is to be achieved in the context of livestock farming.

Perspectives

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 more silage compounds in routine forage analysis to obtain more data about possible relationships among specific compounds. To elaborate more profound causalities the respective features need to be better characterized and 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 fermentation and physiological processes. Silage production will remain a key part in ruminant production systems not only across Europe. This demands continuing approaches for production of high-quality silages involving improvements in management practise to minimise losses and maximise the preservation of the inherent feeding value of the parent crop.

Wilting mainly seems to induce positive responses on fermentation quality, which in turn has a positive impact on DMI of early harvested material treated with inoculants or untreated material. To further minimise the risk of clostridial fermentation, low DM grass should preferentially be wilted to a DM content of 300 g/kg. This is also the level of DM content often recommended giving smallest total losses, i.e., in the field, from fermentation, and from effluent. Changes in chemical composition during ensilage impact the energy supply to ruminal microbes. Paralleled with the increase in degradable N compounds it will affect the balance of absorbed nutrients. Adjusting the ratio of degradable protein and fermentable energy supply in the rumen has been suggested to be the best opportunity when trying to improve the efficiency of N use.

Animal nutrition research can help to improve resource use efficiency given that chemical analysis, *in vitro* methods and animal experiments are combined and used in a strictly standardized way. Focus should be laid upon a hierarchy of methods such that chemical characterization is the first step, if possible paralleled by *in vitro* laboratory methods aiming at closely mimicking *in vivo* conditions in (parts of) the animal's digestive tract. Animal experimentation comprises but is not restricted to quantifying the preference or avoidance of a certain feedstuff or ration, effects on the microbial community in the digestive tract, nutrient digestibility and post-digestive events.

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank everyone who contributed to the successful completion of this work.

Firstly, I would like to thank Prof. Karl-Heinz Südekum for giving me the opportunity to write this dissertation in his working group. I am grateful for your professional guidance and support, as well as for the valuable discussions on any kind of topics. Most of all, I have to thank you for your support during the last and probably one of the most difficult and frustrating phases of finalising this work.

I would also like to thank the members of the examination board, especially Prof. Hubert Spiekers, for kindly agreeing to supervise this work. My gratitude also goes to the chair of the examination board, Prof. Wolfgang Büscher, and to Prof. Katrin Gerlach for her contribution as a subject-related member and for introducing me to the project and for her expert support during our time together at the ITW.

I would also like to thank Dr. Saskia Kehraus and Ernst Tholen for her countless valuable suggestions and her willingness to assist in a wide variety of situations. Further words of gratitude go to Andreas Köster-Schmücker and the staff at Campus Frankenforst, Petra Jacquemien, and all other laboratory staff who analysed a seemingly endless number of samples over many months. In addition, I express my gratitude to thank Dr Christian Maack and the ILT and ITW workshop staff, without their contribution I would not have been able to conduct my experiments.

I would like to express my sincere gratitude to my colleagues, who became friends during our time together at the ITW. Dr Katharina Roth, Dr Haiko Hofmann, Dr Helge Deitmers, Ziade Abraha Kabsay and Prof. Dr Thomas Hartinger have helped me more than once and always supported me in difficult times. Of course, I hope that the wonderful and funny moments we experienced together will remain in my memory for a long time to come. I would also like to express my gratitude to Dr Valérie Schumacher, Dr Klara Schmitz and Dr Beatrix Bonhoff. I spent many great days and hours with you, which I remember with great fondness.

My final words are for my closest family. Without your unconditional support and constant encouragement, this work would not have been possible. Thank you for always believing in me and thank you for your patience during challenging times.