DISS. ETH Nr. 27153

The influence of dietary supplementation of temperate climate tannin-containing plants on digestion and nitrogen metabolism of dairy cows in low-input systems

A thesis submitted to attain the degree of
DOCTOR OF SCIENCE of ETH ZURICH
(Dr. sc. ETH Zurich)

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2020

The greatness of a nation and its moral progress can be judged by the way its animals are treated.

Mahatma Gandhi, 1869 — 1948

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List of Abbreviations

ADF	acid detergent fiber
ADL	ash-free acid detergent lignin
APDE	microbial protein from energy supply and rumen-undegradable protein
APDN	microbial protein from rumen-degradable prteon and rumen-undegradable protein
BW	body weight
CP	crude protein
C(R)	creatinine
CT	condensed tannins
DM	dry matter
ECM	energy corrected milk
HCl	hydrochloric acid
HGT	hohenheim gas test
HT	hydrolysable tannins
IVOMD	in vitro OM digestibility
N	nitrogen
NEL	netto energy lactation
NDF	neutral detergent fiber
NIR(S)	near infrared reflectance (spectroscopy)
NTP	non-tannin phenols
OM	organic matter
PC(A)	principal component (analysis)
PD	purine derivatives
TEP	total extractable phenols
TMR	total mixed ration
TT	total tannins
v/v	volume percent

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Summary

Phenols occurring in plants, especially tannins, are assumed to lower ammonia production in the rumen through binding on dietary protein. Plants with elevated tannin content could, therefore, be used instead of energy-rich diet compounds to lower the metabolic stress for cows in situations of excessive protein in the forage. This could save arable land for food instead of feed production and directs towards sustainable milk production. It was the objective of this thesis to test, if tannin-containing temperate climate meadow plants are able to influence the digestion and nitrogen metabolism of dairy cows and if this approach can be confirmed under practical conditions. One *in vitro*, two *in vivo* experiments on-farm with in total 59 dairy cows and one *in vivo* experiment on research station with 24 dairy cows were conducted during this project.

In the *in vitro* experiment, 35 temperate climate meadow plant species were investigated for their potential to reduce the ammonia formation using the Hohenheim Gas Test. The plants were harvested late during seed production and incubated together with *Lolium perenne* and *Medicago sativa* in a ratio of 0.3:0.4:0.3. They were also analysed for their nutrient and phenolic contents. A negative relationship between total extractable phenols, respectively total tannins and ammonia formation was found. The ammonia concentration in the incubation fluid, the gas production and the *in vitro* organic matter digestibility (IVOMD) were compared with the basal mixture, consisting only of *Lolium perenne* and *Medicago sativa* in a ratio of 0.57:0.43. Nineteen of the tested plants lowered the ammonia concentration in the incubation fluid and 17 plants had a higher IVOMD after 24 hours of incubation than the basal mixture. Among the tested plants, seven (*Galium verum, Leontodon hispidus, Lotus corniculatus, Onobrychis viciifolia, Plantago lanceolata, Sanguisorba minor* and *Scabiosa columbaria)* lowered the ammonia production without impairing the *in vitro* fermentation in general. This indicated their potential as beneficial feed supplements in situations of excessive protein in forage.

Two of the *in vivo* experiments were conducted on an organic low-input dairy cow farm during phases of protein-rich pasture, the first in autumn and the second in spring. In both experiments, all cows were kept together in one herd. In the first one, two of the three groups received daily 2 kg of sainfoin pellets and, with that, approximately 70 g of condensed tannins. One of these groups received the sainfoin pellets during 35 days, the other one intermittently during 2 x 5 days. The analysed parameters were compared to those of a third group, which was fed with ryegrass pellets instead and served as control. Small positive effects on the milk protein and fat yield, as well as higher ratio of purine derivatives to creatinine were found when offering the pellets intermittently over short periods. Both sainfoin treatments adversely impacted the estimated fibre digestion. The results of this first *in vivo* experiment suggest that the application of sainfoin pellets over only short periods is more efficient to influence the protein metabolism than an application over a longer period.

In the second *in vivo* experiment, ryegrass pellets (*L. perenne)* containing 0, 50 or 100 g/kg of chestnut tannin extract (*Castanea sativa* Miller), which consists mainly of hydrolysable tannins, were fed in the amount of 2 kg per day and cow. This resulted in approximately 32.2 and 106.6 additional total tannins per cow and day for the supplemented groups. The voluntary intake of pellets with 100 g/kg chestnut tannin extract was partly compromised in the cows. Feeding pellets containing chestnut tannin extract resulted in no measurable effects on the protein metabolism.

The third *in vivo* experiment was conducted on the research station of AgroVet Strickhof, equipped with facilities allowing to control feed intake and separate sampling of urine and faeces and therefore to determine the nitrogen balance. The experiment was conducted with two consecutive runs, each with 12 dairy cows. As test plants, *S. minor* (small burnet)*, L. corniculatus* (birdsfood trefoil) and *P. lanceolata* (plantain) were chosen based on the result of the *in vitro* experiment (Chapter 2). The experimental plants were mixed at 80 g/kg dry matter (DM) into a total mix ration consisting of grass and maize silage, protein-mix, hay and straw. The feed of the control group was supplemented with ryegrass (*L. perenne).* The mixtures were offered *at libitum* to three cows per run. Per day, the treatment groups (*S. minor, L.corniculatus, P.lanceolata*) had a higher intake of 306, 35 and 31 g total tannins, 38, 36 and 24 g condensed tannins and 268, 0 and 8 g hydrolysable tannins per day compared to the control group, which received ryegrass instead. With *P. lanceolata*, the milk protein content was impaired, whereas *S. minor* shifted the excretion of nitrogen from urine to faeces and reduced the milk urea content.

All three *in vivo* experiments were conducted considering farm practice conditions. It did not seem to be feasible, to feed higher amounts of experimental plants because of limitations from the economic (costs and profit), the animal (intake respectively palatability and performance) and the plant (availability and nutrient content) side. Anyway, integrating *S. minor* into the diet was successful and shifted the nitrogen excretion from urine to faeces. Therefore, this plant could be an interesting option for integrating it into dairy cattle diets to buffer excessive protein in forage.

Zusammenfassung

Sekundäre Pflanzeninhaltsstoffe, insbesondere Tannine, binden an Futterproteine und vermindern so die Ammoniakbildung im Pansen. Pflanzen mit erhöhten Gehalten an Tanninen könnten daher anstelle energiereicher Futterzusätze genutzt werden, um die Stoffwechselbelastung von Kühen während Eiweissüberschüssen im Futter vom Grasland zu reduzieren. Somit könnte wertvolle Ackerbaufläche für den Anbau von Nahrungs- statt Futtermittel genutzt werden und ein weiterer Schritt in Richtung Nachhaltigkeit bei der Milcherzeugung gemacht werden. Das Ziel dieser Dissertation war zu testen, ob mit tanninhaltigen Pflanzen aus der gemässigten Klimazone ein Einfluss auf die Verdauung und den Stickstoffstoffwechsel der Milchkühe erreicht und in die Praxis übertragen werden kann. Während diesem Projekt fanden ein *in vitro*, zwei *in vivo* Versuche auf einem Landwirtschaftsbetrieb mit insgesamt 59 Milchkühen und ein *in vivo* Versuch auf einer Forschungsstation mit 24 Milchkühen statt.

Während des *in vitro* Versuchs wurden 35 einheimische Wiesenkräutern und -leguminosen mittels Hohenheimer Futterwert-Test auf ihr Potential, die Ammoniakbildung zu reduzieren, untersucht. Die Pflanzen wurden in der Phase der Samenbildung geerntet und zusammen mit *Lolium perenne* und *Medicago sativa* im Verhältnis von 0.3 zu 0.4 zu 0.3 inkubiert. Ebenfalls wurden Analysen zu ihren Nährstoff- und Phenolgehalt durchgeführt. Die Untersuchungen ergaben einen negativen Zusammenhang zwischen dem Gehalt an extrahierbaren Phenolen bzw. Gesamttanninen und der Ammoniakbildung. Die Ammoniakkonzentration der Inkubationsflüssigkeit, die Gasproduktion und die *in vitro* Verdaulichkeit der organischen Substanz (IVOMD) wurden verglichen mit den Werten der Kontrollmischung, bestehend aus *Lolium perenne* und *Medicago sativa* im Verhältnis von 0.57 zu 0.43. Im Vergleich zu der Kontrolle, führten 19 der Testpflanzen zu einer geringeren Ammoniakkonzentration der Inkubationsflüssigkeit und 17 Pflanzen zu einer höheren IVOMD nach 24 Stunden Inkubationszeit. Sieben der analysierten Pflanzen (*Galium verum, Leontodon hispidus, Lotus corniculatus, Onobrychis viciifolia, Plantago lanceolata, Sanguisorba minor* and *Scabiosa columbaria*) verminderten die Ammoniakproduktion, ohne die *in vitro* Fermentation im Allgemeinen negativ zu beeinträchtigen. Sie könnten somit möglicherweise eine sinnvolle Futterergänzung bei Proteinüberschüssen im Grundfutter sein.

Zwei der *in vivo* Versuche fanden auf einem biologisch geführten Milchkuhbetrieb statt. Der erste wurde im Herbst und der zweite im Frühling durchgeführt, jeweils also während Zeiten mit hohem Eiweissgehalt der Weidegräser. In beiden Experimenten wurden alle Versuchskühe zusammen in einer Herde gehalten. Im ersten erhielten zwei der drei Versuchsgruppen 2 kg Esparsette pro Tag und damit ungefähr 70 g kondensierte Tannine. Eine der Gruppen erhielt diese Pellets während 35 Tagen, die andere während 2 x 5 Tagen mit einer Unterbrechung von einer Woche. Die analysierten Parameter wurden mit denjenigen einer dritten Gruppe verglichen, die Anstelle von Esparsette Raigras (*Lolium perenne*) in Pelletform erhielt und als Kontrollgruppe fungierte. Bei der Gruppe, die Esparsette über die

zwei kurzen Zeiträume erhielt, wurden gering positive Effekte auf Milchprotein- und Milchfettmenge pro Tag sowie ein höheres Purinderivat zu Kreatinin-Verhältnis im Harn nachgewiesen. Die Faserverdauung war bei beiden Esparsette-Gruppen im Vergleich zur Kontrollgruppe beeinträchtigt. Das Resultat dieses ersten Experiments lässt darauf schliessen, dass eine Verfütterung von Esparsettenpellets über eine kurze Zeitspanne einen deutlicheren Effekt auf den Proteinstoffwechsel hat wie die Verabreichung über einen längeren Zeitraum.

Im zweiten *in vivo* Versuch wurden Raigras-Pellets (*L. perenne*) mit 0, 50 oder 100 g/kg Kastanienextrakt (*Castanea sativa* Miller), der vor allem aus hydrolysierbaren Tanninen besteht, angereichert und in einer Menge von 2 kg pro Kuh und Tag verfüttert. Das führte zu einer erhöhten Aufnahme von 32.2 und 106.6 g Tanninen pro Tag und Kuh für die supplementierten Gruppen. Die Kühe verweigerten teilweise die Aufnahme der Pellets mit 100 g Kastanienextrakt/kg Pellet. Durch das Verfüttern der Pellets mit Kastanienextrakt konnten im Vergleich zu der Kontrolle keine messbaren Einflüsse auf die Stickstoffverwertung festgestellt werden.

Der dritte *in vivo* Versuch fand auf der Forschungsstation AgroVet Strickhof statt, wo die genaue Kontrolle der Futteraufnahme und die separate Sammlung von Kot und Urin und somit die Bestimmung der Stickstoffbilanz möglich war. Dieser Versuch wurde in zwei aufeinanderfolgenden Durchgängen mit je 12 Kühen durchgeführt. Basierend auf den Resultaten des *in vitro* Experiments wurden *S. minor* (kleiner Wiesenknopf), *L. corniculatus* (Hornklee) und *P. lanceolata* (Spitzwegerich) als Versuchspflanzen gewählt. Jeweils 80 g der Testpflanze wurden pro Kilo Mischration, bestehend aus Gras- und Maissilage, proteinreichem Kraftfutter, Heu und Stroh, eingemischt. In das Futter der Kontrollgruppe wurde die gleiche Menge Raigras (*L. perenne*) zugesetzt. Die Mischungen wurden pro Durchgang je drei Kühen *ad libitum* angeboten. Die Zugabe von *S. minor*, *L. corniculatus* und *P. lanceolata* und führte zu einer höheren Aufnahme pro Tag und Kuh von 306, 35 und 31 g Gesamttanninen, 38, 36 und 24 g kondensierten und 268, 0 und 8 g hydrolysierbaren Tanninen im Vergleich zu der Kontrollgruppe. Im Vergleich zu der Kontrollgruppe beeinflusste die Zugabe von *P. lanceolata* den Milchproteingehalt negativ. Mit *S. minor* konnte die Stickstoffausscheidung vom Harn in den Kot verschoben und der Milchharnstoffgehalt gesenkt werden.

Alle drei *in vivo* Versuche wurden im Hinblick auf die mögliche Umsetzung in die Praxis durchgeführt. Der Einsatz höherer Mengen der Versuchspflanzen erschien daher aufgrund der Ökonomie (Kosten und Profit), der Tiere (Schmackhaftigkeit und Leistung) und der Pflanzen (Verfügbarkeit und Nährstoffgehalte) als nicht praktikabel. Trotzdem erwies sich die Zufütterung von *S. minor* erfolgreich und führte zu einer Verschiebung der Stickstoffausscheidung vom Urin in den Kot. Diese bislang kaum beachtete Pflanze kann daher als interessante Option für die Integration in die Milchviehfütterung gesehen werden, um Proteinüberschüsse im Stoffwechsel abzupuffern.

1. General Introduction

1.1 Dairy cows on pasture

Since the domestication of cattle 10'500 years ago (Bollongino et al., 2012), their power was used for agriculture, their skin for leather production and their meat and milk for nutrition. In the year 2018, 97'157 tonnes of meat from cattle was consumed in Switzerland (Proviande Statistik, 2018) and in 2019, 682'909 dairy cows were kept in Switzerland (Bundesamt für Statistik, 2019). Meat is supplying many important nutrients (Pereira & Vicente, 2013) and milk stimulates muscle synthesis, prevents high blood pressure and even cancer, and its calcium is important for bone formation (Haug et al., 2007).

The use of cattle for food production has some advantages. Ruminants were through their whole evolution over millions of years adapted to grazing, even digest cellulose and turn non-protein-nitrogen into animal protein (Knaus, 2009). With this characteristic, they are able to convert plant nutrients also from non-arable land into valuable food for human nutrition (Engelhardt et al., 1985). If considering, that only 11% of the global land area is arable and thus limits the possible food production, permanent pastures (Engelhardt et al., 1985) making up the 23.6% of the area are a promising resource for increasing nutrient production with grazing animals. Switzerland with its extended grassland areas is predestined for feeding cows grassland based. Grassland is valuable for environment, it can improve the biodiversity, prevents desertification, soil erosion and climate change (Steinfeld et al., 2006), is as efficient as mixed feeding systems concerning carbon footprint (O'Mara, 2012) and could perhaps even help fight against antimicrobial-drug resistances (Auffret et al., 2017).

Grazing cows were found to be healthier, had lower levels of lameness and mastitis and also produced more milk compared to cows without access to pasture (Charlton & Rutter, 2017). The milk of grazing cows has a fatty acid composition which is more favourable for human nutrition and a higher content of vitamins than milk of cows which are fed with concentrate and conserved roughages (Haug, 2007). However, in the last decades, dairy cows were bred for high performance and productivity (Harrison et al., 1990; Dobson et al., 2007), resulting in up to 10'000 litres milk per lactation in Holstein cows (Dobson et al., 2007). The original idea had been to improve financial efficiency because the nutrient losses due to maintenance per cow can be distributed on more litres of milk (Gravert, 1985; Dobson et al., 2007). However, this performance cannot be reached with forage from grassland only (Knaus, 2013). If only fed from grassland, the maximum performance is approximately 7000 litres per lactation, depending on the breed (Dobson et al., 2007).

Dairy cows need a high energy density in feed, especially during the first lactation weeks as the peak of highest daily milk yield is reached in the second week but the one of highest feed intake not until week ten (Gravert, 1985). Energy is however the limiting resource in pasture situations (Kolver & Muller, 1998) and additionally, the nutrient composition of pasture grass varies during season and changes with year and management (Kuusela, 2004). Especially during spring and autumn, the protein

content of the pasture grass can be too high compared to the energy content (Pacheco & Waghorn 2008). This problem increases with the potential of dairy cows for high performance, as those cows have to mobilize even more body tissue during this negative energy balance (Pryce et al., 2004). Additionally, with higher performance, fertility and health of dairy cows are impacted (Pryce et al., 1999).

The usual way to avoid adverse impacts on health and performance is to balance the diet with energy- or protein-rich compounds, such as corn silage or concentrate (Hoffman et al., 1993). Since 1940, the production and transport was getting cheaper and therefore affordable to feed concentrate to ruminants (Corah, 2008). However, besides the assumption, that these feed components may negatively impact digestion (Krajcarski-Hunt et al., 2002), it means producing feed for ruminants on the limited productive arable land. This is a net loss for the potentially possible global food supply (Cassidy et al., 2013; Knaus, 2013) and is ecologically not sustainable (Knaus, 2009). For example, more than 80% of protein components for diets of livestock animals in Switzerland are imported and 60% of them derives from soybeans (Grüter, 2016). In the exporting countries, the intensive production of soybean for livestock feeding leads to destruction of natural habitats (Fearnside, 2001). The difference between the growth place of concentrate compounds and the place, where the manure accumulates leads to a separation of the nutrient cycle (Taelman et al., 2015).

Further, the conversion rate of the dietary protein into animal protein has to be considered. It needs four to five times more dietary protein to produce animal protein in form of milk, and the conversion rate with about 5% for producing beef is even much lower (Engelhardt et al., 1985). The feed conversion and milk production efficiency is not depending on the size of dairy cows when fed from pasture (Hofstetter et al., 2011). Each dairy cow excretes – depending on the farm system, nitrogen intake, and performance level – 90 to 150 kg of non-utilized nitrogen per year (Verite & Delaby, 2000). The inefficient use of nitrogen of 0.33 to 0.44 of the input, depending on the farm system (Akert et al., 2020), contributes to the environmental pollution with nitrogen (Broderick, 2006) and means a direct loss of valuable protein resources when producing milk. This can not be completely avoided, although breeding for conversion efficiency could be successful, as it already led to differences between wild and domestic animals (Clauss et al., 2010). However, as mentioned above, ruminants are able to efficiently use and even produce valuable edible protein from protein-poor diets (Broderick, 2006). Together with the reduction of consumption of animal derived food products, to feed dairy cows directly from grassland is therefore an easy way to save resources, lower the environmental impact of livestock and increase the potential for food production with saving cropland (Schader et al., 2018). These cows however have to be bred for this system to reach a performance adequate to the supply of the grassland, and the grazing management has to be adapted. If this is realised, cows are not negatively impacted, even if they do not get concentrate (Ertl et al., 2014; Leiber et al., 2017).

1.2 The protein metabolism of cattle

The metabolizable protein for ruminants is consisting of rumen-bypass protein from the diet and protein of rumen microbes (Kamalak et al., 2005, Figure 1). The bypass protein is not degraded through rumen microbes (Kamalak et al., 2005; Jin et al., 2018). Especially lactating and growing cattle need bypass protein in addition for their performance (Kamalak et al., 2005).

Forty to eighty percent of the dietary protein, depending on kind of protein, solubility in rumen liquid and length of retention time in the rumen, is converted into microbial protein in rumen (Chalupa, 1975). For example, the degradation of protein from lucerne occurs faster than that of protein from *L. corniculatus* (Gierus et al., 2007). Rumen microbes degrade amino acids and proteins of the diet with proteases, deaminases and ureases to ammonia, which is the primary N-containing nutrient for their development (Chalupa, 1975, Jin et al., 2018). The rumen protozoa in addition use the bacterial amino acids for their growth (Chalupa, 1975). This process synthesizes a large proportion of the animal's required protein, as rumen microbes pass through the abomasum to the intestine, where they are digested (Broderick, 2006). It allows the cattle to use also non-protein nitrogen in the diet, such as urea (Chalupa, 1975; Jin et al., 2018) and to survive and even perform with diets containing only urea and ammonium salts (Virtanen, 1966).

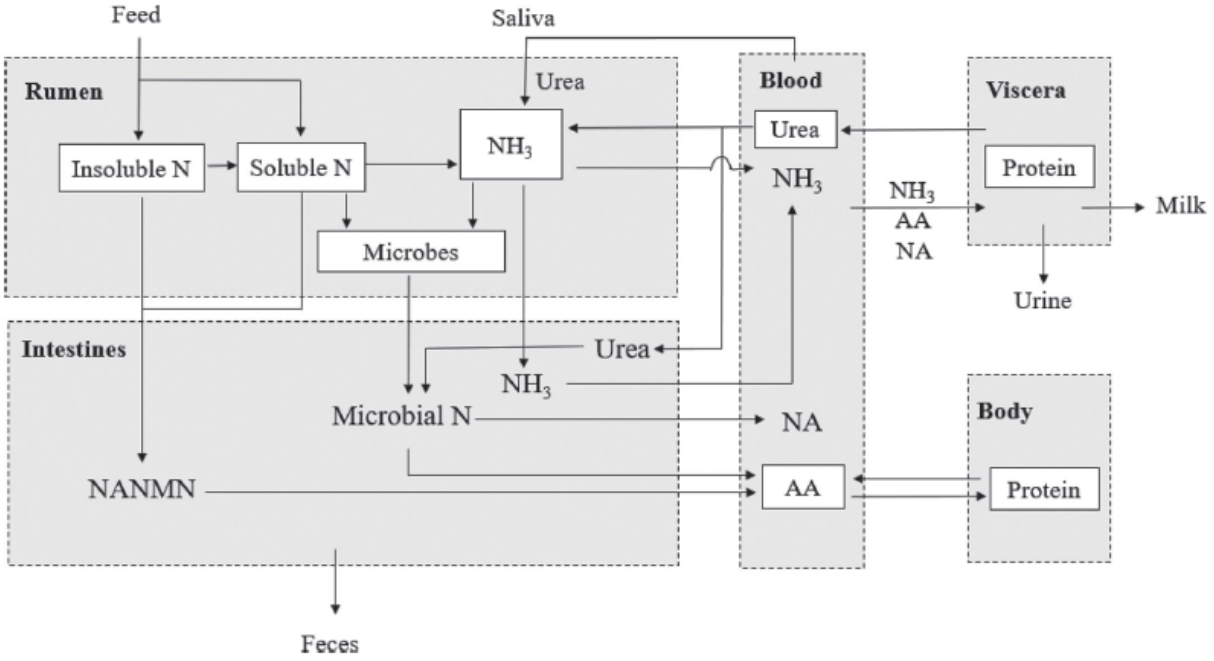

Figure 1. A schematic diagram of the nitrogen metabolism. NANMN= non-ammonia, non-microbial nitrogen; NA = nucleic acids. Boxes with dashed lines are compartments; boxes with solid lines represent pools; arrows indicate fluxes (taken from Li et al., 2019).

However, the rumen microbes are reliant on sufficient energy supply (Chalupa, 1975; Knaus, 2013). If the energy content in diet is too low compared to the content of degradable protein, or the protein degradation in rumen is more rapid than the ammonia can be used for microbial growth, ammonia accumulates in rumen (Cotta & Russell, 1997). It is then absorbed through the rumen wall into

the blood circulation and transformed in the liver to urea under energy consumption (Parker et al., 1995; Cotta & Russell, 1997).

One part of this urea is recycled back with blood circulation to the digestion system via saliva and to the gut (Kennedy & Milligan, 1980, Batista et al., 2017). There, it serves again as nitrogen source for rumen microbes (Reynolds & Kristensen, 2014). The other part is excreted via urine and milk (Reynolds & Kristensen, 2014). Increasing milk urea contents reflects the inefficiency of nitrogen utilization (Nousiainen et al., 2004). The excretion route via urine or faeces depends also on the composition of the diet (Weiss et al., 2009). The excretion of nitrogen via urine is more problematic than excretion via faeces, as the urea in urine is easier transformed to volatile N compounds like urea, nitrate and nitrous oxide (Thompson & Fillery, 1997).

Generally, a correlation between the amount of synthesized urea and the content of crude protein in the diet, respectively the ratio of crude protein to organic matter in the diet exists (Batista et al., 2017). The higher the crude protein content in diet, the higher is the part of urea nitrogen in total urinary nitrogen (Nennich et al. 2006; Batista et al., 2017). The best way to increase the supply of protein to the cow while reducing losses to the minimum, is to maximize microbial protein formation in rumen. The microbial protein is supported through an higher energy supply while minimizing the crude protein in diet (Broderick, 2006). A protein excess situation regularly occurring is that found seasonally in pasture-based diets. This situation means a burden for the cow's metabolism and for the environment.

To reach a better protein supply while reducing losses, manipulating of diet compounds through heat-processing, chemical treatments and encapsulate amino acids or structural manipulation of amino acids to bypass the rumen metabolism were tried (Chalupa, 1975). However, if the advantages of ruminants for food production should be used, a way to deal with the natural occurring imbalances of grassland-based diets has to be found, which can be managed without adding feed produced on croplands.

1.3 The use of tannins in diets for ruminants

Plant compounds are subdivided into primary and secondary plant compounds. Primary plant compounds are essential for growth and reproduction of the plant, whereas secondary plant compounds are important for other functions, for example for attracting the attention of insects for pollination (Dudareva & Pichersky, 2000) or defence against herbivory (Bennett & Wallsgrove, 1994).

One group of secondary plant phenols are the phenols, which can be again divided into non-tannin and tannin-phenols. The tannin-phenols, defined through their protein-binding ability and water-solubility compounds, are grouped into condensed tannins or proanthocyanidines, respectively, and hydrolysable tannins (Constabel et al., 2014). However, these groups are overlapping and some tannins can be assigned to both groups (Makkar, 2003). Tannins are important for the plant concerning defence against insects and also mammals, inhibit bacteria and influence whole ecosystems (Constabel et al.,

2014). Hydrolysable tannins are structurally very diverse, including galloyl glucoses and ellagitannins and occur only in dicotyledonous plants. Condensed tannins are structurally more uniform, they occur also in monocotyledonous plants and are oligomers and polymers of flavan-3-ols (Constabel et al., 2014). There are species, where both, condensed and hydrolysable tannins are produced (Constabel et al., 2014), whereas in other species no tannins at all seem to be formed (Mole, 1993). Condensed tannins occur in the vacuole of the plants (Lees et al., 1993), whereas hydrolysable tannins are affined to cell walls (Grundhöfer et al., 2001). The concentration of tannins in a plant species is also depending on environmental conditions (Constabel et al., 2014). However, for a final verification of the occurrence of tannins respectively the lack of them the analytical method has to be chosen carefully (Schofield et al., 2001; Tarascou et al, 2010).

To predict the effect of tannins on the individual animal species is, based on their both complexities, very difficult (Barbehenn & Constabel, 2011; Constabel et al., 2014). Tannins are known to bind *in vitro* to protein depending on their structure, molecular weight and the pH of the environment (Barbehenn & Constabel 2011). For example, the diet of the brushtail possum consists of varying amounts of eucalyptus rich in tannins, resulting in a poor digestion of protein and probably even influences the general fitness of this animal species (DeGabriel et al., 2009). In ruminants, tannins are also known to reduce the digestibility of proteins, for example in moose (McArt et al., 2009), but not for all ruminants to the same degree. Some browsers and also other mammalian herbivores have enzymes in the saliva, which degrade or bind tannins to ineffective molecules (Shimada, 2006). Cattle as grazers did not develop these enzymes (Shimada, 2006), as tannins rarely occur in grass species (Waghorn & McNabb, 2003).

Consumed with the diet, tannins reach the rumen environment of cattle without being degraded (Figure 2). The protein-binding reaction there depends on different factors. First, it depends on the chemical structure of the plant phenol (Patra & Saxena, 2010), as they largely differ resulting in different biological activities. Second, it depends on different factors of the environment, such as pH of the medium, type of protein and other plant compounds (Perez-Maldonado et al., 1995; McNabb et al., 1998; Min et al., 2002; Min et al., 2005). This makes it difficult to predict possible effects on the nitrogen turnover in cattle. If binding between tannin and protein is successful, proteins could be prevented from degradation to ammonia through rumen microbes. Especially in situations with protein excess in the diet, this effect could be used to lower the ammonia production. In the acidic pH of the abomasum, the bond may be cleaved (McNabb et al. 1998; Piluzza et al., 2014) and, if so, the proteins get available for digestion in the small intestine and perhaps even increase the protein conversion efficiency (Min et al., 2003). However, if this cleavage does not occur, the tannin-protein complexes are at least excreted with faeces (Dschaak et al., 2011) which helps maintaining a closed farm nitrogen cycle. This may reduce the loss of nitrogen as urea which is advantageous for the cow's metabolism, the environment and also from an economic perspective (Constabel et al., 2014).

In low concentrations of 20 – 40 g/kg in dry matter (DM), condensed tannins are known to prevent bloat and improve protein metabolism through shifting its digestion into the intestine, but at high concentrations from 60 – 120 g/kg DM, they can decrease consumption, digestion and performance (Aerts et al., 1999; Min et al., 2003). They may be beneficial for the milk production concerning yield and protein (Huyen et al., 2016), help lowering the milk urea content (Dschaak et al., 2011), and shift the nitrogen excretion from urine to faeces (Min et al., 2003; Chung et al. 2013; Wischer et al., 2014; Huyen et al., 2016).

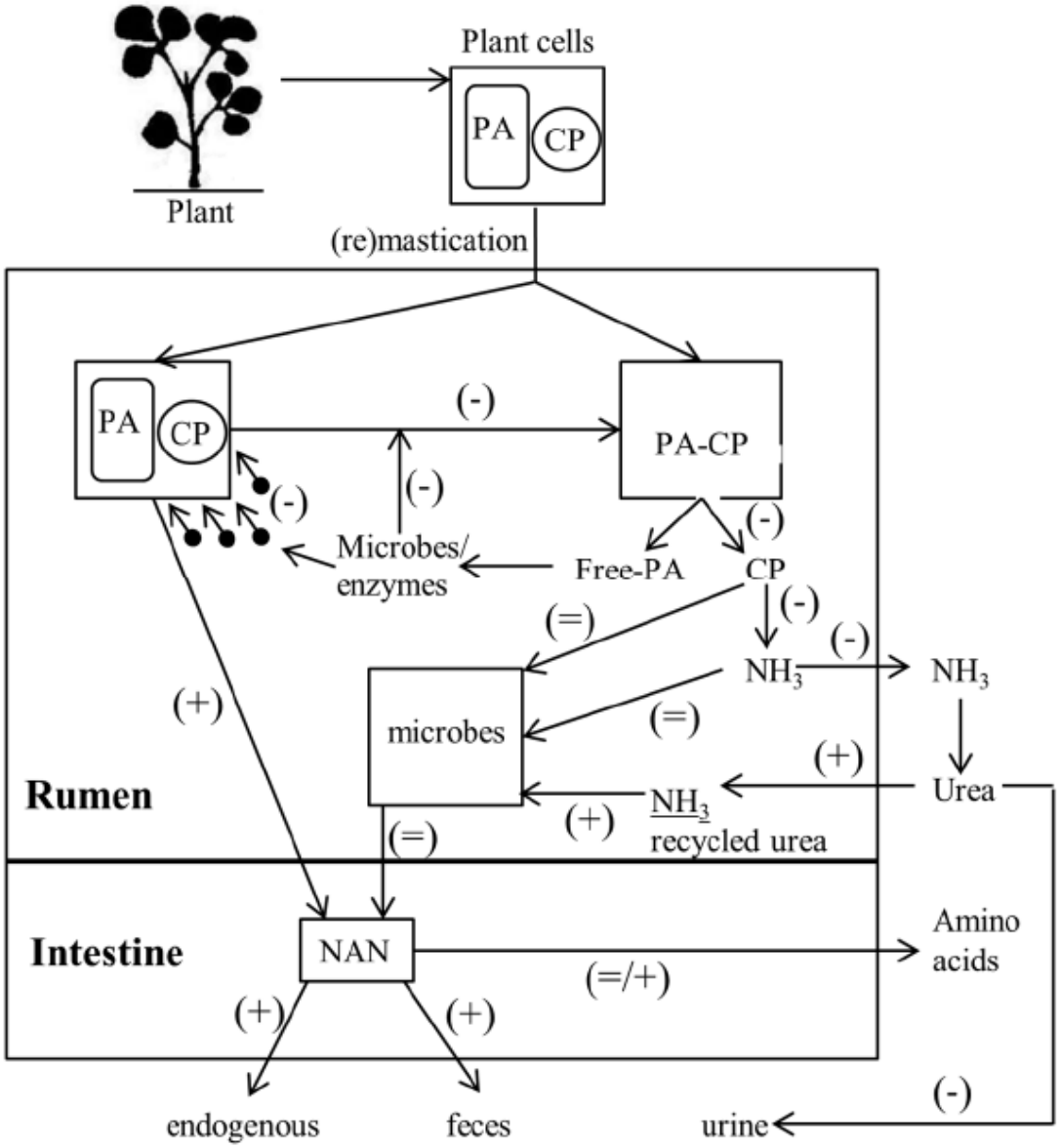

Figure 2. Schematic flow chart of crude protein (CP) digestion from proanthocyanidin (PA)-containing forage. Symbols between brackets represent the effect of PA-containing vs. PA-free forage on protein flow: + represents increased flow, - represents decreased flow and = represents similar flow. NAN: non-ammonia N (taken from Jonker & Yu, 2017).

For hydrolysable tannins, it is also known, that they reduce the ruminal protein degradation and ammonia production *in vitro* without influencing the efficiency of microbial protein synthesis negatively (Wischer et al., 2013). They reduce nitrogen losses over urine, milk (Sliwinski et al., 2004) and manure (Sliwinski et al., 2004; Duval et al., 2016). They are also known to reduce the methane emissions (Duval et al., 2016). Supplemented hydrolysable tannins had no (Sliwinski et al., 2004; Duval et al., 2016) or a positive effect (Ali et al., 2017) on the milk yield.

In many studies, no toxic effects or depression of feed intake of tannins were reported (Sliwinski et al., 2004; Duval et al., 2016). However, other studies found reduced feed intake or toxic effects such as ulcers when diets were supplemented with high concentrations of tannins (Neser et al., 1982; Hervás et al., 2003). For having the desired effects without negative impacts, a carefully choice of tannins and many experiments are needed.

1.4 Objectives and thesis outline

In this thesis, the possibility of mitigating the impact of diets with an excess of protein on cattle and environment, through supplementing temperate climate feed additives was investigated. One *in vitro* and three *in vivo* experiments, on-farm and on research station were conducted. The first experiment was an *in vitro* comparison of 35 different plants harvested at a late growth stage concerning their nutrient and phenolic content. Additionally, they were incubated in the Hohenheim Gas Test to investigate their influence on ruminal gas and ammonia formation (Chapter 2). The second experiment was on-farm, investigating the effect of sainfoin supplementation with two different application durations under practical condition (Chapter 3). The second *in vivo* experiment was also done under on-farm conditions. The supplemented pellets contained two different amounts of chestnut tannin extract (Chapter 4). The third *in vivo* experiment was done on a research station, based on results of the previous *in vitro* test. The supplemented test plants were small burnet, birdsfoot trefoil and plantain, (Chapter 5). The following research questions were investigated:

Experiment 1: Mature herbs as supplements to ruminant diets: effect on *in vitro* ruminal fermentation and ammonia production (Chapter 2)

- How differ temperate climate herbs and legumes in the period of seed ripening concerning their nutritional and phenolic composition?
- Are there differences between the plants in their efficiency to reduce *in vitro* ammonium production in rumen fluids, while maintaining digestibility?
- Which of these plants could be beneficial for supplementing diets of cattle during protein excess in grassland?

Experiment 2: Short-term versus long-term sainfoin supplementation of dairy cows in a low-input feeding system: effects on protein utilization (Chapter 3)

- Does adding sainfoin pellets to the diet of dairy cows improve the nitrogen metabolism also under practical conditions?
- Is a shorter or longer application of sainfoin more efficient for reaching a positive influence on the nitrogen metabolism?
- Can adding sainfoin to the diet during protein excess in grassland be recommended in practice?

Experiment 3: Graded supplementation of chestnut tannins to dairy cows fed protein-rich spring pasture: effects on indicators of protein utilization (Chapter 4)

- Does adding pellets containing chestnut tannin extract to the diet of dairy cows improve protein digestion and metabolism?
- Can different effects be observed, if the pellets contain 50 or 100 g/kg dry matter (DM) of chestnut extract?
- Is this a practicable approach under on-farm conditions during excessive nitrogen supply from spring pasture?

Experiment 4: Efficiency of small burnet, birdsfoot trefoil and plantain in limiting nitrogen losses by dairy cows in a low-concentrate feeding system (Chapter 5)

- Can an effect on the nitrogen metabolism of dairy cows be reached with adding those plants at 80 g/kg DM to the diet?
- Are there effects of the test plants on the nitrogen metabolism of dairy cows and are they different between the treatments?
- May this be an approach for improving the nitrogen metabolism during protein-rich forage-based winter diets?

2. Mature herbs as supplements to ruminant diets: effect on *in vitro* ruminal fermentation and ammonia production

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Animal Production Science, accepted.

2.1 Abstract

High concentrations of crude protein in ruminant diets may lead to excessive ruminal ammonia production, which may stress the animal's metabolism and impact nitrogen efficiency. This may become a problem in zero-concentrate feeding systems when pasture grass is rich in crude protein. Polyphenols like tannins may protect part of dietary protein from ruminal degradation and thus inhibit ammonia formation. The current study screened phenol-rich mature herbs for their potential to mitigate ruminal ammonia formation in cattle, when supplemented to a forage diet. Thirty-five temperate climate meadow plant species (32 herbs and three legumes), which appear in biodiverse natural and sown pastures, were investigated for their effects on ruminal ammonia production. The above-ground material was harvested during ripening of the seeds and analysed for nutrient proximates and phenol concentrations. Net energy and protein absorbable at the duodenum were calculated. Incubations (24 h) with cattle rumen fluid following the in vitro Hohenheim Gas Test protocol were carried out to compare the effects of the test plants on ruminal gas and ammonia formation. Test plants replaced one third of a basal mixture of proportionately 0.57 *Lolium perenne L.* and 0.43 *Medicago sativa L.* (air-dry matter basis). Results were compared to those obtained with the basal mixture alone. According to regression analysis, ammonia concentration after incubation was negatively related to concentrations of total extractable phenols and total tannins in feed mixtures, while the relation was weakly positive with dietary crude protein. In vitro gas production (indicating ruminal organic matter digestibility) and ammonia concentrations in the incubation medium after 24 h were significantly lower in 23 and 19 of the test diets compared to the basal mixture, respectively. Incubations containing *Galium verum, Leontodon hispidus, Lotus corniculatus, Onobrychis viciifolia, Plantago lanceolata, Sanguisorba minor* and *Scabiosa columbaria* maintained gas production and thus estimated in vitro organic matter digestibility, while lowering ammonia concentrations. Seven mature herbs out of a screening of 35 proved a potential to positive effects on ruminal protein utilization without impairing fermentation. These herbs are of particular interest as supplements to dairy cows grazing protein-rich pastures.

2.2 Introduction

When cattle consume diets excessive in rumen-degradable crude protein (CP), the net ammonia production in the rumen may rise due to the fact that fermentable carbohydrate and thus energy supply from these diets limit rumen microbial protein synthesis. As excessive ammonia is absorbed from the rumen, it leads to a burden for the ruminant's metabolism and, additionally, to environmental pollution with easily soluble nitrogen-containing compounds excreted via their urine (Reynolds and Kristensen, 2014; Sinz et al., 2019a). Excess dietary rumen-degradable CP supply occurs for example in low-input ruminant systems in autumn, when grasses with high CP concentrations but limited energy content are abundant (Pacheco and Waghorn, 2008). An alternative approach to compensate for high-protein pasture forage and forage conserves builds on including plants with elevated concentrations of secondary

compounds, such as tannins, in the diet of ruminants. Tannins bind to feed proteins, whereat the bond formation depends on factors such as pH of the medium, type of protein and other plant compounds (Perez-Maldonado et al., 1995). Accordingly, tannin-protein complexes are formed under ruminal pH conditions. These complexes hamper rumen microbial crude protein degradation and thus decelerate ammonia formation. Part of these tannin-protected proteins may be released in the abomasum and digested in the abomasum and small intestine (Pilluza et al., 2014). By this they can contribute to covering the ruminant's amino acid requirements. Alternatively, the complexes remain, or are re-established under small intestinal pH conditions, and the protein is excreted with the faeces (Dschaak et al., 2011). Based on these mechanisms, cattle grazing on herb-rich pastures instead pastures poor in species may increase their protein utilization (Totty et al., 2013). Together with further implications for animal health and welfare (Leiber et al., 2020), this should encourage farmers trying to establish herbs as supplements to cows grazing young grass in spring or autumn. However, it is important to know first the extent to which suitable herb species would contribute to improving protein digestion in ruminants.

A number of screenings of herbal species concerning their nutrient and phenolic contents have been made. Examples are the studies by Macheboeuf et al. (2014), who analysed a large collection of wild plants grown in the French Massif Central Area, Jayanegara et al. (2011), who investigated a number of alpine forages, and Terranova et al. (2018) who screened woody plants grown in temperate climatic conditions. Still data on temperate climate meadow herbs and legumes are scarce. They typically occur on swards established to generate a high biodiversity and are harvested late, often accomplished during seed ripening. In order to assess the suitability of mature herb-rich swards as supplements that influence protein digestion, plants need to be investigated specifically at this mature stage, as concentrations of phenols can either increase or decrease during plant development, depending on species and other factors (Kälber et al., 2014; Stewart et al., 2019).

The present study was conducted to assess a large number of temperate climate herbs and legumes harvested during seed ripening stage. They were compared with respect to their nutrient and phenolic concentrations and their efficiency to reduce in vitro the ammonia concentration in rumen fluid, while maintaining organic matter (OM) digestibility when added to a standard diet. The overall aim was to identify plant species particularly promising at late harvest. Herbs with low rumen ammonia production and high ruminal fermentation rates could then be used as supplements for cattle in periods of dietary CP excess.

2.3 Materials and methods

Swiss ecotypes of 35 plant species from 13 plant families (as listed in T 1) were used. They had been grown as pure cultures in Lenggenwil, Switzerland, located at 47°28'31.34'' N, 9°11'11.67'' E and 580 m above sea level. The harvest took place from July to August 2016, a time when the seeds of the plants ripened. Per plant species, between 5 and 10 kg of wet weight was sampled by cutting 1 cm above

ground, divided in three different batches, dried at 48 °C for 24 h, and milled through a 0.5-mm sieve (Retsch SK 100, Retsch®, Haan, Germany). Pure *Lolium perenne* and *Medicago sativa* were also harvested during the ripening period (first cut from seed production fields) and treated the same way to form the basal mixture.

All analyses were repeated three times per individual plant species by using the different batches of the harvested material, resulting in n = 3 per test plant species. In the dried material, concentrations of dry matter (DM), total ash, CP, crude fiber as well as neutral (NDF) and acid detergent fiber (ADF) were determined with near infrared reflectance (NIR) spectroscopy (NIR-Flex N-500, Büchi, Flawil, Switzerland). This equipment had been previously calibrated with 180 samples from different mixed grass-herb swards analysed by wet chemistry in parallel. For each variable and plant species, the arithmetic mean of three NIR determinations was used. The OM was calculated as DM minus total ash. Using compositional data, net energy for lactation (NEL) and absorbable protein at the duodenum were estimated using regressions of Agroscope (2020). The absorbable protein at the duodenum is either limited with the sum of microbial protein from energy supply and rumen-undegradable protein (APDE) or to the sum of microbial protein from rumen-degradable crude protein and rumen-undegradable protein (APDN; Agroscope, 2020; Colin-Schoellen et al., 2000).

For analyzing total extractable phenols (TEP), 60 mg of the ground plant material was extracted with 6 mL of 70% aqueous acetone (v/v). The supernatant was filtered (Cameo™ syringe filter™, non-sterile, pore size 1.2 µm). From this extract, 0.02 mL respectively 1.0 mL were taken to determine the amount of TEP and the non-tannin phenols (NTP) with the Folin-Ciocalteu method described in detail in the laboratory manual of Makkar (2003). Absorption was measured at 725 nm with a spectrophotometer (Bio Spectrometer Eppendorf D30, Eppendorf AG, Hamburg, Germany). Total tannins (TT) were calculated as the difference of TEP and NTP, and given as equivalents of tannic acid. Condensed tannins (CT) were analysed following the protocol for the butanol-HCl-assay (Makkar, 2003). Hydrolysable tannins (HT) are equivalent to the difference of TT and CT. Absorption was measured at 550 nm and given as leucocyanidin equivalents.

As a control, the basal diet, consisting of *L. perenne* and *M. sativa*, was incubated at a ratio of 0.57:0.43 (114 mg and 86 mg DM) according to the method of Menke and Steingass (1988). In addition, each individual of the 35 test plants was incubated together with the basal diet (*L. perenne* and *M. sativa*) in a ratio of 0.30:0.70 (test plant : basal diet). Two-hundred milligrams of the 36 combinations were incubated in a Hohenheim Gas Test apparatus on two different days (two runs) in three syringes each, leading to a total of six observations per plant. Along with the test mixtures, in each run, six blank syringes and three syringes each filled with either hay or concentrate as Hohenheim Gas Test standards were incubated for later adjustment of the different runs (Menke and Steingass, 1988). Rumen fluid was taken before the morning feeding from a total of four different cannulated lactating Jersey cows (mixture from two cows per run). These cows had been fed with a total mixed ration containing grass and maize

silage, grass hay, barley straw and a concentrate mixture at a forage : concentrate ratio of 0.68:0.32 (on DM basis). Housing of and rumen fluid collection from the rumen-cannulated cows were approved by the Regierungspräsidium Stuttgart, Germany (licence no A401/14 TE). After filtration of the rumen fluid, a buffer solution was added to stabilize the pH. An amount of 30 mL of this mixture was filled in the pre-warmed syringes and air was removed. Subsequently, the syringes were placed into a water bath at 39°C for 24 h. The syringes were gently shaken every hour but this only during the first 6h of incubation. When the gas production exceeded 70 mL after 8 h, syringes were reset to 35 mL by releasing part of the gas produced. After 24 h, the total amount of gas produced was recorded and the incubation fluid was filled into sediment tubes for ammonia analysis by a pH-meter (Model 713, Metrohm, Herisau, Switzerland) equipped with an ion-selective electrode. The ammonia value was corrected for the concentration in the blanks. To calculate the net gas production, the mean gas volume produced from the syringes only containing buffered rumen fluid (i.e., blanks) was subtracted from the volumes measured with the respective test diets. This result was adjusted using a correction factor calculated from the observed and expected net gas productions from the standard hay and standard concentrate. In vitro OM digestibility (IVOMD) was calculated based on the compositional data and the net gas production (Menke and Steingass, 1988) as

IVOMD (mg/g OM) = 148.8 + 8.893 × gas production (mL/200 mg DM) + 0.448 × dietary CP (mg/g DM) + 0.651 × dietary total ash (mg/g DM).

For the statistical analysis, SPSS® Statistics version 24 was used. For the in vitro data, the 35 diets containing the test plants were treated as fixed effects and the two incubation runs in the Hohenheim Gas Test as random effects (n = 6 per test-plant based diet). For assessing differences between the basal mixture and the means obtained with the test plants, Tukey's procedure was used, and $P < 0.05$ was considered to be significant. In addition, a principal component analysis (PCA) was performed including all available data from the 35 test diets (total n = 3 × 35 for nutrient and phenol concentrations and IVOMD and n = 6 × 35 for gas production and ammonia concentration). For this purpose, the compositional data were calculated for the respective diets from composition and proportions of test plants and basal mixture. The first two principal components were used to calculate the factor scores for each plant. Finally, based on the data averaged per test-plant based diet, a regression analysis was performed with gas production and ammonia concentration of the inoculum after 24 h incubation as dependent and CP, TEP, and TT as independent variables. The analyses were at first performed with linear, quadratic, exponential, and logarithmic terms. The best fitting model was displayed in the results, considering $P < 0.05$ as significant. If none of the models was significant, the linear regression was indicated.

2.4 Results

2.4.1 Chemical composition of the test plants

Concentrations of OM covered a range from 832 g/kg DM (*Silene nutans*) to 973 g/kg DM (*Stachys officinalis*, Table 1). The median was at 919 g/kg DM. The CP concentrations ranged from 72 g/kg DM for *Leucanthemum vulgare* to 204 g/kg DM for *Campanula rapunculoides* with its median was 120 g/kg DM. The concentration of crude fiber was highest in *L. vulgare* with 422 g/kg DM and lowest in *C. rapunculoides* with 175 g/kg DM. The median was 304 g/kg DM. The NDF content was very low in *C. rapunculoides* with 115 g/kg DM. The highest NDF content was found with 744 g/kg DM in *Picris hieracioides*, the median was at 452 g/kg DM. The median of ADF was 382 g/kg DM. The lowest ADF content was 84 g/kg DM as determined in *Knautia arvensis*, the highest 555 g/kg DM in *P. hieracioides*. The highest calculated contents of NEL (5.10 MJ/kg DM), APDE (92 g/kg DM) and APDN (131 g/kg DM) were found in *C. rapunculoides*, the lowest NEL (1.47 MJ/kg DM) and APDE values (35 g/kg DM) were calculated for *L. vulgare*. The level of APDN was lowest in *L. vulgare* and *Gallium mollugo* (both 45 g/kg DM). The medians for these variables were 3.45 MJ NEL/kg, 66 g APDE/kg DM and 76 g APDN/kg DM. A large number of individual plants differed in composition ($P < 0.05$) from the basal mixture (values given in bold in T 1).

The highest concentrations of TEP and NTP were detected in *Origanum vulgare* with 192 and 94 g/kg DM, respectively, and the lowest in *Tragopogon pratensis ssp. orientalis* with 6 and 6 g/kg DM, respectively, with medians at 48 and 33 g/kg DM (T 2). The TT concentration was highest in *Sanguisorba minor* with 135 g/kg DM and lowest in *T. pratensis ssp. orientalis* for which no tannins were detected. The median for TT was at 15 g/kg DM. Condensed tannins were detected in eight plants, namely *Campanula rapunculus, L. corniculatus, O. viciifolia, Primula elatior, S. minor, Silene vulgaris, S. officinalis,* and *Thymus pulegioides*. Among these plants, *O. viciifolia* had the highest CT concentration with 38 g/kg DM. The median CT concentrations of these eight plants was 4 g/kg DM. The differences between TT and CT show that most test plants were mainly characterized by HT (data not shown in T because not directly analysed). Except *Anthyllis vulneraria ssp. carpatica*, *Leucanthemum vulgare, Silene flos-cuculi*, all test plants differed at least in one of the concentrations of TEP, NTP, and TT ($P < 0.05$) from those of the basal mixture (values given in bold in T 2).

2.4.2 In vitro organic matter digestibility, gas production and ammonia concentration after incubation

The pH of the incubation fluid after 24 h of incubation was between 6.8 and 6.9 (data not shown). The numerically highest net gas production during 24 h was realized with the basal mixture with 57 mL/200 mg DM (T 2). When one third of the basal mixture was replaced by the test plants, the diet containing *S. pratensis* resulted in (per 200 mg DM) the highest gas production (53 mL/24 h) and it was lowest with 24 mL/24 h with O. vulgare. The median was 43 mL/200 mg DM during 24 h. Compared to the

basal mixture, gas production was lower ($P < 0.05$) from 23 out of the 35 test diets. The median of the IVOMD was 541 g/kg OM, the lowest value was 336 g/kg OM (with *O. vulgare*) and the highest value was 684 g/kg OM with *P. lanceolata*. Compared to the basal mixture, the IVOMD of the test diets was lower ($P < 0.05$) with 18 out of the 35 plants. The median of ruminal ammonia concentration of the incubation fluid after 24 h of incubation was 16 mmol/L. The lowest value was 11 mmol/L (with *P. hieracioides*) and the highest was 22 mmol/L (with *S. flos-cuculi*). Nineteen test diets led to lower ($P < 0.05$) ammonia concentrations after 24 h of incubation compared to the basal mixture alone.

Table 1. Chemical composition (g/kg dry matter) of the collected plants ($n = 3$).ADF, acid detergent fiber; APDE, absorbable protein at the duodenum calculated as the sum of microbial protein from energy supply and rumen undegradable protein; APDN, absorbable protein at the duodenum calculated as the sum of microbial protein from rumen degradable protein and rumen undegradable protein; CF, crude fiber; CP, crude protein; NDF, neutral detergent fiber; NEL, net energy lactation; OM, organic matter; SEM, standard error of the mean. Values in bold differ ($P < 0.05$) from the basal mixture. Means with different superscripts differ significantly at $P < 0.05$.

No	Plant species	Plant family	OM	CP	CF	NDF	ADF	NEL (MJ)	APDE	APDN
01	Basal mixture[A]		898^{m-o}	85^{q}	353^{c}	612^{b}	443^{f-i}	3.44^{hi}	57.2^{l-o}	57.1^{no}
02	*Achillea millefolium*	*Asteraceae*	**932^{e-g}**	107^{l-n}	**346^{d}**	**491^{g-k}**	**469de**	**3.00^{l-o}**	58.7^{l-n}	**67.7^{l}**
03	*Anthyllis vulneraria,* ssp. *carpatica*	*Fabaceae*	**952^{b}**	160^{d}	**268^{qr}**	**438^{m-o}**	**367^{mn}**	**4.13^{ef}**	**78.0^{cd}**	**102.3^{d}**
04	*Aquilegia vulgaris*	*Ranunculaceae*	**910^{j-m}**	196^{b}	**251^{t}**	**295^{r}**	**253^{u}**	**4.83^{b}**	**90.0^{a}**	**125.7^{b}**
05	*Campanula rapunculoides*	*Campanulacea*	**836^{q}**	204^{a}	**175^{y}**	**115^{t}**	**94^{x}**	**5.10^{a}**	**92.0^{a}**	**131.0^{a}**
06	*Campanula rapunculus*	*Campanulacea*	**940^{b-e}**	86^{q}	**314^{hi}**	**488^{h-k}**	440^{g-i}	3.27^{i-k}	57.0^{m-o}	54.0^{op}
07	*Carum carvi*	*Apiaceae*	**948^{bc}**	**160^{d}**	**182^{x}**	**337^{q}**	**308^{r-t}**	**4.57^{c}**	**80.3^{bc}**	**103.0^{d}**
08	*Clinopodium vulgare*	*Lamiaceae*	909^{k-m}	**101^{mn}**	**334^{e}**	**503^{f-j}**	449^{e-i}	**2.80^{o}**	55.3^{o}	**63.7^{m}**
09	*Crepis biennis*	*Asteraceae*	895no	**120^{ij}**	**329^{ef}**	**508^{f-h}**	**461^{d-g}**	**3.10^{k-m}**	**61.3^{k}**	**76.7^{j}**
10	*Daucus carota*	*Apiaceae*	**928^{e-h}**	**129^{gh}**	**263^{rs}**	**423^{n-p}**	**390^{kl}**	**4.10^{e-g}**	**73.0^{f-h}**	**82.3^{hi}**
11	*Galium mollugo*	*Rubiaceae*	**919^{h-k}**	**73^{s}**	**301^{kl}**	**505^{f-i}**	**491^{c}**	**3.13^{k-m}**	**52.3^{p}**	**45.0^{q}**
12	*Galium verum*	*Rubiaceae*	**917^{h-k}**	**119^{j}**	**318^{gh}**	**448^{l-o}**	447^{f-i}	3.57^{h}	**66.3^{ij}**	**75.3^{j}**
13	*Hieracium pilosella*	*Asteraceae*	**855^{p}**	**112^{kl}**	**307^{j}**	616^{b}	329^{o-r}	**2.97^{m-o}**	58.3^{l-n}	**71.3^{k}**
14	*Knautia arvensis*	*Caprifoliaceae*	**916^{h-k}**	**108^{lm}**	**334^{e}**	**554de**	**84^{x}**	**3.03^{l-n}**	59.0^{k-m}	**68.3^{kl}**
15	*Leontodon autumnalis*	*Asteraceae*	899^{l-n}	**164^{d}**	**298^{lm}**	**390^{p}**	**346no**	**4.00^{fg}**	**77.7^{de}**	**105.0^{d}**
16	*Leontodon hispidus*	*Asteraceae*	**920^{g-k}**	**100no**	**307^{jk}**	**524^{e-g}**	449^{e-i}	**3.20^{j-l}**	59.7^{kl}	**62.7^{m}**
17	*Leucanthemum vulgare*	*Asteraceae*	**945^{b-d}**	**72^{s}**	**422^{a}**	**480^{h-l}**	428^{ij}	**1.47^{q}**	**34.7^{r}**	**45.0^{q}**
18	*Lotus corniculatus*	*Fabaceae*	**919^{h-k}**	**136^{fg}**	**279^{p}**	**347^{q}**	**322^{p-s}**	**4.10^{e-g}**	**74.0^{fg}**	**86.3^{fg}**
19	*Onobrychis viciifolia*	*Fabaceae*	887no	**130^{gh}**	**306^{jk}**	**444^{m-o}**	**409^{jk}**	3.60^{h}	**68.7^{i}**	**82.7^{hi}**
20	*Origanum vulgare*	*Lamiaceae*	**922^{f-j}**	**133^{f-h}**	**318^{g-h}**	**336^{q}**	**290^{t}**	3.57^{h}	**68.7^{i}**	**84.3^{gh}**
21	*Picris hieracioides*	*Asteraceae*	**913^{i-k}**	85^{q}	**361^{b}**	**744^{a}**	**555^{a}**	**2.23^{p}**	**46.0^{q}**	**53.3^{n-p}**
22	*Plantago atrata*	*Plantaginaceae*	**923^{f-i}**	**127^{hi}**	**229^{v}**	**278^{rs}**	**255^{u}**	**4.37^{cd}**	**75.3^{ef}**	**80.7^{i}**
23	*Plantago lanceolata*	*Plantaginaceae*	**920^{g-k}**	**101^{mn}**	**226^{vw}**	**260^{s}**	**190^{w}**	**4.53^{c}**	**72.3^{gh}**	**63.0^{m}**
24	*Primula elatior*	*Primulaceae*	**933^{d-f}**	**137^{f}**	**292no**	**423^{op}**	**370^{m}**	**3.90^{g}**	**72.7^{gh}**	**87.0^{fg}**
25	*Prunella vulgaris*	*Lamiaceae*	898^{m-o}	**121^{ij}**	**312^{ij}**	**440^{m-o}**	**381^{lm}**	3.40^{h-j}	**65.0^{j}**	**76.7^{j}**
26	*Rumex acetosa*	*Polygonaceae*	**938^{c-e}**	84^{qr}	**297^{l-n}**	**443^{m-o}**	440^{hi}	3.40^{h-j}	57.3^{l-o}	**52.3^{p}**
27	*Salvia pratensis*	*Lamiaceae*	896no	**180^{c}**	**260^{s}**	**336^{q}**	**335^{o-q}**	**4.37^{cd}**	**82.7^{b}**	**115.7^{c}**
28	*Sanguisorba minor*	*Rosaceae*	**922^{f-j}**	**128^{h}**	**293^{m-o}**	**469^{j-m}**	**308^{r-t}**	3.47^{hi}	**66.7^{ij}**	**81.3^{hi}**
29	*Scabiosa columbaria*	*Caprifoliaceae*	**929^{e-h}**	**94^{op}**	**343^{d}**	590^{bc}	**500^{bc}**	**3.00^{l-o}**	56.3^{no}	58.7^{n}
30	*Silene dioica*	*Caryophyllaceae*	**936^{c-e}**	**139^{f}**	**221^{w}**	**457^{k-n}**	**342^{op}**	**4.30^{de}**	**75.3^{ef}**	**88.7^{f}**
31	*Silene flos-cuculi*	*Caryophyllaceae*	886^{o}	**128^{h}**	**272^{q}**	**470^{i-m}**	**320^{q-s}**	**3.97^{fg}**	**71.7^{gh}**	**81.3^{hi}**
32	*Silene nutans*	*Caryophyllaceae*	**832^{q}**	**148^{e}**	**238^{u}**	**330^{q}**	**231^{v}**	**4.30^{de}**	**77.3^{de}**	**94.3^{e}**
33	*Silene vulgaris*	*Caryophyllaceae*	**913^{i-k}**	**118^{ik}**	**341^{d}**	**534^{ef}**	463^{d-f}	**2.87^{no}**	58.7^{l-n}	**75.0^{j}**
34	*Stachys officinalis*	*Lamiaceae*	**973^{a}**	89^{pq}	**346^{d}**	585^{bd}	**480^{cd}**	**3.20^{j-l}**	57.7^{l-o}	55.3^{n-p}
35	*Thymus pulegioides*	*Lamiaceae*	**911^{i-l}**	**109^{l}**	**328^{f}**	**434no**	**383^{lm}**	**3.10^{k-m}**	59.7^{kl}	69.3^{kl}
36	*Tragopogon pratensis ssp. orientalis*	*Asteraceae*	**968^{a}**	**77^{rs}**	**288^{o}**	**570^{cd}**	**512^{b}**	**3.00^{l-o}**	**52.0^{p}**	48.3^{q}
	SEM		2.9	3.2	4.9	11.5	10.7	0.071	1.16	2.06
	P-value		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[A]*Lolium perenne* and *Medicago sativa* mixed in a ratio of 0.57:0.43 (on dry matter basis).

Table 2. Phenolic contents of the collected plants (n = 3) and *in vitro* fermentation characteristics after 24 h of incubation of 200 mg dry matter of mixtures of test plants with a basal mixture (0.3:0.7; n = 6). CT, condensed tannins; DM, dry matter; ND, not detected; NTP, non-tannin phenols; TEP, total extractable phenols; TT, total tannins; SEM, standard error of the mean. Values in bold differ ($P < 0.05$) in this compositional variable from the basal mixture or the values obtained with the basal mixture as sole substrate. Means with different superscripts differ significantly at $P < 0.05$.

No.	Plant species	TEP (g/kg DM)	NTP (g/kg DM)	TT (g/kg DM)	CT (g/kg DM)	Total gas (mL/200 mg DM)	IVOMD (g/kg OM)	Ammonia (mmol/L)
01	Basal mixture[A]	14.5^{r-t}	12.4^{pq}	2.2st	ND	57.3^{a}	701^{a}	21.3^{ab}
02	*Achillea millefolium*	**88.1^{ef}**	**36.1^{h-l}**	**52.0^{d-f}**	ND	**41.5^{e-l}**	**524^{c-h}**	15.5^{c-j}
03	*Anthyllis vulneraria,* ssp. *carpatica*	24.0^{q-r}	18.3^{n-p}	5.7^{n-t}	ND	**44.9^{b-k}**	562^{a-g}	12.9^{h-j}
04	*Aquilegia vulgaris*	**42.9^{l-n}**	**40.6^{f-i}**	2.3^{r-t}	ND	**44.1^{b-k}**	564^{a-g}	19.6^{a-d}
05	*Campanula rapunculoides*	**46.7^{k-n}**	**37.8^{g-k}**	8.9^{m-t}	ND	**41.5^{f-l}**	**531^{b-h}**	19.1^{a-e}
06	*Campanula rapunculus*	**49.7^{j-l}**	**35.2^{l-m}**	**14.5^{k-n}**	1.9^{e}	**42.7^{d-l}**	**535^{b-h}**	16.1^{k-l}
07	*Carum carvi*	**32.8^{n-p}**	**31.9^{k-m}**	0.9^{o-t}	ND	50.1^{a-i}	620^{a-e}	17.1^{a-i}
08	*Clinopodium vulgare*	**144.9^{c}**	**80.9^{b}**	**64.1^{c}**	ND	**35.5^{k-m}**	**458^{f-i}**	**11.6^{ij}**
09	*Crepis biennis*	**75.5^{gh}**	**43.8^{eg}**	**31.7^{ij}**	ND	**42.2^{d-l}**	**534^{b-h}**	**14.5^{d-j}**
10	*Daucus carota*	**48.4^{k-m}**	**22.2^{n}**	**26.2^{jk}**	ND	49.2^{a-i}	611^{a-f}	17.7^{a-h}
11	*Galium mollugo*	**43.9^{k-n}**	**32.5^{k-m}**	**11.3^{l-q}**	ND	**42.2^{d-l}**	**531^{b-h}**	**13.3^{f-j}**
12	*Galium verum*	**50.0^{j-l}**	**33.0^{k-m}**	**17.1^{k-m}**	ND	50.7^{a-g}	649^{a-d}	**14.7^{d-j}**
13	*Hieracium pilosella*	**43.9^{k-n}**	**33.8^{j-m}**	10.1^{l-s}	ND	**37.8^{j-m}**	**487^{e-i}**	**14.4^{d-j}**
14	*Knautia arvensis*	**44.4^{k-n}**	**31.8^{k-m}**	**12.6^{l-r}**	ND	**32.3^{l-n}**	**422^{g-i}**	**14.5^{d-j}**
15	*Leontodon autumnalis*	**36.1^{n-p}**	21.6^{n}	**14.5^{l-p}**	ND	**40.2^{h-l}**	**513^{c-h}**	**15.7^{c-j}**
16	*Leontodon hispidus*	**37.6^{m-o}**	22.2^{n}	**15.4^{k-o}**	ND	52.0^{a-d}	661^{a-c}	**12.8^{h-j}**
17	*Leucanthemum vulgare*	21.1^{qr}	13.4^{op}	7.6^{m-t}	ND	48.5^{a-i}	570^{a-g}	16.4^{a-j}
18	*Lotus corniculatus*	**65.5^{hi}**	30.7^{lm}	**34.9^{hi}**	34.5^{b}	51.9^{a-e}	615^{a-f}	**15.3^{c-j}**
19	*Onobrychis viciifolia*	**78.0^{fg}**	29.4^{m}	**48.6^{ef}**	37.8^{a}	50.4^{a-h}	622^{a-e}	**14.5^{d-j}**
20	*Origanum vulgare*	**192.2^{a}**	**93.5^{a}**	**98.7^{b}**	ND	**24.5^{n}**	**336^{i}**	**12.9^{h-j}**
21	*Picris hieracioides*	**40.4^{l-n}**	**30.7^{lm}**	9.8^{m-t}	ND	**40.4^{g-l}**	**512^{c-h}**	**11.2^{j}**
22	*Plantago atrata*	**60.7^{ij}**	**49.3^{de}**	11.4^{m-t}	ND	**45.8^{b-k}**	572^{a-g}	17.0^{a-i}
23	*Plantago lanceolata*	**89.9^{e}**	13.1^{op}	**76.9^{c}**	ND	53.4^{a-c}	684^{ab}	**12.3^{h-j}**
24	*Primula elatior*	**97.4^{de}**	**42.5^{f-h}**	**54.9^{ef}**	22.1^{c}	51.0^{a-f}	631^{a-e}	17.0^{a-i}
25	*Prunella vulgaris*	**103.3^{d}**	**54.6^{d}**	**48.7^{ef}**	ND	**42.4^{d-l}**	**536^{b-h}**	16.9^{a-i}
26	*Rumex acetosa*	**74.6^{gh}**	**39.7^{g-j}**	**34.9^{gh}**	ND	**43.1^{c-k}**	**540^{b-g}**	**13.2^{g-j}**
27	*Salvia pratensis*	**108.4^{d}**	**46.4^{ef}**	**62.0^{de}**	ND	53.4^{a-b}	661^{a-d}	16.9^{a-i}
28	*Sanguisorba minor*	**170.8^{b}**	**35.4^{l-m}**	**135.3^{a}**	4.1^{d}	47.1^{a-j}	588^{a-f}	**13.2^{g-j}**
29	*Scabiosa columbaria*	**43.5^{l-n}**	**33.2^{k-m}**	10.3^{n-t}	ND	48.4^{a-i}	576^{a-g}	**14.8^{d-j}**
30	*Silene dioica*	25.5^{p-r}	**18.9^{n-o}**	6.6^{n-t}	ND	**44.8^{b-k}**	557^{a-g}	20.7^{a-c}
31	*Silene flos-cuculi*	17.8^{q-s}	13.6^{op}	4.2^{q-t}	ND	**42.1^{d-l}**	**534^{b-h}**	21.7^{a}
32	*Silene nutans*	**26.6^{o-q}**	**21.7^{n}**	4.9^{p-t}	ND	**42.5^{d-l}**	542^{a-g}	17.0^{a-i}
33	*Silene vulgaris*	**28.6^{o-q}**	**19.5^{no}**	9.1^{m-t}	3.8^{d}	**32.3^{l-n}**	**423^{g-i}**	**15.8^{c-j}**
34	*Stachys officinalis*	**55.0^{i-k}**	**38.0^{g-k}**	**17.0^{kl}**	1.7^{e}	**39.8^{i-m}**	**501^{d-h}**	**13.8^{e-j}**
35	*Thymus pulegioides*	**135.3^{c}**	**65.8^{c}**	**69.6^{cd}**	2.4^{e}	**41.3^{f-l}**	**523^{c-h}**	18.7^{a-g}
36	*Tragopogon pratensis ssp. orientalis*	5.5^{t}	**5.8 ^{r}**	ND	ND	**29.8^{mn}**	**380^{hi}**	**15.6^{c-j}**
	SEM	4.20	1.76	3.00	3.11	0.57	8.7	0.27
	P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[A]*Lolium perenne* and *Medicago sativa* mixed in a ratio of 0.57:0.43.

2.4.3 Results of principal component and regression analyses

The first principal component (PC) explained 38.8 % and the second 21.3 % of the variation, adding up to 60.1 % (Fig. 3). This demonstrates that there is a close relationship among variables of plant composition and in vitro ruminal fermentation variables. Antagonistic relationships were observed between the concentrations of TEP, NTP, or TT and net gas production or ammonia concentrations, and between fibre contents (NDF and ADF) on the one side and CP, APDN, APDE and NEL on the other. Separation of ammonium formation from gas production and IVOMD by principal component analysis was not possible.

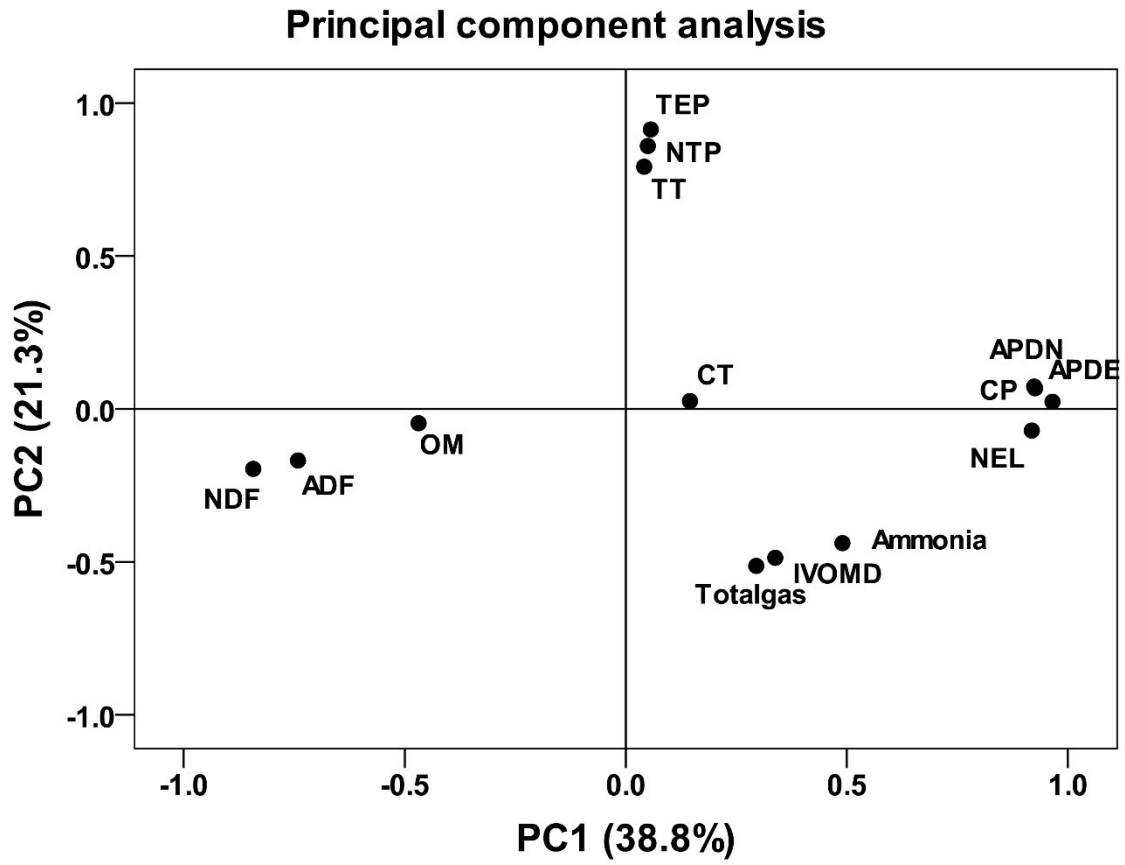

Figure 3. Plot of the first two principal components describing the relationships among variables of plant composition and in vitro ruminal fermentation. ADF, acid detergent fibre; APDE, absorbable protein at the duodenum calculated as the sum of microbial protein from energy supply and rumen undegradable protein; APDN, absorbable protein at the duodenum calculated as the sum of microbial protein from rumen degradable protein and rumen undegradable protein; CP, crude protein; CT, condensed tannins; IVOMD, in vitro organic matter digestibility; NEL, net energy for lactation; NDF, neutral detergent fibre; NTP, non-tannin phenols; OM, organic matter; TEP, total extractable phenols; TT, total tannins.

The plot of the first two PC scores (Fig. 4) describes the association of each plant to the factor scores. In general, those test plants which were situated in the upper half of the plot were rich in phenols, and those in the lower half had high gas production and ammonia concentrations after 24 h when incubated in combination with the basal mixture. The test plant species arranged at the right side of the

plot had high CP concentration and high nutritive values concerning APDE, APDN and NEL, the ones on the left side were characterized by high fibre concentrations. The test plants allocated to the right upper quadrant of the plot were comparably rich in phenols and had a high nutritional value (i.e., rich in CP, NEL, APDE, and APDN). When incubated together with the basal mixture, they had the potential to reduce ammonia concentrations in vitro and, at the same time, improve the nutritional value of the mixed diet concerning NEL, APDE and APDN as compared to the basal mixture alone. In detail, the plants found in this quadrant included *C. rapunculoides, O. viciifolia, O. vulgare, P. atrata, P. lanceolata, P. elatior, P. vulgaris, S. pratensis*, and *S. minor*. However, no herb considerably reached the center or even the right upper corner of this quadrant, which would have meant a clear association of low ammonia production and high nutritional quality. On the opposite, in particular *C. rapunculus, G. mollugo, G. verum, H. pilosella, L. hispidus, L. vulgare, P. hieracioides, S. columbaria, S. vulgaris,* and *T. pratensis ssp. orientalis* had low concentrations of TEP similar to those of the basal diet and resulted in high gas production and ammonia concentrations when incubated together with the basal mixture.

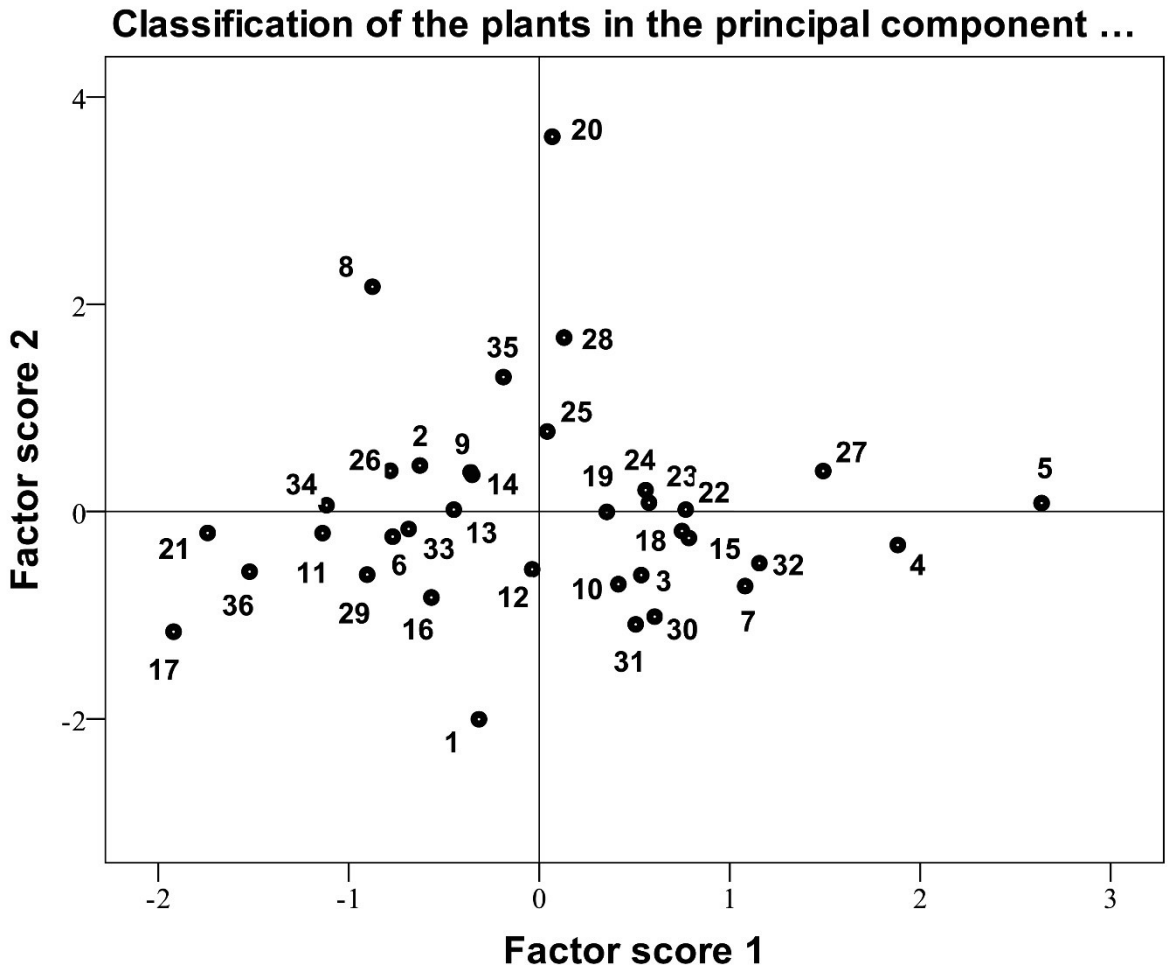

Figure 4. Plot of the first two factor scores describing the classification of each plant within the principle component loading (Figure 3; for number codes of plants see Table 1).

The regression analyses served to identify relationships between CP, TEP and TT concentrations of the test-plant based diets on the one hand and the in vitro gas production and ammonia concentrations on the other hand. As expected, CP concentration was positively related to ammonia concentration after 24 h of incubation (Fig. 5B, $P < 0.05$), whereas the relationship of CP and total gas production was not significant (Fig. 5A). There was an exponential relationship between TEP and gas production with an increase followed by a decrease at higher TEP concentrations of the diet (Fig. 6A, $P < 0.05$). Incubating test plants with the basal mixture reduced ammonia concentrations in a linear relation with increasing contents of TEP (Fig. 6B, $P < 0.05$) and TT (Fig. 7B, $P < 0.05$). No relationship was found between TT and gas production (Fig. 7A).

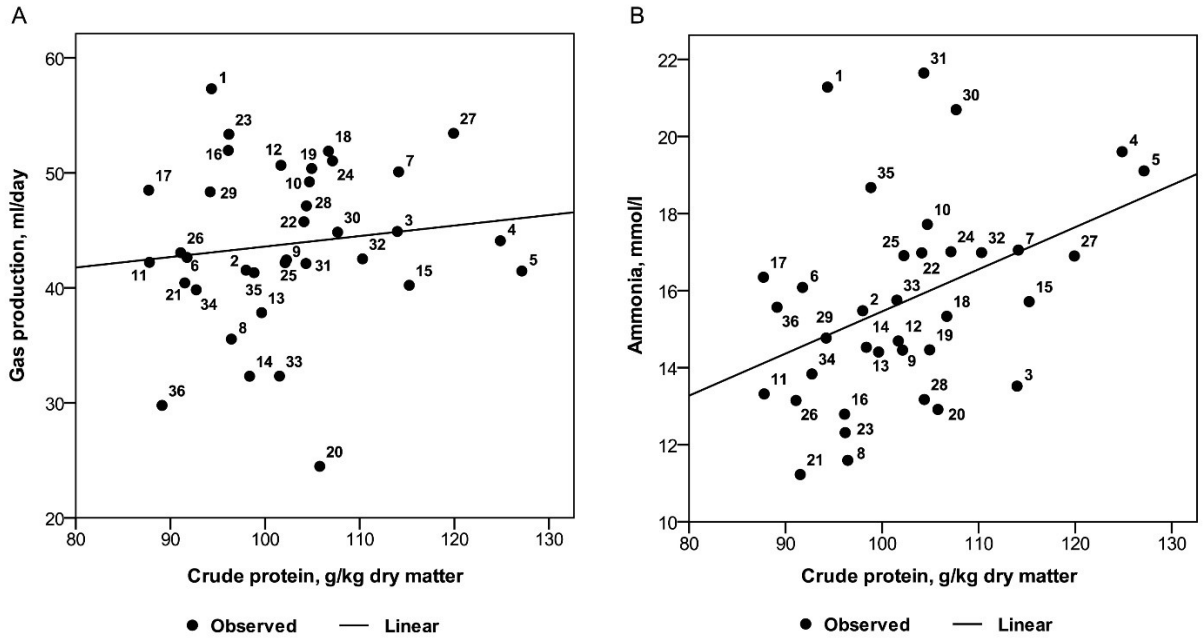

Figure 5. Gas production and ammonia concentration in relation to dietary crude protein (A, y = 34.5 + 0.091 × x; R2=0.016; not significant), respectively ammonia production (B, y = 4.53 + 0.109 × x; R2=0.168; $P < 0.05$). For number codes of plants see Table 1.

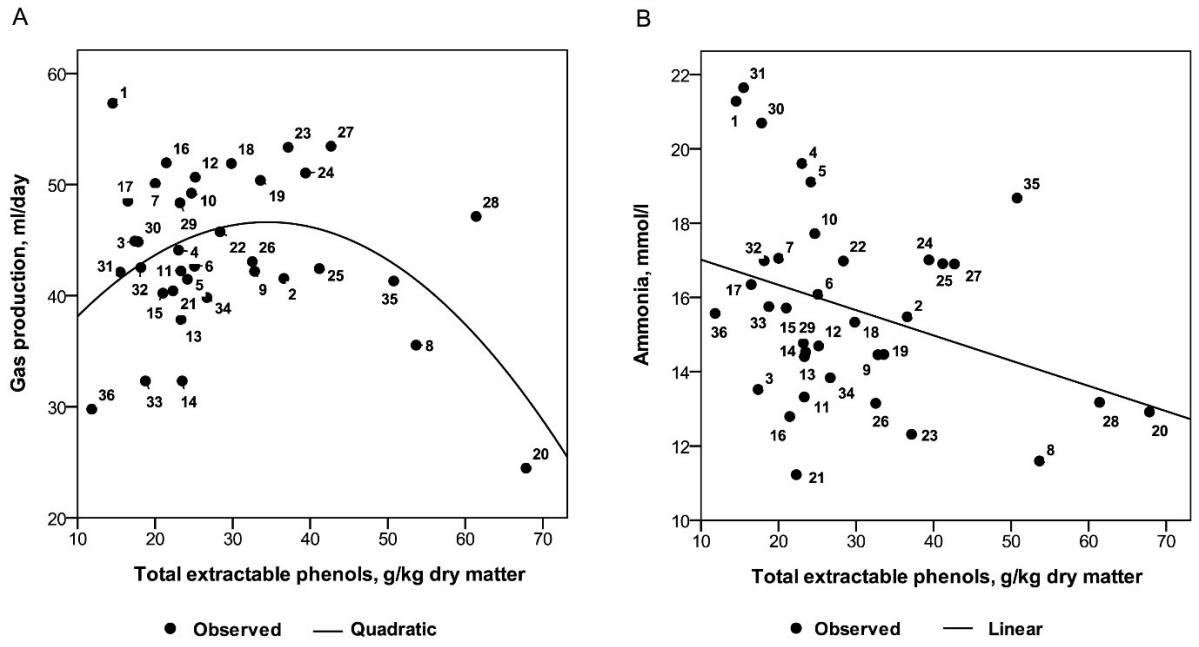

Figure 6. Gas production and ammonia concentration in relation to dietary contents of total extractable phenols (A, y = 29.8 + 0.976 × x – 0.0141 × x2; R2=0.198; P < 0.05; B, y =17.7 – 0.068 × x; R2=0.116; P < 0.05). For number codes of plants see Table 1.

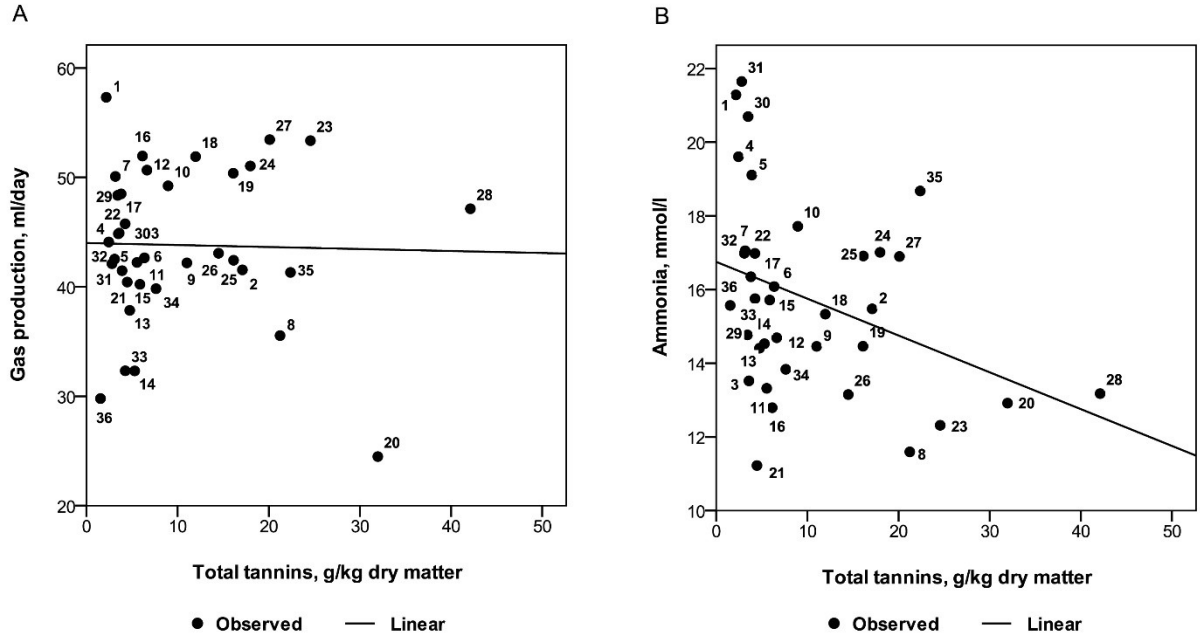

Figure 7. Gas production and ammonia concentration in relation to dietary contents of total tannins (A, y = 44.0 – 0.0181 × x; R^2=0.001; not significant; B, y = 16.8 – 0.0999 × x; R^2=0.128; *P* < 0.05). For number codes of plants see Table 1.

2.5 Discussion

The goal of the present study was to compare different temperate herb species harvested at a late growth stage with respect to their nutrient and phenol concentrations and their influence on gas production and ammonia formation during 24 h of in vitro incubation in rumen fluid together with a basal diet. One intention was to identify herbs which have a high digestibility (estimated as IVOMD from nutrient composition and gas volume), but at the same time, are able to lower ammonia formation in rumen. Such plants would be of value for further investigation in order to develop forage-based feed additives for improving protein utilization by dairy cows. A second objective was to establish relationships between compositional and fermentation variables across a large number of plant species for this late harvest stage. All 35 plants tested were grown at the same time and at the same site, which enhanced comparability among plants but restricted the applicability of the results to these conditions. The dietary inclusion of 30 % was even high with respect to practical feasibility, however too low tannin supplementation levels may fail in achieving effects (Kapp-Bitter et al., 2020). The voluntary intake of plants containing high phenolic contents may be reduced but, in certain metabolic states of the animal, unchanged or even increased (Villalba *et al.,* 2015). However, these effects have to be subject of future investigations and of targeted management on farm.

2.5.1 Ammonia mitigating potential of the test plants

Technically, ammonia could be measured only once, namely at the end point of incubation after 24h. This approach therefore does not reflect temporal dynamics of ruminal N metabolism, but it can serve as an indicator of ammonia formation *in vivo*. The in vitro ammonia concentration in the inoculum after 24 h of incubation increased with increasing CP concentration of the plants as expected (Chanthakhoun et al., 2014; Frank and Swensson, 2002). Ammonia production depends on the rumen-degradable CP concentration of the diet and, simultaneously, on the accessible energy available in the rumen (Reynolds and Kristensen, 2014). Absorbed ammonia has to be considered as a loss, and is even a metabolic burden for the liver (Parker et al., 1995), and it contributes significantly to blood and milk urea concentrations and thus urinary N excretion (Nousiainen et al., 2004). It is expected that tannins will protect dietary proteins from ruminal degradation by the formation of complexes (Perez-Maldonado et al., 1995), which could reduce the generation of ammonia and improve protein efficiency of dairy cows. There is in vitro (Jayanegara et al., 2011; Sinz et al., 2019b) and in vivo evidence (Kälber et al., 2012; Sinz et al., 2019a) that tannin-rich forage herbs may have this effect. It would be of importance to identify forage combinations, which at the same time lower ruminal ammonia concentration and maintain fermentation efficiency including microbial protein synthesis (Jayanegara et al., 2013; Sinz et al., 2019b).

Results of the present study confirmed the expected negative relationship between phenol concentrations and ammonia formation during in vitro incubation. The reasons for this include especially the protein-binding effects of tannins (McSweeney et al., 2001) as discussed above. Although other factors cannot be excluded, the results of the present evaluation suggest that the ammonia production

was negatively related with TT concentrations. Among the tannins, the CT are likely most efficient to lower ammonia production in rumen (Koenig et al., 2018; Naumann et al., 2017). However, as only few of the test plants contained detectable levels of CT, the relationships with CT established in the present study should be considered with care. Accordingly, the position of the CT in the PCA was not clear in one direction, as was also the case in the study by Jayanegara et al. (2011). Rather, it is likely that HT tannins played a major role here. Additionally, of the 19 plants lowering ammonia concentration, all diets except *P. lanceolata, S. vulgaris*, and *T. pratensis ssp. orientalis* had a greater NTP concentration than the basal mixture. Thus, effects of other phenols such as flavonoids (Waghorn & McNabb, 2003; Sinz et al., 2018) or differences concerning other compounds, such as e.g. crude protein content, have to be taken into account. Further, there were also incubation mixtures with a greater TEP content, in which the ammonia concentration in the inoculum after 24 h was not different from the basal mixture even if the TT content of the test plants was greater than that in the basal mixture. This is consistent with results of previous research, where effects on ammonia formation of phenol-rich plants were found to depend also on the plant species, the extracts of which had been investigated by Sinz et al. (2019b). Although not investigated in the present study, plant bioactive lipid compounds may have affected ammonia formation as well (Khiaosa-ard & Zebeli, 2013).

2.5.2 Relationships between ammonia mitigation and nutritive and digestion characteristics

It was not possible to separate effects on the different incubation variables, meaning that ammonia was mitigated together with gas and, consequently, also IVOMD in most cases. The desired candidate would have been an herb, which would have resulted in incubation variables that are displayed in the upper right corner of the principal component analysis, indicating low ammonia formation at high dietary protein. No test plant completely fulfilled this criterion. However, some herbs approached this goal, among them *C. rapunculoides* and *S. pratensis*, which both had rather high protein content and still only average ammonia formation. Both were characterized by high proportions of NTP. On the other hand, *O. vulgare* and *S. minor* expressed low ammonia formation at average nutrient concentrations. These herbs were both characterized by high TEP concentrations but were almost absent in CT, indicating that HT indeed had a major effect, which is well in line with literature (Jayanegara et al., 2011; Aboagye et al., 2019; Stewart et al., 2019). However, again also the fraction of NTP was clearly present in these plants and might have played a role (Khiaosa-ard & Zebeli, 2013; Sinz et al., 2018). The two herbs with high CT concentrations (*L. corniculatus* and *O. viciifolia*) had also quite clear mitigating effects on ammonia formation, as expected (Mueller Harvey, 2006; Dschaak et al., 2011; Ghelichkhan et al., 2018), at fair dietary protein contents. Among the test plants of the present study, *O. viciifolia* was most researched also with respect to ammonia formation. It was found earlier to have clear rumen ammonia-mitigating properties across various cultivars and harvest dates (Scharenberg et al., 2009; Azuhnwi et al., 2012; Grosse Brinkhaus et al., 2016).

In the PCA, the occurring negative relationships between concentrations of NEL, APDE, and APDN vs. fibre (NDF and ADF) concentrations were expected, because fibrous carbohydrates have a lower digestibility than other carbohydrates. There was an exponential relationship between dietary TEP concentrations and gas production, indicating that at low and high TEP concentrations adverse effects will occur, whereas intermediate TEP concentrations may even promote ruminal nutrient degradation. This supports the observation that higher TEP concentrations impair certain rumen bacteria and thus for instance inhibit cellulolysis (Min et al., 2002, McSweeny *et al.,* 2001) and, with this, nutrient degradation. It explains why apparent total tract digestibility declined with increasing dietary TEP concentrations in the in vivo studies of Yang et al. (2017) and Henke et al. (2017).

2.6 Conclusions

Within the 35 mature herbs grown in a temperate climate we did not find one which would decrease ammonia formation and at the same time, improve IVOMD in in vitro rumen fermentation. We found indications that there is a correlation between hydrolysable tannins and non-tannin phenols and ammonia mitigation by this type of plants. The herbs *Galium verum, Leontodon hispidus, Lotus corniculatus, Onobrychis viciifolia, Plantago lanceolata, Sanguisorba minor* and *Scabiosa columbaria* mitigated ammonia and fairly maintained IVOMD. These plants might be beneficial for lowering ammonia production when supplemented to grassland-based diets excessive in CP, and this without impairing nutritional quality. Implementation of such plants in farm practice appears realistic, because all plants investigated were grown for seed production thus making them ready to be cultivated. It has to be investigated in further studies if these plants will exhibit the effects detected also *in vivo* and to test if this is the case also at lower dietary proportions.

3. Short-term versus long-term sainfoin supplementation of dairy cows in a low-input feeding system: effects on protein utilization

Based on Alexandra N. Kapp-Bitter, Uta Dickhoefer, Gerdine Kaptijn, Vasilisa Pedan, Erika Perler Michael Kreuzer, Florian Leiber.
Livestock Science, in revision.

3.1 Abstract

An on-farm study was conducted with the objective to determine the effect of the tanniferous legume sainfoin (*Onobrychis viciifolia*) on protein utilization of dairy cows, which were exposed to excessive dietary crude protein during autumn pasture grazing in a zero-concentrate feeding system. In order to assess a system applicable in practice, comparably small amounts of sainfoin were offered. To compare short-term with long-term supplementation, sainfoin was offered either continuously or in intervals interrupted by periods without sainfoin feeding. Within one dairy herd of 60 Swiss Fleckvieh cows, 30 cows were chosen and randomly allocated to three groups balanced for milk yield, milk urea concentration, days in milk and parity. During 5 weeks, 2 kg /cow and day (as fed) of sainfoin pellets were provided either over 35 days (SLT) or, intermittently, for 2 × 5 days (SST) in weeks 3 and 5 of the experiment. A control group (CON) received 2 kg/cow and day (as fed) of ryegrass pellets during the 5 weeks. The entire herd grazed together on a protein-rich natural sward and was kept overnight in an open space barn receiving fresh grass and hay ad libitum. Experimental pellets were offered individually twice per day in the barn trough. Feed, milk, faeces and urine samples were collected repeatedly in weeks 0 (baseline, prior to experimental feeding), 3 and 5. Milk was analyzed for fat, protein and urea, urine for purine derivatives, creatinine and nitrogen, and faeces for protein, fibre and particle fractions. During three sampling weeks, chewing activity was recorded by sensor halters. Intake was estimated by proxies, and based on feces analyses digestibility estimates were calculated. Eating time was not affected, but SST increased rumination time in the first half of the day. Milk fat and protein yields were greater in SST compared to CON, but no treatment effect on concentration and yield of milk urea was found. The proportion of particle fractions in faeces was smaller but estimated fibre digestibility was lower in both sainfoin treatments compared to CON. In urine, the ratio of purine derivatives to creatinine was higher in SST than in CON, indicating a higher ruminal microbial protein synthesis. In summary, the study revealed small positive effects on protein metabolism and yield when sainfoin was applied in comparably small amounts over two short periods, whereas a long-term application had no effect.

3.2 Introduction

The nutrient composition of pasture grass changes with season, year and management. This means a varying diet with possible deficits or excesses in certain nutrients, when cattle are fed exclusively with forages from grassland (Kuusela, 2004). Such imbalances may lead to problems in performance, health and fertility. An excess of crude protein (CP) on pasture in spring and autumn can be one of these problems (Pacheco and Waghorn, 2008). Due to the lack of energy and carbon skeletons, the high nitrogen (N) concentrations in pasture forages cannot be used by the rumen microbes for their own protein synthesis (Sutter et al., 2017). Instead, ammonia produced during rumen CP degradation is directly absorbed through the rumen wall and metabolized in the liver to urea. Only a small part of this urea is recycled to the rumen; most is excreted with urine and milk (Reynolds and Kristensen, 2014).

The detoxification of absorbed ammonia is an unnecessary energy loss to the cow (Parker et al., 1995) and stresses the liver. In addition, it increases the environmental impact of animal husbandry, because urinary N is more volatile than faecal N (Weiss et al., 2009). Commonly these imbalances in dietary energy and N sources are compensated by providing supplement feeds rich in energy but low in N like corn silage or concentrate. However, to prevent feed-food competition in animal production systems and to reserve arable land directly for food production (Schader et al., 2015), the omission of such feeds in cattle diets is increasingly considered as an option.

One way to lower metabolic urea loads and urinary N excretion while at the same time improving protein use efficiency in dairy cows with grassland-only diets could be the inclusion of tannin-rich herbs in the diets (Totty et al., 2013). Tannins bind to proteins in the rumen and these bonds may partly be released in the abomasum (McNabb et al., 1998). Therefore, these proteins are either available in the small intestine for digestion or are excreted with faeces instead of urine (Dschaak et al., 2011). A promising tannin-containing herb growing under temperate climatic conditions is sainfoin (Onobrychis viciifolia). Sainfoin contains more than 60 different phenolic compounds (Regos et al., 2009), including various condensed tannins (CT) which are produced in the chloroplasts of the cell (Wang et al., 2015). When mixed with alfalfa, sainfoin tannins were found to lower solubility and, therefore, ruminal degradability of CP (Aufrere et al., 2013). For instance, lower ruminal ammonia concentrations have been observed by Kraiem et al. (1990) when sainfoin was added to alfalfa diets of steers. Thus, feeding sainfoin can reduce milk urea and urinary N excretion while increasing feed protein conversion rates into milk protein and faecal N excretion (Chung et al., 2013; Huyen et al., 2016; Min et al., 2003, Scharenberg 2009).

In agricultural practice, high dietary inclusion levels of herbs such as sainfoin are limited by their high production costs, low palatability, moderate nutritive value and their possible anti-nutritional effects. At the same time, too low tannin supplementation levels may fail in achieving the desired effects (Kapp-Bitter et al., 2020). Therefore, the positive effects of tannin-rich herbs at reasonable dietary inclusion levels have to be demonstrated under realistic practice conditions. Furthermore, there may be short-term and long-term palatability constraints (Silanikove et al., 2001), and also effects on ruminal ammonia formation may depend on the duration of the herb feeding (Khiaosa-ard et al., 2012).

Against this background, an on-farm experiment with dairy cows grazing on autumn pasture in a zero concentrate feeding system was designed. With the objective of counterbalancing expected dietary N excess, pelleted sainfoin was offered in feasible dosages, and effects of a continuous versus an interrupted temporary offer on protein conversion efficiency and N excretion via urine and faeces were assessed. The particular challenge of conducting such an experiment under commercial on-farm conditions was addressed by choosing the best available proxies for a sound estimation of digestive and metabolic effects.

3.3 Materials and Methods

3.3.1 Experimental design and protocol

An experiment (approved by the Cantonal Veterinary Office of Aargau, Switzerland; license number AG75689) was carried out on a dairy farm with a zero-concentrate feeding system. The experiment lasted for 6 weeks from September to November 2016. After a baseline data collection week (week 0), 30 Swiss Fleckvieh cows were assigned for 5 weeks to three dietary treatments. These comprised a control treatment (CON) offering per cow 2 kg/day (as fed) of pelleted ryegrass (*Lolium perenne*) and two treatments with 2 kg/day (as fed) of pelleted sainfoin (*Onobrychis viciifolia*). In one sainfoin treatment, the pellets were only fed during the first 5 days each (Sainfoin short-term, 'SST') in weeks 3 and 5 (the same two periods where data and sample collection took place). The other group received the sainfoin pellets during the entire 5 weeks (Sainfoin long-term, 'SLT'). The ryegrass for pellet production was harvested after flowering stage and pelleted by Gebrüder Herzog (Hornussen, Switzerland). The sainfoin pellets were harvested during flowering stage, pelleted by and purchased from Agrobio Schönholzer AG (Neukirch an der Thur, Switzerland). In both cases, the entire above-ground material had been harvested from pure stands and sun-dried. All cows were always kept together either in an open-space barn or on pasture. After each milking (05.00 h and 16.30 h), they were fixed in headlocks during 30 to 45 min where they individually received 1 kg portions (as fed) of the respective experimental pellets. Cows grazed on a natural grass-rich pasture approximately from 07.00 h to 16.00 h. During night in the barn, they were provided with a cut grass-clover mixture consisting of ryegrass (*Lolium multiflorum*) and red clover (*Trifolium pratense*) as well as grass hay from a grass-rich natural meadow. Limited grass growth on pasture due to dry weather conditions forced the farmer to keep the cows in barn during the last 2 days in week 3 and for the first 5 days in week 4. During this time, the intake from pasture was replaced with higher offer of the grass-clover mixture. Sampling was not affected, as it was performed in weeks 0 (baseline), 3 and 5, i.e. before and after these days of complete indoor stay.

The 3 × 10 cows were allocated to the treatment in a complete randomized design. Group averages were balanced for a number of traits. Accordingly, cows of CON, SST and SLT had an initial milk yield (arithmetic mean ± one standard deviation) of 20.3 ± 4.3, 18.8 ± 5.4 and 19.9 ± 5.3 kg/day, respectively. The corresponding initial averages for milk urea concentration, stage of lactation and number of lactations (arithmetic mean ± one standard deviation) were 44.7 ± 2.3, 44.5 ± 3.7 and 45.2 ± 3.6 mg/dL, 167 ± 76, 160 ± 66 and 159 ± 66 days as well as 3.2 ± 2.3, 3.0 ± 1.7 and 3.0 ± 2.1 lactations. Finally, data from one SLT cow had to be excluded from the experiment due to nervous behaviour.

3.3.2 Data and sample collection

Samples of each forage were taken 11 times evenly distributed across the experiment. For this purpose, pasture forage was manually cut 5 cm above ground across 1 m2 on five different sites each. Samples

of the fresh grass-clover mixtures and grass hay fed in barn were taken at five places across the barn trough at each sampling event. Samples of the ryegrass and sainfoin pellets were collected two to three times per week and then pooled to one sample per week. The samples of fresh plants were dried at 40°C during 48 h. Afterwards, all feed samples were milled through a 0.5-mm-sieve (Retsch SK 100, Retsch®, Haan, Germany).

For recording of eating and rumination behaviour, sensor halters (RumiWatch®, Itin + Hoch GmbH, Liestal, Switzerland; Rombach et al., 2018) were mounted on the first and second lactating cows during the sampling weeks (0, 3 and 5). In week 0, from groups CON and SST six halters each could be evaluated, from group SLT only two because of technical problems. In week 4 and 6, groups SST and C wore each five halters, group SLT six. Sensor data were converted to eating and rumination times (min/d and min/h) with the Rumiwatch converter V0.7.3.2 (Rombach et al., 2018). The records collected during the respective sampling weeks were evaluated for the time between 05.00 h on Tuesday until 05.00 h on Friday (i.e., 72 h). Values of eating time, rumination time and number of activity changes were calculated for entire days (min/d or n/d) and for three periods, namely from 05.00 h to 13.00 h, from 13.00 h to 21.00 h and from 21.00 h to 05.00 h (min/h or n/h).

Individual milk yields were quantified and milk samples were taken on Tuesday evening, Wednesday morning, Thursday evening and Friday morning of each sampling week. Milk samples were conserved with Bronopol® and pooled to one sample per animal and week by mixing the samples proportionately to milk amounts obtained at the respective milking events. Along with milk sampling, faeces and urine spot samples were taken. Faeces samples were taken rectally from each cow and stored at 4°C. These samples were pooled at similar portions to one sample per cow and week at the end of each sampling week. Half of the pool sample was dried at 40°C for 48 h and then milled through a 0.5-mm-sieve for laboratory analysis. The other half was stored at -20°C for later sieve washing. Spontaneously excreted urine by each cow was collected during pellet feeding in the headlocks after milking and subsequently acidified to pH 2-3 with sulfuric acid (20%; v/v). An amount of 50 ml of each urine sample was filtered (WhatmanTM filter paper 1, GE Healthcare, Buckinghamshire, Great Britain), and 10 ml of the filtered urine was diluted with 40 ml distilled water (1:5, v/v) and vortexed. Three 15-ml-aliquots were then stored at -20°C.

3.3.3 Laboratory Analysis

Dry matter (DM) concentrations of all fresh feed and faeces samples were determined by drying them at 40°C for 48 h. Concentrations of total ash, CP and fibre fractions (neutral and acid detergent fibre (NDF and ADF)) as well as crude fibre (all ash-corrected) were determined by near infrared spectroscopy (NIRS, NIRFlex N-500, Büchi, Flawil, Switzerland). Calibration of the NIRS device had been carried out with results of wet chemically analysed samples for both, 180 forage samples from grass-herb based swards and 45 faeces samples. Samples for calibration for faeces analysis were taken during the experiment in the present study and additionally from four other farms feeding diets of

different composition. Additionally, ash-free acid detergent lignin (ADL) was determined in all forage and faeces samples by the use of sulphuric acid (72%, v/v) using Fibertherm FT 12 (C. Gerhardt GmbH & Co. KG, Königswinter, Germany). Phenol analysis was performed in all feeds, pooled per week, according to the laboratory manual of Makkar (2003), with slight modifications. Briefly, extracts were prepared with 70%-aqueous acetone (v/v), which were subsequently filtered (Cameo™ syringe filter™, non-sterile, pore size 1.2 µm). Phenols measured included total extractable phenols and non-tannin phenols using tannic acid as a standard. In addition, condensed tannins (CT) were quantified with the butanol-HCl-assay (Makkar, 2003) with leucocyanidin as standard. Total tannins were calculated as total extractable phenols minus non-tannin phenols.

In the thawed faeces samples, particle size distribution was quantified according to Leiber et al. (2015b) using four sieves with different mesh sizes (4, 2, 1 and 0.3 mm diameter). Residues were dried at 105°C for 12 h, and proportions of the fractions were calculated.

Milk samples were analysed at the Swiss routine milk analysis laboratory (Suisselab AG, Zollikofen, Switzerland) for fat, protein, lactose and urea with a Fourier transform infrared spectroscopy (MilkoScan FT 6000, Foss Electric, Hillerød, Denmark).

The purine derivatives (PD) allantoin and uric acid, and creatinine in urine were analysed with high-performance reversed-phase liquid chromatography (Dickhoefer et al., 2015). Allantoin and uric acid were considered as total PD, because hypoxanthine and xanthine are almost absent in cattle urine (Verbic et al., 1989). Total urinary N was analysed by Kjehldahl method (AOAC 991.20; AOAC, 2000) applying the Gerhardt KT20 (C. Gerhardt, Königswinter, Germany) for digestion and a Kjeldahl apparatus (Büchi B324, Büchi Labortechnik, Essen, Germany) for distillation.

3.3.4 Calculations and statistical analysis

Net energy for lactation (NEL) and absorbable protein at the duodenum based on rumen-undegradable nitrogen compounds plus microbial protein either from fermentable energy (APDE) or from rumen-degradable nitrogen compounds (APDN) were calculated according to the regressions of Agroscope (2020) using concentration of DM, total ash, CP and crude fibre as measured in the feeds.

Apparent total tract digestibility of CP, NDF and ADF were estimated by a three-step approach following Leiber et al. (2015a). At first, the average total DM intake at herd level was assumed to be 18 kg/day according to the estimations of Agroscope (2020) for the average milk yield level of the experimental animals. Secondly, we calculated the intake of the hay and fresh grass-clover mixture consumed in barn on the herd level based on test weighing and assumed that the difference between the DM intake in the barn to 18 kg DM/day as intake during grazing on pasture. During the experimental pellet feeding, we also considered the 2 kg /day (as fed) of pellets as part of the 18 kg of total intake per day (Leiber et al., 2015a). Thirdly, ADL was assumed to be indigestible (Jung and Allen, 1995). Then, the following equations were applied:

(1) Faeces amount [kg DM/day] = ADL intake [g/day] / ADL in faeces [g/kg DM]

(2) Faecal CP, NDF or ADF excretion [g/day] = faeces amount [kg DM/day] × CP, NDF or ADF in faeces [g/kg DM]

(3) Apparent total tract digestibility of CP, NDF or ADF [%] = 1 – (CP, NDF or/ADF excretion [g/day] /CP/NDF/ADF intake [g/day]) × 100.

Total urine volumes could not be determined. However, daily urinary creatinine excretion is considered to be constant as it reflects maintenance muscle protein turnover (Chizzotti et al., 2008). Therefore, urine parameters can be related to it, to account for inter-animal and day-to-day variations in urine volume. The PD:C ratio is an indicator for microbial protein synthesis in rumen (Tas and Susenbeth, 2007; Chizzotti et al., 2008):

(4) PD:C ratio = (allantoin [mmol/l] + uric acid [mmol/l]) / creatinine [mmol/l].

For determining the PD:creatinine index (Chen and Ørskov 2004), the individual body weight (BW) of the experimental cows was estimated before and after the experiment with a weighing tape (Vieh- und Schweinemessband 250 cm, Hoechstmass Balzer, Sulzbach, Germany) and the average BW was included in the following formula.

(5) PD:C-index = (allantoin [mmol/l] + uric acid [mmol/l]) / creatinine [mmol/l] × BW [kg0.75].

Next, the PD:N ratio was calculated as an indicator for the efficiency of use of ingested N for microbial protein synthesis in the rumen (Tas and Susenbeth, 2007) as:

(6) PD:N ratio = (allantoin [mmol/l] + uric acid [mmol/l]) / N [g/l].

Finally, the N:creatinine ratio (N:C), an indicator for N excretion in urine (Chizzotti et al., 2008), was computed as:

(7) N:C ratio = N [g/l] / creatinine [mmol/l].

Data were analysed with SPSS® statistical software version 24, using a general linear model. Dietary treatment, sampling week (i.e., 3 and 5) and their interaction were used as fixed factors, while data from week 0 were used for weighted least squares corrections on animal level. Least squares means were calculated and presented in the tables. Multiple comparisons among the least squares means for each treatment across both weeks (not shown in tables) were performed with Tukey's procedure, considering $P < 0.05$ as significant and $P < 0.10$ as tendency.

3.4 Results

3.4.1 Diet composition

The chemical composition of the main forages changed during the course of the experiment (Table 4), which was also due to variable weather conditions. The latter also influenced the daily time cows were turned out to pasture and, thus, estimated DM intake from pasture (lower in week 3 compared to weeks 0 and 5). The CP concentrations of the forage fed in the barn and on pasture developed differently. During the experiment, it decreased from week 0 to 5 in the grass-clover mixture, whereas it increased in pasture towards the end of the trial. In week 5, the concentrations of NDF and ADF in pasture herbage were very low. In week 3, the ratios of APDN to APDE were 1.12 and 1.15 for CON and for the sainfoin groups, respectively. The corresponding ratios were 1.31 and 1.32 in week 5. The concentrations of total tannins in the forages varied with time as well, but were generally very low, and no CT could be detected. The concentrations of the non-tannin phenols were clearly higher than those of the tannins. The concentrations of total extractable phenols, non-tannin phenols and total tannins were much higher in the sainfoin pellets compared to the ryegrass pellets (Table 3), and CT could be detected only in the sainfoin pellets.

Table 3. Chemical composition (g/kg dry matter) of sainfoin and ryegrass pellets in the sampling weeks (mean of two samples with three replicates per sample ± standard deviation).

	Sainfoin	Ryegrass
Analyzed variables		
Dry matter (g/kg fresh weight)	928 ± 4.3	946 ± 5.2
Total ash	111± 3.6	67 ± 2.8
Crude protein	128 ± 4.4	45 ± 2.3
Neutral detergent fibre	444 ± 4.9	697 ± 1.5
Acid detergent fibre	409 ± 4.4	484 ± 2.7
Acid detergent lignin[1]	74.9 ± 10.46	55.8 ± 7.30
Crude fibre	305 ± 6.1	382 ± 7.3
Total extractable phenols[1,2]	78.0 ± 1.53	6.4 ± 0.22
Non-tannin phenols[1,2]	29.4 ± 0.33	5.9 ± 0.11
Total tannins[2,3]	48.6 ± 1.89	0.5 ± 0.39
Condensed tannins[1,4]	37.8 ± 0.71	ND
Calculated variables		
NEL (MJ/kg dry matter)	4.67 ± 0.109	3.56 ± 0.143
APDE (g/kg dry matter)	79.7 ± 1.83	45.7 ± 1.42
APDN (g/kg dry matter)	81.4 ± 3.04	13.7 ± 2.28

APDE/ APDN, absorbable protein at the duodenum consisting of rumen-undegradable protein and microbial protein from fermentable energy/ rumen-degradable protein; NA = not available; ND, not detected; NEL, net energy for lactation.

[1] Samples pooled per week before analysis.

[2] Tannic acid equivalents.

[3] Difference between the overall mean of total extractable phenols and non-tannin phenols.

[4] Leucocyanidin equivalents.

Table 4. Chemical composition of the basal diet components (g/kg dry matter ± standard deviation) in the sampling weeks (mean of two samples with three replicates per sample).

	Fresh grass-clover mixture fed in barn			Grass hay fed in barn			Pasture forage		
Week	0	3	5	0	3	5	0	3	5
Analyzed variables									
Dry matter (g/kg fresh matter)	174 ± 0.0	199 ± 1.8	200 ± 18.6	923 ± 0.2	785 ± 39.8	874 ± 55.4	233 ± 60.7	484 ± 12.1	NA
Ash	105 ± 3.1	129 ± 9.3	138 ± 7.8	98 ± 6.7	97 ± 5.9	115 ± 2.5	82 ± 4.7	82 ± 6.3	118 ± 2.6
Crude protein	210 ± 1.5	202 ± 9.7	173± 5.4	95 ± 9.5	92 ± 2.7	107 ± 2.4	161 ± 13.5	122 ± 5.9	248 ± 1.5
Neutral detergent fibre	266 ± 12.0	264 ± 40.5	284 ± 9.4	489 ± 18.5	479 ± 25.8	520 ± 29.1	498 ± 88.2	486 ± 69.6	213 ± 2.8
Acid detergent fibre	175 ± 9.4	180 ± 16.3	147 ± 12.1	327 ± 9.7	323 ± 16.3	386 ± 17.9	230 ± 29.0	211 ± 30.4	73 ± 2.5
Acid detergent lignin[1]	42.0 ± 2.83	31.6 ± 7.03	36.9 ± 2.80	40.4 ± 2.68	44.0 ± 0.09	52.4 ± 1.59	33.0 ± 1.50	46.8 ± 0.08	27.9 ± 2.82
Crude fibre	201 ± 5.5	225 ± 13.8	222 ± 5.6	288 ± 3.2	289 ± 8.8	324 ± 12.8	269 ± 17.9	276 ± 19.3	157 ± 1.2
Total extractable phenols[1,2]	28.4 ± 0.58	17.1 ± 1.17	24.9 ± 1.77	10.8 ± 0.89	16.2 ± 7.03	10.7 ± 0.26	25.0 ± 2.94	17.6 ± 0.99	22.1 ± 1.50
Non-tannin phenols[1,2]	21.8 ± 1.21	16.8 ± 0.01	21.7 ± 1.98	10.4 ± 0.10	10.2 ± 0.41	9.8 ± 0.60	21.2 ± 0.98	17.2 ± 0.53	20.9 ± 1.07
Total tannins[2, 3]	6.61 ± 1.566	0.35 ± 0.345	3.19 ± 2.372	0.52 ± 0.346	5.99 ± 1.218	0.88 ± 0.192	3.80 ± 1.965	0.34 ± 0.000	1.21 ± 0.832
Calculated variables									
NEL (MJ/kg dry matter)	5.53 ± 0.047	5.25 ± 0.150	5.20 ± 0.058	4.35 ± 0.050	4.26 ± 0.141	3.68 ± 0.254	5.08 ± 0.249	4.72 ± 0.219	5.43 ± 0.047
APDE (g/kg dry matter)	98.3 ± 0.47	93.7 ± 2.69	89.3 ± 1.11	69.3 ± 2.36	67.5 ± 1.89	65.3 ± 3.09	83.0 ± 3.78	78.5 ± 2.87	102.7 ± 0.47
APDN (g/kg dry matter)	134.8 ± 1.21	129.7 ± 6.05	111.2 ± 3.72	59.7 ± 6.05	58.0 ± 1.83	67.8 ± 1.46	103.0 ± 8.67	77.7 ± 3.82	158.7 ± 0.94

APDE/APDN, absorbable protein at the duodenum consisting of rumen-undegradable protein and microbial protein from fermentable energy/ rumen-degradable protein; NA = not available; NEL, net energy for lactation.

[1] Samples pooled per week before analysis

[2] Tannic acid equivalents.

[3] Difference between the overall mean of total extractable phenols and non-tannin phenols.

3.4.2 Eating and rumination behaviour

Eating time per day and per hour did not differ among groups (Table 5, overall treatment means across both weeks not shown in table). Only, SST resulted in a shorter ($P < 0.10$) eating and longer rumination time ($P < 0.05$) between 05.00 h and 13.00 h compared to CON, but no other group differences occurred. The number of activity changes remained unaffected by the treatments.

3.4.3 Milk yield and composition

Milk yield did not differ among groups (Table 6). Still, milk fat and milk protein yields were greater ($P < 0.05$) with SST than with CON, with SLT being intermediate. This was the effect of the combination of numerical differences in both, milk yield as well as milk fat/and protein concentrations. Treatment SST led to a lower ($P < 0.05$) lactose concentration in milk compared with SLT, whereat values of both groups did not differ from CON. Milk urea concentration decreased ($P < 0.001$) from week 3 to 5, and there was a trend ($P < 0.10$) towards lower values with both sainfoin treatments, irrespective of the sampling week, compared to CON. However, milk urea yield did not differ among groups. The BW of cows did not differ among groups.

3.4.4 Faeces-related variables

The faecal CP concentration was higher ($P < 0.05$) in both sainfoin groups compared to CON, and the NDF concentrations were lower with SLT compared to CON ($P < 0.05$; Table 7; overall treatment means across both weeks not shown in table). The proportion of total particles > 0.3 mm in faeces DM was lower ($P < 0.05$) in both sainfoin groups compared to CON, which was mainly caused by differences in the fraction > 0.3 to 1 mm. The proportion of the particles > 4 mm showed a tendency ($P < 0.10$) to be lower in SST compared to CON. In this fraction also an interaction of treatment and week ($P < 0.05$) was found, where the proportion decreased ($P < 0.05$) from week 3 to week 5 in SST and CON, but increased ($P < 0.05$) with advancing experimental duration in SLT. The estimated apparent total tract digestibility of CP tended ($P < 0.10$) to be lower in group SST than in CON. The estimated digestibility of NDF and ADF was lower ($P < 0.05$) with both sainfoin groups compared to CON.

3.4.5. Urine-related variables

Urinary PD:C ratio and index (i.e., adjusted for BW) tended ($P < 0.10$) to be higher with SST compared to CON. Group SLT tended ($P < 0.10$) to have a higher ratio of N:C than CON (Table 8). There was a treatment and week interaction ($P < 0.05$) in the PD:N ratio, where the increase from week 3 to week 5 was greater with CON than with both sainfoin groups. Moreover, SST tended ($P < 0.10$) to have a higher PD:N ratio than SLT, both without difference to the group CON.

Table 5. Effect of short-term versus long-term sainfoin supplementation on eating and rumination time as well as number of activity changes.[1]

Treatment group (G)	CON		SST		SLT			*P*-value		
Sampling week (W)	3	5	3	5	3	5	SEM	G	W	G × W
Eating time										
min/day	524	609	510	573	516	603	11.5	0.561	0.003	0.873
05.00-13.00 h (min/h)	28.0	31.3	24.5	27.8	28.6	28.4	0.64	0.047	0.112	0.551
13.00-21.00 h (min/h)	29.7	32.6	30.6	31.3	29.5	33.6	0.84	0.965	0.143	0.687
21.00-05.00 h (min/h)	8.6	12.3	8.6	12.4	6.5	13.8	0.47	0.957	< 0.001	0.328
Rumination time										
min/day	494	415	530	448	505	457	10.4	0.262	0.003	0.819
05.00-13.00 h (min/h)	18.6	13.7	22.9	17.7	17.9	17.6	0.56	0.005	0.006	0.236
13.00-21.00 h (min/h)	13.1	11.9	13.1	12.8	17.4	11.1	0.52	0.465	0.024	0.119
21.00-05.00 h (min/h)	29.8	26.0	29.9	25.6	28.9	29.0	0.58	0.740	0.032	0.356
Activity changes										
n/day	178	150	155	141	144	144	5.2	0.219	0.200	0.555
05.00-13.00 h (*n*/h)	7.8	6.5	7.4	5.9	7.3	5.8	0.30	0.584	0.031	0.987
13.00-21.00 h (*n*/h)	7.7	6.1	6.6	6.4	6.7	7.0	0.25	0.711	0.343	0.261
21.00-05.00 h (*n*/h)	6.6	5.9	5.6	5.5	3.9	5.3	0.33	0.160	0.768	0.482

[1] Mean of six (SLT), respectively five (CON and SST) measurements per group with recording time of 72 h.

Table 6. Effect of short-term versus long-term sainfoin supplementation on milk yield and composition.[1]

Treatment group (G)	CON		SST		SLT			*P*-value		
Sampling week (W)	3	5	3	5	3	5	SEM	G	W	G × W
Daily yield										
Total milk (kg/cow and day)	17.3	16.8	19.1	19.0	18.9	18.1	0.518	0.291	0.646	0.955
Fat (g/cow and day)	672	649	778	769	752	738	0.015	0.012	0.616	0.982
Protein (g/cow and day)	590	596	653	681	621	639	0.012	0.047	0.468	0.935
Urea (g/cow and day)	5.57	4.73	5.83	4.94	5.96	4.84	0.174	0.808	0.009	0.945
Composition										
Fat (g/100 g milk)	4.13	4.19	4.33	4.38	4.20	4.29	0.084	0.638	0.684	0.993
Protein (g/100 g milk)	3.58	3.74	3.69	3.88	3.52	3.75	0.063	0.564	0.133	0.971
Lactose (g/100 g milk)	4.60	4.62	4.53	4.57	4.69	4.69	0.016	0.007	0.591	0.896
Urea (mg/dL milk)	31.6	27.4	30.2	25.6	30.2	25.9	0.332	0.099	<0.001	0.974
Body weight (kg)	NA	638	NA	649	NA	634	10.9	0.849	NA	NA

NA = not available

[1] All parameters are means of nine (SLT), respectively ten samples (CON and SST, each pooled of four samples per cow and week).

Table 7. Effect of short-term versus long-term sainfoin supplementation on composition of faeces, estimated apparent total tract digestibility and particle size distribution in the faeces.

Treatment group (G)	CON		SST		SLT			*P*-value		
Sampling week (W)	3	5	3	5	3	5	SEM	G	W	G × W
Feces composition (g/kg dry matter)[1]										
Crude protein	141	149	147	157	142	161	0.1	0.006	<0.001	0.076
Neutral detergent fibre	424	422	412	406	415	398	0.3	0.021	0.108	0.491
Acid detergent fibre	459	456	457	452	468	444	0.2	0.794	0.015	0.116
Acid detergent lignin	177	185	174	179	176	177	0.1	0.058	0.005	0.199
Particles (g/100 g dry matter)[2]										
∑ particles > 0.3 mm	50.2	49.3	44.8	44.8	43.9	44.8	0.82	0.015	0.990	0.900
> 0.3 to 1 mm	36.0	33.1	29.7	30.8	30.2	31.4	0.71	0.026	0.880	0.407
> 1 to 2 mm	8.91	10.1	8.07	9.45	11.1	10.9	0.47	0.128	0.393	0.723
> 2 to 4 mm	3.85	3.37	3.64	3.60	2.79	3.28	0.133	0.145	0.970	0.348
> 4 mm	3.61	1.85	2.38	1.51	2.35	2.40	0.134	0.058	0.002	0.020
Estimates of apparent total tract digestibility (g/100 g)										
Crude protein	76.1	81.7	74.9	80.4	76.1	79.7	0.002	0.039	<0.001	0.099
Neutral detergent fibre	76.7	74.6	74.1	71.0	74.2	71.3	0.001	<0.001	<0.001	0.180
Acid detergent fibre	59.2	52.5	54.8	46.1	54.3	46.3	0.001	<0.001	<0.001	0.011

[1] Mean of nine (SLT), respectively ten samples (CON and SST) with three values per sample (each pooled of four samples per cow and week).
[2] Means per group, made of four samples per cow and week.

Table 8 Effect of short-term versus long-term sainfoin supplementation on urine variables.[1]

Treatment group (G)	CON		SST		SLT			*P*-value		
Sampling week (W)	3	5	3	5	3	5	SEM	G	W	G × W
Total N[2] (g/L)	6.25	4.57	4.89	4.59	6.18	5.41	0.091	< 0.001	<0.001	0.010
Allantoin (mmol/L)	11.16	11.04	9.55	10.81	11.65	11.85	0.190	0.005	0.243	0.304
Uric acid (mmol/L)	1.62	1.66	1.38	1.69	1.72	1.76	0.044	0.192	0.146	0.348
Creatinine (mmol/L)	4.10	3.84	3.28	3.60	3.89	3.93	0.087	0.029	0.849	0.381
PD:C ratio[2]	3.16	3.35	3.46	3.65	3.53	3.53	0.061	0.090	0.307	0.765
PD:C index[2,]	397	423	449	475	442	441	8.9	0.065	0.355	0.773
PD:N ratio[2] (mmol/g)	2.05	2.83	2.26	2.76	2.16	2.50	0.037	0.069	<0.001	0.023
N:C ratio[2] (g/mmol)	1.57	1.21	1.54	1.31	1.67	1.41	0.028	0.080	<0.001	0.579

[1] All parameter means of nine (SLT), respectively ten samples (CON and SST) with three values per sample (each pooled of four samples per cow and week).
[2] PD, purine derivatives; N, nitrogen; C, creatinine.
[3] Adjusted by body weight of the cows.

3.5 Discussion

3.5.1 On-farm experimental approach

The main goal of the present study was to confirm the known influence of a feasible level of sainfoin supplementation on the protein metabolism of dairy cows under on-farm conditions with easily

measurable indicators. The real conditions of a commercial dairy farm are different from the controlled, constant and technical environment of a research station. Clearly, the results of such on-farm experiments are restricted to the specific and partly unpredicted conditions under which they are produced. However, this is also true for pasture-based experiments on research stations when natural grasslands with high heterogeneity are investigated (Leiber et al., 2019) and reflects the extremely high variability in forage composition in and between pasture-based systems. This general problem cannot be overcome only by standardized station experiments, but rather by large numbers of similar experiments under varying on-farm conditions (Richter et al., 2009). A constraint of on-farm experiments is that methods of data and sample acquisition, which are invasive or require fixing the animals in their stand, such as for blood and rumen fluid sampling or complete excreta collection, can seldom be applied in privately owned animals. The easiest-to-obtain and likely most comprehensive indicators are those derived from milk, where information about individual milk yield is available from routine testing and where almost always an aliquot of the whole volume milked in 24 h can be recorded. In case of faeces and urine only spot sampling is feasible on farm, meaning that variables derived from these samples can have only proximate character but may still bear complementary information. The digestibility data provided in this study are therefore very clearly just estimates, which give orientation, and the absolute digestibility values have limited accuracy. The chewing sensors used provided a further information related to intake and rumination (Kovacs et al., 1997; Rombach et al., 2019). In the present context, they may be informative proxies of animal responses to herbal feed additives. Despite milk yield and composition, all parameters used in this experiment have to be considered as proxies with the aim to reveal a best possible overall description of the effects of sainfoin supplementation.

3.5.2 Suitability of basal diet, sainfoin pellets and supplementation schedule

During the experimental period, the basal diet showed, as anticipated, a clear excess of APDN compared to APDE, which indicates that the supply with rumen-degradable protein was too high in relation to the energy available for rumen microbial protein synthesis. Sainfoin as model plant for providing tannins to counteract a poor ruminal CP utilization contained 37 g/kg DM of CT. This concentration was in a similar range like in other studies (Aufrere et al., 2008; Bouchard et al., 2013; Scharenberg et al., 2008). Per day, each cow in the sainfoin groups received 70 g CT (equivalent to approximately 4 g CT/kg dietary DM when assuming a total intake of 18 kg DM/day). The CT mainly, if not exclusively, came from the sainfoin because CT concentrations in the other feeds were not detected. With this dietary CT level no adverse impact on feed intake, nutrient digestion and animal health is expected (Aerts et al., 1999a; Bouchard et al., 2013; Scharenberg et al., 2009). The daily CT supplementation was at the lower threshold of dietary CT concentrations at which even positive effects could be anticipated (Scharenberg et al., 2009). On the other hand, the quantity was in a realistic range for on-farm use, because a supply of more than 2 kg /day (as fed) of sainfoin per cow does not appear feasible due to the high costs of production.

A true shortcoming in the present study was that the control pellets made from ryegrass contained unexpectedly low crude protein and energy. This for sure affected yields and limited the conclusions based on comparison with the control. However, a direct comparison between continuous and intermittent sainfoin supplementation appears still valid. In the present study, continuous and intermittent addition sainfoin pellets to the diet were compared in order to determine whether effects persist across a longer adaptation period (Aerts et al., 1999b; Khiaosa-ard et al., 2012). Indeed, effects of the interrupted supplementation of sainfoin (SST) were more pronounced compared to feeding continuously over 3 and up to 5 weeks. This observation points towards a limited effect of continuous supplementation of sainfoin in affecting protein digestion and metabolism in cows through adaptation (Matthews et al., 2019). A successful application of sainfoin supplementation may thus be restricted to periods no longer than a week.

3.5.3 Eating and rumination behavior, digestibility estimates and urinary indicators

Daily eating and rumination times as observed in our study were similar to those in other on-pasture studies (Rombach et al., 2018; Rombach et al., 2019). On average, daily rumination and eating times did not differ between dietary treatments, although during the first half of the day rumination time was longer in SST compared to the other groups. This could be assumed as explanation for the lower proportion of particle size in group SST (Clauss et al., 2009; Kornfelt et al., 2013). The fiber digestibility estimates were nonetheless lower with both sainfoin treatments than in CON, which might be related to an adverse effect of the sainfoin tannins on fibre digestion of the basal diet (Silanikove et al., 2001).

In the present study, ADL concentrations in feed and faeces were used to estimate apparent total tract nutrient digestibility. Lignin was considered to be indigestible (Jung and Allen, 1995). According to the expectations that tannins would bind to protein in the digestive tract (McNabb et al., 1998), the estimated apparent total tract CP digestibility should have been lower with sainfoin supplementation as compared to CON, this, however was not found with a sufficient clarity. Acknowledging the approximate character of the digestibility estimates, the differences were not large enough to consider them relevant.

Indicators for metabolic protein utilization and excretion in urine can be grouped into urinary N and PD. A part of urinary N origins also from the ammonia formed during CP degradation by rumen microbes, which is absorbed, detoxified in the liver and excreted mainly as urea. Thus, it depends on the composition of the diet, mainly on its N to energy balance (Dijkstra 2013). The PD in urine mainly originate from microbial nucleic acids digested and absorbed in the small intestine. They are are used to estimate microbial protein synthesis in rumen (Tas and Susenbeth, 2007).

With sainfoin supplementation during short periods, the PD:C ratio and index tended to be higher compared to CON, indicating an increase in rumen microbial growth in SST as compared with CON. On the one hand, this might be due to the very low CP and NEL supply from the ON pellets. On the other hand, such effect was not found when sainfoin was supplemented continuously. Also, urinary

nitrogen was lower with SST compared to SLT, and accordingly group SST tended to have a higher urinary PD:N ratio than group SLT. This indicates, that intermittent supplementation of sainfoin resulted in a better N utilization by rumen microbes than a continuous supply.

3.5.4 Effects on milk yield and composition

Associated with a higher synthesis of microbial protein and a lower metabolic load of ammonia absorbed from the rumen, supplementing sainfoin as a tannin-rich herb was expected to increase the yield of milk protein and decrease that of milk urea (Totty et al., 2013). In the present study, milk protein yield actually changed as anticipated, but part of this effect was likely due to the poor quality of the supplement in CON. However, in both sampling weeks the effect of SST was stronger than that of SLT, implying a weak hint that particularly the intermittent supply might be of advantage. The milk urea yield was not affected by dietary treatment, likely due to the comparably low level of tannins in sainfoin (Scharenberg et al., 2009). Hence, the chosen levels of sainfoin supplementation were too low to induce pronounced effects on production and protein metabolism, indicating that there is a clear trade-off between production costs for sainfoin supplements and their efficacy/effectiveness in practice. This is in line with similar findings regarding hydrolysable tannin supplements (Kapp-Bitter et al., 2020). Thus, applicable solutions to manipulate nitrogen efficiency in grazing cows remain to be a challenge.

3.6 Conclusions

An experiment was conducted under practical farming conditions with a feasible amount of sainfoin in diets for dairy cows fed high-protein autumn herbage. With interrupted supplementation of sainfoin in two 5-day periods, the milk protein yield slightly increased and the urinary PD indicated an improved microbial growth rate compared to longer lasting sainfoin feeding. Both sainfoin treatments impacted the estimated fiber digestion. Results suggest that sainfoin might affect cattle protein conversion more efficiently when applied interruptedly for short periods. However, the effects were generally small, and there is obviously a trade-off between applicably low levels of sainfoin supplementation and the expected benefits. The limitations regarding the measured parameters of the experiment, which were due to the practice-oriented on-farm situation of the experiment, do not contradict this conclusion.

4. Graded supplementation of chestnut tannins to dairy cows fed protein-rich spring pasture: effects on indicators of protein utilization

Based on Alexandra N. Kapp-Bitter, Uta Dickhoefer, Enyonam Suglo, Livia Baumgartner, Michael Kreuzer, Florian Leiber.
Journal of Animal and Feed Sciences 29, 97-104, 2020.

4.1 Abstract

An on-farm experiment was conducted in order to evaluate effects of graded supplementation with chestnut tannin extract to cows in situations of excessive dietary protein supply on a low-input organic dairy farm. Respectively 10 Swiss Fleckvieh cows received twice per day 1 kg of experimental pellets containing either 0, 50 or 100 g/kg of chestnut extract (targeted at approximately 0, 5 and 10 g extract/kg of total dietary dry matter). Experimental feeding lasted for 21 days. Measurements and collection of milk, feces and urine spot samples were performed in weeks 0 (baseline), 1 and 3. All cows were kept in one herd on pasture; fresh grass and grass hay were provided in the barn during night. Milk yield was recorded and cows wore sensor halters for recording chewing activity. In urine, total nitrogen and purine derivatives were measured; faeces were analysed for protein, fibre and particle fractions; in milk, solid concentrations were determined. The data was analysed with a general linear model. Cows did not show differences in general eating and rumination behaviour, but needed time to accept the tannin-containing pellets. Milk yield and composition were not affected by treatment, except for lactose content. No relevant differences between treatments were found for urinary and faecal parameters. In conclusion, although technically easy to supplement, pellets containing chestnut tannin extract were not readily accepted by the cows and effects on protein digestion and metabolism were not found. Successful on-farm application of chestnut extract in order to improve nitrogen efficiency therefore seems questionable.

4.2 Introduction

Against the background of the increasing ecological pressure due to land-use for feed concentrate production (Wilkinson, 2011; Schader et al., 2015), low or zero feeding of concentrates to ruminants is one option in discussion (Ertl et al., 2015; Leiber et al., 2017). One of the associated challenges is to improve the conversion of feed nitrogen (N) into milk N when nutritional imbalances in the forage cannot be counterbalanced with concentrates. In low-input systems protein excess in pasture grass often occurs during spring and autumn (Pacheco and Waghorn, 2008). This excess protein is degraded to ammonium in the rumen and is at least partly not used by the rumen microbes. Ammonium has to be metabolized in the liver under energy consumption (Parker et al., 1995) and is excreted largely as easily volatile urea via urine. Hence, protein excess in cattle diets is a burden for the cow's metabolism and for the environment.

A possible solution could be to feed tannin rich plants and profit from potentially positive effects on the protein metabolism of ruminants (Mueller-Harvey, 2006). Tannins may form complexes with dietary proteins in the rumen and thereby reduce proteolysis; the protein could be released in the abomasum (McSweeney et al., 2001). One promising tannin-containing feed supplement is the extract from the chestnut tree (*Castanea sativa* Miller), consisting primarily of hydrolysable tannins (Mueller-Harvey, 2006). *In vitro*, chestnut extract was found to reduce ruminal protein degradation without influencing microbial protein synthesis (Wischer et al., 2013). Chestnut tannin extract therefore may

reduce the metabolic load from excessive ammonium *in vivo* and either increase the supply with protein released from the tannin-protein complex or enhance its excretion as quite stable faecal compounds (Mueller-Harvey, 2006). Supplementing chestnut extract was found to reduce N losses (Śliwiński et al., 2004) and methane emissions (Duval et al., 2016) from the manure of dairy cows. Concomitantly, milk yield was not (Duval et al., 2016) or even positively affected (Ali et al., 2017), and no toxic effects or decline in feed intake was reported (Śliwiński et al., 2004; Duval et al., 2016). As the protein excess in grass is usually timely limited, chestnut extract could be supplemented on a farm with reasonable costs. It is, however, unknown, if the effects of such extracts are substantial and can be detected and quantified also under practice conditions of a commercial farm.

The aim of this study was, therefore, to investigate if adding graded levels of chestnut tannin extract results in an improved protein digestion and metabolism under practical conditions with excessive N supply from spring pasture. For this purpose, dehydrated grass pellets were produced as a vehicle for chestnut extract supplementation. Data and samples related to chewing behaviour, milk performance, and faeces and urine composition were obtained. The hypothesis was that the supplemented chestnut extract would lower ruminal protein degradability and eventually improve protein efficiency by better utilization in the duodenum. Under commercial on-farm conditions, this should be indicated by reduced milk urea concentrations and eventually either higher milk protein yields or decreased apparent protein digestibility, or both. Purine derivatives in urine should be indicative for changes in ruminal microbial protein synthesis.

4.3 Materials and Methods

4.3.1 Experimental design and protocol

This experiment took place from April until May 2017 on an organic dairy farm and was approved by the cantonal veterinary office of Aargau, Switzerland (AG75689). A pre-treatment sampling week (week 0) served to collect baseline data. For the following 21 days (weeks 1-3), 30 lactating Swiss Fleckvieh cows were randomly assigned to three groups of ten animals each and allocated to treatments with pellets containing 0, 50 or 100 g/kg of chestnut tannin extract (TAN0, TAN50 or TAN100; targeted at approximately 0, 5 or 10 g extract/kg of total dietary dry matter (DM) based on supplementation of 2 kg pellets/day and an estimated total daily feed intake of 20 kg DM for all cows (Leiber et al., 2015a). Data and sample collection were carried out in weeks 0, 1 and 3. The initial average milk yield before the experiment for the three groups of ten animals each (± standard deviation) was 25.4±5.2, 25.3±4.9 and 25.5±5.4 kg/day, respectively. The corresponding values for days in milk were 131±58, 118±54 and 116±75; cows had 3.6±1.7, 3.6±1.9 and 3.4±2.0 lactations, and milk urea content was 19.5±5.0, 20.7±5.0 and 19.4±4.6 mg/dl. Cows were kept together in one herd in an open-space barn and went to pasture (natural grass-rich pasture on a nutrient rich ley soil) during the day between milking times from 05.00 to 16.30 h. During night, a freshly cut grass-clover mixture consisting of *Lolium multiflorum* and red clover (*Triticum pratense*) was offered in barn *ad libitum*. Because of sudden frost and snowfall,

duration of pasture access had to be halved in the last six days of the experiment, with the cows receiving more of the fresh grass-clover mixture and 3 kg DM/day of extra meadow hay (*L. perenne*) per cow in barn for compensation.

After morning and evening milking, cows were fixed in headlocks for 30 min and were individually fed 1 kg of the experimental pellets (totally 2 kg/cow per day) produced by UFAG (Herzogenbuchsee, Switzerland). Pellets consisted of dehydrated *L. perenne*, 100 g/kg of molasses and either 0, 50 or 100 g/kg of chestnut tannin extract (as fed). The rather low levels of supplementation had been chosen in order to avoid detrimental effects (Jayanegara et al., 2011; Henke et al., 2017) and to be economically feasible under practice conditions. The tannin extract (powder) had been prepared from *Castanea sativa* Miller (Farmatan 75®, Tanin Sevnica, Slovenia), and contained (g/100 g of extract): pure tannins (75, including castalagin, vescalagin, castalin and vesaclin) as well as sugar (16), water (7) and crude ash (2), according to the producer.

4.3.2 Data and sample collection

Grazed and mowed grasses were sampled twice per week during the experiment; the hay was sampled twice in the last week, as it was only fed then. For each sampling, samples of the pasture forage were taken from five randomly distributed places, each over 1 square meter, and cut 5 cm above the ground. Samples of the grass hay and fresh grass-clover mixture fed in the barn were taken from five places distributed over the whole length of the feeding bank. Pellet samples were taken three times. Forage samples were dried at 40°C for 48 h. All samples were milled through a 0.5-mm-sieve (Retsch SK 100, Retsch®, Haan, Germany).

In all sampling weeks, pressure sensor halters (RumiWatch®, Itin + Hoch GmbH, Liestal, Switzerland) were mounted on six cows per group for 5 days, except for TAN100, of which seven animals were chosen. Data obtained between 05.00 h on Tuesday and 04.59 h on Friday of each sampling week were converted to eating and ruminating time and number of activity changes (Rumiwatch converter V0.7.3.2; Rombach et al., 2018). Based on this, the mean values per day and hour were calculated. Days were also subdivided into diurnal phases (05.00 to 13.00 h, 13.00 to 21.00 h and 21.00 to 05.00 h) in order to analyse more details of behavioural patterns throughout the day.

Samples of milk, faeces and urine were taken on Tuesday and Thursday evenings, and Wednesday and Friday mornings in the sampling weeks. Milk yield was recorded at each milking. The corresponding evening and morning milk samples were mixed proportionate to milk amounts per cow and conserved with Bronopol®. Individual body weight was estimated in weeks 0 and 3 of the experiment with a weighing tape (Vieh- und Schweinemessband 250 cm, Hoechstmass Balzer GmbH, Sulzbach, Germany). Faeces samples were taken from the rectum of each cow and were stored at 4°C. On Fridays of each week, samples were pooled at equal proportions to one sample per cow per week. Half of the pooled sample was kept at -20°C, the other half was dried at 40°C for 48 h and milled to 0.5-mm-particle size afterwards. Urine was collected individually from cows when spontaneously excreted

or after manual stimulation. To avoid pollution, approximately 200ml of urine were taken three seconds after starting of excretion (Chizzotti et al., 2008). Afterwards, the samples were acidified to pH 2-3 with 20% sulphuric acid (v/v). Afterwards, the urine samples were filtered (Whatman™ filter paper 1), diluted with distilled water (1:5, v/v) and frozen at -20°C. Before analysing, the samples were pooled to one sample per cow and week.

4.3.3 Laboratory analysis

With near infrared reflectance (NIR) spectroscopy (NIRFlex N-500, Büchi, Flawil, Switzerland) the concentrations of dry matter (DM), total ash, crude protein and fiber fractions (i.e., neutral and acid detergent fibre, NDF and ADF) in forage and faeces samples were determined. The NIR device was calibrated with 180 forage (from different grass-herb swards) and 45 faeces samples (from five different farms, including samples from the present study), which had been analysed for proximate compounds with standard methods (Leiber et al., 2015a). The content of ash-free acid detergent lignin (ADL) in forage samples was determined with sulphuric acid (75%, v/v) (Fibretherm FT 12, C. Gerhardt GmbH & Co. KG, Königswinter, Germany). For phenol analysis, the protocols of Makkar (2003) were followed, and concentrations of condensed tannins (CT) were determined with the butanol-HCl-assay. Total extractable phenols (TEP) and non-tannin phenols (NTP) were expressed as tannic acid equivalents, CT were expressed as leucocyanidin equivalents. Total tannins were calculated as TEP minus NTP. The particle size distribution of faeces was determined according to Leiber et al. (2015b) by washing faeces samples sequentially through sieves with mesh sizes of 4.0, 2.0, 1.0 and 0.3 mm diameter and drying the residues for 12 h at 105°C. Milk samples were analysed for fat, protein, lactose and urea with a Fourier transform infrared spectroscopy (MilkoScan FT 6000, Foss Electric, Hillerød, Denmark). The total urine N was determined with a Kjeldahl apparatus (Büchi B324, Büchi Labortechnik GmbH, Essen, Germany) after digestion with the Gerhardt KT20 (C. Gerhardt GmbH & Co, Königswinter, Germany). Allantoin, uric acid and creatinine (CR) were determined by high-performance reversed-phase liquid chromatography following Dickhoefer et al. (2015). The sum of allantoin and uric acid was considered to reflect total purine derivatives (PD). The CR excretion via urine was considered to be constant relative to the animals' protein and thus body mass (Chizzotti et al., 2008). Hence, CR concentration in urine was used to correct N and PD concentrations for differences in urine volume and dilution rate.

4.3.4 Calculations

Net energy for lactation (NEL) and absorbable protein at the duodenum, based on rumen-undegradable protein plus microbial protein either from fermentable energy (APDE) or from rumen-degradable protein (APDN) were calculated following Agroscope (2019), using the feeds' concentrations of DM, total ash, crude protein and crude fibre. Apparent total tract nutrient digestibility was estimated according to Leiber et al. (2015a). Assumptions made were a total DM intake of 20 kg/cow and day using a regression of Agroscope (2019) in relation to average milk yield of the cows. The DM intake

during grazing on pasture was estimated from total DM intake minus grass, hay and pellet intakes in barn, which were determined by test weightings of the feed offered. Furthermore, ADL was assumed to be indigestible (Jung and Allen, 1995). The following equations were used:

(1) Faeces amount [kg DM/day]=ADL intake [g/day]/ADL in faeces [g/kg DM]

(2) Faecal nutrient excretion [g/day] =faeces amount [kg DM/day]×nutrient in faeces [g/kg DM]

(3) Apparent total tract nutrient digestibility [g/100 g] = (1– (nutrient excretion [g/day])/nutrient intake [g/day])) ×100.

From urine data, four ratios were calculated:

(1) PD:CR ratio=(allantoin [mmol/l]+uric acid [mmol/l])/CR [mmol/l]

(2) adjusted PD:CR index=(allantoin [mmol/l]+uric acid [mmol/l])/CR [mmol/l]×body weight [kg0.75]

(3) PD:N ratio=PD [mmol/l]/N [g/l]

(4) N:CR ratio =nitrogen [g/l]/CR [mmol/l].

The PD:CR ratio and index are indicators for the ruminal microbial protein synthesis (Chizzotti et al., 2008). The PD:N ratio indicates the efficiency of feed N use for rumen microbial growth (Tas and Susenbeth, 2007), and the N:CR ratio is an indicator for the N excretion with the urine (Chizzotti et al., 2008).

4.3.5 Statistical analysis

Data were analysed with SPSS® (Version 24) statistical software, using a general linear model with treatment (level of chestnut tannins in pellets), week (1 or 3) and their interaction as fixed factors, where the data from week 0 was used for weighted least squares-correction on animal level. As no significant interaction between treatment and week occurred (except for pellet intake) and no significant week effects could be detected, tables only display treatment means and *P*-values across both sampling weeks. With the Tukey's method, multiple comparisons among treatment means were accomplished.

4.4 Results

The crude protein content of pasture and barn-fed fresh forage was highest at the start of the experiment, and decreased towards the end of the experiment (Table 9). The APDN:APDE ratio concomitantly declined from 1.16 and 1.15 (weeks 0 and 1) to 1.07 in week 3. The experimental pellets offered to the different treatment groups clearly differed in the contents of phenolic fractions, whereas they were similar in the other constituents. The amount of TEP and TT in the forage varied little throughout the experiment, and no CT could be detected. With 16.2 and 53.3 g/kg, the concentration of TT in the 50 g- and the 100 g-pellets was lower than targeted.

Table 9. Chemical composition of the basal diet components (g/kg dry matter; means ± standard deviation) in the sampling weeks (mean of two samples with three replicates per sample) and ryegrass pellets including 100 g/kg of molasses, and 0, 50 and 100 g/kg chestnut tannin extract (mean of three samples with three replicates per sample).

	Fresh grass-clover mixture fed in barn			Pasture forage			Grass hay fed in barn	Treatment pellets		
Week	0	1	3	0	1	3	3	TAN0	TAN50	TAN100
Proportion in diet (g/kg)[1]	317	290	390	683	616	362	151			
Analysaed variables										
Dry matter (g/kg fresh weight)	191 ± 8	191 ± 11	174 ± 11	221 ± 27	260 ± 5	312 ± 95	916 ± 0	895 ± 3	888 ± 4	879 ± 4
Total ash	103 ± 8	107 ± 1	95.2 ± 1.5	97 ± 0	98 ± 3	93 ± 9	126 ± 23	129 ± 2	125 ± 4	118 ± 2
Crude protein	155 ± 4	148 ± 2	113 ± 1	156 ± 5	141 ± 11	141 ± 7	111 ± 2	209 ± 3	207 ± 1	211 ± 1
Neutral detergent fibre	377 ± 17	372 ± 45	388 ± 3	382 ± 12	391 ± 18	402 ± 35	490 ± 11	225 ± 57	235 ± 34	239 ± 31
Acid detergent fibre	206 ± 8	204 ± 9	255 ± 4	193 ± 10	202 ± 14	206 ± 1	311 ± 7	155 ± 22	169 ± 17	170 ± 9
Acid detergent lignin[2]	33.9 ± 0	21.7 ± 0	20.9 ± 1	19.2 ± 0	17.3 ± 0	16.5 ± 1	23.1 ± 1	76.5 ± 6	80.9 ± 2	82.2 ± 6
Crude fibre	252 ± 1	258 ± 6	260 ± 1	254 ± 6	260 ± 2	261 ± 6	295 ± 6	223 ± 6	242 ± 2	254 ± 2
Total extractable phenols[2,3]	26.7 ± 0	28.0 ± 3	29.3 ± 1	23.6 ± 1	23.3 ± 4	21.5 ± 2	11.9 ± 1	17.2 ± 3	33.9 ± 4	78.2 ± 4
Non-tannin phenols[2,3]	19.7 ± 2	16.8 ± 2	20.7 ± 1	16.1 ± 1	19.2 ± 2	18.5 ± 1	11.2 ± 3	17.2 ± 1	17.8 ± 1	25.0 ± 1
Total tannins[2,3,4]	7.1 ± 2	11.2 ± 3	8.6 ± 1	7.5 ±2	4.1 ± 3	3.0 ± 0	0.6 ± 1	0.0 ± 0	16.1 ± 4	53.3 ± 5
Condensed tannins[2,5]	ND	ND	ND	ND	ND	ND	ND	ND	0.70 ± 0.0	0.96 ± 0.0
Calculated variables										
NEL (MJ/kg dry matter)	5.02 ± 0	4.85 ± 0	4.67 ± 0	5.02 ± 0	4.97 ± 0	4.86 ± 0	3.93 ± 0	5.23 ± 0.05	5.04 ± 0.05	4.89 ± 0.03
APDE	86.2 ± 0	83.5 ± 1	75.7 ± 1	85.8 ± 1	82.5 ± 2	82.4 ± 0	68.3 ± 4	94.3 ± 1	92.3 ± 0	91.3 ± 1
APDN	99.7 ± 3	94.7 ± 1	71.3 ± 1	99.8 ± 3	89.7 ± 7	90.0 ± 5	70.7 ± 1	134 ± 2	133 ± 0	135 ± 1

APDN, absorbable protein at the duodenum consisting of rumen-undegradable protein, APDE, microbial protein from fermentable energy/rumen-degradable protein; NEL, net energy for lactation; ND = not detected

[1]The difference to 1000 g is the proportion of fed pellets in week 1 and 3

[2]Samples pooled per week before analysis

[3]Tannic acid equivalents

[4]Difference between the overall mean of total extractable phenols and non-tannin phenols

[5]Leucocyanidin equivalents

The intake of the TAN100 pellets was significantly lower than in the other treatments (Table 10). This was more pronounced in week 1 (96.8, 82.1 and 57.9 g/100 g of pellets offered consumed in TAN0, TAN50 and TAN100, respectively) than in week 3 (98.9, 99.3 and 87.1 g/100 g; (as fed)), when there was only a tendency ($P < 0.10$) of a difference in pellet intake. Daily eating time, rumination time and number of activity changes were not influenced by dietary treatments.

Table 10. Effect of feeding pellets containing different amounts of chestnut tannin extract on eating and rumination time as well as number of activity changes averaged across two experimental sampling weeks.

	TAN0	TAN50	TAN100	SEM	*P-value*
Pellet intake (% of offered)	98.5[a]	89.8[a]	74.6[b]	1.69	< 0.001
Eating time[1]					
min/day	584	583	608	9.9	0.509
05.00-13.00 h (min/h)	29.7	28.1	34.1	1.40	0.203
13.00-21.00 h (min/h)	32.3	33.2	39.2	2.14	0.351
21.00-05.00 h (min/h)	10.2	11.1	11.3	0.45	0.567
Rumination time[1]					
min/day	527	545	558	6.3	0.139
05.00-13.00 h (min/h)	17.0	19.2	20.7	1.22	0.449
13.00-21.00 h (min/h)	18.0	17.8	20.6	0.88	0.348
21.00-05.00 h (min/h)	31.3	31.2	37.1	1.46	0.159
Activity changes[1]					
n/day	130	120	122	4.1	0.527
05.00-13.00 h (*n*/h)	5.31	4.82	6.14	0.432	0.454
13.00-21.00 h (*n*/h)	5.74	4.87	5.99	0.350	0.423
21.00-05.00 h (*n*/h)	5.09	5.16	5.51	0.280	0.799

[a b]Group-means with different superscripts differ significantly at $P < 0.05$.
[1]Mean of six measurements per group (seven with 100 g extract /kg) with total recording time of 72 h.

Milk yield and milk solid composition also did not differ among treatments (Table 11), except for lactose content, which was lower in TAN50 compared to TAN0 and TAN100.

Table 11. Effect of the pellets containing different amounts of chestnut tannin extract on milk yield and composition averaged across two experimental sampling weeks.

	TAN0	TAN50	TAN100	SEM	*P-value*
Daily milk yield					
total milk (kg/cow and day)	25.7	24.3	25.4	0.51	0.520
fat (g/cow and day)	872	873	896	0.0	0.803
protein (g/cow and day)	801	792	797	0.0	0.969
lactose (kg/ cow and day)	1.21	1.19	1.21	0.024	0.900
urea (g/cow and day)	4.73	4.55	4.47	0.155	0.767
Milk composition					
fat (g/100 g milk)	3.63	3.67	3.57	0.044	0.685
protein (g/100 g milk)	3.30	3.32	3.24	0.029	0.490
lactose (g/100 g milk)	4.74[a]	4.88[b]	4.77[a]	0.018	0.005
urea (mg/dL)	18.3	18.1	18.2	0.563	0.985

[a b] Group means with different superscripts differ significantly at $P < 0.05$.

Faeces composition and estimated apparent total tract digestibility estimates were not different between treatments (Table 12). Particle size distribution in faeces differed only in the proportion of the fraction >4 mm, which was significantly higher in TAN50 than TAN100. Total N concentration and

PD:CR ratio in urine were greater in TAN50 than TAN0. Urinary allantoin concentration tended ($P < 0.10$) to be higher in TAN50 than TAN100. The other urine parameters did not differ between treatments.

Table 12. Effect of the pellets containing different amounts of chestnut tannin extract on faecal and urine variables averaged across two experimental sampling weeks.

	TAN0	TAN50	TAN100	SEM	*P-value*
Feces composition (g/kg of DM)[1]					
crude protein	163	162	165	1.1	0.680
neutral detergent fibre	409	398	413	4.7	0.460
acid detergent fibre	397	379	383	4.4	0.201
acid detergent lignin	164	162	166	1.5	0.620
Estimated apparent digestibility (%)					
crude protein	81.7	81.2	81.1	0.00	0.395
neutral detergent fibre	83.9	83.9	83.7	0.00	0.382
acid detergent fibre	72.4	73.2	73.4	0.00	0.306
Particles in faeces (g/100 g of DM)[2]					
∑ particles > 0.3 mm	46.4	49.4	44.9	2.43	0.743
> 0.3 to 1 mm	30.8	31.8	29.8	1.36	0.842
> 1 to 2 mm	7.33	6.68	7.49	0.549	0.816
> 2 to 4 mm	4.73	5.73	4.63	0.420	0.496
> 4mm	4.08^{ab}	5.79^{a}	3.69^{b}	0.354	0.034
Urine variables					
total N (g/l)	7.12^{a}	9.31^{b}	8.72^{ab}	0.295	0.015
allantoin (mmol/l)	12.7	13.8	12.2	0.29	0.063
uric acid (mmol/l)	0.917	0.975	0.898	0.0220	0.327
creatinine (CR; mmol/l)	4.47	4.24	4.16	0.116	0.533
purine derivatives (PD):CR ratio	3.14^{a}	3.58^{b}	3.35^{ab}	0.069	0.038
PD:CR index[3]	421	448	430	9.6	0.505
PD:N ratio (mmol/g)	2.60	2.54	1.58	0.236	0.175
N:CR ratio (g/mmol)	1.86	2.26	2.32	0.123	0.297

[a,b] Group means with different superscripts differ significantly at $P < 0.05$
[1]Mean of ten samples with three values per sample (each from four samples pooled per cow and week)
[2]Means per group, made of four samples per cow and week
[3]Adjusted for body weight of individual cows

4.5 Discussion

4.5.1 Suitability of the on-farm approach

It was the main goal to investigate, if supplementing pellets enriched with chestnut tannin extract is a method to be established on farm in order to improve protein digestion and metabolism under conditions of dietary N excess in pasture-only feeding situations. For this purpose, an organic dairy farm with a zero-concentrate feeding strategy at spring season was chosen and the results would apply for these conditions. Limitations of an on-farm situation are that, except for yield of milk and milk constituents, only few quantitative measures are available and therefore estimates with restricted accuracy have to be used. Although data of the chewing sensors used are related with feed intake (Rombach et al., 2018), they are useful for relative estimation of treatment effects rather than absolute intake quantification

under practical conditions (Leiber et al., 2016). Also regarding digestibility, only rough estimates could be calculated, which represent rather relative than absolute values. Still, there is quite a set of on-farm parameters, including the urinary N compounds, which may give information about the influence of the chestnut tannin extract on the nutrient digestion and protein metabolism of the dairy cows. The important advantage of this kind of on-farm experiments is that results are closer to real practice conditions than it might be obtained on station.

4.5.2 Experimental feeds

In the present study, pellets with increasing contents of chestnut tannin extract, providing approximately 0, 5 and 10 g extract per kg of total diet, were fed during 21 days. Unfortunately, this resulted in clearly lower measured tannin concentrations than had been targeted (0, 1.6 and 5.3 g total tannins, respectively, as analyzed). Considering also the incomplete intake of the pellets (90% for TAN50, 75% for TAN100), the realized intake was only 29 g of hydrolysable tannin/day for TAN50 and 80 g/day for TAN100. However, with these concentrations of chestnut tannins in the diet, effects on N metabolism in ruminants had been achieved in other studies (Ali et al., 2017; Aboagye et al., 2018). For on-farm conditions a risk of detrimental effects due to too high tannin supplements (Jayanegara et al., 2010; Henke et al., 2017) cannot be accepted. Therefore, even though the realized tannin levels were at the lower end, it must be considered that, given an obvious difficulty of exact dosage, there would be not much margin to increase them without negative effects. Thus, if the actual supplementation levels in this study would not work, this would mean a fail of concept for practice.

Although milk urea concentrations were not high, there was still potential to reduce them. The basal diet indeed had an excess of crude protein with APDN > APDE and CP/NEL > 25, suggesting that crude protein supply for the rumen microbial synthesis was too high in relation to their energy supply.

4.5.3 Palatability, intake, and milk production

The immediate palatability of the pellets was limited, especially with the highest level of chestnut extract. In week 1, only slightly more than half of the amount offered in TAN100 was consumed, but even with treatment TAN50, 20% of offered amount was refused. This was likely owed to the astringent taste of tannins (Kumar and Singh, 1984) as, in order to limit need for pellets, extract concentration was quite high. In other studies, the extract was given via concentrate (e.g. Ali et al., 2017) or mixed in total ration or silage (Śliwiński et al., 2004; Colombini et al., 2009; Aboagye et al., 2018) to ensure high and stable intake. These approaches, however, were impossible under the given on-farm conditions.

Regarding total feed intake indicators, we found no significant differences in eating and rumination time among groups and the data recorded was in the expected range (Rombach et al., 2018). Therefore, it could be assumed that total intake on pasture and during the night in the barn did not substantially differ among TAN0, TAN50 and TAN100, and that there was no effect on rumination time. Supplementing chestnut tannin extract did also not affect milk yield. This is consistent with some other studies, where even higher concentrations of chestnut tannins were used (Śliwiński et al., 2004;

Colombini et al., 2009). Contrary to the literature (Śliwiński et al., 2004; Ali et al. 2017; Colombini et al., 2019), in the present study the protein and fat concentrations were not affected. Most importantly, falsifying part of the hypothesis, no effects on milk urea occurred. The results indicate that the supplementation level chosen was likely too low to provoke the desired effects. However, higher tannin supplementation levels could also have led to milk yield depressions (Henke et al., 2017), which has to be avoided in practice. Thus, it appears to be very critical to find the right dosage of tannins if clear but only positive effects on protein efficiency are the target.

4.5.4 Indicators of chestnut extract effects in faeces and urine

Purine derivatives in the urine allow for estimation the amount of rumen microbial protein produced and digested in the duodenum, as they origin mainly from rumen microbial nucleic acids and their derivatives (Tas and Susenbeth, 2007; Henke et al., 2017). In the present study, almost no effects of treatment on these indicators were found, which is in line with the absence of effects on milk urea, and indicates once again that even 5.3 g hydrolysable tannins per kg DM of feed (as realized with TAN100) is below the effective level. The urinary N concentrations and excretion in ruminants partly depend on the amount of ammonium formed in the rumen, which increases with surplus of protein available to the rumen microbes in diet compared to their energy supply (Nousiainen et al., 2004). When urinary N concentration was related to CR concentrations to correct for dilution in spot samples (Chizzotti et al., 1998), this parameter was also not significantly different between groups. Also, the N content of feces was not influenced by supplementing the chestnut extract. This was also opposite to our expectation, that tannins would shift the N excretion from urine to faeces (Mueller-Harvey, 2006). The apparent total tract nutrient digestibility estimates had been calculated under the assumption, that DM intake was adequately estimated and that ADL is fully indigestible (Jung and Allen, 1995). These digestibility estimates were not affected by treatment. Also, no effects were found on faecal fibre fractions, which are considered to be indicative for fibre degradation (Leiber et al, 2015b). This indicates that no detrimental effects on digestion were provoked by the given chestnut tannin supplementation levels.

4.6 Conclusions

From a theoretical concept, supplementing ryegrass pellets containing chestnut tannin extract in a low-input system at the times of excessive dietary crude protein could be quite easily realized on a farm. The present study, however, revealed two major setbacks. Even at a dosage too low to reach the desired effects, palatability of the pellets was impaired. Despite this, no clear improvements in N utilization of the cows were found. Based on these results, the supplementation of hydrolysable tannins to dairy cows in low-input systems does not appear to improve protein digestion and N metabolism, and is therefore not recommended.

5. Efficiency of small burnet, birdsfoot trefoil and plantain in limiting nitrogen losses by dairy cows in a low-concentrate feeding system

Based on Alexandra N. Kapp-Bitter, Jöel Berard, Sergej L. Amelchanka, Cem Baki, Carmen Kunz, Andrea Steiner, Michael Kreuzer, Florian Leiber.
In preparation for submission to Animal Nutrition.

5.1 Abstract

In the present study the tanniferous forage plant, small burnet (*Sanguisorba minor*), was compared with the reasonably well researched birdsfoot trefoil (*Lotus corniculatus*) and plantain (*Plantago lanceolata*) in their efficiency to abate urinary nitrogen losses and to enhance nitrogen utilization of dairy cows. The plants were mixed into a grass/maize-based forage diet at 80 g/kg dietary dry matter, aimed at reflecting an inclusion level applicable in farm practice. The plants replaced English ryegrass (control diet). Twenty-four multiparous dairy cows were randomly allocated to the four diets. Complete intake, milk yields and urine and faeces excretion were recorded individually, and representative samples of milk and excreta were taken over eight days, following ten days of adaptation. During sampling days, jaw movements were recorded with Rumiwatch® sensors. The diets supplemented with birdsfoot trefoil, plantain or burnet contained, per kg of dry matter, 1.8, 1.7 and 15.5 g total tannins; 1.8, 1.2 and 1.9 g condensed tannins; and 0.0, 0.5 and 13.6 g hydrolysable tannins, respectively. Treatment effects were observed for patterns of activity change between eating, rumination and idling. Burnet reduced the digestibility of acid detergent fibre, but not of neutral detergent fibre, and lowered the occurrence of small particle fractions in faeces. Milk yields and composition were not affected, apart from a decline in milk protein content when feeding plantain. Milk urea content was reduced with burnet by 26% compared to control, plantain and birdsfoot trefoil. Burnet, but not birdsfoot trefoil and plantain, substantially shifted nitrogen excretion from urine to faeces (about 30% lower urine-N losses), but did not concomitantly improve utilization of dietary nitrogen for milk protein synthesis. The results indicate that burnet is an interesting forage plant for reducing the N burden of the animal's metabolism and the environment. A similar effectiveness of feeding birdsfoot trefoil or plantain seems to need cultivars with higher phenol contents or larger dietary additions.

5.2 Introduction

Dairy production systems, which are based on low dietary concentrate proportions (Leiber et al., 2017), may experience a varying nutrient composition, since system definitions or production standards restrict counterbalancing imbalance in the forage with cereals or oilseed cakes. This is particularly a problem when there is an excess of dietary nitrogen (Powell et al., 2012), e.g. in spring or autumn pasture. High forage crude protein (CP) concentrations may be associated with a relative lack of energy in the rumen and an increase in ruminal production of ammonia with the consequence of low nitrogen use efficiency, metabolic stress and increased urine N excretion (Nousiainen et al., 2004; Pacheco and Waghorn, 2008; Powell et al., 2012). One approach to minimise ruminal ammonia production and thus N losses could consist in protecting excessive protein from ruminal degradation by plant phenols, especially tannins, which build complexes with proteins at ruminal pH (Mueller-Harvey et al., 2019). At abomasal pH, tannin-protein complexes may disintegrate, thus making the protein available for small intestinal digestion (Mueller-Harvey et al., 2019). In this case, N utilization would be improved and urinary N excretion would be reduced (Tseu et al., 2020). Even in the event that the protein-tannin complex would

remain indigestible and be excreted with the faeces in a form slowly mineralised in the soil, this would at least slow down N-leakage from the nutrient cycles to water bodies. Besides condensed tannins (CT; Barry and McNabb, 1999; Mueller-Harvey et al., 2019), hydrolysable tannins (HT) have also been demonstrated to lower ruminal ammonia production (Jayanegara et al., 2011) and milk urea concentrations (Ali et al., 2017), the latter being indicative for urinary N excretion (Nousiainen et al., 2004).

However, such approaches need to be implemented in farm practice. One straightforward way could be the integration of tannin-rich herbs into pasture swards (Cheng et al., 2017). We focused on three tannin-rich herb species, proven to be well capable of being established in grasslands (Hamacher et al., 2012). Small burnet (Sanguisorba minor), present in natural pastures, is characterised by a high content of secondary plant compounds (Hamacher et al., 2012) and was shown to affect rumen fermentation (Meissner et al., 1993; Kaplan et al., 2014). Plantain (Plantago lanceolata), a further forage plant with comparably high phenol contents, was already investigated on pasture or in diets, and somewhat contrasting results concerning urine-N losses and milk yield were found (Cheng et al., 2017; Minneé et al., 2017; Bryant et al., 2018; Ineichen et al., 2019). Similarly, birdsfoot trefoil (Lotus corniculatus), containing CT, is of interest in this context as it was shown to reduce ruminal protein degradation (Molan et al., 2001) and urinary N excretion (Ghelichkhan et al., 2018).

The aim of the present study was to test, in a fully controlled experiment, the potential effect of the mentioned herbs on the nitrogen balance of cows fed forage-based diets slightly excessive in dietary protein. In a wider perspective, we considered scenarios of establishment of such herbs in pastures up to 10% biomass as realistic. Therefore, we introduced wild-sampled herb materials into standard diets at 80 g/kg DM and evaluated the effects on: intake behaviour; digestibility; milk yield and quality; and nitrogen partitioning to milk solids and excreta. The main hypothesis was that the herb additives would at best increase protein use efficiency or at least shift nitrogen excretion from urine to faeces.

5.3 Materials and methods

5.3.1 Dietary treatments, cows and housing

This experiment was carried out in spring 2019 at the Research Station AgroVet-Strickhof, Eschikon-Lindau, Switzerland. It was approved by the cantonal veterinary office of Aargau, Switzerland (AG75689).

The basal diet consisted (g/kg DM) of grass silage (balanced ryegrass dominated sward, harvested at panicle formation stage), 377; maize silage, 327; commercial protein-rich mineral-supplemented concentrate, 154; ryegrass hay (similar sward type and harvest stage as the grass silage), 41; wheat straw, 21; and experimental plant material, 80. The concentrate was composed of soybean meal, maize gluten, rapeseed meal, rapeseed cake, triticale, sugar beet molasses, sunflower meal, wheat starch and minerals. In the control treatment, part of the diet (80g/kg) was dried English ryegrass (*Lolium perenne*; purchased from Urs Knecht, Brütten, Switzerland). The experimental plant materials comprised birdsfoot trefoil,

plantain or burnet (all purchased from Phyzolaboratoire, 26400 Aouste-sur-Sye, France; collected from natural swards in France, Albania and China, respectively). They were also added to the respective diets at 80g/kg DM. To prevent selection of single components, all diets were offered as total mixed rations. Feed was offered at ad libitum access. Before providing new portions, refusals were always weighed and removed.

Twenty-four multiparous dairy cows (12 Brown Swiss and 12 Holstein) were split into two runs investigated consecutively for 25 days each. Per run, three cows each were assigned to one of the four dietary treatments in a randomised design ensuring balanced means of milk yield (overall initially 26.4 ± 5.7 kg/day) and days in milk (263 ± 89), as well as contents of protein (3.84 ± 0.29) and urea (25.0 ± 4.9 mg/dL) in milk. Across both runs, breeds were balanced among treatments.

In the first four experimental days, cows were kept in a loose housing system and adapted to the basal experimental diet. Thereafter, cows were kept in a tied stall for another ten days of adaption where the treatment plants were added to the diet. Stands were randomly assigned in position in the barn with respect to the diets. Over four days in each of the following two weeks, sampling was performed. Feeds were renewed twice daily at 05:00 and 16:00 h and milking at the stands took place simultaneously.

Samples of all diet components were taken ten times (five times equally distributed over each run). Due to lower variation, concentrate, mineral mix and herb meals were pooled to one sample per run. After collection, grass and maize silage were dried at 40°C for 48 h. Subsequently, all feeds were milled through a 0.5-mm sieve (Retsch SK 100, Retsch®, Haan, Germany). Feed intake was registered with balances mounted below the individual feed plates at each stand (Mettler-Toledo, 8606 Greifensee, Switzerland). Cows wore RumiWatch® halter sensors (Itin + Hoch GmbH, Liestal) for recording of jaw movements. Data from the sensors were resolved to eating, ruminating, and idling (Rumiwatch converter® V0.7.3.2; Rombach et al., 2018). Frequencies of changes between the different activities were calculated. Data obtained between 12.00 h on Monday and 11.59 h on Thursday in the respective sampling week were taken to calculate means per day and per hour. The daytimes were subdivided into three phases, 04.00 to 12.00 h, 12.00 to 20.00 h and 20.00 to 04.00 h.

During the four sampling days per week, milk, urine and faeces amounts were recorded daily and samples were drawn from each individual cow. Evening and morning milk samples were pooled corresponding to milk yield and conserved with Bronopol®. Faeces were collected in trays arranged below a grid at the rear end of the stands (Leiber et al., 2004). Representative samples were drawn daily and stored at 4°C. Urine was collected with urinals attached to the skin around the vulva with Velcro straps. The urine was ducted by a tube into canisters containing sulphuric acid adjusted to prevent gaseous N-losses (Leiber et al., 2004). From each canister, one sample per day was taken and frozen at –20°C. Later, faeces and urine samples each were pooled to one sample per cow and sampling week. An aliquot part of the faeces was dried at 60°C for 48 h and milled to 0.5 mm diameter, the remainder was frozen at –20°C.

5.3.2 Laboratory analyses

The contents of dry matter (DM), total ash and CP of feed items and faeces were analysed with standard methods (AOAC, 2005). Dry matter and total ash were determined with a thermogravimetric device model TGA 701 (Leco Corporation, St. Joseph, MI, USA) and N was analysed on a C/N-analyser (TruMac CN, Leco Corporation, St. Joseph, Michigan, USA, AOAC index no. 968.06). Crude protein contents of feed items were calculated as 6.25 × N. The contents of neutral (NDF) and acid detergent fibre (ADF) of the concentrate were determined on a Fibretherm analyser (Gerhardt, Königswinter, Germany; method 6.5.1 and 6.5.2, respectively, VDLUFA Methodenbuch, 2012). Fibre fractions were expressed without residual ash, NDF was assayed with heat-stable amylase and without sodium sulphite. The contents of fibre fractions in all other feed items and faeces were determined with near infrared spectroscopy (NIRFlex N-500, Büchi, Flawil, Switzerland). The NIR device was calibrated with 180 forage (from different grass-herb swards and silages) and 45 faeces samples (from five different farms).

Total extractable phenols (TEP), non-tannin phenols (NTP) and CT of the feed items were analysed as described by Makkar (2003). Briefly, 60 mg were put into 70% aqueous acetone (v/v) and were pressed with a syringe (Braun Omnifix® syringe 5ml) through a filter (Cameo™ syringe filter™, non-sterile, pore size 1.2 µm). Absorption was measured at 725 nm and 550 nm (CT) with a spectrophotometer (Bio Spectrometer Eppendorf D30). The results were expressed as tannic acid equivalents and for CT as leucocyanidin equivalents. Total tannins (TT) were calculated as TEP minus NTP, hydrolysable tannins (HT) as TT minus CT.

Particle size distribution in the faeces was quantified with a sieve washing method (Leiber et al., 2015). Exactly 100 g of sample was put on top of four sieves with 4, 2, 1 and 0.3 mm mesh sizes. Each sieve was rinsed for 10 s with water. Afterwards, the residues were dried for 12 h at 105°C and weighed.

Urine-N was analysed in the acidified samples with the Dumas-method (Trumac CN, Leco Corporation, St. Joseph, MI, USA). Milk was analysed at Suisselab (Zollikofen, Switzerland) for fat, protein, lactose and urea using Fourier transform infrared spectroscopy (MilkoScan FT 6000, Foss Electric, Hillerød, Denmark).

5.3.3 Calculations and statistical analysis

Contents of net energy for lactation (NEL) and of absorbable protein at the duodenum, based on rumen-undegradable protein plus microbial protein either from fermentable energy (APDE) or from rumen-degradable protein (APDN), were estimated applying equations from Agroscope (2020) from measured contents of DM, total ash, CP and crude fibre. Milk protein-N content was calculated as protein/6.38. For considering milk urea in the N-balance, it was multiplied by 0.467 for N-concentration, and 1.03 kg of milk was counted as 1 L. According to Agroscope (2020), energy corrected milk (ECM) was calculated as follows:

ECM = Milk yield [kg] × (0.38 × fat [%] + 0.24 × protein [%] + 0.17 × lactose [%]) / 3.14

Data were analysed with SPSS® version 24, applying a general linear model, with treatment, run and their interaction as fixed factors, and animal as the experimental unit. As the sampling weeks within the runs were not significant in a first model run, this effect was omitted from the model. Milk-related data obtained before the experiment were used as covariate. Multiple comparisons among treatment means were performed with Tukey's procedure, considering $P < 0.05$ as significant and $P < 0.10$ as tendency. The tables display treatment means and standard errors of the mean.

5.4 Results

5.4.1 Feed characteristics, intake and eating behaviour

The ryegrass used for the control treatment was of unexpectedly poor quality (low in CP, high in fibre; Table 13). There was a gradient from low to high CP content from burnet to plantain and birdsfoot trefoil, with a gradient in the opposite direction in TEP and TT contents. The high TEP content of burnet was mostly resulting from HT. The complete control, birdsfoot trefoil, plantain and burnet diets as consumed contained per kg DM: 14.0, 15.6, 17.2 and 32.1 g TEP; 0.1, 1.9, 1.7 and 15.5 g TT; 0.0, 1.8, 1.2 and 1.9 g CT; and 0.1, 0.1, 0.5 and 13.6 g HT, respectively.

Intakes of DM and organic matter were similar in all groups. Differences in nutrient intake therefore resulted from compositional differences. Accordingly, there were tendencies for higher CP and APDN intakes with birdsfoot trefoil compared to control (Table 14). The APDN-to-APDE ratio was 1.15, 1.19, 1.13 and 1.14 for control, birdsfoot trefoil, plantain and burnet, respectively. The TEP intake was twice as high and that of TT ten times as high with burnet than with birdsfoot trefoil and plantain. Intake of TEP did not differ from control with the latter two, but TT intake was significantly higher. Apart from HT, the burnet group also consumed most NTP (not significantly different from plantain).

Eating and rumination times did not differ among groups across the day nor in the three 8-h phases (Table 14). Nevertheless, the number of activity changes was significantly lower with burnet than with birdsfoot trefoil (across the entire day). Between 12.00 h and 20.00 h, the number of activity changes tended to be lower with burnet compared to plantain.

Table 13. Composition of the diet components (g/kg dry matter) and the diets (means ± standard deviations).

	Basal diet components						Test plants		
Item	Grass silage[1]	Maize silage[1]	Mineralised concentrate[2]	Grass hay[1]	Wheat straw[1]	English ryegrass[2]	Birdsfoot trefoil[2]	Plantain[2]	Burnet[2]
Proportion in diet realised	377	327	154	41	21	80[8]	80[8]	80[8]	80[8]
Analysed variables									
Dry matter (g/kg wet weight)	378 ± 25	321 ± 28	918 ± 1	918 ± 1	920 ± 2	920 ± 2	901 ± 0	908 ± 0	848 ± 0
Organic matter	782 ± 19	895 ± 6	872 ± 3	843 ± 4	851 ± 4	875 ± 0	823 ± 4	743 ± 1	840 ± 0
Crude protein	170 ± 8	73 ± 4	372 ± 17	72 ± 1	44 ± 1	43 ± 0	196 ± 0	120 ± 0	54 ± 0
Neutral detergent fibre	373 ± 40	360 ± 44	183 ± 8	412 ± 10	790 ± 25	724 ± 20	347 ± 3	308 ± 15	441 ± 2
Acid detergent fibre	274 ± 17	206 ± 8	104 ± 6	214 ± 3	421 ± 7	485 ± 3	277 ± 4	274 ± 1	246 ± 1
Crude fibre	255 ± 16	220 ± 6	117 ± 7	363 ± 1	439 ± 3	360 ± 15	242 ± 3	252 ± 0	256 ± 2
Total extractable phenols[3,4]	18.2 ± 0.3	15.0 ± 0.2	7.5 ± 1.4	10.1 ± 1.4	7.5 ± 0.3	7.1 ± 1.2	26.0 ± 1.8	45.7 ± 1.2	243.6 ± 24.8
Non-tannin phenols[3,4]	18.2 ± 1.1	15.0 ± 0.3	7.2 ± 0.7	9.8 ± 0.3	7.5 ± 0.2	6.7 ± 1.0	3.6 ± 0.3	26 ± 1.0	40 ± 1.6
Total tannins[3,4,5]	0 ± 0.86	0 ± 0.18	0.26 ± 1.29	0.26± 0.90	0.00 ± 0.34	0.37 ± 1.23	22.38 ± 1.76	20.09 ± 1.23	203.47 ± 24.91
Condensed tannins[3,6]	ND	ND	ND	ND	ND	ND	22.4 ± 0.9	14.6 ± 0.7	25.2 ± 1.2
Hydrolysable tannins[3,7]	0 ± 0.81	0 ± 0.22	0.26 ± 1.38	0.26 ± 0.46	0 ± 0.39	0.37 ± 1.36	0 ± 1.79	5.50 ± 1.19	178.3 ± 0.19
Calculated variables									
NEL	5.74 ± 0.13	6.32 ± 0.06	6.57 ± 0.00	4.01 ± 0.03	2.99 ± 0.05	3.95 ± 0.25	5.85 ± 0.05	4.80 ± 0.00	5.2 ± 0.0
APDE	77.1 ± 1.2	64.2 ± 0.4	198.2 ± 6.0	61.7 ± 0.5	43.9 ± 0.7	53.0 ± 2.0	102.0 ± 0.0	79.0 ± 0.0	68.0 ± 0.0
APDN	106.6 ± 5.2	45.4 ± 2.4	271.9 ± 11.8	44.5 ± 0.7	26.8 ± 0.7	26.0 ± 0.00	126.0 ± 0.0	76.0 ± 0.00	32.5 ± 0.5

APDE/APDN, absorbable protein at the duodenum consisting of rumen-undegradable protein and microbial protein from fermentable energy/rumen-degradable protein; NEL, net energy for lactation; ND = not detected.

[1]Mean of ten samples with three replicates per sample.

[2]Mean of two samples with three replicates per sample.

[3]Samples pooled before analysis to one sample per run.

[4]Tannic acid equivalents.

[5]Difference between total extractable phenols and non-tannin phenols.

[6]Leucocyanidin equivalents.

[7]Difference between total tannins and condensed tannins.

[8]For calculation, mean of dry matter amount of supplements was taken

Table 14. Intake, digestibility and chewing behaviour (n = 6 per treatment; calculated from 2 × 72 h individual recording time per cow).

	Treatment					
	Control	B. trefoil	Plantain	Burnet	SEM	*P*-value
Daily intake per cow						
Dry matter (kg)	18.9	19.9	19.8	19.8	0.36	0.765
Organic matter (kg)	16.0	16.7	16.5	16.7	0.30	0.841
Crude protein (kg)	2.88$^{(a)}$	3.27$^{(b)}$	2.88$^{(a)}$	3.04$^{(ab)}$	0.0551	0.055
Neutral detergent fibre (kg)	7.17	6.90	7.02	7.22	0.134	0.833
Acid detergent fibre (kg)	4.62	4.50	4.69	4.43	0.086	0.734
NEL (MJ)	110	118	114	117	2.104	0.551
APDE (kg)	1.67	1.83	1.67	1.77	0.032	0.222
APDN (kg)	1.92$^{(a)}$	2.17$^{(b)}$	1.89$^{(a)}$	1.92$^{(ab)}$	0.037	0.042
Total extractable phenols (g)	266^{a}	310^{ab}	341^{b}	635^{c}	6.5	<0.001
Non tannin phenols (g)	265^{a}	273^{ab}	307^{bc}	328^{c}	5.2	<0.001
Total tannins (g)	2^{a}	37^{b}	33^{b}	308^{c}	2.1	<0.001
Condensed tannins (g)	0^{a}	36^{c}	24^{b}	38^{c}	0.4	<0.001
Hydrolysable tannins (g)	2^{a}	1^{a}	10^{a}	270^{b}	1.8	<0.001
Eating time						
min/day	417	421	378	412	8.7	0.292
04.00-12.00 h (min/h)	21.0	21.5	18.6	21.5	0.56	0.209
12.00-20.00 h (min/h)	22.1	22.7	20.9	21.4	0.50	0.597
20.00-04.00 (min/h)	9.00	8.46	7.79	8.59	0.291	0.532
Rumination time						
min/day	495	498	501	501	6.3	0.981
04.00-12.00 h (min/h)	17.6	15.4	16.2	16.9	0.40	0.213
12.00-20.00 h (min/h)	17.7	19.1	18.3	18.7	0.36	0.573
20.00-04.00 (min/h)	26.4	27.8	28.1	27.0	0.44	0.533
Activity changes						
n/day	151^{ab}	174^{b}	166^{ab}	139^{a}	4.4	0.040
04.00-12.00 h (*n*/h)	7.46	8.19	7.44	6.88	0.181	0.102
12.00-20.00 h (*n*/h)	6.93$^{(ab)}$	8.16$^{(a)}$	8.25$^{(a)}$	6.34$^{(b)}$	0.259	0.031
20.00-04.00 (*n*/h)	4.48	5.37	5.08	4.21	0.200	0.169

$^{a-d}$Means within a row with different superscripts differ at $P < 0.05$.
$^{(a)-(b)}$Means within a row with different superscripts differ at $P < 0.10$.
APDE/APDN, absorbable protein at the duodenum consisting of rumen-undegradable protein and microbial protein from fermentable energy/rumen-degradable protein; NEL, net energy for lactation

5.4.2 Amounts and composition of faeces and urine, and digestibility

Faeces amounts (DM) were similar in all groups, while urine amounts were lowest with burnet compared to all other groups (Table 15). There was no treatment effect on faecal DM content and CP content in DM. Faeces of the burnet-fed cows had the lowest NDF content (compared to control and the plantain group) and, concomitantly, the highest ADF content (compared to the birdsfoot trefoil group). Urine-N content was not affected by the test plants. Apparent ADF digestibility had a tendency to be higher with plantain than with burnet, but treatments did not differ in apparent OM, CP and NDF digestibility. The sum of particles > 0.3 mm was lower with plantain and burnet compared to control. This was most

pronounced in the fractions of particles > 0.3 and < 1 mm length and > 1.0 and < 2.0 mm length (tendency for a difference of plantain vs. control).

Table 15. Characteristics of faecal and urinary excretion as well as apparent total tract digestibility (n = 6 per treatment; calculated from 2 × 72 h individual total amount sampling per cow).

	Treatment					
	Control	B. trefoil	Plantain	Burnet	SEM	*P*-value
Daily amounts (kg/cow)						
Faeces (DM)	4.95	5.28	4.65	5.49	0.148	0.283
Urine	29.1[a]	32.3[a]	29.4[a]	23.1[b]	0.80	0.002
Faeces composition (g/kg)						
Dry matter (DM)	126	127	119	113	2.9	0.291
Organic matter in DM	752[ab]	757[b]	737[a]	743[ab]	1.955	0.005
Crude protein in DM	157[a]	163[a]	161[a]	173[b]	1.115	<0.001
Neutral detergent fibre in DM	495[a]	482[ab]	497[a]	473[b]	0.2	0.004
Acid detergent fibre in DM	428[ab]	414[b]	431[ab]	437[a]	0.3	0.025
Particle fractions						
∑ 0.3 mm	360[a]	348[ab]	311[b]	317[b]	5.2	0.004
> 0.3 to 1 mm	185[a]	163[ab]	151[b]	145[b]	3.0	<0.001
> 1 to 2 mm	109[(a)]	100[(ab)]	93[(b)]	94[(ab)]	2.3	0.078
> 2 to 4 mm	47.1	60.7	49.0	48.4	2.26	0.134
> 4 mm	20.2	25.0	17.2	29.8	2.02	0.149
Urine-N (g/kg)	5.75	5.25	5.25	4.94	0.116	0.113
Apparent total tract nutrient digestibility (%)						
Organic matter	76.5	75.8	79.3	75.6	0.648	0.164
Crude protein	72.5	73.5	74.1	69.6	0.767	0.180
Neutral detergent fibre	65.3	62.7	68.2	63.2	0.985	0.200
Acid detergent fibre	53.6[ab]	51.2[ab]	57.9[a]	46.4[b]	1.292	0.025

[a-b]Means within a row with different superscripts differ at $P < 0.05$.
[(a)-(b)]Means within a row with different superscripts differ at $P < 0.10$.

5.4.3 Milk yield and composition

Yields of milk, ECM, fat, protein, and lactose did not differ among groups (Table 16). Milk protein content was highest in control and with birdsfoot trefoil, lowest with plantain and intermediate with burnet. Lactose content was significantly higher with plantain than with birdsfoot trefoil. Milk urea content was up to 26% lower with burnet compared to ryegrass and plantain, with birdsfoot trefoil being intermediate.

5.4.4 Nitrogen balance

The daily N secretion with milk did not differ among groups; however, the urinary N-excretion was significantly lower with burnet than with all other groups by about 30% on average. Nitrogen excretion with faeces was higher with burnet than with birdsfoot trefoil but did not differ from the group control (Table 17). Relative to intake, the urinary N losses via urine on average declined by 102 g/kg with burnet compared to the other treatments. This also affected the ratio of urine-N and faeces-N to total-N excretion accordingly. The utilization of total dietary N and apparently digested N did not differ among groups.

Table 16. Milk yield and milk composition (n = 6 per treatment; each pooled from 2 × 2 samples per cow).

	Treatment					
	Control	B. trefoil	Plantain	Burnet	SEM	*P*-value
Daily yield						
Total milk (kg)	21.2	18.2	21.1	21.6	0.818	0.479
Energy corrected milk (kg)	24.1	21.1	22.9	23.8	1.000	0.683
Fat (g)	1059	924	989	1025	47.667	0.735
Protein (g)	795	717	725	804	30.369	0.659
Lactose (g)	975	816	1013	985	39.483	0.299
Urea (g)	5.85	4.70	5.67	4.59	0.242	0.129
Milk composition						
Fat (%)	4.98	5.06	4.94	4.66	0.071	0.233
Protein (%)	3.93[a]	3.94[a]	3.64[b]	3.73[ab]	0.031	0.003
Lactose (%)	4.61[ab]	4.43[b]	4.72[a]	4.52[ab]	0.029	0.007
Urea (mg/dL)	27.8[a]	25.0[ab]	27.0[a]	20.6[b]	0.63	0.001

[a-b]Means within a row with different superscripts differ at $P < 0.05$.

Table 17. N-balance and N-utilization of nitrogen (n = 6 per treatment; calculated from 2 × 72 h individual total amount sampling/recording).

	Treatment					
	Control	B. trefoil	Plantain	Burnet	SEM	*P*-value
N-balance (g/day per cow)						
Intake	461[(a)]	523[(b)]	461[(a)]	486[(ab)]	8.8	0.055
Faeces	124[ab]	138[ab]	120[a]	149[b]	3.8	0.033
Urine	166[a]	167[a]	152[a]	114[b]	4.2	<0.001
Milk[c]	122	111	111	122	5.1	0.755
Unexplained[d]	49[(a)]	107[(b)]	79[(ab)]	101[(ab)]	7.8	0.051
N-losses (g/kg of N intake)						
Faeces	273[(ab)]	266[(ab)]	258[(a)]	310[(b)]	7.7	0.098
Urine	362[a]	322[a]	332[a]	237[b]	8.0	<0.001
Urine-N : faeces-N ratio	56.9[a]	54.6[a]	56.3[a]	43.7[b]	1.1	<0.001
N-utilization (g milk N per kg of intake of)[c]						
Total N	264	214	239	253	9.5	0.293
Apparently digested N	365	292	326	370	14.6	0.213

[a-b]Means within a row with different superscripts differ at $P < 0.05$
[(a)-(b)]Means within a row with different superscripts differ at $P < 0.10$
[c]Sum of milk protein N and milk urea N
[d]Including body retention

5.5 Discussion

5.5.1 Experimental design and feeds

It was the main goal of the present investigation to extend the possibilities for farm practice to abate urine-N excretion and improve N utilization in dairy cows in low-concentrate feeding systems during periods of dietary N excess. Therefore, we studied cows fed a common forage-based diet with moderate protein excess resulting in dietary CP contents in the range of 145-165 g/kg DM and a moderate surplus of APDN over APDE. The aim was to counteract this excess by replacing ryegrass (control) with tannin-

containing meals of birdsfoot trefoil, plantain and burnet, provided at dietary proportions of 80 g/kg DM. The level of the herb supplements in the present study was chosen to target practicability. We assumed that up to 10% of such herbs in pastures might be integrated at reasonable costs, but not more. Also, cultivating such herbs in monocultures dedicated to supplementation of cows in dietary N excess could hardly be possible at costs affordable for more than 10% of a temporary diet.

The ryegrass for the control group was of unexpectedly low quality, while the tannin-rich test plants were of higher nutritive value. These herbs differed little in nutrient composition, but substantially in TEP content and composition. Although all three test plants contained relatively similar amounts of CT, burnet was by far richer in TEP, NTP and especially HT than birdsfoot trefoil and plantain, which were comparable regarding phenol fractions. Compared to literature, the phenol and tannin concentrations analysed in all three plants were in a usual range (Hamacher et al., 2012; Ghelichkhan et al., 2018; Kara et al., 2018; Stewart et al., 2019).

5.5.2 Effects of the test plants on feed intake, digestibility and performance

DM intake did not differ among groups suggesting all plants were of similar palatability. This is comparable to other studies where intake was not impaired by the substantial dietary inclusion of birdsfoot trefoil (Broderick et al., 2017; Stewart et al., 2019), plantain (Cheng et al., 2017; Ineichen et al., 2019) or burnet (Stewart et al., 2019), although some refusal had been expected due to the bitter and astringent taste properties of tannins (Kapp-Bitter et al., 2020).

In contrast to the study of Tseu et al. (2020), intake and duration of eating and ruminating were not affected by the test plants in the present study, but we found a lower frequency of activity changes in cows fed burnet. The main distinctive property of burnet was its high concentration of HT which might have changed ruminal fermentation patterns (Jayanegara et al., 2011), and indirectly influenced eating behaviour (Tseu et al., 2020), even if the total intake and duration of eating were not affected.

As an indicator of fibre degradation, we assessed the abundance of particle fractions in faeces. Cows fed plantain and burnet had a lower total content of particles >0.3 mm in faeces DM than control, especially due to the small fractions, which points towards an improved fibre degradation (Kornfelt et al., 2013). However, other than expected, the differences could not be explained by a correspondingly different time spent for rumination. Perhaps, differences in fibre structure from the test plants may have led to variation in ruminal degradation and retention times (Owens et al., 1998; Kornfelt et al., 2013). Anyway, the assumption of an improved fibre digestion of the burnet diet is in contrast to the ADF digestibility which was lowest in that diet. The latter suggests a lower energetic value of burnet even compared to the low-quality ryegrass, different from what was calculated from proximate contents (NEL). However, if true, this was not distinctive enough to affect milk yield. All diets had a similar apparent digestibility of OM, CP and NDF. The similar digestibility values with partly negative effects of burnet are generally consistent with reports from other studies (Hymes-Fecht et al., 2013; Cheng et al., 2017; Stewart et al., 2019). However, it has to be taken into account that phenolic concentrations in herbs largely depend on

their phenological stage (Kälber et al., 2014; Stewart et al., 2019) and thus results may not be generalised.

The tested herbs had been expected to improve feed protein utilization and to increase milk protein yield. The lack of such effects indicates that the feed CP protected by tannins from ruminal degradation did not contribute noticeably to metabolic protein supply. Consequently, neither did we find any effects on feed nitrogen utilization. However, the reduced milk urea content in the group treated with burnet gives a rather strong indication that the HT from this herb indeed protected feed protein from ruminal degradation (Pacheco and Waghorn, 2008). Consistent with our findings, supplementing 200 g/kg DM of birdsfoot trefoil did not improve the N-utilization in cows in the study of Grosse Brinkhaus et al. (2016). Broderick et al. (2017) observed an improved N-efficiency, however at considerably higher dosages (51% of DM). Concerning plantain, Ineichen et al. (2019) also found no improvement in N-utilization for milk protein formation, neither did Box et al. (2017) even when feeding plantain exclusively.

5.5.3 Effect of the test plants on the route of N excretion, and associated effects on faeces and urine amounts as well as composition

With increasing dietary contents of phenols, tannins and especially CT, N-excretion was expected to shift from urine to faeces which would then be associated with a higher faeces-N content and lower apparent CP digestibility (Mueller-Harvey et al., 2019; Dschaak et al., 2011; Ghelichkhan et al., 2018). A shift like this was indeed observed, but, similarly to Stewart et al. (2019), only with burnet. Therefore, HT obviously also exhibit a CP-protecting activity in the rumen. Apart from that, it seems that the phenol amounts provided by the supplementation of birdsfoot trefoil and plantain in the present study were too small to cause a significant rerouting of dietary N from urine to faeces. At an unexpectedly low CT content, Grosse Brinkhaus et al. (2016) also found birdsfoot trefoil supplementation to be ineffective in mitigating ruminal ammonia concentration and urinary N losses in dairy cows. Ineichen et al. (2019) reported a lack of effect also for plantain, whereas other authors found considerable effects of plantain on N-metabolism of ruminants (e.g. Minnée et al., 2017, Cheng et al., 2017). These contradictory results highlight the fact that generalisation has to be made cautiously since phenolic concentrations in herbs largely depend on phenological stages (Kälber et al., 2014; Stewart et al., 2019) and tannins may differ in their effectiveness due to differences between cultivars (Mueller-Harvey et al., 2019).

The milk urea content turned out to be an excellent indicator of the effectiveness of the test plants in the present study, not only in principle but also concerning the level of effect, as the average declines in urine-N excretion and milk urea content with burnet were similar with 30% and 26%, respectively. With birdsfoot trefoil, the same effect was also described by Broderick et al. (2017), whereas neither variable were affected by birdsfoot trefoil in the experiment of Grosse Brinkhaus et al. (2016).

5.6 Conclusion

In an experiment designed to evaluate the effects of tannin-rich herb supplements in dairy cow diets at dosages relevant to farm practice, birdsfoot trefoil, plantain and burnet did not improve feed nitrogen utilization for milk N secretion. However, burnet was very effective in shifting the N-excretion from urine to faeces, thus ameliorating the N-burden of animals and the environment. Milk urea content turned out to be indicative not only for the basic effectiveness, but also for the level of effect in this respect. To feed this amount of burnet on-farm would require establishing this herb in pastures or cultivating it separately for inclusion in barn-fed diets. Cost-effectiveness assessments still have to be made. Care has to be taken concerning burnet addition owing to the observation that its phenols are mostly hydrolysable tannins which may become harmful to the animal when consumed at too high levels. Similar comparative studies including burnet are rare; thus, broader experimental evidence is needed for this particular herb.

6. General Discussion and Conclusion

The present thesis was based on the following considerations, which built on former research and experiences:

- Commonly, energy-rich compounds are supplemented for balancing diets with excessive crude protein (Hoffman et al., 1993). Such supplements affect the fermentation in the rumen of cows (Krajcarski-Hunt et al., 2002) and have to be grown on arable land, which is lost for food production (Cassidy et al., 2013; Knaus, 2013).
- Milk from dairy cattle can be produced with feed exclusively originating from grassland (Broderick 2006, Leiber et al., 2017).
- Concerning requirements of the cows, the protein content of the forage from grassland is too high in some seasons in relation to the content of fermentable energy (Pacheco & Waghorn 2008) resulting in an inefficient use and therefore loss of nitrogen resources (Broderick 2006).
- Some secondary plant compounds, such as tannins, have the ability to bind to dietary protein in the rumen (Barbehenn & Constabel, 2011) and thus reduce their degradation to ammonia and therefore reduce nitrogen losses through excretion via urine (Dschaak et al., 2011). In some cases, tannins may even improve the nitrogen conversion efficiency (Min et al., 2003).
- Tannins also occur in some temperate climate meadow plants (Jayanegara et al., 2011; Terranova et al., 2018) and thus it could be an option to foster their cultivation in grassland.
- Therefore, supplementing the forage-based diet of dairy cows with temperate climate phenol-containing plants during protein excess in the diet may be an approach to alleviate the burden on the cow's metabolism, to reduce nitrogen losses and even to increase biodiversity (Constabel et al., 2014).

To test whether these findings can be transferred to and confirmed under practical conditions was the motivation to perform one *in vitro* and three *in vivo* experiments. They were conducted with feasible dietary proportions of experimental plants, two were conducted on a low-input organic dairy farm and the third on a research-station.

6.1 Considerations concerning the suitability of the approach for farm practice

As it was the aim of this thesis to test the effects of tannin containing plants under practical conditions, two *in vivo* experiments (Chapter 3 and 4) were conducted on a commercial organic dairy farm. These dairy cows were already adapted to exclusively grassland-based feeds during the vegetation period. Thus, the conditions were optimal for testing the practical implementation of supplementing the animals with tannin-containing plants while grazing on pasture. During the third *in vivo* experiment, which was conducted on a research station (Chapter 5), cows were fed with a total-mixed ratio and stayed during sampling periods in the barn.

The biggest disadvantage of experiments conducted on-farm, was the inaccurate estimation of feed intake on pasture and also in barn. In contrast to the experiment on research-station, where the forage was placed as total-mixed diets on individual balances in front of each cow and each weight change was registered automatically, the amount of nutrients consumed could not be calculated exactly on farm. Consequently, also the apparent digestibility could only be estimated. To have at least proxies, cows were equipped with chewing sensors, which worked very well except in week one of experiment 1, building on the findings that these data are related with feed intake (Rombach et al., 2018). Alternatively, to increase the accuracy when cattle are grazing on pasture, methods such as markers, herbage disappearance or differences in animal performance could be implemented in the experiments (Macoon et al., 2003).

During the on-farm experiments, only spot samples of faeces and urine could be taken in contrast to the research station, where the available facilities allowed complete excretion collection. In the case of faecal samples, this again led to inaccurate estimates concerning the excreted nutrients. However, variations in the dilution of urine samples can be corrected with the creatinine content (Chizzotti et al., 2008), and therefore the results were assumed to be approximately the same as those obtained with total collection. The applied complete collection method on research station had the disadvantage, that the cows had to stay at their places during sampling period and therefore, experiments with cows grazing on pasture were excluded.

Measuring and analysing of milk parameters did not differ between on-farm and research experiments and the obtained results can therefore be compared.

Even if it was tried to minimize them, different impacts influenced the conditions during the experiments. This includes for example animal characteristic, such as individual stress resistance, influences from individual handling of the animals and samples through involved persons, and influences from nature which could not completely be avoided nor be balanced. Therefore, the environmental impacts during the conducted experiments in this project, on-farm as well as on research station, restrict the results to the specific conditions which occurred during the experiment (Leiber et al., 2019; Richter et al., 2009).

6.2 Tanning-containing plants and their integration in dairy cattle diets under practical conditions

In the *in vitro* study seven plants harvested at a late growth stage were identified to lower the ammonia formation without impairing IVOMD compared to incubations of *L. perenne* and *M. sativa* alone (Chapter 2). Negative correlations between phenolic contents and ammonia production were found as they were also described by Bhatta et al. (2013). However, probably influences on the ammonia formation of bioactive plant compounds other than from phenols (e.g. Navarette et al., 2016) or other plant ingredients, which were not analysed during this experiment, could not be excluded.

For a transfer of the approach into farm practice, factors of experimental plants, of animals, management and also economic aspects had to be considered. Compared to high quality forage (e.g. van

Dorland et al., 2007) which meets the genetic need and potential of modern dairy cow breeds, the nutritional values of the tested plants, harvested at a late growth stage (Chapter 2), were low. Additionally, high-bred forage plants, such as ryegrass, red and white clover or lucerne, tolerate an intensive utilisation and achieve a high harvest. Therefore, an effective supplementation amount as low as possible would be most interesting from an economic point of view, to avoid replacing a bigger part of the regular diet with the plants tested in the *in vitro* experiment.

Further, the acceptance by the cows of higher amounts of tannin-containing plants in the diet could be compromised, as secondary plant compounds may limit the palatability (Silanikove et al., 2001). The acceptance of pellets with 100 g/kg of chestnut tannin extract was indeed reduced, and cows refused them especially at the start of the experiment (Chapter 4). In contrast, the sainfoin pellets (Chapter 3), the pellets with 50 g/kg of chestnut tannin extract (Chapter 4) and the total mixed rations containing *S. minor, L. corniculatus* and *P. lanceolata* (Chapter 5) were accepted by the cows. Preference trials with different forages or different conservation methods (e.g. Lombardi et al., 2015) would be an interesting option for further investigations.

For the purpose of good control and for ensuring intake, the application in form of pellets (Chapter 3 and 4) and the mixing into a total mix ratio (Chapter 5) were chosen in the experiments. Both kinds of application were successful but go along with high production costs and the need of cropland, as plants have to be grown, harvested and processed separately. Less expensive possibilities, such as conservation methods without pelleting (fresh, hay or silage) or sowing directly into pasture, should be investigated further concerning their successful applicability.

The effects seem also to depend on the duration of supplementing. With sainfoin, an effect was only observed when supplemented over short periods (Chapter 3). This suggest that rumen microbes adapt to the exposure to tannin-containing plants, when supplements were fed over longer time (Matthews et al., 2019). Therefore, adding tannin-containing supplements to the diet should be limited to the time period of high protein content in forages; this also to ensure economic feasibility. Perhaps, also the time point of offering the supplemented plants during the day or even in dependence of the feeding itself could have an impact, whereas the harvesting time of the forage is probably negligible (Lombardi et al., 2015).

Adding tannin-containing plants in higher amounts has to be done with caution, as toxicity can not be excluded, especially in case of the digestible hydrolysable tannins (Neser et al., 1982; Hervás et al., 2003). However, none of the test plants led to any change concerning the measured or estimated amount of total dry matter intake or milk yield, and no signs of toxicology were observed during the experiments (Chapter 3 – 5).

6.3 Influence of supplementing tannin-containing plants to the diet of dairy cattle on nitrogen-related parameters

It was the main goal of the present thesis to investigate, if effects of temperate climate tannin containing plants on the nitrogen metabolism of dairy cows could be detected under farm practice conditions. Four of the promising plants (harvested at late growth stage) out of the *in vitro* experiment and additionally chestnut tannin extract, were investigated during the *in vivo* experiments. Approximately 10% of the diet was replaced with the experimental plants, which seemed to be a feasible dosage for practical conditions. However, only small effects on the nitrogen metabolism were detected.

When fed during a short time, sainfoin led to a higher milk protein yield and to a trend of a decreased milk urea content. Independent of feeding duration, it led to a higher crude protein content of the faeces. These are indicators for lower amounts of ammonia absorbed and an improved protein conversion efficiency. However, when fed over a longer period, the ratio of nitrogen to creatinine in urine was even higher, which suggests, that a longer supplementation of sainfoin leads to increased nitrogen excretion via urine (Chapter 3). Therefore, no shift of nitrogen excretion from urine to faeces occurred with sainfoin feeding.

With pellets containing chestnut tannin extract, no effects on the nitrogen metabolism were detected at all (Chapter 4).

Adding *P. lanceolata* to the diet led to a lower milk protein content compared to control group, which was an undesired effect (Chapter 5).

The aim of the thesis could best be achieved with the supplementation of *S. minor.* This plant occurs in many parts of the world, including Europe and Asia, is resistant against drought and has a good forage quality with high total phenol content. Additionally, it has antimicrobial and antiviral abilities, which are used in traditional human medicine (Karkanis et al., 2014). Its feeding led to a significantly lower milk urea content and a shift of nitrogen excretion from urine to faeces compared to the control group (Chapter 5).

The promising *in vitro* results concerning *O. viciifolia, L. corniculatus* and *P. lanceolata* could not be confirmed *in vivo*. Prediction from *in vitro* to *in vivo* was in this case not reliable, which is for example also known for digestibility characteristics (Bruineberg et al., 2002). Possible reasons could have been the different dietary proportion of the tannin-containing plants (30% *in vitro*, 10% *in vivo*), the likely different harvest stages of the plants or the differences in total diet composition (lucerne and ryegrass in contrast to grass and maize silage, hay and straw).

The lack of clearer effects on the nitrogen metabolism in all *in vivo* experiments is most probably due to the low concentration of tannins in the experimental diets. In several studies (e.g. Chung et al., 2013, Ghelickhan et al., 2018, Minneé et al., 2017), effects of tannin-containing plants on the nitrogen metabolism of dairy cows were observed. In these experiments, the concentration of tannins in the diets exceeded those applied in the present studies. Anyway, the results with *S. minor* also demonstrate the possibility to influence the metabolism with a feasibly small amount of forage in the diet. The question

is open, if there are other temperate climate plants which can be cultivated on pasture show the desired effects on the nitrogen metabolism also in small, feasible dosages. Alternatively, breeding could perhaps increase the concentration of tannins in plants, so that the amount in the diet is feasible from economic and animal aspects. For sainfoin, breeding success with a higher harvest yield while maintaining the tannin concentration could already be achieved (Kölliker et al., 2017).

6.4 Further parameters indicating changes concerning rumen microbes and digestion in general

Additional to the indicators of the nitrogen metabolism, other parameters were obtained which indicated effects of the experimental plants on the rumen metabolism.

Some urine parameters were influenced by the treatment diets. With sainfoin supplementation during short periods, the purine derivative to creatinine ratio and index as well as the purine derivative to nitrogen ratio tended to be higher compared to the control group, indicating an increase in rumen microbial growth (Chapter 3). When supplementing pellets containing 50 g/kg chestnut tannin extract, the purine derivative to creatinine ratio in urine was also greater than in the control group (Chapter 4).

In all experiments, treatment influences on the milk lactose content were observed. Short-term feeding of sainfoin led to a lower content of lactose compared to longer lasting feeding (Chapter 3), pellets containing 50 g/kg of chestnut tannins led to a higher lactose content compared to the control group (Chapter 4), and it was significantly lower when birdsfoot trefoil was fed instead of plantain (Chapter 5). These observations direct to different developments of the rumen pH (Hanusovsky et al., 2018), also indicating changes in the rumen metabolism.

The different diets also influenced the estimated apparently digestibility of fibre and the proportion of individual faeces particle sizes. With sainfoin the digestibility of ADF and NDF was reduced (Chapter 3). Including *S. minor* in the diet reduced the digestibility of ADF. Compared to the respective control group, sainfoin (Chapter 3), *P. lanecolata* and *S. minor* groups (Chapter 5) had a lower proportion of particles > 0.3 mm in the faeces.

Even if it was not the main aim of this project to observe the effect of treatment plants on these parameters, they anyway indicate that with small changes of diet ingredients the digestion and metabolism of cows indeed can be influenced.

6.5 The value of tannin-containing plants in pasture for the biodiversity and the life quality of dairy cattle

Biodiversity in agriculture decreased in the last decades and the diversity of crop species decreased to only few plants (Ceccarelli, 2009). These monocultures led to a loss of living spaces for many different creatures. To maintain and again increase biodiversity means not only to save species from getting extinct but also to maintain food security (Ceccarelli, 2009). It is even known that cultures with mixed plant species are more productive, cover the soil and conserve the nitrogen better (Schmid, 2003).

With reduced biodiversity, the variety of plants available for feeding of dairy cattle also got lost. Even cows, which have access to pasture, often face cultures consisting only of grass and clover. On more heterogeneous pastures, cows have the possibility to decide, if and where they want to graze. Thus, they can choose which plant and which part of this plant they want to eat. This decision may also be influenced by their nutritional state. For example, cows fed a low crude protein supplement grazed more high-protein clover than cows, getting a high crude protein supplement. This selection belongs to their natural behaviour and was described to be important for their welfare (Leiber et al., 2020; Soder et al., 2009). Unfortunately, cows do not select their diet to support a high milk yield (Soder et al., 2009). However, the milk and meat produced from cattle grazing on heterogenous swards with a high biodiversity may have a higher nutritional quality (Rook, 2006).

Grazing animals in general but also the kind of grazing species influence the sward structure differently. They selective defoliate plants, which alters light and available soil nutrients. They trample gaps between the plants, where other plants have the chance to grow. They put dung onto more or less specific places, which means location advantages for some species. They influence the distribution of seeds, through exozoochorous or endozoochorous routes. The resulting heterogeneous patches through grazing animals increase floral and faunal biodiversity (Rook et al., 2003).

Tannins are known to active antimicrobial, depending on the kind of tannins and the interaction with the organism. For example, different tannins of both, condensed and hydrolysable groups, have inhibitory effect on *Campylobacter jejuni* (Constabel et al., 2014). Sowing tanning-containing plants directly into pasture, could therefore probably have a positive effect on biodiversity (Constabel et al., 2014), animal health and also welfare.

6.6 General conclusions and outlook

In general, the results from the present doctoral project demonstrated that tannin-containing plants, such as *S. minor* or *O. viciifolia,* provided in dietary proportions feasible in farm practice may influence the nitrogen metabolism of dairy cattle. It also appears that the implementation of such plants in farm practice is realistic, because all plants investigated are already grown for seed production, thus they are ready to be cultivated in grassland or pure swards. However, this project also showed, that not every tannin-containing plant is suitable for this purpose and that the transfer from *in vitro* results to *in vivo* may fail. If and which tannin-containing plants reliably influence the ammonia production in rumen in a way that less ammonia is absorbed through the rumen wall and therefore reduce the stress for the cow's metabolism and reduce nitrogen losses, has to be further tested *in vivo*. Different factors concerning animal, diet, supplementing plant and management side have to be considered. Besides scientific measurements and results, the appropriate nutrition of our livestock animals is trapped between economics, sustainability and animal welfare. Which of these aspects will have the highest importance, is not least a question of ethics.

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Danksagung

Von Anfang bis Ende dieses Projektes wurde ich von vielen Menschen tatkräftig und moralisch unterstützt – danke euch allen!

Im Speziellen gilt mein Dank Dr. Florian Leiber, der mir mit der Anstellung am FiBL ein Dissertationsprojekt zu einem spannenden Thema ermöglichte. Vielen Dank Florian für deine Unterstützung in sämtlichen Bereichen während des gesamten Projektes!
Ein besonderer Dank geht an Prof. Dr. Michael Kreuzer, der mir jederzeit und bei jeder Frage zur Seite gestanden ist, mit unerschöpflicher Geduld und unglaublicher Geschwindigkeit meine vielen Skripte korrigiert und wertvolle Inputs gegeben hat: Herzlichen Dank Michael für deine grossartige Betreuung!
Ebenfalls möchte ich Dr. Uta Dickhöfer danken, für die Unterstützung in Sachen Harnanalysen und das ausführliche Korrekturlesen meiner Manuskripte. Vielen Dank Frau Dickhöfer für Ihre Hilfe!
Mein Dank gilt auch Prof. Dr. Martin Gierus für die Übernahme des Korreferats: Vielen Dank, Herr Gierus!
Weiter möchte ich besonders Alois Huber danken, der mir für zwei der Versuche seine Kuhherde zur Verfügung gestellt und gelassen die ungewohnte Betriebsamkeit ertragen hat.
Das Durchführen der Tierversuche wären ohne helfende Hände nicht möglich gewesen – vielen Dank, Gerdine Kaptijn, Livia Baumgartner, Markus Leubin, Cem Baki, Enyonam Suglo und Andrea Steiner für euren grossen Einsatz auch zu den unmöglichsten Tageszeiten! Ohne euch hätte ein grosser Teil Spass und Freude gefehlt!
Nebst der Hilfe im Stall haben mich die gleichen Personen und zusätzlich auch Erika Perler, Carmen Kunz, Herrmann Baumgärnter und Vasilisa Pedan im Labor angeleitet und unterstützt – danke euch allen für die schönen Stunden im Labor!
Weiter möchte ich dem Stallteam der Acroscope danken für Ihre Unterstützung während des dritten Versuches.
Ebenso möchte ich Johannes Burri danken, der mit mir stundenlang über die Felder gefahren ist, um meine Pflanzensammlung zu komplettieren.
Einen speziellen Dank für seine mentale Unterstützung möchte ich auch an Dr. Simon Ineichen richten, der jederzeit die exakt richtigen Worte für mich gefunden hat und an Dr. Melissa Terranova, die mir jede Frage mit unendlicher Geduld beantwortet und tatkräftig beim Basteln geholfen hat.
In meinem privaten Umfeld halfen mir speziell mein Mann, Daniel Bitter, und mein Vater, Robert Kapp, aus jedem Tief und freuten sich mit mir über jedes Hoch. Danke euch von ganzem Herzen für eure unermüdliche Unterstützung!

Ohne finanzielle Mittel kann ein solches Projekt nicht durchgeführt werden. Vielen Dank an den Schweizerischen Nationalfonds und die Stiftung Edith Maryon für die Finanzierung dieses Projektes.