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Suitability of forage stands with contrasting species richness to improve the nitrogen use efficiency and the milk fatty acid profile of dairy cows

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List of Abbreviations

ALA	α-linolenic acid (C18:3 *cis*-9, *cis*-12, *cis*-15), an omega-3 (*n*-3 fatty acid)
ADF	acid detergent fiber
ADL	acid detergent lignin
BW	body weight
CT	condensed tannin
CLA	conjugated linoleic acid
CP	crude protein
DIM	days in milk
DM	dry matter
DMI	dry matter intake
ECM	energy-corrected milk
FA	fatty acid
FAME	fatty acid methyl ester
HT	hydrolysable tannins
IVOMD	*in vitro* organic matter digestibility
LA	linoleic acid (C18:2 *cis*-9, *cis*-12), an omega-6 (*n*-6 fatty acid)
MP	metabolizable protein
MUFA	monounsaturated fatty acids
NEL	net energy for lactation
NDF	neutral detergent fiber
N	nitrogen
NUE	nitrogen use efficiency
non-fiber-CHO	non-structural carbohydrates
NTP	non-tannin-phenols
OA	oleic acid (C18:1 *cis*-9)
OM	organic matter
PPO	polyphenol oxidase
PUFA	polyunsaturated fatty acids
RDP	rumen degradable protein
RA	rumenic acid (C18:2 *cis*-9, *trans*-11)
SFA	saturated fatty acids
SD	standard deviation
TEP	total extractable phenols
TT	total tannins
uCP	utilizable CP
VA	vaccenic acid (C18:1 *trans*-11)

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Zusammenfassung

Wiesenfutter stellt in den gemässigten Zonen für Wiederkäuer die wichtigste Futterquelle dar. Intensiv bewirtschaftete Wiesen und Weiden sind reich an Protein. Die Rohproteingehalte im Wiesenfutter sind oftmals höher als für eine optimale Versorgung der Wiederkäuer im Pansen benötigt wird. Diese Überschüsse gehen mit erhöhten Ausscheidungen an Stickstoff einher und führen zu einer Reduktion der Stickstoffnutzungseffizienz. Hohe Ausscheidungen an ungenutztem Stickstoff belasten nicht nur den Stoffwechsel des Tieres, sondern auch die Umwelt. Bedingt durch die hohe ruminale Abbaubarkeit des Rohproteins aus dem Wiesenfutter, kann trotz übermässiger Versorgung an Rohprotein eine ungenügende Versorgung an Protein für das Tier entstehen.

Kräuter weisen im Vergleich zu Gräsern oftmals höhere Gehalte an Phenolen (sekundäre Pflanzeninhaltsstoffe) auf. Eine bedeutende Gruppe an Phenolen stellen die Tannine, bzw. deren Untergruppen der kondensierten (CT) und hydrolysierbaren Tannine (HT) dar. Gewisse CT können die ruminale Abbaubarkeit des im Wiesenfutter enthaltenen Proteins senken, während HT oftmals mit einer Verringerung des Futterverzehrs und der Verdaulichkeit des Futters in Verbindung gebracht werden. Tannine, sowohl CT wie HT können das Milchfettsäuremuster positiv beeinflussen. In dieser Dissertation wurden spezifische Kräuter respektive kräuterreiche Wiesenbestände untersucht, um deren Eignung in der Wiederkäuerernährung hinsichtlich Futterwert, Stickstoffumsatz, Milchleistung, Milchinhaltsstoffen und Milchfettsäureprofil zu evaluieren.

In einem ersten Experiment (Kapitel 2) wurden kräuterreiche Wiesenbestände auf ihren Futterwert, den Gehalt und die Zusammensetzung an Phenolen untersucht. Dabei wurde über zwei Jahre Probenmaterial aus Wiesenbeständen aus drei Langzeitdüngungsversuchen im Schweizer Berggebiet gesammelt (Bremgarten, 2 Nutzungen pro Jahr, 930 m.ü.M., Kanton SO, angelegt 1972; Orsière, 2 Nutzungen pro Jahr, 1‘190 m.ü.M., Kanton VS, angelegt 1984 und Eggenalp, 3 Nutzungen pro Jahr, 1‘340 m.ü.M., Kanton BE, angelegt 1956). Die untersuchten Düngungsvarianten umfassten an allen Standorten sowohl ungedüngte Verfahren wie auch solche mit Phosphor und Kalium (PK) bzw. mit Stickstoff, Phosphor und Kalium (NPK) gedüngte Parzellen. Durch Unterschiede der Düngungsversuche hinsichtlich geographischer Lage, Standort, Höhenlage und Nutzungsintensität haben sich in Kombination mit der Düngung eine Vielzahl von artenreichen Wiesenbeständen etabliert. Die Futterproben dieser kräuterreichen Wiesenbestände wurden bezüglich ihrer chemischen und mineralischen Zusammensetzung untersucht. Zusätzlich wurde der Ertrag bei jeder Ernte bestimmt. Ausschliesslich im ersten Erhebungsjahr wurde ausserdem die botanische Zusammensetzung

der Parzellen, sowie der Gehalt an Phenolen und deren Zusammensetzung ermittelt. Dieselben Proben wurden mittels des modifizierten Hohenheimer Futterwerttests auf ihre *in vitro* Verdaulichkeit, sowie die Gehalte an Energie (Netto-Energie-Laktation, NEL) und nutzbarem Rohprotein analysiert.

Am Beispiel der untersuchten Bergwiesenbestände der Langzeitdüngungsversuche mit einem Kräuteranteil zwischen 18.4 bis 45.1 % zeigte sich, dass artenreiche Bestände je nach Düngung und Nutzungsintensität eine hohe Futterqualität erreichen können. Die Gehalte an Phenolen der kräuterreichen Bestände waren deutlich erhöht. Im Besonderen am Standort Bremgarten wurden durch den hohen Anteil an *Geranium sylvaticum* L. die Gehalte an Phenolen und auch der HT deutlich erhöht und konnten somit auf eine bestimmte Kräuterart zurückgeführt werden. Der Anteil an Leguminosen wurde durch Düngung von Phosphor und Kalium gefördert. Leguminosen wie *L*otus *corniculatus L.* oder *Lathyrus partensis* L. erhöhten den Gehalt an CT im Wiesenfutter.

Aufbauend auf den Versuchen der Wiesenbestände der Düngungsexperimente, wurde die Wirkung von kräuterreichen Wiesenbeständen aus dem Berggebiet (Kapitel 3) oder einer Kunstwiese, die sich durch einen hohen Anteil an Spitzwegerich (*Plantago lanceolata* L.) (Kapitel 4) auszeichnete an Milchkühen in je einem Stickstoffbilanzversuch mit vollständig getrennter Sammlung von Kot und Urin untersucht. Zusätzlich zur Wirkung auf den Stickstoffumsatz wurden die Verdaulichkeit, die Milchleistung, sowie die Milchinhaltsstoffe und das Milchfettsäuremuster bestimmt. In beiden Versuchen wurden vier unterschiedliche Rationen an Gruppen mit je sechs Kühen verfüttert. Im ersten Versuch (Kapitel 3) wurden als Heu konservierte Wiesenbestände ohne die Zugabe von Kraftfutter an laktierende Milchkühe verfüttert. Dabei wurde Heu von zwei Wiesenbeständen aus dem Berggebiet mit unterschiedlichem Kräuteranteil und zwei gräserreichen Wiesenbestände aus dem Talgebiet verglichen. Ein höherer Kräuteranteil des Bergheus war dabei mit einem höheren Gehalt an Phenolen und CT verbunden. Als Vergleichsrationen wurden zwei Rationen mit vorwiegend Englischem Raigras (*Lolium perenne* L.) mit tiefen Gehalten an Phenolen und CT, jedoch unterschiedlichen Rohproteingehalt verfüttert. Im zweiten Versuch (Kapitel 4) wurde die Wirkung von Kräutern anhand von Spitzwegerich im Kunstfutterbau auf intensiv genutzten landwirtschaftlichen Flächen im Talgebiet untersucht. Dabei wurden mit siliertem Wiesenfutter aus Kunstwiesenbeständen folgende drei Rationen erstellt, die mit zunehmenden Gehalten an Phenolen verbunden waren: Englischem Raigras; Englischem Raigras und Rotklee (*Trifolium pratense* L.*)*, sowie Englischem Raigras, Rotklee und Spitzwegerich. Diese Rationen wurden *ad libitum* mit einer Gabe von 3 kg Weizen pro Tag und Kuh

verfüttert. Als Kontrollration diente eine Ration bestehend aus Maissilage und Sojaextraktionsschrot.

In beiden Fütterungsversuchen hat sich gezeigt, dass durch den Verzehr von Kräutern die Menge an aufgenommen Phenolen erhöht wurde. Die Gehalte an CT in den an Milchkühe verfütterten Rationen, die in dieser Arbeit verwendet wurden, konnten die Stickstoffnutzungseffizienz nicht verbessern. Allerdings wurde im Fall der Ration mit Bergheu mit höherem Kräuteranteil die Stickstoffausscheidung vom Urin in den Kot verlagert, was mit der Aufnahme an CT korreliert. Damit eröffnet sich Potential, kräuterreiche Rationen gezielt dafür zu nutzen, die Belastungen des Stoffwechsels des Tieres bzw. der Umwelt an Stickstoff zu reduzieren. In beiden Fütterungsversuchen mit kräuterreichen Rationen konnten die Milchleistung und Ausscheidungen an Milchfett und Protein nicht gesteigert werden. Die Rationen mit Bergheu erhöhten den Transfer an α-Linolensäure ins Milchfett. Die Verfütterung von Spitzwegerich führte zu einer Milchfettsäurezusammensetzung mit erhöhten Gehalten an Vakzensäure, konjugierten Linolsäuren und gesamthaft höheren Gehalten an mehrfach ungesättigten Fettsäuren. Insofern führte die Verfütterung von Kräutern zu einem höheren Gehalt an Fettsäuren mit potentiell gesundheitsfördernder Wirkung.

Summary

Forage from grasslands (in the following referred to as forage) represents the most important feed for ruminants in temperate climates. Intensively managed meadows and pastures are rich in crude protein. The contents of crude protein of forage are often higher than required by ruminants for ideal ruminal conditions. Excess of crude protein increases the excretion of nitrogen and reduces the nitrogen use efficiency. The supply of protein to the animal may however be limited even under conditions of excessive supply of crude protein due to the high ruminal degradability of the forage protein. A high ruminal protein degradability is challenging for the metabolism of the animal and may contribute to environmental pollution.
When compared to grasses, herbs often contain higher amounts of phenolic compounds (plant secondary metabolites). A major group of phenolic compounds consists of tannins with its two subclasses of condensed (CT) and hydrolysable tannins (HT). Some condensed tannins can reduce the ruminal degradability of the forage protein, while HT are often related to reduced palatability and forage digestibility. Tannins, both CT and HT may have a beneficial impact on the milk fatty acid profile. In this dissertation, specific herbs or grassland swards rich in herbs were evaluated for their suitability in ruminant nutrition with respect to feed value, nitrogen use efficiency, yield and composition of milk and milk fatty acid profile.

In a first experiment (Chapter 2), swards rich in herbs were analyzed for their feed value and the content and composition of phenols. Over a period of two years, forage samples were obtained from swards of three long-term mineral fertilization field experiments. The experimental fields were located in the mountain area of Switzerland: Bremgarten, two cuts per year, 930 m a.s.l., Canton Solothurn, established in 1972; Orsière, 2 cuts per year, 1‘190 m a.s.l., Canton Valais, established in 1984 and Eggenalp, 3 cuts per year, 1‘340 m a.s.l., Canton Berne, established in 1956. At each of the three experimental sites, three different treatments of fertilization were investigated including unfertilized swards, those fertilized with phosphorus and potassium (PK) and swards fertilized with nitrogen, phosphorus and potassium (NPK). A variety of species rich swards have been established due to the differences of the long-term mineral fertilization treatments with respect to geographical location, site, altitude and cutting frequency. Forages samples were analyzed for their chemical and mineral composition. During the first year of sampling (2015), the species composition of each sward was determined, and samples were analyzed for the content and composition of phenolic compounds and the *in vitro* assessed digestibility, the content of energy (net-energy for lactation, NEL) and protein (utilizable crude protein) with the modified version of the Hohenheim gas test.

Based on the investigation of the mountain grassland swards with proportions of herbs between 18.4 to 451. % of the long-term mineral fertilization field experiments, a high forage quality can be achieved depending on fertilization and cutting intensity of the swards. Species rich mountain swards contained elevated contents of phenolic compounds. At site Bremgarten, the content of phenolic compounds and those of HT were particularly elevated due to the high proportion of *Geranium sylvaticum* L. present in these swards. The proportion of legumes was increased when fertilizing phosphorus and potassium. Legumes species such as those of *Lotus corniculatus* L. and the one of *Lathyrus pratensis* L. probably increased the content of CT in the forage, respectively.

Based on the experiments conducted with swards obtained from the three long-term mineral fertilizer experiments, effects of swards rich in herbal species obtained from mountain meadows (Chapter 3) or an artificially established sward containing high proportions of plantain (*Plantago lanceolata* L.), a herb described for its elevated tannin content (Chapter 4) were evaluated *in vivo* with lactating dairy cows. In two separate feeding experiments with complete collection of feces and urine respectively, the effects of these swards were evaluated with respect to the digestibility, nitrogen turnover, the milk yield and composition as well as the milk fatty acid profile. In each of the two experiments, four distinct diets were created, and each diet was fed to six cows. In the first feeding experiment (Chapter 3), swards conserved as hay were fed to lactating dairy cows with supplementation of concentrates. Two hay swards differing in their proportion of herbs were obtained from mountain meadows and compared to two grass dominated hay swards from the lowland area. A higher proportion of herbs in the swards obtained from mountain meadows was related to higher contents of phenolic compounds and CT. Two reference diets based on ryegrass (*Lolium perenne* L.) with low contents of phenols and CT were created. While one of the reference diets had a similar content of crude protein as the two diets containing the hay swards from mountain meadows, the other reference diet was characterized by a distinct excess of crude protein. In a second feeding experiment (Chapter 4), the effects of herbs were investigated in intensely managed production systems with the example of plantain. Therefore, three diets with ensiled forage from artificially established swards related to increasing contents of phenolic compounds were designed: Ryegrass; ryegrass and red clover (*Trifolium pretense* L.) and ryegrass-red clover and plantain. These three diets were fed *ad libitum* and supplemented with 3 kg of wheat per day and cow each and compared to a reference diet based on corn silage and soybean meal.

In both feeding experiments, it could be shown that feeding diets containing herbs increased the intake of phenolic compounds. The contents of CT of swards which were fed within the experiments of this thesis were unable to improve the nitrogen use efficiency in dairy cows. However, in the case of the diet with the sward obtain from the mountain meadow with the highest proportion of herbs; the excretion of nitrogen could be displaced from urine to feces, which was related to the intake of CT. This finding indicates potential to feed swards rich in herbs to reduce the metabolic load of nitrogen for the animal as well as the environmental pollution of nitrogen. The feeding of diets rich in herbs did not result in increased milk yield or excretion of milk fat or protein. The diets containing hay from mountain meadows increased the transfer of α-linolenic acid to the milk fat. Feeding a diet containing plantain resulted in a milk fatty acid profile with increased contents of vaccenic acids, conjugated linoleic acids and overall higher contents of polyunsaturated fatty acids. In this sense, feeding of diets containing herbs increased the content of fatty acids related to potentially beneficial health effects.

Chapter 1

General Introduction

1.1 Nitrogen metabolism in ruminants

The primary source of nitrogen (N) for ruminants is metabolizable protein (MP) at the duodenum in the form of amino acids derived from microbial protein synthesized in the rumen rather than from feed protein itself (Leng and Nolan, 1984) (Figure 1). The microbial degradation of feed protein in the rumen follows the enzymatic cascade of proteolysis, peptidolysis and deamination and eventually the formation of ammonia which is the main N source for ruminal bacteria. The ruminal ammonia concentration is determined by the amount of feed protein in the rumen, the degree of ruminal degradability of the feed protein and the (simultaneous) availability of fermentable organic matter to rumen microbes (Hristov et al., 2005). The presence of fermentable organic matter is essential for microbes to synthesize microbial protein from ammoni-N (Nocek and Russell, 1988; Keim and Anrique, 2011), resulting in a decrease in ruminal ammonia concentration. Hence, feed compoments rich in (readily) fermentable organic matter such as corn silage or concentrates (containing elevated amounts of sugars or starch) to diets with excess N supply (e.g. to cows grazing pasture) is a potential strategy to increase the incorporion of ammonia-N into microbial N and thus increase the amount of MP. There are two further types of ammonia sinks in the rumen including the outflow of ammonia from the rumen via rumen fluid and the absorption of ammonia through the rumen wall (Leng and Nolan, 1984). Maintaining microbial protein synthesis requires a minimal supply of N in the form of at least 110 g ruminal degradable protein kg DM^{-1} and a ruminal concentration of ammonia of 3.1 mg dl^{-1} (Kröber et al., 1999). Under excessive N supply, ammonia surpluses will be detoxified in the liver to urea at, however, high metabolic costs to the animal (Twigg and Van Gils, 1988) and is eventually excreted in the urine. In lactating animals, a minor proportion of urea is also excreted in the milk. In manure, urea is readily transformed into ammonia by the ubiquitous microbial enzyme urease. High secreation of urea-N in urine therefore increases the N emission potential of the manure by releasing ammonia and nitrous oxides to the air and leaking of nitrate to soil and ground water (Tamminga, 1992).

Under limited supply of N to the rumen, which restricts the proliferation of ruminal microbes, recycling of N becomes important by secretion of urea into the saliva (Lapierre and Lobley, 2001), with this cycle being referred to as the ‘rumino-hepatic cycle’.

The N use efficiency (NUE), i.e. the ratio of milk-N to N intake for lactating dairy cows accounts on average for 25 % (Tamminga, 1996). From the beginning to the end of the lactation, the NUE is decreasing (Münger, 1997), as protein is mobilized from the body

during early lactation and again deposited toward the end of the lactation. On herd level, the NUE is strongly related to the number of lactations and increases with increasing number of lactations (Tamminga, 1996; Ryan et al., 2011). In dairy cows, the NUE can be quantified using the following calculation:

$$N_{intake} = N_{excretion\ in\ milk} \pm N_{retention} + N_{feces} + N_{urine.}$$

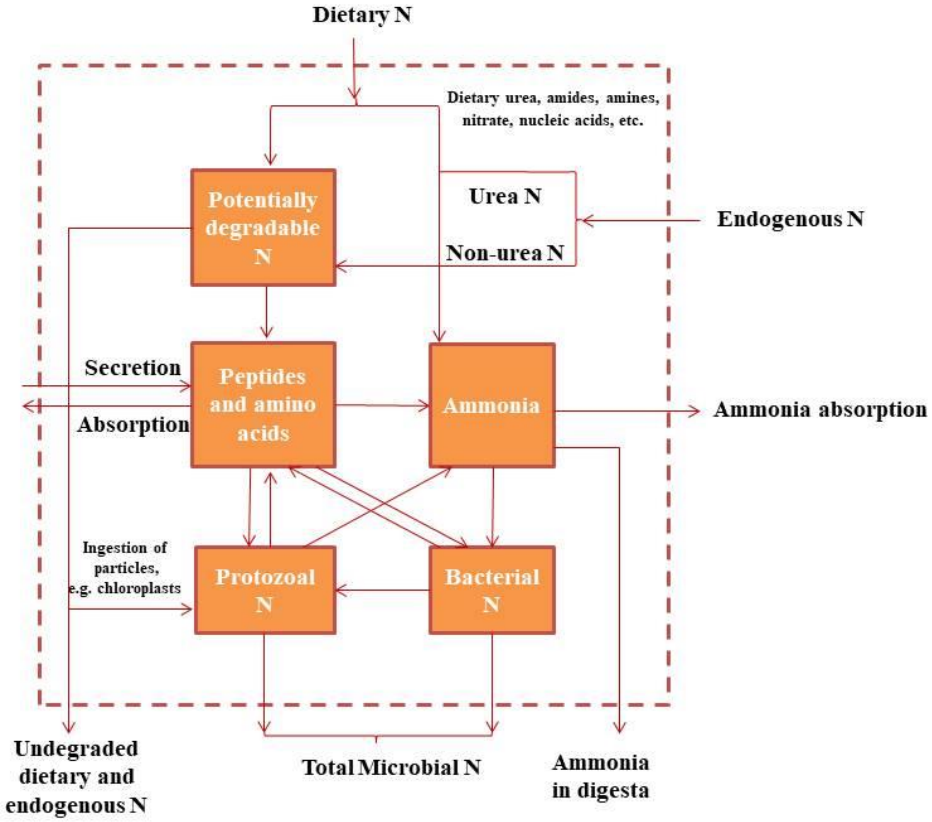

Figure 1: A reproduction of the model of the nitrogen metabolism in the rumen by *Leng and Nolan* (1984).

1.2 Protein supply and nitrogen use efficiency in dairy cows in grassland-based production systems

Forage from grassland (in fresh, ensiled or dehydrated form) is the main feed component of dairy cows in Switzerland (Schmid and Lanz, 2013) and accounts for approximately three quarters of total dry matter (DM) intake (Ineichen et al., 2015). Consequently, the supply of forage N to ruminal microbes and eventually the supply of MP to dairy cows is essential. The supply of N from forage of grassland often exceeds the minimal supply of N required for ruminal fermentation (Kröber et al., 1999). However, the high ruminal degradability of the forage protein (Givens and Rulquin, 2004), the main source of forage N, may result in a low NUE in dairy cows fed primarily or solely forage from grassland (van Dorland et al., 2007a). Consequently, the supply of MP to dairy cows is limited, requiring supplementation of MP by other feedstuffs than those from grasslands in the form of non-forage protein carriers such as soy bean meal. The ruminal degradability of the protein from soybean meal is comparably low, resulting in a potentially high supply of MP and a high NUE, when not supplemented with energy rich component such as corn silage or cereals increasing the supply of fermentable organic matter. Therefore, and in addition to meet the increasing needs for MP of dairy cows with higher milk yields, there has been a growing importation of protein carriers in Switzerland during the last two decades, among of which soybean meal is of major importance. As the production of soybean meal is related to deforestation of rain forests, which are transformed on a big scale into agricultural land, the utilization of soy bean meal is more and more criticized due to environmental and ecological concerns.

Increasing the supply of MP from forage from grassland is therefore a key element in grassland-based ruminant production systems for both milk and meat. Therefore, it is of importance to find mechanisms to increase the NUE in dairy cows (and other ruminants) fed forage from grassland as the major or sole feedstuff. Several approaches where tested to increase the NUE of forage from grassland. These include forage with reduced protein contents (Miller et al., 2001; Moorby et al., 2006), increased proportion of rumen undegradable protein (Woodward et al., 2009) or increased content of fermentable organic matter (i.e. sugars) (Miller et al., 2001; Edwards et al., 2007). Although the various approaches tested are promising, there is still need to increase the NUE in grassland based ruminant production. A further approach to increase the NUE is to decrease the ruminal degradability of the forage protein through plant secondary metabolites as discussed in the following section.

1.3 Diversity, functions and determination of plant secondary metabolites

With more than 100'000 different chemical structures, plant secondary metabolites represent a variety of heterogenous molecules and are generally more concentrated in dicotyledonous (including legumes and herbs) than in monocotyledonous plant species (including grasses) (Duncan and Poppi, 2008). Based on their chemical structures, plant secondary metabolites are differentiated in various distinct groups including cyanogenic glucosides, alkaloids, terpenoids, steroids, benzoic acids or phenols and others. Altough non-essential for the plant organism for either growth or reproduction (Salminen et al., 2011), plant secondary metabolites serve the plant in various ways including i) defense against viruses, bacteria and amoebae, fungi or plants and herbivorous insects and higher animals), ii) transport of metals, iii) symbiotic interactions between microbes and plants, nematodes, insects, and other animals and iv) as pheromones (Demain and Fang, 2000; Zaynab et al., 2018). Plant secondary metabolites play a major role in the chemical defense against herbivores (Salminen et al., 2011); however, various secondary metabolites are involved in attracting plant pollinators and are relevant for flower scent and pigmentation (Harborne, 2001).

When analyzing plant secondary metabolites in plant species or forage, specific compounds can be identified (Fraisse et al., 2007; Besle et al., 2010) or groups of structurally and chemically similar compounds can be determined (Makkar, 2003). Within the following thesis, the method of Makkar (2003) and modifications indicated by Jayanegara et al. (2012) are applied. This method allows to chemically differentiate phenolic compounds determined as total extractable phenols (TEP), which can further be differentiated into non-tannin phenols (NTP) and total tannins (TT) with the latter one consisting of two fractions including condensed tannins (CT) and hydrolysable tannins (HT). The group of CT is of particular interest due to the ability to decrease the ruminal degradability of forage protein (Waghorn, 2008).

1.4 Tannins – chemistry and nutritional relevance to ruminants

Condensed tannins – also referred to as pro-anthocyanidins are non-branched polymers of flavonoid units, whereas HT are polymers of gallic acids esterified to the hydroxyl groups of a sugar compound (McSweeney et al., 2001) (Figure 2). Condensed tannins have a much higher molecular weight (2 to 7 times) than HT (Mueller-Harvey and McAllan, 1992). The phenologic stage of plants affects tannin concentrations: Plants in flowering stage increase tannin concentration as carbon is no longer a limiting factor for the production of phenolic compounds (Iason et al., 1993). Higher tannin contents increase the capability of the plant for

chemical defense of vulnerable parts relevant for reproduction (Salminen et al., 2011). Consequently, tannins are more concentrated in leaves and flowers than in stems of plants (van Soest, 1994). Environmental factors and abiotic stress such as increasing temperature or light intensity and water stress may increase the concentration of tannins as well (van Soest, 1994).

In ruminants, microbial enzymes were shown to degrade HT, whereas CT with carbon-carbon bonds of their sub-units are non-degradable under anaerobic conditions (McSweeney et al., 2001). Tannins may basically exhibit two different types of chemical activity which include the binding of CT to proteins at neutral pH or the pro-oxidant activity at alkaline conditions (Salminen et al., 2011), leading to oxidative stress in plant herbivores (Appel, 1993). Particularly, HT have been related to elicit toxicity or anti-nutritional effects in ruminants due to the release and absorption of their degradation products in the small intestine (Reed, 1995; McSweeney et al., 2001). Condensed tannins form complexes with proteins through the formation of hydrogen bonds which are stable at ruminal pH conditions, however, dissociate at pH levels of below 3.5 in the abomasum or > 8 in the duodenum (Mueller-Harvey and McAllan, 1992). These CT-protein complexes inhibit at least partially the degradation by rumen microbial enzymes (Makkar, 2003; Mueller-Harvey, 2006). The CT also can inhibit both fungal and bacterial enzymes (McSweeney et al., 2001) or directly inhibit fiber degrading bacteria such as strains of *Butyrivibrio fibrisolvens* (Jones et al., 1994). Condensed tannins can also bind to hemicellulose, cellulose, starch and pectins (Schofield et al., 2001) which can reduce fiber degradation (McSweeney et al., 2001).

A decrease in the ruminal protein degradability may result in an increase of the supply of MP or essential amino acids at the duodenum (Min et al., 2003) and increases animal performance (Waghorn, 2008). Condensed tannins were also shown to increase the outflow of non-ammonia N from the rumen (Min et al., 2003), causing a shift from urinary N excretion towards fecal N excretion (Carulla et al., 2005; Hess et al., 2006) and may therefore decrease the N emission potential of the manure N (Lee et al., 2012). The reactivity of tannins in the animal depends on the amount ingested, the structure and molecular weight of the tannins (Hagerman and Butler, 1991). Both the effect of CT and HT on voluntary feed intake are variable (Frutos et al., 2004). However, CT concentration higher than 55 g per kg DM were found to reduce DM intake (Min et al., 2003). Attachment of CT to taste receptors or reaction with salivary mucoproteins may decrease palatability (McLeod, 1974). Concentration of 20 to 45 g CT per kg DM were shown to be effective in reducing ruminal degradation of forage protein (Min et al., 2003). An adaptation strategy to inactive CT is proline rich saliva found in

some ruminants as deer, goats and sheep complexing CT prior to reaching the rumen (Estell, 2010).

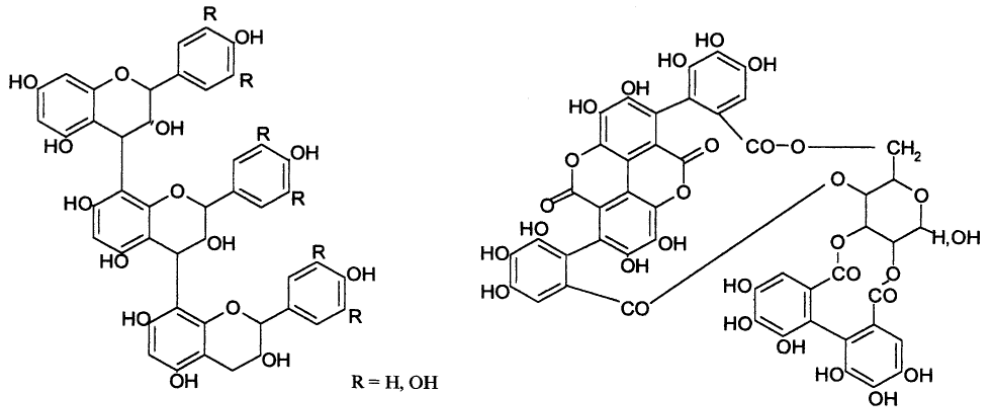

Figure 2: Examples of chemical structures of condensed tannins (left) and hydrolysable tannins (right) (*McSweeney et al*. 2001).

1.5 Ruminal biohydrogenation, milk fatty acid profile and relation to phenolic compounds

The milk fatty acid (FA) profile found in dairy cows is determined by numerous factors including breed, stage of lactation, composition of diet as well as environmental factors (Palmquist et al., 1993). The milk FA composition is, however, strongly influenced by the dietary FA composition (Boufaied et al., 2003; Leiber et al., 2005). Approximately 90 % of the total FA's present in forage from grasslands account for palmitic acid (C16:0, a saturated FA), oleic acid (OA, C18:1 *cis*-9, a monounsaturated FA (MUFA)), linoleic acid (LA, C18:2 *cis*-9, *cis*-12) and α-linolenic acid (ALA, C18:3 *cis*-9, *cis*-12, *cis*-15) with LA and ALA being polyunsaturated FA's (PUFA) (Boufaied et al., 2003). Particularly in higher concentrations, unsaturated FA such as LA or ALA may have a "toxic effect" on ruminal microorganisms. These FA are therefore detoxified by saturation to stearic acid (C18:0) in a variety of isomerization and saturation steps producing numerous FA intermediates (Figure 3). This process of saturation of dietary PUFA is referred to as ruminal biohydrogenation and is primarily due to the activity of specific ruminal bacteria (Martin and Jenkins, 2002) and to a lesser extent to protozoa or association of protozoa with bacteria (Boeckaert et al., 2007). During ruminal biohydrogenation, a number of FA intermediates are produced, including

vaccenic acids (VA, C18:1 *trans*-11) and rumenic acid (RA, C18:2 *cis*-9, *trans*-11). Ruminal biohydrogenation therefore decreases the escape of dietary LA and ALA from the rumen and increases the one of stearic acid and depending on the extent of biohydrogenation, the one of VA and RA. Rumenic acid is formed through different pathways both in the rumen and the mammary gland, with the former one, however, being less significant. Hence, RA is primarily synthesized in the mammary gland from the precursor VA (Griinari et al., 2000) and to a lesser extent by cellulolytic bacteria from the biohydrogenation of LA (*Butyrivibrio fibrisolvens*) (Kepler and Tove, 1967). In the rumen, RA is formed either by isomerization of LA through *cis*-12, *trans*-11-isomerase (Kepler and Tove, 1967) or in case of the precursor ALA, first isomerized to C18:3 *cis*-9,*trans*-11,*cis*-15 and further hydrogenated to C18:2 *trans*-11, *cis*-15 and eventually VA and transformed in the mammary gland to RA through delta-9-desaturase (Griinari et al., 2000). The initial step of biohydrogenation occurs at a faster rate than the hydrogenation of VA to stearic acid (C18:0), leading to an accumulation of VA in the rumen (Griinari et al., 1997). Consequently, higher ruminal concentrations of VA increase concentrations of RA in the milk (Griinari et al., 2000). Oleic acid does not contribute to the formation of either VA or RA, respectively (Collomb et al., 2006).

Rumenic acid is one of the major conjugated linolenic acids (CLA) found in milk fat of dairy cows (Collomb et al., 2002a). There are several health-related benefits attributed to the consumption of CLA and therefore, higher concentrations of RA in milk fat are desired. Health-related benefits from CLA include inhibition of carcinogenesis (Ha et al., 1987; 2004; Lee and Lee, 2005), anti-diabetic effects (Khanal, 2004) and lowering body weight (lowering fat mass reviewed by Roche et al. (2001)). The CLA content in milk fat of dairy cows can vary from 2 to 37 mg per g fat (Parodi, 1999). Increased contents of CLA were found in milk from pasture fed dairy cows, with RA accounting for 75 to 90 % of total CLA's (Bauman et al., 2003). Conjugated linoleic acids were shown to vary seasonally with greater contents in spring and with decreasing contents towards autumn (Banni et al., 1996). Besides, CLA, omega-3 (*n*-3) FA are considered essential to the human body (Nakamura and Nara, 2003). Fatty acids such as ALA (*n*-3) or LA omega-6 (*n*-6) FA serve as precursors for other desired long chain *n*-3 FA (eicosapentaenoic or docosahexaenoic) essential to human health (Barcelo-Coblijn and Murphy, 2009). Although, *n*-3 FA are highly desired in both the animal and human diet, the ratio of *n*-3 to *n*-6 consumed needs to be consideres, as a low ratio of *n*-3 to *n*-6 has been related to health issues (Barcelo-Coblijn and Murphy, 2009).

Increasing the content of CLA in milk and the escape of ALA from the rumen to prevent biohydrogenation of PUFA is therefore desired. With increasing altitudes from 600 m a.s.l. to

2’120 m a.s.l., the milk FA composition of dairy cows increased in the concentration of PUFA and particularly the one of CLA (Collomb et al., 2002a). These differences where related to increasing proportions of herbs in the swards with increasing altitude (Collomb et al., 2002b).

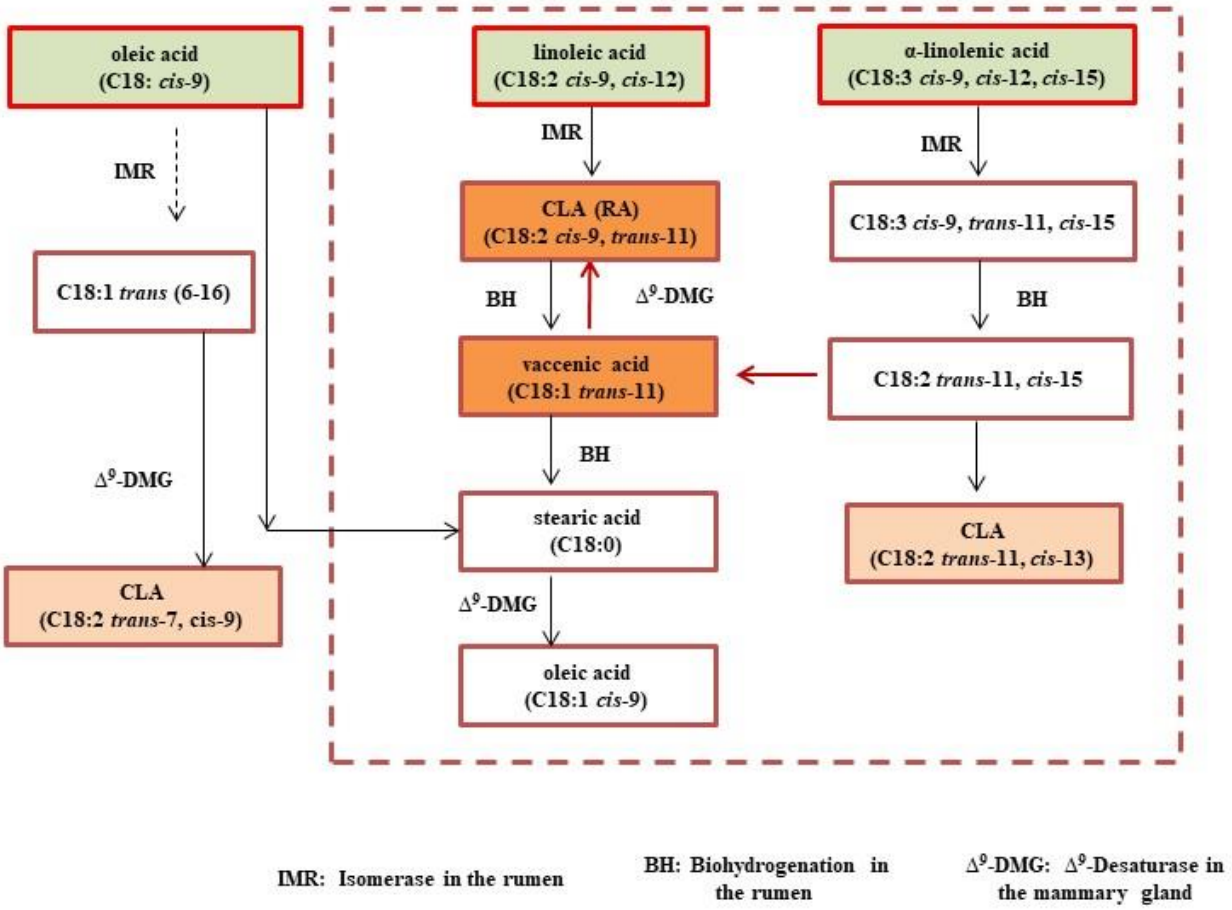

Figure 3: Pathways of the formation of vaccenic (C18:1 *trans*-11) and rumenic (C18:2 *cis*-9, *trans*-11) acid in lactating ruminants (reproduced from Collomb et al. 2006).

The botanical composition was shown to affect the rate of ruminal biohydrogenation. Feeding herbs decreased the rate of biohydrogenation when compared to a diet composed of primarily corn silage (Petersen and Jensen, 2014). Although the concentration of ALA in the milk is related to the concentration of ALA in the forage (Hebeisen et al., 1993), higher *n*-3 FA contents in milk when fed forage from species rich mountain meadows when compared to diet based on corn silage and ryegrass were related to plant secondary metabolites inhibiting ruminal biohydrogenation (Leiber et al., 2005). Phenolic compounds were shown to interfere in ruminal biohydrogenation and alter the FA profile of the ruminal fluid and consequently the one in the milk fat. Both CT (Khiaosa-Ard et al., 2009; Vasta et al., 2009a, 2011) and HT

(Jayanegara et al., 2011a; Jayanegara et al., 2012) were shown to affect ruminal biohydrogenation but at different steps of the biohydrogenation cascade, with CT at the terminal step (Khiaosa-Ard et al., 2009) and HT at the initial step (Jayanegara et al., 2011a). Forage from alpine pasture fed to a dairy cow increased the concentration of VA in the ruminal fluid compared to feeding of forage from lowland origin, which was related to the content of phenolic compounds of the alpine forage (Khiaosa-ard et al., 2011).

1.6 Objectives and thesis outline

In this thesis, the potential of swards with contrasting species richness to improve the protein supply, the nitrogen use efficiency and the milk fatty acid profile of dairy cows was evaluated in a combination of *in vitro* and *in vivo* experiments. The first experiment assessed the forage quality of species rich swards harvested from three long-term mineral fertilization field experiments located mountain area of Switzerland. These swards differed in botanical composition with varying proportion of herbs (Chapter 2). In addition, two feeding experiments with dairy cows tested the effects of diets containing herbs with elevated content of phenolic compounds on the nitrogen use efficiency and the milk fatty acid profile. The first feeding experiment included swards of mountain origin (Chapter 3). The second experiment tested the effect of a herb, plantain (*Plantago lanceolata*), in an artificially established sward sown together with ryegrass and red clover (Chapter 4). The following research questions were addressed with these three experiments:

Experiment 1: Feeding value of herbage from species-rich mountain grasslands subjected to zero, PK and NPK mineral fertilization for 40 years (Chapter 2)

- Do swards fertilized with either PK or NPK for decades have a higher nutrient and energy content than unfertilized swards?
- Does long-term fertilization reduce species-richness and, along with that, the content of phenols in the forage?
- Is the legume proportion in swards fertilized for long with only PK higher and these swards richer in phenols compared to swards fertilized by NPK or remaining unfertilized?

Experiment 2: Digestibility, nitrogen utilization and milk fatty acid profile of dairy cows fed hay from species rich mountainous grasslands with elevated herbal and phenolic contents (Chapter 3)

Do dairy cows fed hay from mountain grasslands with elevated herbal and phenolic content

- have a lower forage digestibility
- an improved nitrogen use efficiency
- and milk fatty acid profile compared to cows fed grass hay with low phenolic content?

Experiment 3: Milk fatty acid profile and nitrogen utilization of dairy cows fed ryegrass-red clover silage containing plantain (*Plantago lanceolata*) (Chapter 4)

Does increasing silages botanical composition (either ryegrass alone, ryegrass and red-clover or ryegrass, red-clover and plantain (*Plantago lanceolata*)) supplemented with concentrate

- decrease forage digestibility
- improve the nitrogen use efficiency
- increase the content of polyunsaturated and conjugated linolenic fatty acids of milk compared to a silage prepared from corn and soy bean meal to dairy cows?

Both for the hypothesis tested in Experiment 2 and 3, effects observed are related to diet chemical composition and the amount of phenolic compounds ingested, determined according to the procedure described in Makkar (2003).

Chapter 2

Feeding value of herbage from species-rich mountain grasslands subjected to zero, PK and NPK mineral fertilization for 40 years

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2.1 Abstract

Yield, nutrient and mineral contents of herbage from species-rich mountain grasslands were assessed in 2015 and 2016 on sites where three fertilization regimes (0, PK and NPK) had been established for >40 years. Levels of mineral fertilization had considered soil analyses. In vitro organic matter digestibility (IVOMD) and contents of net energy, utilisable crude protein (uCP) and different phenolic fractions were assessed in 2015. The sites covered a gradient in altitude from 930 (L) to 1190 (M) and 1340 m a.s.l. (H) and geographically the southern (L), central (H) and northern (L) Swiss Alps). Forage was harvested either twice (L or M) or three times (H) per year. Sward legume proportions were higher in case PK had been fertilised for >40 years compared to 0 and NPK fertilization, whereas swards fertilized differently did not differ in proportions. At all sites, the CP content was lower in the first harvest than in the following harvests. The fibre content was affected the number and time of harvest and was specific for each field experiment. The mineral composition was affected by interactions of harvest and fertilization. Fertilization did not affect IVOMD and uCP contents. The particularly high contents of phenolic compounds observed on site L independent of fertilization and the high CT contents observed in the first harvest of the PK-fertilized swards were likely species-dependent effects. The study shows that effects of long-term mineral fertilization on nutrient and energy content were expressed differently at each site.

2.2 Introduction

Intensification of grassland utilization for livestock production has evolved in swards composed of particularly high yielding grass (i.e. *Lolium* spp.) either sown alone or combined with one or few high-performance legume species (Dietl, 1995). Herbage produced from intensively managed grasslands is therefore characterized by a high nutrient density and digestibility (Bruinenberg et al., 2002; Tilley & Terry, 1963). This helps promoting animal performance when compared to animals fed with herbage from species-rich grassland (Bruinenberg et al., 2003; Hammond et al., 2014). However, there is considerable interest in increasing plant species richness also in intensively managed grasslands. The intention is to increase resilience of such swards to varying environmental conditions, the conservation of biodiversity and to profit from specific dietary properties of such grasslands (French, 2017). There has been particular interest in the research of herbal species as dietary component for improving the sustainability of grassland based ruminant productions systems (Hammond et al., 2014). A number of herbal species, but also various legumes, are characterized by elevated contents of phenolic compounds which are widely absent in grass species (Fraisse et al., 2007; Jayanegara et al., 2011b; Jeangros et al., 1994a). Specific phenolic compounds like the tannins have the potential to improve nitrogen (N) metabolism (Waghorn, 2008) and to mitigate methane emissions in ruminants (Hristov et al., 2013). However, high levels of phenolic compounds may decrease herbage palatability (Pfister et al., 1997) and digestibility (Scehovic, 1995a) and, for instance in the case of hydrolysable tannins (HT), could even get toxic to ruminants (McSweeney et al., 2001). This indicates that the balance between favorable and unfavorable effects of phenols in forage is delicate.

Mountainous grasslands are rarely managed as leys and therefore are often naturally rich in herbs and legumes (Jeangros et al., 1999; Collomb et al., 2002b). However, mineral fertilization may reduce species richness in these swards as plant species, performing better once nutrient limitations are removed, get promoted and, in case of N fertilization, as the natural N2 fixing ability of legumes is no competitive advantage any more. This may not be necessarily expressed, or not fully expressed, in the short term but may generate characteristic plant communities in the long term. This is of interest, as farmers' fertilization management decisions may persist over an extended period of time. However, really long-term studies (such as that by Schellberg et al., 1999) are extremely rare due to the necessity to ensure that no deviations from the scheme are happening.

Therefore, the present study built on including forages harvested from mountain fields where different schemes of mineral fertilizer treatment had been established >40 years ago (Baumberger et al., 1996; Carlen et al., 1998; Thomet and Koch, 1993). In detail, the following hypotheses were tested. (1) Swards fertilized with either PK or NPK for decades have a higher nutrient and energy content than unfertilized swards. (2) Long-term fertilization reduces species-richness and, along with that, the content of phenols in the forage. (3) The legume proportion in swards fertilized for long with only PK is higher and these swards are richer phenols compared to swards fertilized by NPK or remaining unfertilized.

2.3 Materials and Methods

2.3.1 Characteristics of experimental swards and sampling procedure

The herbage investigated in the present study was obtained from mountain swards which are part of three long-term mineral fertilization field experiments located in the mountain region of Switzerland: Bremgarten, canton of Solothurn at 930 m a.s.l. (47° 33'N, 7° 67'E) established in 1972 on a gleyic cambisol, 5.0°C annual mean temperature, northern exposition located in the Jurassic arc (L: low) (Thomet and Koch, 1993); Orsière, canton of Valais at 1190 m a.s.l. (46° 05'N, 7° 16'E) established in 1984 on a brown soil on lime with 6.6°C annual mean temperature and western exposition located in the southern Alpes (M: medium) (Carlen et al., 1998) and Eggenalp, canton of Berne at 1340 m a.s.l. (46° 57'N, 7° 36'E) established in 1956 on an acid brown soil, 4.5°C annual mean temperature, north western exposition located in the central Alpes (H: high) (Baumberger et al., 1996). At each site, the fertilizer regime consisted of three different treatments, including no fertilization (0), fertilization of phosphorus and potassium (PK) and fertilization of nitrogen, phosphorus and potassium (NPK). Amounts of fertilizers applied differed between sites for both PK and NPK treatments (kg ha and year^{-1}): 80 P_2O_5, 240 K_2O (L), 63 P_2O_5, 200 K_2O (M), 60 P_2O_5, 180 K_2O (H) in case of treatment PK and 75 N, 80 P_2O_5, 240 K_2O (L), 60 N, 63 P_2O_5, 200 K_2O (M), 85 N, 60 P_2O_5, 180 K_2O (H) in case of treatment NPK. Nitrogen was applied in the form of ammonium nitrate (NH_4NO_3), phosphorus in the form of triple super phosphate (46 weight % P_2O_5) and potassium in the form of 60 % water soluble K_2O. At sites L and M, the total dose of phosphorus and potassium was applied at the beginning of the vegetation period while N was applied in half at the beginning of the vegetation and at the day of the first harvest once the herbage had been removed. At site H, potassium was applied entirely at the beginning of the vegetation while phosphorus was applied as stock fertilization every third year only. Nitrogen was fertilized in a third of the yearly amount prior to the beginning of the vegetation

and the two remaining parts where fertilized within one week after the first harvest. Exclusively at site M, which is located in the southern Alpes, the experimental field was irrigated according to the local practices which included 50 to 90 mm of water per year during summer periods with limited precipitation (Carlen et al., 1998).

Swards were harvested during the years 2015 and 2016, respectively, according to the long-term harvesting regime specific for each site. At sites L and M, there were two harvests per year while at site H three harvests were conducted per year. The harvest dates during the first year (2015) were 30th of June and 29th of September for L; 22nd of June and 27th of August for M; 17th of June, 21st of July and 17th of September for H and for the second year (2016) were 30th of June and 5th of October for L, and 22nd of June and 29th of August for M, 24th of June, 30th of July and 14th of September for H, respectively. Plant species composition of each plot was assessed according to Daget and Poissonet (1969) prior to the first harvest in spring (Table 1). Functional groups of plants were categorized into grass, legume and herbs. The number (nb) of plot replicates per fertilizer treatment and the plot size (m^2) varied between sites (nb/m^2): 3/15.9 L, 4/50 H and 4/15 M. At site L, the plot size from 6 x 2.65 m was decreased to 6 x 1.30 m (7.8 m^2) to determine DM yield (DMY) and collect herbage samples. At sites M and H, DMY and samples were collected from the material of the whole plot area.

At each site, plots were mowed mechanically at 4 cm above ground level and the herbage yield in kg fresh matter per plot was recorded using a spring feather balance. The material was formed to a pile and a herbage sample of 1000 to 1500 g was taken with a drill rod to create particle sizes of no longer than 5 cm. The obtained samples were put in plastic bags along with cooling elements until further processing at the same day. One part of each sample was dried at 105°C for 24 hours for dry matter determination. A further sample was immediately dried at 60 °C during 24 h for chemical analyses. Dried samples were ground to pass a 1 mm sieve and stored in the absence of light at cool temperatures (approximately 4 °C) to prevent light degradation of phenolic components. Of each herbage sample, an amount of approximately 10 g was further ground to a sieve size of 0.75 mm as requested for incubation with the Hohenheim gas test (Menke and Steingaß, 1988).

2.3.2 Laboratory analysis

The following standard procedures (van Soest et al., 1991; AOAC, 1997) were used to analyse the nutrient contents of the obtained herbage samples. Both DM and ash content were

assessed by an automatic thermogravimetric determinator (TGA-701, Leco. St. Joseph, MI, USA). To determine the N content, a C/N analyser (Type TruMac CN, Leco Coperation, St. Joseph, MI, USA. AOAC index No. 968.06) was used. The N content was multiplied by the factor of 6.25 to calculate the content of crude protein (CP). A fibertec System M (Tecator, 1020 hot extraction, Foss Hillerød, Denmark) was used to determine neutral (NDF) and acid detergent fiber (ADF) and acid detergent lignin (ADL). Fiber contents were assessed without residual ash. In the case of NDF, 100 µL of a heat stable α-amylase was added to the NDF solution. Anhydrous diethyl ether was used for determination of the content of ether extract (EE) using an extraction equipment B-811 (Büchi, Flawil, Switzerland; AOAC index No. 963.15). Mineral contents (Ca, P, K, Mg) were determined using system MARS6 with MarsXpress (CEM,3100 Smith Road, Matthes, NC 28106) and a thermo iCAP 6300 inductively coupled plasma radial spectrometer (Thermo Fisher Scientific.Inc, 81 Wyman Street, Waltham, MA 02454).

2.3.3 Measurement of phenolic components and *in vitro* incubation using the Hohenheim gas test

Exclusively for herbage harvested in the first year (2015), the content of phenolic components, its fractions and *in vitro* assessed feed parameters were obtained. The content of phenolic components was determined using the basic analytical procedure given by Makkar (2003), following the modifications listed in detail by Jayanegara et al. (2012). The phenolic fractions included total extractable phenols (TEP), non-tannin phenols (NTP) and CT and were measured photo-metrically at 725 nm for TEP and NTP and at 550 nm for CT, respectively. Total tannins (TT) were calculated as the difference of TEP and NTP. The content of HT was calculated as the difference of TT and the one of CT. Phenolic fractions are expressed as gallic acid ($C_7H_6O_5$, 170.12 g mol^{-1}) equivalents.

In order to simultaneously determine the content of net energy for lactation (NEL) and utilizable crude protein (uCP) the modified procedure of the Hohenheim gas test method (Steingaß and Südekum, 2013) as described in Edmunds et al. (2012) was applied with incubation times of 24 h. Rumen fluid was obtained from a ruminally fistulated Brown Swiss cow from the Vetsuisse Faculty of the University of Zurich, Switzerland (approval no ZH 38/14). Each herbage sample was incubated in two consecutive runs with 200 mg (± 10 mg) DM (n = 1 per run). In every run, blanks and standards of hay, concentrate and protein (n = 3 per run each) were included (Edmunds et al., 2012). Upon incubation, gas production was recorded immediately and a volume of 15 mL of the incubated rumen-fluid buffer mixture

was obtained and put on ice to terminate fermentation. Accordingly, 150 µL of 5 M H_2SO_4 were added to prevent volatilization of N. Acidified samples were frozen and stored at – 20 °C. Ammonia concentrations of blanks, standards and herbage samples were detected using an NH_3 selective electrode (Metrohm AG, Herisau, Switzerland). The electrode was calibrated using NH_3Cl at 0.1, 1 and 10 mmol NH_3/L. The content of uCP was calculated according to Edmunds et al. (2012), the digestibility of organic matter (IVOMD) and NEL was calculated based on Menke and Steingaß (1988). Incubation of samples was repeated in case of a deviation higher than 5 % of the NEL content or, in the case of uCP, higher than 10 % for the same sample assed during two runs, respectively.

2.3.4 Statistical analysis

Data were analysed with SAS (version 9.4 SAS Institute Inc., Cary, NC) using a linear mixed-effects model (proc function). A statistical model was applied for each site individually to account for the site-specific set-up and characteristics of each field experiment. Harvest (*h*), fertilization (*f*) and year (*y*) were used as fixed effects and a random effect estimate was created for plot. Interactions were included for $h \times f$. Multiple comparisons among means were considered significant at $p < 0.05$. Values are shown as averages of the two years as arithmetic means. Multiple comparisons among means were conducted using the method based on Tukey-Kramer. Homogeneity of variances and normality of residuals were checked graphically. If data were transformed before analysis of variance using log transformations, *p* values and superscripts are based on transformed values. The parameters assessed *in vitro*, and the phenolic components presented in Table 5 were determined in year 2015 only, whereas the statistical model did not include the factor year for this data. The number of species (*n*) and functional groups (grass, legume, herb) were assessed prior to the first harvest at all sites in year 2015 only whereas this statistical model omitted harvest, year and its interaction. Annual DMY (DMY_A) was using the model including fertilization and year as fixed effects and plot as random intercept.

$$Y = h + f + y + h \times f + 1|plot$$

h	*fixed effect harvest*
f	*fixed effect fertilization*
y	*fixed effect year*
$h \times f$	*interaction between harvest × fertilization*
$1\|plot$	*random intercept*

Table 1 Botanical family and species composition (%) of swards from mountain grassland differently fertilized (0, PK, NPK) of three long-term mineral field experiments located at increasing altitudes from 930 m a.s.l. (L) to 1190 m a.s.l. (M) to 1340 m a.s.l. (H) shown for species if they occurred in one swards at values larger than 5.0 %.

Experimental site		L			M			H		
Botanical family	Species/Fertilization	0	PK	NPK	0	PK	NPK	0	PK	NPK
Poaceae	*Agrostis capillaris*							4.3	5.2	
Poaceae	*Arrhenatherum elatius*	1.8	15.6	18.8	1.6	4.8	2.8			
Poaceae	*Briza media*				6.3	2.8	2.3			
Poaceae	*Bromus erectus*	17.3							1.4	
Cyperaceae	*Carex montana*							10.4		
Poaceae	*Dactylus glomerata*		3.7	3.2	3.1	9.4	12.1		1.0	6.4
Poaceae	*Festuca pratensis*				6.3	6.6	8.7	6.7	9.7	3.7
Poaceae	*Festuca rubra*	33.1			14.8	6.3	10.3	9.2	20.6	18.6
Poaceae	*Holcus lanatus*	1.8	17.7	25.2						
Poaceae	*Trisetum flavescens*		14.0	25.2	9.7	11.1	16.5		1.7	6.2
Fabaceae	*Lathyrus pratensis*		11.6		1.1	5.3	1.7	1.1		
Fabaceae	*Lotus corniculatus*	5.7								
Fabaceae	*Trifolium pratense*	1.7	9.3		4.7	7.1	1.5	1.7	1.3	
Fabaceae	*Trifolium repens*					4.3			10.0	4.8
Fabaceae	*Vicia sepium*		6.8							
Asteraceae	*Crepis aurea*							10.1	2.7	
Geraniaceae	*Geranium sylvaticum*	5.4	11.9	17.5	3.2	6.6	7.9		1.1	6.2
Asteraceae	*Leontodon hispidus*				3.8	5.3	1.1			
Apiaceae	*Pimpinella major*				4.5	7.0	4.8			
Plantaginacae	*Plantago lanceolata*	1.5			3.4	2.2	1.0	2.9	4.9	6.5
Rosaceae	*Sanguisorba minor*	11.8						3.5		
Lamiaceae	*Thymus serpyllum*							1.5	8.4	6.5
Asteraceae	*Tragopogon pratensis*	5.1				1.1				

2.4 Results

2.4.1 Species composition and dry matter yield

Due to the long-term mineral fertilization treatments swards with contrasting species compositions have evolved at the different experimental sites (Table 1). Species number was affected by fertilization at site H only and was higher in unfertilized than fertilized swards (+6.3 PK, +7.5 NPK) (Table 2). The proportion of grass (%) was not affected by fertilization at site H but was higher when fertilized with NPK compared to PK at sites L (+24.3) and M (+11.7). Fertilizing PK increased the proportion of legumes (%) compared to unfertilized swards at sites L (+20.3) and H (+7.38) or NPK at all sites (L: +26.6, M: +13.5, H: 6.78). The proportion of herbal species (%) was not affected by fertilization and increased from site L (23.7) to M (35.6) and H (43.0).

Table 2 Annual dry matter yield (DMY_A, dt ha^{-1}) and average number of species (*n*) and proportions of functional groups (% grass, legume, herb) of swards from differently fertilized mountain grassland (0, PK, NPK) of three long-term mineral field experiments located at 930 m a.s.l. (L), 1190 m a.s.l. (M) and 1340 m a.s.l. (H).

site	fertilization	*n*	grass	legume	herb	DMY_A
L	0	20.3‡	60.7[b]	7.45[b]	31.9	33.8[c]
	PK	16.3	53.9[b]	27.7[a]	18.4	63.3[b]
	NPK	16.0	78.2[a]	1.13[b]	20.7	76.9[a]
	SEM	1.29	3.85	4.166	2.80	5.08
	*p-values**					
	f	0.453	0.006	0.003	0.113	<0.001
	y	-	-	-	-	<0.001
M	0	28.3	53.1[a]	14.8[a]	32.1	51.8[b]
	PK	26.8	43.2[b]	20.3[a]	36.4	71.7[a]
	NPK	25.0	54.9[a]	6.77[b]	38.4	72.2[a]
	SEM	1.07	2.35	2.180	2.09	3.56
	p-values					
	f	0.408	0.025	0.009	0.560	0.017
	y	-	-	-	-	0.206
H	0	36.3[a]	49.5	5.32[b]	45.1	27.3[c]
	PK	30.0[b]	47.2	12.7[a]	40.1	44.5[b]
	NPK	28.8[b]	49.8	5.92[b]	43.7	74.7[a]
	SEM	1.45	1.53	1.307	1.50	4.69
	p-values					
	f	0.019	0.495	0.021	0.323	<0.001
	y	-	-	-	-	0.018

‡Values show arithmetic means and within a column at the same site, means of different superscripts are significantly different at $p < 0.05$.
**h*: harvest; *f*: fertilization; *h* × *f*: harvest × fertilization.

Annual DMY (dt ha^{-1}) increased from unfertilized swards to PK and from PK to NPK at sites L (+29.5, +13.6) and H (+17.2, +30.2). At site M, DMY_A was similar with PK and NPK, but lower in unfertilized swards (-20.2). Other than at site M (61.3, 69.1), DMY_A was lower in the first year compared to the second year of assessment (L: 48.4, 67.6 and H: 43.1, 54.3). Both harvest and fertilization influenced the formation of DMY at each site (Table 3). At site L, DMY was significantly higher in the first (43.0) compared to the second (15.0) harvest. Swards fertilized with PK (31.7) or NPK (38.4) were similar in DMY, while unfertilized swards (16.9) yielded lower DMY. At site L, DMY was lower in the first year of sampling (24.2 and 33.8). At M, the DMY decreased from the first (35.9) to the second (29.3) harvest and was higher in PK (35.9) and NPK (36.1) compared to unfertilized swards (25.9). At site H, the DMY was higher in the first (24.9) compared to the following harvests (12.3 and 11.5, respectively). The DMY increased significantly from unfertilized swards (9.10) to PK (14.8) to NPK (24.8).

2.4.2 Nutritional composition

At site L, the herbage contained higher contents of CP (g kg DM $^{-1}$) in the second (103) compared to the first (89.2) harvest (Table 3). Swards fertilized with PK (107) had higher contents of CP compared to NPK (85.2) but not than unfertilized swards (96.2). In the second year of sampling, the content of CP was higher (102) compared to the first year (90.6). At site M, the content of CP was higher in the first year of sampling (180 and 122) and increased from the first (111) to the second (191) harvest. At site H, the content of CP was higher in the third harvest (190) compared to the first (124) or second (153) harvest. In the first year of sampling, the CP content was higher than in the second year (167 and 144).

The fiber contents consisting of NDF, ADF and ADL (g kg DM $^{-1}$) varied with respect to fertilization, harvest and year differently at each site. At the lowest site, L, both the contents of NDF (527 and 415) and ADF (337 and 266) were higher in the first compared to the second harvest in contrast to those of ADL. The content of NDF between PK (457) and NPK (510) was similar, while unfertilized swards (445) had lower contents of NDF compared to NPK swards. The content of ADL was lower in the first year of sampling than in the second year (56.3 and 67.4). At site M, contents of NDF and ADF were higher in the first (486 and 337) compared to the second (441 and 278) harvest. Additionally, in the second year of sampling, the content of NDF was higher (476) than in the first year (451).

Table 3 Forage dry matter yield (DMY, dt ha^{-1}) and chemical composition (g kg^{-1} DM) of forage from differently fertilized mountain grassland (0, PK, NPK) of three long-term mineral field experiments located at 930 m a.s.l. (L), 1190 m a.s.l. (M) and 1340 m a.s.l. (H).

site	harvest	fertilization	DMY	CP	NDF	ADF	ADL	EE
L	1st	0	25.5^{b‡}	86.7^{bc}	507^{a}	309	59.8	23.4^{b}
		PK	47.4^{a}	102^{abc}	521^{a}	350	72.3	21.2^{b}
		NPK	56.2^{a}	78.9^{c}	552^{a}	353	61.7	22.0^{b}
	2nd	0	8.38^{d}	106^{ab}	384^{b}	244	55.2	35.8^{a}
		PK	15.9^{c}	112^{a}	392^{b}	265	64.0	35.0^{a}
		NPK	20.7^{bc}	91.4^{abc}	467^{ab}	288	57.9	34.9^{a}
		SEM	1.538	6.13	25.6	32.3	6.94	2.69
		*p-values**						
		h	<0.001	0.006	<0.001	0.008	0.258	<0.001
		f	<0.001	0.004	0.033	0.338	0.297	0.870
		y	<0.001	0.026	0.099	0.816	0.030	0.053
		h×*f*	0.699	0.740	0.610	0.927	0.919	0.936
M	1st	0	32.2^{ab}	105^{c}	469^{ab}	326^{abc}	75.5^{ab}	23.6^{c}
		PK	36.2^{a}	115^{bc}	488^{ab}	340^{ab}	65.8^{ab}	24.3^{c}
		NPK	39.2^{a}	113^{c}	502^{a}	346^{a}	75.3^{a}	25.3^{bc}
	2nd	0	19.5^{b}	203^{a}	425^{b}	266^{c}	62.7^{ab}	29.8^{ab}
		PK	35.5^{a}	196^{a}	444^{ab}	284^{bc}	71.9^{ab}	30.3^{ab}
		NPK	33.0^{ab}	175^{ab}	454^{ab}	283^{bc}	62.2^{b}	31.8^{a}
		SEM	3.66	20.3	16.2	18.1	4.69	1.31
		p-values						
		h	0.012	<0.001	0.001	<0.001	0.012	<0.001
		f	0.032	0.704	0.136	0.288	0.997	0.327
		y	0.124	<0.001	0.046	0.677	0.124	<0.001
		h×*f*	0.153	0.469	0.984	0.950	0.006	0.974
H	1st	0	15.3^{bcd}	114^{b}	466^{bd}	289^{bc}	55.2	25.0^{c}
		PK	22.0^{b}	118^{ab}	475^{abc}	313^{b}	52.1	25.8^{bc}
		NPK	37.4^{a}	139^{ab}	547^{a}	348^{a}	56.6	24.4^{c}
	2nd	0	7.11^{cd}	136^{ab}	419^{bcde}	267^{cd}	54.6	29.0^{abc}
		PK	11.8^{bcd}	148^{ab}	374^{e}	261^{cd}	56.7	32.9^{a}
		NPK	18.0^{bc}	175^{ab}	402^{bcde}	265^{cd}	50.3	32.5^{ab}
	3rd	0	4.88^{d}	195^{a}	409^{cef}	244^{d}	56.1	32.1^{ab}
		PK	10.6^{bcd}	188^{ab}	375^{e}	257^{d}	59.9	35.6^{a}
		NPK	19.0^{bc}	188^{ab}	399^{def}	256^{d}	53.6	34.8^{a}
		SEM	2.832	17.0	16.0	7.3	3.07	1.52
		p-values						
		h	<0.001	<0.001	<0.001	<0.001	0.465	<0.001
		f	<0.001	0.351	0.089	0.002	0.603	0.090
		y	0.055	0.044	0.612	0.060	0.833	<0.001
		h×*f*	0.327	0.744	0.001	<0.001	0.220	0.686

CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; EE, ether extract.

‡Values show arithmetic means and within a column at the same site, means of different superscripts are significantly different at $p < 0.05$.

**h*: harvest; *f*: fertilization; *h* × *f*: harvest × fertilization.

At sites L and H, the content of ADL was not affected by treatment, but at site M, within harvests, the content of ADL did not differ between treatments, but was higher in the first harvest compared to the second harvest when fertilized with NPK. At site H, however, there were significant interactions of $h \times f$ for both the contents of NDF and ADF. wards fertilized with NPK had higher contents of NDF than unfertilized swards from the first harvest, whereas in the second and third harvest, there were no differences in the contents of NDF between all treatments. Swards fertilized with NPK had further higher contents of ADF than unfertilized swards or PK in the first harvest, whereas in the second and third harvest, there were no differences in the content of ADF between treatments. The content of ADL was not affected by treatment.

The content of EE (g kg DM^{-1}) was affected by harvest at site L, by harvest and year at M and by harvest, fertilization and year at H. At L, the content of EE was lower in the first (22.2) compared to the second (35.2) harvest, as well as at M, where the content of EE increased from the first (24.4) to the second (30.7) harvest and from the first year (23.2) to the second year (31.9) of sampling. At site H, both in the second (31.5) and third (34.2) harvest, the content of EE was higher compared to the first harvest 25.1). In the first year of sampling, the content of EE was lower (27.2) compared to the second year of sampling (33.3).

2.4.3 Contents of ash and minerals

The content of ash (g kg DM^{-1}) was affected differently at each site (Table 4). At site L, the ash content was higher in the second (97.1) compared to the first (79.5) harvest and was higher in PK (105) compared to unfertilized (84.5) or NPK swards (75.2). In the second year of sampling (93.8), the ash content was higher than in the first year (82.8). At M, the ash content was significantly higher in the second (164) compared to the first (124) harvest. At site H, the content of ash was higher in the third harvest (151) compared to the first (104) or second (116) harvest as well as in the first year (140) compared to the second year (107) of sampling.

For the content of Ca (g kg DM^{-1}) at site L, an interaction of $h \times f$ was found. In the first harvest, the content of Ca was similar for all types of fertilization, whereas in the second harvest, the content of Ca was lower for swards fertilized with NPK compared to PK or unfertilized swards. At site M, the content Ca was higher in the second (17.4) compared to the first (12.2) harvest. The content of Ca was higher in unfertilized swards (16.5) compared to NPK (13.3) but not different to PK (14.6). At site H, the content of Ca was higher in

unfertilized swards (11.2) and PK (10.5) compared to NPK (8.33) and was higher in the second (11.7) and third (11.2) harvest compared to the first (7.12) harvest.

Table 4 Mineral composition (g kg–1 DM) of forage of from differently fertilized mountain grassland (0, PK, NPK) of three long-term mineral field experiments located at 930 m a.s.l. (L), 1190 m a.s.l. (M) and 1340 m a.s.l. (H).

site	harvest	fertilization	Ash	Ca	P	K	Mg
L	1st	0	70.9^{c‡}	9.59^{b}	1.35^{c}	11.7^{bc}	1.41^{bc}
		PK	97.4^{ab}	9.21^{b}	2.57^{ab}	17.6^{a}	1.44^{c}
		NPK	70.3^{c}	6.97^{b}	1.97^{bc}	15.3^{a}	1.28^{c}
	2nd	0	98.1^{ab}	15.7^{a}	1.31^{c}	11.0^{c}	2.14^{a}
		PK	113^{a}	14.9^{a}	2.78^{a}	15.4^{a}	1.81^{ab}
		NPK	80.1^{bc}	9.28^{b}	2.14^{ab}	14.7^{ab}	1.55^{bc}
		SEM	6.42	1.029	0.171	0.77	0.103
		*p-values**					
		h	0.002	<0.001	0.305	0.017	<0.001
		f	<0.001	0.001	<0.001	<0.001	0.027
		y	0.033	0.240	<0.001	0.153	0.003
		h×*f*	0.352	0.046	0.596	0.261	0.004
M	1st	0	134	13.5^{cd}	1.47^{c}	10.4^{b}	2.57^{b}
		PK	124	12.3^{d}	2.45^{b}	18.6^{a}	1.90^{c}
		NPK	113	10.9^{d}	2.54^{b}	18.3^{a}	1.81^{c}
	2nd	0	181	19.6^{a}	1.91^{b}	10.3^{b}	4.14^{a}
		PK	159	16.9^{ab}	3.59^{a}	19.6^{a}	2.44^{b}
		NPK	151	15.7^{bc}	3.56^{a}	18.8^{a}	2.66^{b}
		SEM	17.4	0.80	0.154	0.74	0.132
		p-value					
		h	0.006	<0.001	<0.001	0.391	<0.001
		f	0.314	0.006	<0.001	<0.001	<0.001
		y	0.517	0.625	0.917	0.912	0.081
		h×*f*	0.925	0.434	0.002	0.724	0.001
H	1st	0	88.0^{c}	7.96^{cd}	1.95^{c}	13.6^{c}	2.89^{cde}
		PK	104^{bc}	7.33^{cd}	3.76^{b}	25.2^{b}	2.17^{ef}
		NPK	120^{abc}	6.05^{d}	3.86^{b}	27.0^{ab}	1.93^{df}
	2nd	0	102^{bc}	12.8^{a}	1.97^{c}	13.1^{c}	4.22^{ab}
		PK	118^{abc}	12.3^{a}	4.53^{a}	29.3^{a}	3.14^{bcd}
		NPK	127^{abc}	9.89^{abc}	4.46^{a}	30.2^{a}	3.01^{bce}
	3rd	0	161^{a}	12.8^{a}	2.00^{c}	13.2^{c}	4.86^{a}
		PK	148^{ab}	11.7^{ab}	4.63^{a}	27.4^{ab}	3.25^{abcd}
		NPK	146^{ab}	9.04^{bc}	4.48^{a}	28.8^{ab}	2.76^{bce}
		SEM	11.50	0.677	0.133	0.81	0.361
		p-value					
		h	<0.001	<0.001	<0.001	0.005	<0.001
		f	0.115	0.001	<0.001	<0.001	0.011
		y	<0.001	0.201	0.685	0.002	0.002
		h×*f*	0.180	0.492	0.006	0.076	0.003

‡Values show arithmetic means and within a column at the same site, means of different superscripts are significantly different at $p < 0.05$.

**h*: harvest; *f*: fertilization; *h* × *f*: harvest × fertilization.

Phosphorus and K fertilization had strong effects on herbage contents of P and K (g kg DM^{-1}). At site L, the content of P increased from unfertilized swards (1.33) to NPK (2.05) and to PK (2.68). The content of P was further lower in herbage harvested in the first year of sampling (1.79 and 2.25). Herbage from the first harvest at site L, had higher contents of K (14.9) than from the second harvest (13.7). Swards fertilized with PK (16.5) or NPK (15.0) were similar in K content, but unfertilized swards had significantly lower contents of K (11.4). At site M, the content of P increased for all treatments from the first to the second harvest. Within harvests, it was lower for unfertilized swards compared to PK or NPK. Swards fertilized with PK (19.1) or NPK (18.5) at site M, had higher contents of K than unfertilized swards (10.3). At site H, there was a in interaction of $h \times f$ for the content of P. The P content of unfertilized swards did not differ between harvests and was lower than those of PK and NPK in all harvests. The content of P increased from the first to the following harvest for PK and NPK but did not differ between the treatments. The content of K was lower in unfertilized swards (13.3) compared to PK (27.3) or NPK (28.7) and lower in the first (22.2) compared to the second (24.0) year of sampling at site H. The content of K significantly increased from the first (21.9) to the second (24.2) harvest, while the third (23.2) harvest did not differ to the other harvests.

At each site, there was an interaction of $h \times f$ for the content of Mg (g kg DM^{-1}). At site L, Mg content was higher in the second year (1.69) compared to the first year of sampling (1.52). In the first harvest, there was no difference in the content of Mg between swards of different fertilization, but in the second harvest, swards fertilized with NPK had lower contents of Mg compared to unfertilized swards. At site M, the content of Mg increased from the first to the second harvest for all treatments and was higher in unfertilized swards compared to PK or NPK within each harvest, respectively. At site H, the content of Mg was higher in the first (3.29) compared to the second (2.98) year of sampling. Within the first and second harvest, respectively, the content of Mg was not affected by fertilization, whereas within the third harvest, unfertilized swards had higher contents of Mg than NPK.

2.4.4 *In vitro* determined feed value and phenolic fractions.

Other than at site M, IVOMD (%) was affected by harvest. At site L, IVOMD was higher in the second than in the first harvest (57.7 vs. 6.56) and at H, the IVOMD was similar for the first to the second harvest but higher in the last harvest (67.5, 67.4 and 72.8) (Table 5). At each site there was an effect of the harvest on the content of NEL (MJ kg DM^{-1}). At site L, the content of NEL was higher in the second (5.41) compared to the first (4.53) harvest.

Table 5 Digestibility of organic matter (IVOMD, %), contents of net energy for lactation (NEL; MJ kg DM^{-1}), utilizable crude protein (uCP; g kg DM^{-1}) and of different phenolic fractions (g kg DM^{-1}) of forage from differently fertilized mountain grassland (0, PK, NPK) of three long-term mineral field experiments located at 930 m a.s.l. (L), 1190 m a.s.l. (M) and 1340 m a.s.l. (H).

site	harvest	fertilization	IVOMD	NEL	uCP	TEP	NTP	TT	CT	HT
L	1st	0	60.1‡	4.86^{b}	162^{abc}	62.4	30.8	31.6	5.93^{ab}	25.6
		PK	56.1	4.35^{b}	148^{bc}	64.0	27.9	36.1	11.6^{a}	24.5
		NPK	56.1	4.39^{b}	125^{c}	35.9	17.5	18.4	2.39^{b}	16.0
	2nd	0	64.5	5.47^{a}	173^{abc}	58.1	35.9	22.2	2.31^{b}	19.9
		PK	64.5	5.35^{a}	174^{ab}	79.4	35.8	43.7	3.47^{b}	40.2
		NPK	61.4	5.43^{a}	191^{a}	65.1	32.1	33.0	2.15^{b}	30.9
		SEM	1.945	0.171	8.3	12.60	7.15	7.20	1.337	7.05
		*p-value**								
		h	0.009	0.001	<0.001	0.239	0.167	0.497	0.003	0.193
		f	0.260	0.226	0.689	0.312	0.486	0.173	0.043	0.391
		h×*f*	0.587	0.422	0.001	0.458	0.799	0.304	0.022	0.287
M	1st	0	61.6	4.88	148	39.2	27.4	15.1	2.27	13.0
		PK	63.2	4.87	143	35.1	25.1	10.1	2.42	7.67
		NPK	62.7	4.82	147	49.5	27.5	22.1	3.32	18.7
	2nd	0	62.9	3.75	140	26.7	22.2	4.85	2.81	2.42
		PK	62.3	4.01	147	28.7	19.6	9.08	2.42	6.66
		NPK	61.7	4.20	144	30.1	21.0	9.15	3.62	5.48
		SEM	2.40	0.439	5.7	5.78	2.29	4.86	1.065	4.554
		p-value								
		h	0.915	0.021	0.597	0.006	0.012	0.047	0.645	0.038
		f	0.962	0.892	0.976	0.398	0.493	0.399	0.644	0.475
		h×*f*	0.845	0.805	0.516	0.330	0.945	0.332	0.934	0.296
H	1st	0	66.3	5.52^{ab}	172	30.4	22.5	7.98	2.03	5.85
		PK	69.0	5.72^{a}	163	29.2	22.4	6.82	0.72	6.10
		NPK	67.1	4.91^{ab}	156	26.9	23.0	6.48	0.52	5.96
	2nd	0	64.1	5.08^{ab}	157	35.3	26.5	8.74	1.45	7.29
		PK	69.5	5.66^{ab}	165	42.3	27.6	14.7	0.90	13.8
		NPK	68.6	5.34^{ab}	166	36.6	26.6	12.5	0.64	11.8
	3rd	0	72.1	4.72^{b}	155	37.6	26.5	11.2	N.D.	11.1
		PK	72.5	5.11^{ab}	167	31.1	24.0	7.04	N.D.	7.02
		NPK	73.9	5.30^{ab}	157	31.9	25.2	7.96	N.D.	7.94
		SEM	1.711	0.192	5.2	3.53	2.23	2.733	0.384	2.558
		p-value								
		h	0.001	0.042	0.487	0.010	0.059	0.064	0.554	0.049
		f	0.124	0.120	0.329	0.673	0.951	0.971	0.076	0.899
		h×*f*	0.592	0.020	0.125	0.377	0.908	0.284	0.122	0.217

IVOMD, *in vitro* assessed digestibility of organic matter; TEP, total extractable phenols; NTP, non-tannin phenols; TT, total tannins; CT, condensed tannins; HT, hydrolysable tannins; N.D., not detected.

‡Values show arithmetic means and within a column at the same site, means of different superscripts are significantly different at $p < 0.05$.

**h*: harvest; *f*: fertilization; *h* × *f*: harvest × fertilization.

At H, there was an interaction of *h* × *f*. Within each harvest, there were no differences in the content of NEL between treatments, however swards fertilized with PK had a higher content

of NEL in the first harvest compared to unfertilized swards of the third harvest. At M, the content of NEL decreased from the first (4.86) to the second (3.99) harvest. There was an interaction of $h \times f$ for the content of uCP (g kg DM^{-1}) at L. Within harvests, the content of uCP was similar between treatments, however swards fertilized with NPK had higher contents of uCP in the second harvest compared swards fertilized with PK and NPK of the first harvest. There was no effect of treatment at H and L for the content of uCP.

The effect of harvest and fertilization on the phenolic fractions (g kg DM^{-1}) strongly differed between sites: At site L, there was significant interaction on the content of CT, while other phenolic fractions (TEP, NTP, TT and HT) did not differ between treatments. In the first harvest, PK had a significantly higher content of CT than NPK but not than unfertilized swards, whereas in the second harvest, there was no difference in the content of CT between treatments. At site H, the contents of TEP and HT increased from the first to the second harvest, while there was no difference to the third harvest, respectively (TEP: 28.8, 38.1 and 33.5; HT: 5.97, 11.0 and 8.70). There were no effects on the content of NTP, TT or CTP at H, however, there were not CT detectable for the third harvest. In contrast, at site M, the contents of TEP, NTP, TT and HT decreased significantly form the first to the second harvests, respectively (TEP: 41.3 and 28.5; NTP: 26.6 and 20.9; TT: 15.7 and 7.69 and HT: 13.1 and 4.85).

2.5 Discussion

2.5.1 Species richness and dry matter yield of mountain swards

The objectives of this study were to assess the herbage quality and its seasonal variation of mountain grassland swards with contrasting species richness and compositions. The swards obtained from the fertilization field experiments located at different geographical regions and altitude in the Swiss mountain area represented considerable differences with respect to the amount of fertilizer applied, the timing and number of harvests per year, the regrowth duration between cuts, climatic, edaphic and topographic variations. Due to the long-term effect of mineral fertilization of more than four decades at each site, established botanical compositions within individual plots of the fertilization field experiments have established. Environmental factors including altitude, temperature or soil texture, moisture, pH and nutrients content were found to be the main factors influencing plant species richness and composition (Gusmeroli et al., 2012). With increasing altitude from lowland to mountain grasslands, plant species richness increases (Jeangros et al., 1999; Collomb et al., 2002b), which was also observed with increasing altitude of the field experiments in both unfertilized

and fertilized swards. Janssens et al. (1998) related soil phosphorus content to species richness with levels of phosphorus higher than 5 mg per 100 g of soil decreasing plant species richness. Fertilization is associated with decreasing species richness (Dietl, 1995) and therefore, it would be expected, that, swards fertilized with PK or NPK would be lower in species richness than unfertilized swards. However, the effect of fertilization on species richness in our study was minor in contrast to species composition.

Fertilization of PK or NPK increased the proportion of (primarily) tall growing grass species; as the one of *Arrhenatherum elatius*, *Holcus lanatus* and *Trisetum flavescens* at site L, the one of *Dactylus glomerata* and *T. flavescens* at site M and the one of *Festuca rubra* at site H. This response was reported by several other studies (Schellberg et al., 1999; Tallowin et al., 1999; Ĉop et al., 2009). Jacot et al. (2000) showed that the proportion of legume species in swards is decreasing with increasing altitude. The proportion of legumes at the mountain sites numerically decreased from L to H, however, at M it was almost doubled in unfertilized swards. Higher annual temperatures at M may have favored the occurrence of legume species which is a most likely reason for this difference (Carlen et al., 1998). The proportion of legumes in swards benefited when fertilized with PK but not when additionally fertilized with N. Generally, the proportion of herbal species increases with increasing altitude at the expense of grass species (Jeangros et al., 1999; Collomb et al., 2002b). This pattern could also be observed in our study with decreasing average proportions of grasses and increasing proportion of herbal species at sites with increasing altitude. The proportions of herbal species increased with increasing altitude of the field experiments, however, they were largely unaffected by the different fertilization treatments. The composition of herbal species appears to be site specific. At L, the high proportion of *Geranium sylvaticum* in both fertilized treatments may have resulted of disequilibrium of harvest intensity and fertilization (Jeangros et al., 1994a) while at site M, the similar proportions of herbal species in all treatments may by an effect of higher annual temperatures and irrigation (Carlen et al., 1998). At site H, similar herbal species occur within the fertilization treatments, with however some dominant herbal species when fertilized with NPK as seen by the proportions of *G. sylvaticum*, *Plantago lanceolata* and *Thymus serphyllum*.

The potential of herbage yield differs between sites. Unfertilized swards at L and H had similar annual herbage yield in contrast to unfertilized swards at M, where annual herbage production was by approximately 20 dt ha^{-1} higher. This indicates the influence higher annual temperatures and irrigation during dry summer periods present at this site likely resulting in higher proliferation of minerals by the soil. Similar to the reports by Schellberg et al. (1999),

Tallowin et al. (1999) or Ĉop et al. (2009), annual herbage yield increased in fertilized swards and were lower in unfertilized swards. There was a seasonal variation in herbage yield with higher yields in the first compared to the following harvests. This is related to the phenological stages of especially grasses and common for swards with late herbage removal of the first harvest (Tallowin et al., 1999).

2.5.2 Nutrient composition, herbage digestibility and net energy content species rich swards

Species rich swards are characterized with species being present at different phenological stages when compared to species poor grasslands (Schubiger et al., 2001; Bruinenberg et al., 2002). Swards of the different fertilization treatments were harvested at the same cutting dates and consequently the phenological stages varied between the swards. The mountain swards in this study further contained significant proportions of herbal species. Some herbal species may be of similar herbage quality as grass species of intensively utilized grasslands (Bovolenta et al., 2008; Jayanegara et al., 2011b; Jeangros et al., 1994a). Daccord and Arrigo (1992) determined the herbage quality of two mountain meadows harvested three or four times during several consecutive years. Although there was a difference in the proportion of herbal species of 12 % when harvested three or four times, besides differences in lignin content, the meadows showed a similar chemical composition.

However, the effect of the number and timing of harvests is numerously reported to be more relevant than the type of fertilization on herbage quality both in semi-natural (Ĉop et al., 2009; Wyss, 2002) or artificially established grasslands (Schubiger et al., 1999). Consequently, the nutrient composition of swards from different treatments must be interpreted with respect to the time of the first harvest specific for each site. Swards fertilized with NPK which favour the occurrence of tall growing grasses, which during the first harvest decreases at a faster rate than in consecutive harvests due to morphological changes reflected by a decline in the ratio of leaves to stems (Duru et al., 2000; Andueza et al., 2015). Swards fertilized with particularly NPK might have benefited from more frequent harvest and particularly an earlier first harvest (Andueza et al., 2015) as observed in other long-term fertilization field experiments (Schellberg et al., 1999; Tallowin et al., 1999). Differences in fiber content between the first and the following harvest(s) are likely to explain the increase in herbage digestibility form the first to the following harvest(s). Contents of NEL observed in this study were similar to those observed by Schellberg et al. (1999) who reported NEL

contents of herbage from semi-natural grasslands between 4.8 to 5.6 for the first and between 4.2 to 5.3 NEL kg DM^{-1}, respectively.

The CP content of herbage from the first harvest at all sites was lower than the one of the subsequent harvest(s) and limiting for the CP requirements for optimal fermentation. In contrast, the CP content of the second (M) and/or third harvest (H) exceeded these recommendations and were similar to those from intensively managed grasslands (van Dorland et al., 2007a). The CP content of herbage from species-rich grasslands (Hammond et al., 2014; Bruinenberg et al., 2003) can be limiting when regarding the minimal requirements of lactating dairy cows (Kröber et al., 2000). Andres et al. 2005 reported a successive decline in CP content with increasing phenological stage. The decline in CP content takes place at a higher rate during the first harvest than during the following harvests (Schellberg et al. 1999). Exclusively a site L, there was an effect of fertilization on the CP level of the herbage with higher CP contents in PK fertilized swards compared to NPK, which was likely due to the large presence of legumes contributing to high CP contents. Other studies found decreasing CP content with increasing N fertilization (Schubiger et al., 1999). Due to similar proportions of tall growing grasses in swards fertilized with either PK or NPK, this effect was not observed in this study.

2.5.3 Mineral contents of biodiverse swards

Herbage mineral contents are influenced by several factors including plant species composition (Pirhofer-Walzl et al., 2011), growth stage and harvest (Schlegel et al., 2016) or fertilization (Pirhofer-Walzl et al., 2011; Jeangros and Sinaij, 2018; Tallowin and Jefferson, 1999; Schellberg et al., 1999) but only few studies assessed mineral composition of mountain swards (Daccord and Arrigo, 1992). The mineral composition of the herbage harvested from the fertilizer experiments showed a large variation for all minerals determined. Daccord and Arrigo (1992) listed mineral composition of herbage from mountain origin of 7.75 g Ca, 4.60 g P, 2.10 g Mg and 29.2 g K for the first and of 10.7 g Ca, 3.58 g P, 2.43 g Mg and 21.7 g K of the second harvest, respectively. At site L, compared to the findings of Daccord and Arrigo (1992), the contents of P and K appear particularly low. This again may be related to the late harvest and long regrowth duration of these swards respectively and is supported by the finding from Schlegel et al. (2016), who found decreasing contents of P, K, Mg with increasing sward maturity. At all sites, Ca contents were rather low when fertilized with NPK, although Schlegel et al. (2016) reported similar Ca contents although with increasing growth stages. In the experiment described by Jeangros and Sinaj (2018), the P and K concentration

in herbage increased with increasing fertilization level. At all sites, potassium was fertilized prior to the first harvest. Only at sites L and H, there were higher potassium contents in the herbage of the first harvest, whereas at site M, potassium contents did not differ between harvests. Both ash and mineral contents were higher in subsequent harvests than in the first harvest. As reported in other studies (Schlegel et al., 2016), herbage contents of Mg increased from the first to following harvests.

2.5.4 Phenolic contents of swards rich in herbs

As expected from species rich mountain herbage, phenolic contents were between two and four times higher compared to those observed in grass monocultures (14.4 g kg DM^{-1}; Besle et al., 2010). Fraisse et al. (2007) found phenolic contents of mountain pastures between 19 and 32 g kg DM^{-1}. Phenolic compounds may protect sensitive plant tissues from UV light and are therefore expected to be more concentrated in swards of higher altitudes due to increasing UV light exposure (Iason et al. 1993). However, phenolic contents were particularly highly concentrated in herbage harvested at the lowest site L, whereas phenolic contents from herbage harvested at higher located sites M or H were lower and similar to those reported by Fraisse et al. (2007). At site M, the phenolic content and its fractions decreased from the first to the second harvest, which could not be observed at the other sites. However, there were several tendencies for a harvest effect at site H, with numerically higher contents during the first harvest, indicating an increase in contents of phenolic fractions during flowering stage of the first harvest when plants are in reproductive stages as phenolic compounds are more concentrated in leaves and flowers than in stems (van Soest, 1994).

The particularly high contents of phenolic compounds observed at site L within all treatments and CT contents observed in the first harvest of swards fertilized with PK are likely species-dependent. An assessment of the phenolic content of selected single species including, *G. sylvaticum* revealed particularly high contents of TEP and HT, with this herbal species being particularly present in all swards irrespective of the type of fertilization (134 g TEP, 18.1 g NTP, 116 g TT, 12.8 g Ct and 103.1 g HT). High contents of HT are, however, critical, as they were shown to decrease palatability (Pfister et al. 1997) or being toxic when consumed in high amounts by ruminants (McSweeney et al. 2001) and reduce herbage digestility (Scehovic, 1995b). Other than herbal plant species, particularly high contents of CT at site L when fertilized with PK during the first harvest can be related to the presence of *L. pratensis* (70.0 g TEP, 15.3 g NTP, 54.8 g TT, 43.5 g CT and 11.3 g HT) or *L. corniculates* (57.0 g TEP, 21.0 g NTP, 36.1 g TT, 25.3 g CT and 10.7 g HT) and to a lesser extent to *V.*

sepium (26.8 g TEP, 19.5 g NTP, 7.3 g TT, 2.2 g CT and 5.0 g HT). The presence of CT containing legume species may therefore be relevant with regards to effects of CT on N metabolism, animal performance (Waghorn, 2008) or mitigation of methane emissions in ruminants (Hristov et al., 2013) then large proportions of herbal species containing low contents of CT.

2.6 Conclusions

The focus of this study was to characterize the nutritional composition of swards with contrasting species richness and species composition from mountain grassland of different long-term mineral fertilizer experiments in the mountain region of Switzerland with proportions of herbs varying between 18.4 and 45.1 %. Plant species composition differed between mountain sites. The findings show that mountain grasslands display a large variation in herbage quality. Herbage form the first harvests was limited in CP contents, rich in fibre and low in digestibility. In contrast, herbage from the following harvests increased in quality, particularly when cut three times and was comparable to intensively utilized species poor grasslands. Although, late first harvests reduced herbage quality, flowering swards are essential to conserve plant species rich swards. High contents of phenolic compounds and condensed tannins observed are likely species-dependent. A further understanding of utilization of herbage from species-rich swards by ruminants may contribute to increase sustainability of ruminant productions systems and balance agro-environmental interests.

Chapter 3

Digestibility, nitrogen utilization and milk fatty acid profile of dairy cows fed hay from species rich mountainous grasslands with elevated herbal and phenolic contents

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3.1 Abstract

In the present study, the influence of mountain hay diets with elevated proportions of herbs and phenolic contents on nitrogen utilization and milk fatty acid profile of dairy cows was investigated. Two grass hay-based diets, either low or high in nitrogen (N) content (GN^-, GN^+) and similar in fiber content and lignification, and two diets consisting of hays with high proportion of herbs (H) with either low or high phenolic (P) content (HP^-, HP^+) were fed iso-energetically and without concentrate. All diets contained a *Lolium perenne* dominated basal hay and experimental hay in proportions of 0.45:0.55, except for the N-excess diet GN^- (0.80:0.20). Feed intake, milk yield and total amount of feces and urine were recorded and sampled from 24 multiparous mid-lactation cows (eight Brown Swiss, 16 Holstein) producing on average 33 kg/day of energy-corrected milk. The experiment was performed in three runs with two cows per diet. Data was analyzed by a general linear model considering diet and run as effects. Intake was highest in GN^+ and HP^+ but lower with GN^- and HP^-. No condensed tannins (CT) were detected in GN^- and GN^+. Intake of phenolic compounds was high in HP+ (402 g/day per cow) lower with HP^- (302 g/day) and lowest with GN^- and GN^+ (ca. 190 g/day). The intake of CT was higher in HP^+ (115 g/day) compared to HP^- (31 g/day). Yield of milk and energy-corrected milk as well as gross milk constituents were not affected by diet. Apparent total tract nutrient digestibility was higher for the grass-based diets (GN^-, GN^+) than for the diets with high herbal proportion (HP^-, HP^+). With GN^+, absolute urinary N losses and those in proportion of total excreta N were higher than in the other diets and was lowest with HP^+. Utilization of N was lower with GN^+ and HP^+ compared to HP^-. The milk fat of cows fed HP^+ had higher proportions of polyunsaturated fatty acids compared to that of GN^-. The transfer rate of C18:3 *n*-3 from feed to milk was highest for the two herbal hay diets. The secretion of C18:1 *trans*-11 in relation to the amount of C18:2 *n*-6 + C18:3 *n*-3 ingested was highest for diet HP^-. In conclusion, mountain hay rich in herbs was found to be a dietary means to lower the N emission potential of the manure urinary N excretion which demonstrates that inclusion of herbs into grasslands may be beneficial.

3.2 Introduction

Intensively managed grassland, often composed of a limited number of high-yielding grasses and clovers, often provides more forage N than required for optimal ruminal fermentation when fed as the main feed in dairy cow diets (van Dorland et al., 2007a). In addition, due to the typically high ruminal degradability of the protein of these forages (Givens and Rulquin, 2004), the nitrogen (N) utilization is often relatively low in such diets as compared to diets based on maize silage, cereals and soybean meal. If not supplemented with energy rich feeds to increase microbial N utilization (Nocek and Russell, 1988), excess herbage protein will result in high ruminal ammonia N formation and lead to increased urinary urea excretion and promote the potential of the manure for emissions of ammonia and nitrous oxides to the air and nitrate to soil and ground water (Tamminga, 1992).

In contrast to intensively managed grasslands, the forage from species-rich lowland (Hammond et al., 2014) or from mountainous grasslands (Leiber et al., 2004) is characterized by a lower and sometimes limiting N content for the requirements of lactating dairy cows (Kröber et al., 2000). A number of dicotyledonous non-leguminous forage species (herbs), present at mountainous meadows, are characterized by an elevated content of phenolic compounds (Jeangros et al., 1994a; Jayanegara et al., 2011b). Forage from mountainous grassland was found to contain more phenols (35.3 g/kg dry matter (DM); Fraisse et al., 2007) compared to ryegrass silage (14.4 g/kd DM; Besle et al., 2010). Cows were shown to ingest up to 0.5 kg/day of phenolic compounds when grazing mountainous pastures, but this more at the start of the grazing period as phenolic contents decrease with increasing growth stage of the sward (Fraisse et al., 2007). Although a number of phenolic compounds may have favorable properties in ruminant nutrition, they may also impair digestibility (Scehovic, 1995b) and palatability, and may even be toxic (Pfister et al., 1997). A major class among phenolic compounds are the condensed tannins (CT). These polymers of flavonoids which cannot be degraded by ruminal microbes (McSweeney et al., 2001) and have been shown to form complexes with dietary proteins and fiber as well as enzymes secreted by microbes at ruminal pH conditions (Makkar, 2003; Mueller-Harvey, 2006). The formation of protein-CT complexes may therefore reduce ruminal forage protein degradation and increase the supply of protein at the duodenum (Waghorn, 2008). A decrease in the formation of ammonia in the rumen and its energy consuming detoxification in the liver (Twigg and van Glis, 1988) eventually reduces urinary N losses and thus the N emission potential of the manure (Carulla et al. 2005).

The distinct botanical composition of mountainous pastures is a relevant key factor for the sensory quality of various dairy products (Farruggia et al., 2014; Peiretti et al., 2016), with some being foods of Protected Designation of Origin, providing mountainous dairy farmers with a higher income (Sturaro et al., 2013). Feeding dairy cows on mountainous or alpine pasture with elevated phenolic compounds was found to result in milk fat with a higher proportion of α-linolenic acid (ALA, C18:3 *n*-3) compared to the milk fat of dairy cows when fed lowland ryegrass pasture (Collomb et al., 2002b; Leiber et al., 2005). Fatty acids (FA) such as ALA or linoleic acid (LA; C18:2 *n*-6) are essential *n*-3 and *n*-6 FA, respectively, and serve as precursors for long-chain *n*-3 and *n*-6 FA having various biological effects in relation to human health (Barcelo-Coblijn and Murphy, 2009). Specific classes of phenolic compounds may also inhibit ruminal biohydrogenation at different steps promoting other FA like vaccenic acid (VA; C18:1 *trans*-11) and rumenic acid (RA; C18:2 *cis*-9, *trans*-11) (Khiaosa-Ard et al., 2009; Vasta et al., 2009a; Jayanegara et al., 2012), where RA is the predominant conjugated linoleic acid (CLA) in ruminant-source foods (Collomb et al. 2006).
The use of species-rich swards in ruminant nutrition is getting an increasing interest (French et al., 2017; Hammond et al., 2014) as it is a potential strategy to improve the sustainability of grassland utilization by ruminants. The aim of the present study was, therefore, to make a direct comparison of the effects of different forages prepared from species-rich mountainous grasslands composed of different proportions of herbs and phenolic contents with those of species-poor grass-based diets on i) forage digestibility, ii) the utilization of N and iii) the milk FA profile of dairy cows.

3.3 Material and methods

3.3.1 Animals

The experiment was approved by the cantonal veterinary office of Zug, Switzerland (licence no. ZG 69/15) and lasted 20 days in total for each cow. Twenty-four multiparous cows were selected from the > 60 dairy cows of the experimental herd of the ETH Research Station Chamau (47° 12’ 32’’ N, 8° 24’ 31’’). Cows were in mid lactation to allow feeding without supplementation of concentrates. To fulfill this primary goal, cows of two breeds had to be selected (eight Brown Swiss and 16 Holstein Friesian). The cows were investigated in three subsequent runs each comprising eight cows with always two cows assigned to one of four diets prior to the start of the experiment. This was done in a complete randomized design where group averages were balanced for breed types (two Brown Swiss and four Holstein Friesian per diet type), body weight, number of lactations, yield of energy-corrected milk

(ECM), yield of milk protein and fat (kg per day) and stage of lactation. In groups GN^-, GN^+, HP^- and HP^+, the cows had initial body weights of 661 ± 64 kg, 653 ±40, 676 ± 45 and 662 ± 29 (mean ± standard deviation of the four created diet groups respectively), had performed 3.17 ± 1.94, 3.50 ± 1.64, 4.17 ± 1.17 and 3.50 ± 1.52 lactations, had a yield (per cow and day) of 32.2 ± 4.1, 33.0 ± 4.4, 32.5 ± 5.9 and 32.2 ± 12.7 kg ECM, 1.09 ± 0.15, 1.11 ± 0.13, 1.11 ± 0.18 and 1.08 ± 0.40 kg protein and 1.29 ± 0.18, 1.30 ± 0.22, 1.32 ± 0.25 and 1.32 ± 0.48 kg fat, and were 144 ± 89, 167 ± 119, 176 ± 97 and 161 ± 84 days in milk, respectively. These data were measured at the last routine milk quality assessment prior to the experiment. At that time, the cows had been housed in a free-stall barn with timely limited access to an outside, uncovered area (ca. 90 m^2). The pre-experimental diet consisted (g/kg DM) of grass silage (460), maize silage (362), hay (94), soybean meal (70), NaCl (6), urea (4) and the same vitaminized mineral mix (4) as that used in the experiment (see below). Additionally, the cows had received concentrates either rich in net energy for lactation (NEL) or utilizable crude protein CP (uCP; conceptually similar to metabolizable protein) which were allocated in individual amounts to meet the cows' requirements for maintenance and milk yield (Agroscope, 2017).

3.3.2 Hay types and experimental diets

Hay material prepared from five meadows differing in species diversity and composition was used in the present experiment (Table 6). The species composition of the meadows where the hay types were harvested from was determined latest 1 week prior to harvesting following Dietl (1995). All diets contained hay from a ryegrass (*Lolium perenne*) dominated sward (basal hay) harvested from a ley in the second year after sowing at Chamau. All other hay types were produced from semi-natural grasslands and purchased from private dairy farms and had been harvested as first cut in early summer 2016. Both the basal hay and the hay from semi-natural grasslands were air dried with ventilated systems on the farm after field drying (except for hay type 'grass G1', which was field dried exclusively). Two of the four semi-natural grassland were mainly composed of different grass species (grass hay types G1 and G2), whereas two hays had higher proportions of herbs (herbal hay types H1 and H2) (Table 6). Concerning energy and protein contents, the hay types varied, per kg of DM, from 4.5 MJ NEL (grass hay G1) to 5.6 MJ NEL (basal hay) and from 87.7 g crude protein (CP) (grass hay G1) to 196 g CP (basal hay). The two herbal hays H1 and H2 contained 24.9 g and 34.9 g of total phenols per kg of DM respectively, whereas the grass hay types G1 and G2 and the basal hay had total phenol contents below 15 g per kg DM. Condensed tannins were only detected

in the herbal hay types and amounted to 3.4 g (H1) and 12.6 g (H2) per kg of DM, respectively.

Table 6 Botanical composition and estimated proportions of plant species (g/kg of dry matter); only values ≥ 3 g/kg present in the experimental hay types.

Hay type	Basal	Grass 1	Grass 2	Herbal 1	Herbal 2
Altitude of hay origin, m a.s.l.	400	573	1100	1921	1668
Total plant species, *n*	8	6	28	27	44
Grasses, % / *n*	0.935 / 5	0.950 / 3	0.950 / 14	0.600 / 6	0.150 / 16
Agrostis stolonifera				0.030.0	
Bromus erectus			0.237		
Cynosurus cristatus			0.152		
Dactylis glomerata			0.076		
Festuca pratensis				0.030.0	
Holcus lanatus			0.047		
Lolium multiflorum		0.925			
Lolium perenne	0.905		0.171		0.060
Nardus stricta				0.516	
Poa pratensis					0.030
Trisetum flavescens			0.161		
Herbs, % / *n*	0.015 / 1	+[1] / 1	0.025 / 10	0.375 / 17	0.800 / 24
Crepis aurea				0.158	
Crepis biennis					0.040
Crepis paludosa					0.040
Geranium sylvaticum					0.032
Plantago alpina				0.094	
Plantago lanceolata					0.040
Polygonum bistorta					0.240
Ranunculus acris				0.075	
Rhinanthus minor					0.200
Taraxacum officinale					0.040
Veratrum album					0.036
Legumes, proportion / *n*	0.050 /2	0.050 / 2	0.025 / 4	0.025 / 4	0.050 / 4
Trifolium pratense	0.040	0.050			0.040

[1] + = Single herbal species detected.

Four experimental diets were created, which were mixtures of the basal hay and one of the grass or herbal hay types from semi-natural grasslands. The basal hay was used to balance the experimental hays for NEL and uCP across diets, except for diet GN^-, where the proportion of basal hay was increased to create a diet with excess dietary N. Diets GN^- and GN^+ were formulated to be similar in contents of neutral (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL). The hay mixtures generated were composed (in terms of DM) as follows: GN^-, basal hay: grass hay G1, 0.45:0.55; GN^+, basal hay: grass hay G2, 0.80:0.20;

HP^-, basal hay: herbal hay H1, 0.45:0.55; HP^+, basal hay: herbal hay H2, 0.45:0.55. Two grass dominated diets were either low or high N in content and low in plant species richness as well as phenolic contents (GN^- vs. GN^+). In contrast, the two diets with elevated proportions of herbs from hay of mountainous origin were either moderate or high in phenolic content (HP^- vs. HP^+) with an overall high species richness. In addition to the hay mixtures, cows received daily 50 g of NaCl and 100 g of a commercial vitaminized mineral mix (Kroni Locher and Co. AG, Altstaetten, Switzerland). The latter contained, per kg, 160 g Ca, 80 g P, 100 g Mg, 35 g Na, 1 g S, 1'200'000 I.E. vitamin A, 200'000 I.E. vitamin D_3, 3 g vitamin E, 8 g Zn, 4 g Mn, 1 g Cu, 30 mg Se, 100 mg I, and 30 mg Co.

3.3.3 Experimental protocol

The experiment lasted for 20 days for each cow. The first 13 days served as an adaptation period and the last 7 days for data and sample collection. During the first 5 days of adaptation, the pre-experimental diet was gradually replaced with the experimental diet from 1000 g/kg (day 1) to 0 g/kg (day 6). From then onwards, the cows were fed on the experimental hay diets only. During the first 12 days of the adaptation phase, the two cows assigned to a distinct diet and run were kept together in a free range deep litter pen. Afterwards, the cows were transferred to individual tie stalls (day 13 of adaptation period) where they were kept on comfort mattresses. A grid facilitated complete feces collection. In the tie stalls, cows were fed individually. Milking took place at 05:15 h and 16:30 h and feeding was accomplished at 05:45 h and 16:00 h. From day 13 onwards all cows were offered a restricted amount of feed to achieve the same NEL intake in all cows. The allowance, therefore, corresponded to the NEL intake of the cows with the lowest feed intake in the first run, where deliberately the cows latest in lactation had been included. In detail, feed allowance was defined as the average intake of the cow pair receiving the least palatable diet (HP^+) from days 8 to 12 of the adaptation phase, which amounted to 85 MJ NEL/day. Cows had unrestricted access to fresh water during the whole experiment.

3.3.4 Data and sample collection

During the collection period, samples of each hay type were collected three times per run, pooled for each of the three runs and stored until further processing. The amount of leftover hay, if any, was registered prior to the morning feeding of the next day. In the sampling period, at each milking, milk yield was registered, and samples were collected in flasks containing 2-bromo-2-nitropropane-1,3, diol (Bronopol®, D and F Inc., Dublin, CA, USA)

for later analysis of milk gross constituents. Separate aliquot milk samples were taken at each milking for later N and milk FA analysis and frozen at –20°C. Amounts of feces and urine were determined and samples were collected once per day after the morning feeding. Feces were collected in chromium steel trays partially placed below the grid. After weighing, samples amounting to proportionately 0.005 of total feces were taken daily, pooled per cow and stored at –20 °C until being pooled after thawing to one mixed sample per cow and subjected to determination of N and DM. The remaining homogenized pooled material was dried at 60 °C during 48 h for analysis of fiber contents. Additionally, 100 g of feces were collected daily per cow for immediate determination of DM content. Urine was separated from feces by urinals which were attached around the previously shorn vulva of the cows using Velcro straps (IBZ Industrie AG, Adliswil, Switzerland) and a glue (ergo.500 Universal, Kisling AG, Wetzikon, Switzerland). Urine was collected in containers and weighed once per day. During collection, a subsample of urine was diverted from the main part of the urine by using a special T-shaped outlet to generate a subsample at every urination event. This subsample was collected in a canister containing 50 g of 5 M sulfuric acid to prevent gaseous N losses. Samples of acidified urine were stored at –20 °C until being analyzed for N content. Samples of hay and dried feces were ground to pass a 1-mm screen. Different from that, the standards and hay samples used in a separate *in vitro* incubation diet with rumen fluid had a particle size of 0.75 mm.

3.3.5 Laboratory analyses

Proximate contents of hay types and feces were analyzed using standard procedures (van Soest et al., 1991; AOAC, 1997). Determination of DM and total ash was conducted with an automatic thermogravimetric determinator (TGA-701, Leco, St. Joseph, MI, USA). Nitrogen contents of hay types, non-dried feces, milk and acidified urine were determined using a C/N analyzer (Type TruMac CN, Leco Coperation, St. Joseph, MI, USA: AOAC index No. 968.06). The CP was calculated as 6.25 × N. Ether extract was determined in the hay samples using anhydrous diethyl ether (extraction equipment B-811, Bück Flawil, Switzerland; AOAC index No. 963.15). Fiber fractions including NDF and ADF were assessed in hay types and feces, and additionally ADL in the hay types on a Fibertec System M (Tecator, 1020 hot extraction, Foss Hillerød, Denmark) excluding residual ash. The NDF was determined using a heat stable α-amylase (100 µL). The fraction of non-structural carbohydrates (non-fiber-CHO) was calculated as organic matter (OM) – NDF – CP – ether extract. Contents of Ca, Mg, P, Na and K of the hay samples were analyzed using a microwave accelerated reaction system

(MARS6) with MarsXpress (CEM,3100 Smith Road, Matthes, NC 28106) and a thermo iCAP 6300 inductively coupled plasma radial spectrometer (Thermo Fisher Scientific.Inc, 81 Wyman Street, Waltham, MA 02454). Total extractable phenols (TEP), CT and non-tannin phenols (NTP) were analyzed in the hay types using the basic protocol of Makkar (2003) with modifications described by Jayanegara et al. (2012). Briefly, a modified Folin-Ciocaltheu method was applied using polyvinylpolypyrrolidone to separate NTP from total tannins (TT). Condensed tannins were assessed using the butanol-HCl-iron method. The TT were calculated as the difference of TEP and NTP, the hydrolysable tannins (HT) as the difference of TT and CT. All phenol fractions are expressed as gallic acid monohydrate equivalents (188.33 g/mol). Bronopol stabilized milk was analyzed for fat, protein, lactose and urea with infrared spectrophotometry (Milkoscan 4000, Foss Elcetric, Hillerød, Denmark). Based on Agroscope (2017), ECM (kg) was calculated as Milk yield (kg) × [(0.038 × fat (g/kg) + 0.024 × protein (g/kg) + 0.017 × lactose (g/kg)]/3.14.

Contents of NEL and uCP of feeds were estimated using a modified Hohenheim Gas Test method (Steingaß and Südekum, 2013) where ammonia N content is measured in an incubated rumen-fluid buffer mixture. The modified method followed the same procedure as that developed by Menke and Steingaß (1988) except for providing excess nitrogen for microbial fermentation throughout the incubation. This was accomplished by omitting 2 g/l $NaHCO_3$ and adding 2 g/l NH_4CO_3 in the buffer. A protein standard (254 g CP/kg DM) as described by Edmunds et al. (2012) was incubated in addition. The uCP value of the protein standard of 283 g uCP/kg DM after 24 h of incubation indicated by the University of Hohenheim was divided by the amount of uCP recorded in each of two runs. When the resulting factor was between 0.9 and 1.1, runs were considered valid and these factors were used to adjust uCP estimates within run. Rumen fluid was collected from a rumen fistulated Brown Swiss cow maintained according to Swiss guidelines for animal welfare (licence no. ZH 38/14). After 24 h, gas volume was recorded, and 15 ml of incubated rumen fluid buffer sample was collected and immediately put on ice to stop fermentation. After allowing to cool for 15 min, 150 µL 5 M H_2SO_4 was added to prevent NH_3 losses. The acidified samples were stored at –20°C until analysis. Ammonia concentrations were measured using an NH_3 selective electrode (Metrohm AG, Herisau, Switzerland) which was calibrated using NH_3Cl at 0.1, 1 and 10 mmol NH_3/L.

Fatty acids were extracted from the hay samples by using a solvent extractor (ASE 200, Dionex Corporation, Sunnyvale, CA, USA) and a hexane:propane-2-ol mixture (3:2 v/v). Fatty acids were transformed to FA methyl esters (FAME) according to IUPAC (1987)

method 2.301 and cleaning was done as described by Wettstein et al. 2001. A gas chromatograph (model HP 6890 equipped with a FID detector, Hewlett Packard, Palo Alto, CA, USA) equipped with a CP7421 column (200 m × 0.25 mm, 0.25 µm; Varian Inc., Darmstadt, Germany) after split injection (1:5) was employed to analyze FAME. As an internal standard the FA C11:0 (Fluka, Steinheim, Germany) was used, while sunflower oil was used as an external standard in order to calculate the response factor. A volume of 1 µL was injected with a constant hydrogen flow of 1.7 mL/min. The initial temperature was set to 170 °C and held for 1 h followed by an increase by 5°C/min to 230 °C, isotherm for 32 min at 203 °C, increase by 5°C/min to 250 °C, and isotherm for 15 min at 205 °C. In the milk, FA were analyzed from one pooled sample per cow. Internal standards (5 mL of n-heptane containing triundecanoin, tetradecenoic methylate and trivaleranoin) were mixed with 0.5 mL of milk. Sodium methylate was used for cold transesterification to FAME (Suter et al., 1997). The response factors made from triglyceride standards (C6:0, C13:0 and C19:0) were used to adjust to individual FA accordingly. The same gas chromatograph and column were used as for the FA analysis in the hay types. The FAME were injected at a volume of 1.0 µL at a split of 1:1 with a hydrogen flow of 1.7 L/min. The initial temperature was set to 60 °C and held for 12 min followed by an increase of 5°C/min to 170°C, isotherm for 60 min at 170°C, increase of 5°C/min to 250°C, and isotherm for 20 min at 250 °C. Identification of FAME was performed using a Supelco 37 Component standard (Supelco Inc., Bellefonte PA, USA). To confirm correctness of peak identification, the peaks were crosschecked with chromatograms of Collomb and Bühler (2000).

3.3.6 Statistical analysis

Data was subjected to analysis of variance performed with a general linear model using R version 3.3.1 (R Core Team, 2017) with diet as fixed effect and run as block factor. The R-package used was nlme (Pinheiro et al., 2017). Tukey's procedure was used for multiple comparisons among means, considering $P < 0.05$ as significant. Both homogeneity of variances and normality were controlled graphically and numerically with the test procedures of Shapiro-Wilk for normality and Bartlett for homogeneity of variance. In case normality and homogeneity were not given, values were log transformed before analysis of variance. For transformed data, P values and superscripts are based on transformed values. Results are displayed as arithmetic means and standard errors of the mean. Two animals from diet HP^+ were only eating a small proportion of the amount of herbal hay H2 offered. Therefore, the average NEL intake of the six cows of the HP^+ group was considerably lower compared to the

other diets. For this reason, the analysis of variance was performed only after excluding the two HP^+ animals with these large refusals. Still the arithmetic means including all HP^+ cows were displayed in brackets in the tables.

3.4 Results

3.4.1 Composition of the hay types

The hay types differed largely in botanical composition and species number (Table 6). Grass hay G2 was dominated by grasses other than *Lolium* spp. and was composed of 28 different species. Compared to the grass hays, the herbal hays H1 and H2 had much lower grass proportions and substantial proportions of herbs (375 and 800 g/kg total DM, respectively). The CP content ranged from 87 g (G2) to 196 g/kg DM (basal hay) and was intermediate for the other three hay types (Table 7). Contents of NDF were < 500 g/kg DM for the basal hay and the herbal hays but greater for the two grass hays. Contents of ADF were greater than 300 g/kg DM for the two grass hays and hay H2. Acid detergent lignin content was lowest for the basal hay and hay G1 (<45 g/kg DM), about 60 g/kg DM in hay G2 and H1 and high (>70 g/kg DM) in hay H2. The calculated content of non-fiber-CHO was higher in the herbal hays than in the basal and the grass hays. The content of ether extract was low in hay G2 (<20 g/kg DM), intermediate in G1 and H2 and comparably high (>30 g/kg DM) in the basal hay and hay H1. The two herbal hays contained more Ca than the other hay types. The P content was high in the basal hay, but lower in the other hay types. The content of TEP was high with 35 g/kg DM in hay H2, followed by H1 (25 g/kg DM), respectively, and low (< 15 g/kg DM) in the other hay types. No CT were detected in the grass hay types. Consequently, TT obviously consisted only of HT. The amount of TT was 16.6 g and that of CT 12.6 g/kg DM for hay H2. The corresponding values for H1 were 9.1 g and 3.4 g/kg DM. The NEL content was high in the basal hay, intermediate in hays G1 and H1 and low in hays G2 and H2. The gradient from high to low in uCP content was from basal and the two herbal hays, followed by hay G1 and then hay G2. The basal hay and the two herbal hays were similar in ALA and LA proportion of total lipids. Hay G1 lipids contained less LA and G2 hay less ALA compared to the other hay types. The C18:1 *n*-9 proportion was high in the two herbal hays.

Table 7 Composition of the experimental hay types in dry matter (n = 3; means ± standard deviations).

Hay type	Basal	Grass 1	Grass 2	Herbal 1	Herbal 2
Dry matter, g/kg original substance	890 ± 4	892 ± 4	885 ± 7	885 ± 6	878 ± 1
Chemical composition, g/kg					
Organic matter	892 ± 3	884 ± 12	934 ± 3	908 ± 8	909 ± 22
Crude protein	196 ± 1	111 ± 15	87.7 ± 7	126 ± 9	125 ± 6
Neutral detergent fiber	492 ± 1	545 ± 8	609 ± 9	452 ± 20	436 ± 29
Acid detergent fiber	286 ± 5	324 ± 8	391 ± 11	297 ± 10	320 ± 21
Acid detergent lignin	40.2 ± 3.5	44.5 ± 3.0	59.0 ± 2.8	59.9 ± 5.4	71.1 ± 7.9
Hemicellulose	206 ± 4	221 ± 4	218 ± 9	155 ± 10	116 ± 8
Cellulose	246 ± 2	280 ± 7	332 ± 11	237 ± 14	249 ± 26
Non-fiber carbohydrates	166 ± 3	200 ± 29	221 ± 5	296 ± 5	322 ± 22
Ether extract	37.7 ± 0.6	28.7 ± 4.2	16.0 ± 2.6	34.3 ± 1.5	26.0 ± 1.7
Ca	6.16 ± 0.17	4.58 ± 0.25	5.09 ± 0.41	9.74 ± 0.97	10.9 ± 0.5
P	4.63 ± 0.10	3.32 ± 0.77	2.27 ± 0.16	1.57 ± 0.97	2.67 ± 0.48
Mg	2.01 ± 0.03	1.77 ± 0.23	1.39 ± 0.20	2.13 ± 0.15	4.29 ± 0.55
Na	0.18 ± 0.01	0.30 ± 0.26	0.64 ± 0.36	0.07 ± 0.02	0.14 ± 0.06
K	25.9 ± 0.9	22.8 ± 3.2	14.1 ± 0.6	15.2 ± 0.6	9.34 ± 0.94
Phenolic contents, g/kg					
Total extractable phenols	11.6 ± 1.46	13.5 ± 1.49	11.7 ± 1.81	24.9 ± 0.01	34.9 ± 2.06
Non-tannin phenols	9.18 ± 2.77	8.13 ± 3.14	7.24 ± 2.39	15.5 ± 0.62	17.63 ±1.73
Total tannins	2.73 ± 1.33	5.60 ± 2.23	4.59 ± 3.94	9.07 ± 0.29	16.62 ± 1.06
Condensed tannins	ND[2]	ND	ND	3.39 ± 0.89	12.56 ± 0.76
Hydrolyzable tannins	2.73 ± 1.33	5.60 ± 2.23	4.59 ± 3.94	5.68 ± 1.11	4.06 ± 1.62
Calculated contents[1]					
Net energy lactation, MJ/kg	5.63 ± 0.06	5.24 ± 0.20	4.53 ± 0.11	5.25 ± 0.03	4.72 ± 0.11
Utilizable crude protein, g/kg	152 ± 4	124 ± 4	102 ± 8	137 ± 5	145 ± 7
Fatty acids (FA), g/kg total FA methyl esters					
C12:0	3.62 ± 0.58	3.15 ± 0.22	3.12 ± 0.67	3.99 ± 0.31	1.93 ± 0.61
C14:0	6.77 ± 0.25	6.90 ± 0.22	9.05 ± 0.25	11.5 ± 1.4	8.44 ± 0.22
C16:0	203 ± 3	187 ± 1.02	226 ± 11	183 ± 1	191 ± 5
C18:0	21.6 ± 0.6	19.3 ±1.1	28.2 ± 0.8	24.8 ± 2.6	26.7 ± 0.7
C18:1 *n*-9	28.8 ± 0.7	25.9 ± 3.8	32.2 ± 3.4	37.1 ± 6.0	45.0 ± 4.2
C18:2 *n*-6 (LA)	172 ± 5	138 ± 7	170 ± 4	171 ± 10	183 ± 7
C18:3 *n*-3 (ALA)	435 ± 13	486 ± 24	362 ± 5	422 ± 25	394 ± 17
Saturated FA	293 ± 4	277 ± 12	352 ± 12	298 ± 6	304 ± 6
Monounsaturated FA	81.0 ± 5.9	82.4 ± 12.1	91.3 ± 4.2	72.8 ± 19.5	88.1 ± 14.4
Polyunsaturated FA	626 ± 7	641 ± 19	557 ± 8	629 ± 20	608 ± 16

[1] Assessed according to Menke and Steingaß (1988) as well as Steingaß and Südekum (2013).
[2] ND = not detected.

3.4.2 Feed intake, milk production and gross milk constituents

The NEL content of the feed actually consumed was similar in three of the four experimental diets (GN^-, GN^+ and HP^-), but lower ($P < 0.05$) in HP^+ due to the preference of the basal hay compared to hay H2 by some animals (Table 8). The uCP content was highest in HP^+, lowest in GN^- and intermediate in GN^+ and HP^- ($P < 0.05$), whereas the CP content was similar across diets except of GN^+ where it was higher. Cows fed mountainous hay ingested forage with lower NDF ($P < 0.05$) and higher ADL content than cows fed the grass diets ($P < 0.05$). Cows fed diet HP^+ consumed forage of a lower ADF content than cows fed the other diets ($P < 0.05$). The diets as consumed reflected the intended gradient in TEP intake with increasing herbal proportions in the diet. There was no difference between diets GN^- and GN^+ in TEP intake. Total DM intake was higher ($P < 0.05$) with diets GN^+ and HP^+ than with diets GN^- and HP^-. The NEL intake differed ($P < 0.05$) between GN^+ (89 MJ) and GN^- and HP^- (84 MJ). Intake of CP was highest in GN^+, intermediate in HP^+ and lowest in GN^- and HP^- ($P < 0.05$). Milk yield and composition did not differ ($P > 0.10$) between diets. Milk urea-N content was higher ($P < 0.05$) in GN^+ cows compared to cows of the other diets. Feed conversion efficiency was not affected ($P > 0.10$) by diet type.

3.4.3 Apparent total tract digestibility and nitrogen balance

The apparent digestibility of OM, NDF and ADF was highest ($P < 0.05$) and similar with GN^- and GN^+ compared to cows fed the herbal hay types (Table 9). Cows fed HP^- and HP^+ had a similar OM digestibility, but HP^+ cows had a lower ($P < 0.05$) digestibility of NDF and ADF than HP^- cows. Nitrogen intake was highest ($P < 0.05$) in GN^+ cows and lowest ($P < 0.05$) with GN^- and HP^-. Compared to diet GN^-, fecal N losses were higher ($P < 0.05$) with GN^+ and HP^+, but similar with HP^-. The urinary N losses were highest ($P < 0.05$) in GN^+ cows compared to the cows of the other diets. The proportion of fecal N relative to N intake was highest ($P < 0.05$) with HP^+ (not significantly different compared to the HP^- diet). The proportionate urinary N losses did not differ between diets GN^-, GN^+ and HP^-, but were lower ($P < 0.05$) in with HP^+ compared to GN^+. The urine N proportion of total fecal and urinary N excretion was highest with GN^+, intermediate with GN^- and HP^- and lowest with HP^+ ($P < 0.05$). The N utilization for milk protein synthesis calculated either relative to N intake or relative to N apparently digested differed between diet types ($P < 0.05$). The proportion of milk N of N intake was highest with HP^- (not significantly different from GN^-). When related to apparently digested N, N utilization was higher ($P < 0.05$) in cows fed HP^- compared to cows fed on GN^+, whereas there were no significant differences between GN^- and HP^+.

Table 8 Composition of the experimental diets either dominated by grasses (G) low and high in nitrogen content (N^- or N^+) or by herbs (H) low and high in total phenol (TEP) content (P^- or P^+) as well as intake, milk yield and composition of the cows.

Experimental diet[1]	GN^-	GN^+	HP^-	HP^+		
n	6	6	6	4 (6[2])	SEM	*P*-value
Content in dry matter (DM)						
Net energy for lactation, MJ	5.42[a]	5.42[a]	5.41[a]	5.12[b] (5.18)	0.027	<0.001
Utilizable crude protein, g	137[c]	143[b]	143[b]	147[a] (148)	1.1	<0.001
Crude protein, g	151[b]	175[a]	155[b]	156[b] (161)	2.4	<0.001
TEP content, g	12.7[c]	11.7[d]	19.4[b]	24.2[a] (23.2)	1.074	<0.001
Neutral detergent fiber	520[a]	515[a]	468[b]	459[b] (464)	6.0	<0.001
Acid detergent fiber	306[a]	306[a]	292[b]	305[a] (303)	1.8	0.003
Acid detergent lignin	42.5[c]	43.8[c]	51.7[b]	56.8[a] (55.2)	1.33	<0.001
Intake, kg/cow and day						
Hay (DM)						
Total hay (diet)	15.4[b]	16.4[a]	15.6[b]	16.7[a] (15.1)	0.166	<0.001
Basal hay	7.12[b]	13.23[a]	6.50[c]	7.34[b] (7.34)	0.616	<0.001
Grass or herbal hay	8.27[b]	3.19[c]	9.10[a]	9.30[a] (7.74)	0.568	0.005
NEL, MJ	83.5[b]	88.9[a]	84.4[b]	85.3[ab] (77.9)	0.80	0.007
Crude protein	2.32[c]	2.88[a]	2.42[c]	2.59[b] (2.41)	0.052	<0.001
Total phenols, g	195[c]	191[c]	302[b]	402[a] (353)	17.9	<0.001
Condensed tannins, g	ND[4]	ND	30.9[b]	115[a] (96.5)	13.81	<0.001
Milk yield, kg/cow and day						
Absolute	17.3	18.5	18.5	17.1 (14.8)	0.73	0.821
ECM[3]	19.0	19.3	21.3	18.4 (16.6)	0.63	0.350
Milk composition, per 100 g						
Fat, g	4.94	4.36	5.16	4.82 (5.19)	0.146	0.168
Protein, g	3.63	3.48	3.72	3.51 (3.74)	0.111	0.747
Lactose, g	4.56	4.70	4.71	4.49 (4.43)	0.047	0.316
Urea-N (mg/dL)	10.9[b]	14.4[a]	12.3[b]	12.1[b] (12.2)	0.33	<0.001
Feed conversion efficiency						
kg ECM/kg DM	1.24	1.17	1.37	1.11 (1.10)	0.039	0.108

[1] Hay type composition of the diets as offered: GN^- = basal hay: grass hay 1 as 0.45:0.55; GN^+ = basal hay: grass hay 2 as 0.80:0.20; HP^- = basal hay: herbal hay 1 as 0.45:0.55; HP^+ = basal hay: herbal hay 2 as 0.45:0.55.

[2] Without two cows with decreased feed intake during the collection period. In brackets the values for all six cows are displayed.

[3] ECM (energy-corrected milk yield, kg) = milk yield (kg) × [(0.038 × fat (g/kg) + 0.024 × protein (g/kg) + 0.017 × lactose (g/kg)]/3.14 (Agroscope, 2017).

[a-c] Means within row with different superscripts differ significantly ($P < 0.05$).

[4] ND = not detected.

Table 9 Digestibility and nitrogen balance of cows fed hay either dominated by grasses (G) low and high in nitrogen content (N^- or N^+) or by herbs (H) low and high in total phenol content (P^- or P^+).

Experimental diet[1]	GN^-	GN^+	HP^-	HP^+		
N	6	6	6	4 (6[2])	SEM	*P*-value
Apparent total tract nutrient digestibility, proportion						
Organic matter	0.698[a]	0.694[a]	0.669[b]	0.658[b] (0.673)	0.004	<0.001
Neutral detergent fiber	0.678[a]	0.687[a]	0.629[b]	0.570[c] (0.609)	0.010	<0.001
Acid detergent fiber	0.672[a]	0.674[a]	0.611[b]	0.521[c] (0.557)	0.013	<0.001
N balance, g/cow and day						
N intake	370[c]	461[a]	387[c]	415[b] (385)	8.3	<0.001
Fecal N	127[b]	159[a]	147[ab]	156[a] (152)	4.0	<0.001
Urinary N	86[b]	128[a]	92[b]	83[b] (81)	4.7	<0.001
Milk N	94	98	104	88 (81)	2.6	0.275
N losses, g/kg of N intake						
Fecal N	343[b]	346[b]	379[ab]	396[a] (395)	7.1	0.020
Urine N	233[ab]	278[a]	238[ab]	201[b] (211)	8.6	0.007
N loss, g/kg total fecal and urinary N loss						
Urine N	402[b]	446[a]	386[b]	336[c] (348)	9.9	<0.001
N utilization, g milk N per kg						
N intake	255[ab]	213[b]	267[a]	213[b] (210)	7.7	0.009
N apparently digested	388[ab]	327[b]	431[a]	354[ab] (348)	12.9	0.012

[1] Hay type composition of diets as offered: GN^- = basal hay: grass hay 1 as 0.45:0.55; GN^+ = basal hay: grass hay 2 as 0.80:0.20; HP^- = basal hay: herbal hay 1 as 0.45:0.55; HP^+ = basal hay: herbal hay 2 as 0.45:0.55.

[2] Without two cows with decreased feed intake during the collection period. In brackets the values for all six cows are displayed.

[a-c] Means within row with different superscripts differ significantly ($P < 0.05$).

3.4.4 Milk fatty acid profile and secretion relative to intake

In general, there was little influence of the experimental diets on the FA composition of the milk fat (Table 10). Feeding GN^- resulted in a lower ($P < 0.05$) LA proportion than HP^+, but there were no differences to GN^+ and HP^-. The proportion of RA was higher ($P < 0.05$) in HP^+ compared to GN^+ and HP^-, but did not differ from GN^-. The milk fat of cows on HP^- and HP^+ did not differ in ALA or 22:5 *n*-3 FA proportion, but HP^+ resulted in higher proportions ($P < 0.05$) of both FA compared to GN^-. The proportion of polyunsaturated FA (PUFA) was higher ($P < 0.05$) with HP^+ compared to GN^-. Total *n*-3 and *n*-6 FA proportions were lowest with GN^+ ($P < 0.05$), intermediate with HP^- and highest with HP^+. There were no differences in diet effects on the secretion of LA with the milk relative to dietary LA intake. The secretion of ALA was higher ($P < 0.05$) relative to dietary intake of ALA in cows fed HP^- and HP^+ compared to GN^- and GN^+. The secretion of VA, but not of RA, relative to dietary intake of LA and ALA, was higher ($P < 0.05$) with HP^- compared to all other diets.

Table 10 Fatty acid (FA) composition of milk fat (g/kg total FA methyl esters), *n*-6:*n*-3 fatty acid ratio and fatty acid secretion with the milk in relation to dietary fatty acid (g/kg FA ingested) of cows fed hay either dominated by grasses (G) low and high in nitrogen content (N^- or N^+) or by herbs (H) low and high in total phenol content (P^- or P^+).

Experimental diet[1]	GN^-	GN^+	HP^-	HP^+		
n	6	6	6	4 (6[2])	SEM	*P*-value
Individual FA						
C4:0	12.7	12.0	13.8	12.0 (11.4)	0.29	0.049
C6:0	14.1	13.4	14.2	13.6 (12.6)	0.39	0.511
C8:0	11.0	9.8	10.1	10.3 (9.6)	0.52	0.432
C10:0	25.8	23.3	21.7	23.7 (21.6)	1.35	0.317
C12:0	31.1	27.1	25.1	27.3 (25.1)	1.44	0.244
C14:0	114	107	103	109 (103)	3.5	0.380
C16:0	316	305	321	319 (315)	5.9	0.138
C16:1	20.1	21.6	20.8	19.3 (20.3)	1.63	0.774
C18:0	76.8	82.2	80.0	84.3 (83.3)	3.25	0.407
C18:1 *n*-9	232	243	238	221 (246)	7.1	0.724
C18:1 *trans* (total)[3]	16.0	17.8	19.5	17.7 (17.0)	0.540	0.115
C18:1 *trans*-11 (VA)	12.3	14.4	14.9	13.5 (13.0)	1.39	0.350
C18:2 *n*-6 (LA)	12.8^{b}	15.0^{ab}	14.9^{ab}	17.6^{a} (17.0)	0.52	0.022
C18:2 CLA (total)[4]	6.48	6.67	6.76	7.33 (7.20)	0.123	0.164
C18:2 *cis*-9, *trans*-11(RA)	3.26^{ab}	3.07^{b}	3.05^{b}	3.91^{a} (3.80)	0.123	0.024
C18:3 *n*-3 (ALA)	9.2^{b}	10.5^{b}	11.9^{ab}	14.2^{a} (13.2)	0.68	0.003
C22:5 *n*-3	0.58^{b}	0.76^{ab}	0.73^{ab}	0.90^{a} (0.81)	0.091	0.011
C22:6 *n*-3	0.21	0.12	0.10	0.16 (0.14)	0.015	0.303
Sums of FA						
Saturated FA	644	625	630	644 (622)	7.3	0.602
Monounsaturated FA	316	331	324	303 (328)	7.6	0.539
Polyunsaturated FA	40.5^{b}	44.1^{ab}	46.2^{ab}	52.9^{a} (50.2)	0.92	0.010
n-3 FA	11.2^{bc}	11.0^{c}	14.3^{b}	15.5^{a} (15.7)	0.65	<0.001
n-6 FA	19.5^{bc}	20.2^{c}	22.3^{b}	24.5^{a} (24.5)	0.75	<0.001
n-6:*n*-3 FA ratio	1.76	1.92	1.57	1.61 (1.58)	0.055	0.066
Transfer of fatty acids from diets to milk fat						
LA	0.242	0.207	0.278	0.265 (0.254)	0.0123	0.098
ALA	0.055^{b}	0.055^{b}	0.081^{a}	0.084^{a} (0.078)	0.0038	0.001
C18:1 *trans*-11: LA + ALA	0.056^{b}	0.053^{b}	0.071^{a}	0.056^{b} (0.054)	0.0025	0.006
C18:2 *cis*-9, *trans*-11: LA + ALA	0.017	0.012	0.016	0.016 (0.016)	0.0008	0.066

[1] Hay type composition of the diets as offered: GN^- = basal hay: grass hay 1 as 0.45:0.55; GN^+ = basal hay: grass hay 2 as 0.80:0.20; HP^- = basal hay: herbal hay 1 as 0.45:0.55; HP^+ = basal hay: herbal hay 2 as 0.45:0.55.

[2] Without two cows with decreased feed intake during the collection period. In brackets the values for all six cows are displayed.

[3] Sum of C18:1 *t*9; *t*10; *t*11 and *t*12.

[4] Conjugated linoleic acids. Sum of C18:2 *c*9, *t*12; *c*9, *c*15; *c*9, *t*11; *c*9, *c*11 and *t*9, *t*11.

[a-c] Means within row with different superscripts differ significantly ($P < 0.05$).

3.5 Discussion

The objectives of the present study were to investigate the effects of diets including hay from species-rich swards of mountainous origin varying in the proportion of herbs along with an increased content of phenolic compounds on feed intake, diet digestibility, N turnover, animal performance and the milk FA profile of dairy cows. The grass species *Nardus stricta* L. was present in a large proportion in one herbal hay type, and the corresponding diet (HP^-) was characterized by a moderate phenolic content. The second herbal hay type was particularly rich in a variety of herbal species and the corresponding diet (HP^+) had a comparably high phenolic content. As expected, the basal hay and the grass hay types (G1 and G2) were poor in phenolic compounds in contrast to the herbal hay types. Together the combinations with always the same basal grass hay, offered the opportunity to cover a wide range of diets in species abundance, phenolic contents and, eventually, N content.

3.5.1 Feed and phenolic intake as well as diet digestibility

Cows fed the grass-hay based diets ingested similarly low amounts of phenolic contents as described by Besle et al. (2010) for ryegrass or grassland hay diets. The phenolic content of herbal hay H2, fed with diet HP^+, was similar to contents measured by Fraisse et al. (2007) from a mountainous pasture, whereas diet HP^- did not reach this level, likely due to the lower proportion of herbs in this sward. However, cows consuming the herbal diets containing also the grass-hay basal diet ingested lower amounts of phenols per day (300 and 400 g/day and cow, respectively) as would be expected from cows grazing mountainous pasture as the sole source of feed (Fraisse et al., 2007). The use of the basal diet was intended to balance the concentrate-free diets for net energy (NEL) and metabolisable protein (uCP) contents.

A likely reason for the almost complete refusal of herbal hay H2 in diet HP^+ by two of the six cows was the relatively large proportion of little yellow-rattle (*Rhinanthus minor* L.) in this hay type. The feeding value of this plant species is described as poor because its palatability and digestibility are low and, furthermore, the presence of a glucoside deems this forage potentially toxic for ruminants (Jeangros et al., 1994a). Another plant species present in this herbal hay potentially causing a reduction in DM intake was *Veratrum album* L. containing various alkaloids (Heretsch and Giannis, 2015). Diets containing CT levels higher than 50 g/kg DM (Min et al., 2003) were reported to reduce DM intake as well; however, in the present study the CT content was lower than 10 g/kg DM in all diets. Also the HT among the phenolic compounds have been related to elicit toxicity or antinutritional effects in ruminants (McSweeney et al., 2001) because they are degradable in the rumen and their degradation products can be absorbed. However, the content of HT in herbal hay H2 was comparable to the other herbal hay and the grass hays, excluding HT as a primary reason for the refusal of the two cows. Cows are known to regulate the consumption of potentially toxic feeds to sub-lethal levels. Cyclic intake pattern with days of higher intake followed by days of lower intake have been described (Pfister et al., 1997) in order to cope with the metabolic load due to detoxification. The lack of a

similar refusal of this hay type by four of the six cows, however, illustrated the large individualism in palatability perception of cows as was reported in a similar context recently (Costa et al., 2017). In case the cows would have been able to graze the sward in question, much less variation in intake between cows would have been expected as with grazing the critical plants could be avoided.

The lower digestibility of OM and fiber of the diets containing herbal hay (HP^-, HP^+) compared to the diets containing grass hay (GN^-, GN^+) can be primarily associated with the chemical composition of the swards used in the diets and particularly elevated proportions of less well digestible herbs in the mountain hay swards. The ADL contents of herbal hay H1 and especially of herbal hay H2 were clearly higher than that of the basal hay, as was expected as some herbal species are particularly rich in ADL (Daccord et al., 2001; Jayanegara et al., 2011b). Maturation of the sward is one reason for elevated ADL contents, and both herbal hays had been harvested at the stage of flowering. Hammond et al. (2014) reported a reduction of OM and fiber digestibility of 0.121 for OM, 0.176 for NDF and 0.282 for ADF in dairy heifers fed a ryegrass sward oversown with a wild flower mixture when compared to a pure ryegrass pure sward. Also Bruinenberg et al. (2006) described a lower OM digestibility in dairy cows fed a species-rich sward harvested from semi-natural grassland when compared to an intensively managed ryegrass pure stand. This indicates that there would be a clear advantage with an early harvest, as also suggested also by Hammond et al. (2014), but this will only be possible at cost of a limited yield per hectare. It has to be pointed out that in the case of herbal hay H1, used in diet HP^-, rather the high proportion of the fibrous grass species *N. stricta* (Bovolenta et al., 2008; Jayanegara et al., 2011b) than the herbs may have been the factor main responsible for the low digestibility of this diet. By contrast, feeding herbal hay H2 in diet HP^+ where fiber digestibility was lower than with herbal hay H1, these high concentrations of the phenolic compounds might have played a role as they were reported to adversely act on fiber digestibility by reducing fiber breakdown (Scehovic, 1995b).

In the present study, feed intake was deliberately restricted to intake levels found with the least palatable diet (HP^+) in order to be able to compare diets in an iso-energetic feeding situation. Without this restriction, a higher milk yield would have been expected in the grass-based hay diets compared to the species-rich diets (Bruinenberg et al. 2006; Hammond et al., 2014). Contrarily, in early season, a higher milk production of cows grazing a species-rich mountainous pastured compared to cows grazing a primarily grass-based mountainous pasture was observed by Farruggia et al. (2014), and this effect was reversed over the course of the season. Differences in species composition and morphological traits including the leaf-to-stem ratio of the regrowth of the mountainous swards are therefore necessary to account for the potential of mountainous forage for dairy production.

3.5.2 Nitrogen utilization

All diets exceeded the recommended minimum dietary supply of 28 to 32 g CP/MJ NEL to ensure undisturbed ruminal fermentation (Agroscope, 2017). This was important as the purpose of the

experiment was to determine effects of phenolic compounds on the N utilization under situations of sufficient vs. excessive N supply. Feeding the different hay-based diets did not alter milk production and composition, except for the milk urea-N content that was higher with diet GN^+ being higher in CP but supply not more metabolizable protein compared to the other diets. Kröber et al. (2001) found that urinary N and milk urea-N excretion were highly correlated. It could, therefore, be expected that feeding cows diet GN^+, having a higher milk urea-N content, would result in increased urinary N losses.

Compared to the grass-based diet with low contents of phenolic compounds and N (GN^-), the N utilization did not improve with the diets including herbal hays at proportions of ~ 200 and ~ 400 g/kg DM respectively, despite higher contents of CT present in the herbal diets. It was assumed that CT could increase the supply of uCP and, along with that, the essential amino acids at the duodenum (Min et al., 2003) and by this way increase animal performance (Waghorn, 2008). Instead, there was a lower urine N proportion of total manure N in the cows fed the diet with the greatest proportion of herbal hay (HP^+). This may have resulted from association of the protein with cellulose and lignin which makes them less well ruminally degradable (Sniffen et al., 1992; Licitra et al., 1996) which was a characteristics of this diet. Consequently, the ammonia emission potential of the urine of cows fed this diet was reduced (Kröber et al., 2000). The higher phenol intake with diet HP^+ might have been important in this respect as well. Powell et al. (2009) observed a high proportion of N bound to fiber in a diet containing birdsfoot trefoil, a temperate climate legume species containing elevated CT contents. Carulla et al. (2005) reported that feeding extract of *Acacia mearnsii* rich in CT decreased urinary N losses but increased fecal N losses. Due to the capacity of CT to bind to forage protein and prevent it from ruminal degradation (Makkar, 2003; Mueller-Harvey, 2006) this shift towards a higher fecal N excretion indicates that these bonds are not completely released in the lower gut. Fecal N excretion, compared to urinary N excretion, is considered less critical for the animal's metabolism and for environmental issues (Tamminga, 1992). While detoxification of excess ruminal ammonia N into urea N is energy demanding (Twigg and Van Gils, 1988), fecal N losses do not result in an immediate release of N in the form of ammonia (Powell et al., 2009). Min et al. (2003) reported that 20 to 45 g CT/kg diet are required to elicit effects on ruminal protein degradation. In both diets with herbal hay, the concentrations of CT per kg feed were lower, but the structural properties and the reactivity of the CT present in the diets is relevant for exhibition and level of effect as well (Waghorn, 2008). Also HT have been reported to shift the N secretion pattern from urinary N to fecal N (Yang et al., 2016). However, the HT were less prevalent in herbal hay H2 compared to herbal hay H1 and even the two grass hay types.

3.5.3 Milk fatty acid composition

Only few FA were affected by diet type which was unexpected. The most prominent variations included the promotion of the proportion of total PUFA including total *n*-3 and *n*-6 FA as well as ALA

and LA with increasing herbal abundance. Consistent with the present results, Petersen et al. (2011) found an increase in *n*-3 and *n*-6 FA in the milk FA profile when herbs were included in the diet compared to feeding clover or a total mixed ration based on maize silage, with the latter two known to promote the proportions of saturated FA and monounsaturated FA (Collomb et al., 2002a; Leiber et al., 2005). Apart from the proportion of herbs in the diet, a high transfer efficiency of distinct FA is a major cause of elevated concentrations of *n*-3 FA in milk fat (Dewhurst et al., 2006). Mountainous grasslands are often characterized by a high species richness, like those tested in the present study. Collomb et al. (2002a) and Leiber et al. (2005) reported a selective enhancement of *n*-3 FA proportion in the milk fat of cows fed hay from mountainous origin compared to cows fed swards dominated by grass from lowland origin. Leiber et al. (2005) hypothesized that phenolic compounds are relevant for the milk FA profile as they may reduce ruminal biohydrogenation; this explains increased proportions of ALA in milk from cows fed hay from mountainous origin compared to cows fed hay from lowland origin or maize silage-based diets. Various groups of phenols were associated with a partial inhibition of biohydrogenation of PUFA such as LA and ALA (Khiaosa-Ard et al., 2009; Jayanegara et al., 2012); these are, however, active at different steps of the biohydrogenation cascade (Vasta et al., 2009a,b). Khiaosa-Ard et al. (2009) reported that some types of CT inhibited the last step of ruminal biohydrogenation which decreased the proportions of C18:0 and increased that of VA. However, no accumulation of VA and no decrease of C18:0 was observed with the diets richer in phenols (HP^-, HP^+). The high transfer efficiency of ALA in cows receiving herbal hay compared with grass hay is, therefore, likely due to an inhibition of the first step of biohydrogenation by phenolic compounds. This is especially supported by the observation, that diet GN^- included a high proportion of basal hay, which was rich in ALA but did not promote transfer of ALA into milk.

3.6 Conclusions

The present study showed that feeding hay from mountainous grassland as a model for different species richness, herbal abundance and phenolic content is an interesting alternative to feeding of dairy cows. This type of production system would also result in milk characterized by elevated proportions of desired fatty acids. Especially, the possibility to substantially lower urinary N excretion and thus reducing the N emission potential from such production systems was advantageous. Both aspects offer benefit to the farmers by opening up marketing milk for a higher price under label conditions and to fulfill governmentally imposed fines against, or rewards for, differently environmentally friendly dairy cow feeding systems. Drawbacks for the use of swards with herbal abundance include the often lower digestibility and the possible occurrence of unpalatable and potentially toxic plants. The latter could be counteracted by monitoring, the first by targeted inclusion of particularly promising herbal species with elevated phenolic contents in artificially established swards.

Chapter 4

Milk fatty acid profile and nitrogen utilization of dairy cows fed ryegrass-red clover silage containing plantain (*Plantago lanceolata*)

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4.1 Abstract

In this study the effect of plantain (*Plantago lanceolata* L.) ensiled at flowering stage on milk fatty acid profile and nitrogen utilization of dairy cows was tested. Plantain was composed with ryegrass and red-clover silage to form diet GCP. This diet was compared to diets based on ryegrass and red clover silage (GC) or ryegrass silage alone (G) and a total mixed ration based on maize silage, soybean meal and hay of a late first cut (TMR). All cows fed grass silage diets were supplemented with 3 kg wheat/day. In a completely randomized design, 24 multiparous dairy cows were fed one of the experimental diets during 13 days of adaptation and 7 days of sampling. Dry matter intake was reduced in G cows compared to all other groups. Crude protein intake (kg/day) was higher for cows fed diet GCP (3.54) compared to diets GC (2.84), G (1.79) and TMR (2.76). Yield of energy corrected milk (ECM) (27.1 kg/day) and milk protein (910 g/day) of cows fed on diet GCP was higher compared to that of cows fed on diet G (20.2 kg ECM/day, 680 g milk protein/day), but did not differ from cows fed on the GC or TMR diet. Urinary nitrogen (N) excretion relative to N intake and relative to total manure N did not differ between treatments. Utilization of dietary N for milk N formation (g/kg) was lowest for diets GCP (251) and GC (257), highest for diet G (357) and intermediate for diet TMR (313). The proportion of polyunsaturated fatty acids in milk fat was highest for cows fed GCP followed by GC, G and TMR. The proportions of total *n*-3 fatty acids, 18:1 *trans*-11 and 18:2 *cis*-9, *trans*-11 were higher in the milk fat of cows fed GCP than with any other diet. The ratio of *n*-6 to *n*-3 fatty acids was lower for diet GCP (1.5:1) compared to G (2.5:1) and TMR (5.7:1). Compared to G, GC and TMR, the cows fed GCP had both a higher excretion of 18:1 *trans*-11 and 18:2 *cis*-9, *trans*-11 with the milk relative to dietary 18:3 *n*-3 + 18:2 *n*-6 consumed. The results indicate that ensiled plantain fed in a mixture with ensiled ryegrass and red clover is a suitable dietary ingredient for dairy cows and it is an efficient means to increase the nutritive value of the milk fat.

4.2 Introduction

In grassland-based dairy production systems, forages provide large amounts of crude protein (CP). As this type of CP is mostly of high ruminal degradability, the nitrogen (N) use efficiency (NUE; proportion of N consumed as recovered in milk) is rather low (Givens and Rulquin, 2004). Excessive amounts of ammonia may be produced during ruminal CP degradation that have to be detoxified to urea and excreted in urine at high metabolic costs for the animal (Twigg and Van Gils, 1988) and leading to manure with high potential N emissions (Carulla et al., 2005). Ways to improve the NUE from forage species include a reduction of their CP content (Moorby et al., 2006), an increase in the proportion of rumen undegradable protein (Woodward et al., 2009) and the use of forage with high contents of rapidly fermentable organic matter, such as sugars (Moorby et al., 2006) enhancing ammonia use for formation of microbial protein (Nocek and Russell, 1988). Other attempts build on enzymes such as polyphenol oxidase (PPO) which promotes the formation of quinones complexing with proteins and thus protecting them from ruminal degradation (Copani et al., 2014). As PPO is prevalent in red clover, NUE was higher in dairy cows fed this legume when compared to alfalfa (*Medicago sativa*) (Broderick et al., 2001) or timothy (*Phleum pratense*) (Copani et al., 2014).

Among the possibilities to increase the NUE is also the inclusion of feeds or extracts containing bioactive compounds, which are often plant secondary metabolites, in particular condensed tannins (CT). These compounds may form protein-tannin complexes at neutral ruminal pH which reduces their accessibility to and at least partially inhibit protein degradation by rumen microbial enzymes (Makkar, 2003). Potentially, these complexes may disassociate at the low abomasal pH and the protein may be digested in the small intestine thus increasing the supply with metabolizable protein (MP) (Waghorn, 2008). In case the protein-tannin complexes remain stable, this at least leads to a shift in the excretory pattern from urinary N to fecal N (Carulla et al., 2005). The perennial herb species plantain (*Plantago lanceolata*) contains substantial amounts of plant secondary metabolites (Jeangros et al., 1994b; Fraisse et al., 2007; Al-Mamun et al., 2008). These were shown to act anti-bacterial (Ishiguro et al., 1982) and inhibitory to enzymes (Nishibe and Murai, 1995) and may, therefore, be relevant to ruminal fermentation, too, as shown *in vitro* (Navarrete et al., 2016). Plantain has a high nutritive value with a digestibility of 0.70 to 0.80 g/kg organic matter (Jeangros et al., 1994b; Minneé et al., 2017). Plantain is given particular attention in pasture pasture-based dairy production systems in New Zealand and Australia because, different from

ryegrass-clover swards, plantain produces biomass even during conditions of summer drought and heat (Stewart, 1996). Major cultivars are ‘Ceres Tonic’ and ‘Grasslands Lancelot’ which have been improved in their forage value in particular in combination with grass-legumes mixtures (Stewart, 1996). Feeding cut plantain was shown to decrease urinary N concentration and excretion in dairy heifers (Cheng et al., 2017). Adding plantain to ryegrass and white clover reduced rumen ammonia and urinary N concentrations in an experiment with lactating dairy cows (Minneé et al., 2017). Feeding a ryegrass-white clover mixture also containing plantain led to a lower dietary N concentration, improved NUE and milk yield and reduced urinary N excretion in dairy cows (Totty et al., 2013; Pembleton et al., 2016).

Apart from N metabolism, bioactive compounds may modify the milk fatty acid (FA) profile by affecting the ruminal biohydrogenation of monounsaturated FA (MUFA) and polyunsaturated FA (PUFA), and the transfer of these FA and their biohydrogenation intermediates into milk fat. Accordingly, an elevated dietary proportion of fresh herbs was shown to increase the proportion of *n*-3 and *n*-6 FA of the milk fat of dairy cows compared to cows fed a total mixed ration based on maize silage and concentrate (Petersen et al., 2011). Even feeding generic grass-silage instead of maize-silage is known to enrich the milk fat in MUFA and PUFA such as linoleic acid (LA, 18:2 *n*-6) and α-linolenic acid (ALA, 18:3 *n*-3) (Leiber et al., 2005). These two FA cannot be synthesized by the human body, act as precursors for other long chain FA and have been related to various beneficial health promoting effects in humans (Barcelo-Coblijn and Murphy, 2009). Besides LA and ALA, conjugated linoleic acids (CLA) are of major interest (Collomb et al., 2006) as they are presumed to have anticarcinogenic effects. Among the CLA isomers, especially rumenic acid (RA, 18:2 *cis*-9, *trans*-11) is considerably favorable. It is primarily produced from vaccenic acid (VA, 18:1 *trans*-11) in the mammary gland (Griinari et al., 2000). Several studies showed that distinct bioactive compounds affect biohydrogenation, but at different steps (Khiaosa-Ard et al., 2009; Vasta et al., 2009a,b; Jayanegara et al., 2012) either protecting part of LA and ALA or leading to a particular increase in VA. However, it is not yet known if, and to which extent, feeding plantain affects the milk FA profile in dairy cows.

In the present study, two hypotheses were tested: (1) The inclusion of plantain in a ryegrass and red clover based forage improves the NUE compared to forages without plantain. (2) In addition, it leads to a modification of the milk FA profile towards higher proportions of *n*-3 FA and CLA than feeding only ryegrass or a ryegrass and red clover mixture or when compared to a maize-silage soybean meal based total mixed ration.

4.3 Materials and methods

The experiment was approved by the Cantonal Veterinary Office Zug, Switzerland (licence no. ZG 2015/69). Four experimental diets were tested: ryegrass silage only (G), a ryegrass-red clover silage (GC), a ryegrass-red clover plantain silage (GCP) and a total mixed ration (TMR) composed of maize silage, soybean meal and a hay (first cut, harvested at the end of the flowering stage) in a ratio of 0.76:0.15:0.09 on a dry matter (DM) basis. The estimated milk production potential of the TMR diet was 30 kg energy corrected milk (ECM)/day per cow as calculated from anticipated intakes and contents of net energy for lactation (NEL; Agroscope, 2018) and MP (where MP is approximated by the 'utilizable CP; Steingaß and Südekum, 2013). All diets were offered at ad libitum access. The grass silage diets were complemented with 3 kg/d of ground wheat to balance diets for NEL content.

4.3.1 Experimental swards

The experimental swards were established at the ETH Research Station Chamau (400 m a.s.l., Zug, Switzerland). The experimental grass silages were prepared using five individual silages (S1 to S5). Diet G was based on a silage from a pure perennial ryegrass (*Lolium perenne*) stand (S1) sown at 40 kg/ha containing equal proportions of the cultivars 'Salamandra' and 'Alligator' (Eric Schweizer Samen AG, Thun, Switzerland). Diet GC was composed of a 1:1 mixture (DM basis) of two different ryegrass-clover silages (S2 and S3). For that, commercial grass-legume mixtures either obtained from Otto Hauenstein Samen AG (Rafz, Switzerland; S2) or from UFA Samen fenaco (Sämereienzentrum Niderfeld, Winterthur, Switzerland; S3) were sown at 38 kg/ha each. At harvest, silages S2 and S3 contained (g/kg DM) 878 g and 479 g perennial ryegrass, 0 g and 513 g red clover (*Trifolium pratense*), and 122 g and 80 g other species, respectively. Diet GCP was a 1:1 mixture (DM basis) of silages from a ryegrass-clover mixture (UFA Samen fenaco; S4) and a ryegrass-clover-plantain mixture (S5). Mixture S5 was obtained by combining plantain seed material (cultivar 'Herkules', Ceres Tonic, Eric Schweizer Samen AG, Thun, Switzerland) in a ratio of 0.09: 0.91 with the commercial ryegrass-legume mixture S2. At harvest, silages S4 and S5 contained (g/kg DM) 693 g and 126 g perennial ryegrass, 266 g and 30 g red clover, 0 g and 861 g plantain (*P. lanceolata*), and 41 g and 29 g other species, respectively. Per kg DM, the plantain material consisted of 449 g stems, 324 g flowers and 227 g leaves. Slurry and mineral N fertilizer were applied at 150 kg N/ha and year on all swards. Weeds were extinguished chemically (Blacken Star, Lussolin, 363 g/L MCPA – 30 g/L Dicamba, Hygiene GmbH, Langnau, Switzerland). All grass silages were ensiled without additives in round bales (250 to 400 kg). The maize

silage was stored in a tower silage container (Huber Silobau and Kunststoffwerk AG, Lengnau, Switzerland).

4.3.2 Diets

The TMR diet as consumed had a CP content of 145 g/kg DM, which ensured a sufficient supply with RDP. The three grass silage diets were intended to have increasing CP contents from G to GC and to GCP (realized: 128, 165 and 174 g/kg DM, respectively). At the same time, a gradient in total extractable phenols (TEP) (g/kg DM) was established (realized: 9.6 for TMR, 13.4 for G, 13.9, for GC and 14.8 for GCP. With this arrangement, diets with increasing TEP supply at concomitantly increasingly excessive CP supply were established as opposed to diets TMR and G. Each cow was supplemented daily with 50 g of NaCl and 100 g of a vitaminized mineral mix (Kroni Locher and Co. AG, Altstaetten, Switzerland). The mix contained per kg: 160 g Ca, 80 g P, 100 g Mg, 35 g Na, 1 g S, 1'200'000 I.E. vitamin A, 200'000 I.E. vitamin D_3, 3 g vitamin E, 8 g Zn, 4 g Mn, 1 g Cu, 30 mg Se, 100 mg I and 30 mg Co. The pre-experimental diet consisted of grass silage, maize silage, hay, soybean meal, NaCl, urea and a commercial vitaminized mineral mix (Ca: P = 2:1) in proportions of 460, 362, 94, 70, 6, 4, and 4 g/kg DM, respectively. In addition, concentrate was provided in an amount meeting the cows' requirements for maintenance and milk yield (Agroscope, 2018).

4.3.3 Cows

Twenty-four multiparous cows (eight Brown Swiss and 16 Holstein Friesian) were selected from > 60 dairy cows of the ETH Research Station and assigned to one of four treatments (TMR, G, GC and GCP). The cows were split into blocks of eight cows for three subsequent runs with always two cows being assigned to one of four treatments. The assignment was performed in a complete randomized design where block averages were balanced for breed type (two Brown Swiss and four Holstein Friesian per treatment), body weight (BW), days in milk (DIM), and yields of ECM, fat and protein. Accordingly, the cows in TMR, G, GC and GCP had an initial BW of 614 ± 57, 648 ± 55, 625 ± 53 and 668 ± 60 kg (means ± SD) respectively. The corresponding data for DIM were 182 ± 42, 171 ± 78, 162 ± 77 and 171 ± 84 days and for lactation number they were 3.33 ± 1.21, 3.83 ± 1.17, 4.00 ± 1.58 and 2.83 ± 0.98. At the last routine milk quality control prior to the experiment, the cows of the dietary treatments TMR, G, GC and GCP yielded per d 39.9 ± 5.6, 39.7 ± 6.6, 38.9 ± 5.8 and 38.7 ± 6.1 kg ECM, 1.23 ± 0.18, 1.25 ± 0.14, 1.24 ± 0.17 and 1.21 ± 0.15 kg protein and 1.51 ± 0.16, 1.47 ± 0.33, 1.42 ± 0.26 and 1.41 ± 0.27 kg fat, respectively. The experimental period had a

duration of 20 days for each animal and was divided into an adaptation period (13 days) and a data and sample collection period (7 days). During the first 12 days of adaptation, the two cows per treatment and block were kept together in a free range deep litter pen. On day 13 of the adaptation period, cows were transferred to individual tie stalls equipped with troughs on balances, comfort mattresses and a grid allowing complete feces collection. At the start of the adaptation period, the pre-experimental diet was gradually replaced with the experimental diets from proportions of 1 (day 1) to 0 (day 5). The grass-silage fed cows already received the complementary wheat. Cows of the TMR diet did no longer receive wheat from day 6 of adaptation onwards. Milking took place at 05:15 h and 16:30 h, silage and TMR were supplied at 06:00 h and 16:00 h, and the wheat was offered at 06:30 h. Cows had ad libitum access to water during the entire 20-day period.

4.3.4 Data recording and sample collection

Plant species composition of the five swards was assessed in five to ten subsamples (depending on field size) from 0.5 m^2 plots randomly distributed across the field on the day of cutting or one day earlier. The fresh material was sorted for individual plant species. Species proportions were determined after drying at 105°C for 24 h. Feed intake was determined daily during the collection period. Refusals were collected directly before the morning feeding. The DM content of all forages, soybean meal, wheat, and refusals was determined by oven drying at 105°C for 24 h. Proportionate subsamples of refusals per cow per day were dried at 60°C for 24 h and pooled. In each block of collection period, samples of silages, hay, soybean meal and wheat were collected three times and pooled. The pooled silage samples were subdivided and either stored at –20°C or dried at 60°C for 24 h and ground to pass a 1-mm sieve for chemical analysis.

In the collection period, milk yield was registered and milk was sampled at each milking. These samples were pooled per cow per day proportionate to the respective milk amounts. One subsample was collected in a flask containing 2-bromo-2-nitropropane-1,3-diol (Bronopol®, D & F Inc., Dublin, CA, USA). Two further subsamples were frozen at –20°C. Feces were collected quantitatively in chromium steel trays placed below the grid. After morning feeding, proportionately 0.005 of total feces were taken and pooled per cow. Part of it was frozen (for N analysis), another part was dried at 60°C for 48 h (for all other analyses) and a third sample was dried at 105°C for 24 h to determine DM content. The urine was separated from feces by urinals, which were attached around the vulva of the cows with hook-and-loop fastener straps, the counterpart of which was glued (ergo.500 Universal, Kisling AG,

Wetzikon, Switzerland) onto the shorn skin. Urine was collected in containers and weighed daily. A subsample was diverted into a canister containing 50 g of 5 M sulfuric acid to prevent gaseous N losses. Samples were stored at –20°C for later analysis of urinary N content.

4.3.5 Laboratory analysis

The composition of feeds, leftovers and feces was determined using standard procedures (van Soest et al., 1991; AOAC, 1997). Contents of DM and ash were assessed with an automatic thermogravimetric determinator (TGA-701, Leco, St. Joseph, MI, USA; AOAC index no. 942.05). Contents of N of feeds, leftovers, fresh feces, milk and acidified urine were determined using a C/N analyzer (Type TruMac CN 2000, Leco Cooperation, St. Joseph, MI, USA: AOAC index no. 968.06). Crude protein was calculated as either 6.25 or 6.38 × N for either feeds, feed leftovers and dried feces or milk. Ether extract was determined in feeds by the Soxhlet method using the extraction system B-811 (Büchi, Flawil, Switzerland, AOAC index no. 963.15). Contents of neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) without residual ash were analyzed in feeds, leftovers and dried feces on a Fibertec System M (Tecator, 1020 hot extraction, Foss Hillerød, Denmark) excluding residual ash. The NDF content was determined using heat stable α-amylase (100 µL). The content of non-fiber carbohydrates was calculated as organic matter – NDF – CP – ether extract (Hall, 2000). Contents of Ca, Mg, P, Na and K in feeds were analyzed using a microwave accelerated reaction system (MARS6) with MarsXpress (CEM, 3100 Smith Road, Matthes, NC, USA) on a thermo iCAP 6300 inductively coupled plasma radial spectrometer (Thermo Fisher Scientific.Inc, Waltham, MA, USA). Contents of TEP, non-tannin phenols, total tannins, condensed tannins and hydrolysable tannins were analyzed in feeds according to Makkar (2003) as modified by Jayanegara et al. (2012). They were expressed as gallic acid equivalents. Bronopol stabilized milk was analyzed for fat, protein, lactose and urea-N with mid infrared spectrophotometry (Milkoscan 4000, Foss Electric, Hillerød, Denmark). Thawed silage samples were analyzed according to VDLUFA (2018) for pH (method III, 18.1), ammonia N (method II, 3.2.6), acetic acid, butyric acid and lactic acid (method III, 18.2) at the LUFA North-West (Oldenburg, Germany). Results were evaluated according to the standards of the DLG (2006). The ECM (kg) was calculated as milk yield (kg) × [0.38 × % fat + 0.24 × % protein + 0.17 × % lactose]/3.14 MJ NEL (Agroscope, 2018).

Fatty acids were extracted from the feeds by a solvent extractor (ASE 200, Dionex Corporation, Sunnyvale, CA, USA) using a hexane: propane-2-ol mixture (3:2 v/v). They

were transformed to FA methyl esters (FAME) according to IUPAC (1987) method 2.301. Cleaning was performed as described in Wettstein et al. (2001). For FAME analysis, a gas chromatograph (model HP 6890 equipped with a FID detector, Hewlett Packard, Palo Alto, CA, USA) equipped with a CP7421 column (200 m x 0.25 mm, 0.25 µm; Varian Inc., Darmstadt, Germany) was used. Split injection (1:5) was applied. The internal standard was 11:0 (Fluka, Steinheim, Germany), the external standard to calculate the response factor was sunflower oil. A volume of 1 µL was injected with 1.7 mL H_2/min. The temperature program was set to 170°C for 60 min, increase by 5°C/min to 230°C, ramp for 32 min, increase by 5°C/min to 250°C and ramp for 15 min. Milk FA were analyzed in one pooled sample per cow. Internal standards (5 mL of n-heptane containing triundecanoin, tetradecenoic methylate and trivaleranoin) were mixed with 0.5 mL of milk. Sodium methylate was used for cold transesterification to FAME (Suter et al., 1997). The response factors were obtained from 6:0, 13:0 and 19:0 triglyceride standards. The same gas chromatograph and column were used as for FA from feeds. A volume of 1.0 µL FAME was injected at a split 1:1 with 1.7 L H_2/min. The temperature program stared with an initial temperature of 60°C, ramp for 12 min, increase of 5°C/min to 170°C, ramp for 60 min, increase by 5°C/min to 250°C and ramp for 20 min. Identification of FAME was performed using a Supelco 37 component standard (Supelco Inc., Bellefonte PA, USA). Peak identification was further confirmed using chromatograms from Collomb and Bühler (2000).

Contents of NEL and MP (determined as utilizable CP; Steingaß and Südekum, 2013) of the feeds were determined using a modified Hohenheim Gas Test method where the ammonia N content is measured in the incubated rumen fluid buffer sample (Steingaß et al., 2001). For this purpose, samples were additionally ground to pass a 0.75-mm sieve. The modified method followed the same procedure as the standard test except for providing excess nitrogen for microbial fermentation throughout the incubation by omitting 2 g/l $NaHCO_3$ and adding 2 g/l NH_4CO_3 in the buffer solution. A protein standard with 254 g CP/kg DM with an assumed content of 183 g MP/kg DM when determined after 24 h of incubation was included in each run (Edmunds et al., 2012). Rumen fluid was collected from a ruminally fistulated Brown Swiss cow kept at the Vetsuisse Faculty of the University of Zurich, Switzerland (approval no ZH 38/14). After 24 h, gas volume was recorded and 15 ml of incubated rumen fluid buffer mixture was collected and immediately put on ice to stop fermentation. After allowing to cool for 15 min, 150 µL 5 M H_2SO_4 were added to prevent loss of ammonia N. Ammonia concentrations of blanks, standards and samples were measured using an ammonia selective electrode (Metrohm AG, Herisau, Switzerland).

4.3.6 Statistical analysis

The three blocks (3 × 2 cows per treatment) each consisting of an adaptation phase of 13 days followed a collection period of 7 days were slightly overlapping resulting in a total experimental duration of 49 days. A general linear model with dietary treatment as fixed effect and block as block factor was used for ANOVA applying the software R (V3.3.1) and the R package nlme (Pinheiro et al., 2017). Normality and homogeneity of variances were controlled both graphically by controlling the residuals and their distribution and with the test procedures of the Shapiro-Wilk-test for normality and the Bartlett-test for homogeneity of variance. Multiple comparisons among means were assessed with Tukey's procedure considering $P < 0.05$ as significant. Results are displayed as Least Square means and standard error of the mean. Data from one cow of group G were omitted from the statistical evaluation as this cow had to be treated against mastitis.

4.4. Results

4.4.1 Experimental Silages

There was a difference in the chemical composition and plant species composition of the five silages from which the experimental diets were prepared (Table 11). Types S1, S2 and S5 were similar in CP (between 120 and 130 g/kg DM) and fiber content, but the fiber in S5 was more lignified compared to S1 and S2. Silages S3 and S4 were numerically richer in CP (194 to 220 g/kg), poorer in fiber and intermediate in lignin. Contents of ether extract were similar in S1, S2 and S5, and higher in S3 and S4. The Ca content was numerically highest in S5, intermediate in S3 and S4 and lowest in S1 and S2, while contents of K were rather similar across all grass silages. The contents of TEP were not very different in the silages being slightly higher in S5 than in the others. The estimated NEL contents did not differ much between the silages except for S5 (–1 MJ NEL/kg DM). Silages S1, S2 and S5 were lower in estimated MP than S3 and S4. The FA profile did not differ much between S1, S2, S3 and S4. Compared to that, S5 had numerically higher proportions of total SFA, C14:0, C16:0 and C18:0, total MUFA, C18:1 *n*-9 and LA and lower proportions of C12:0, total PUFA and ALA. The fermentation quality of the grass silages greatly differed. More lactic acid and acetic acid was found in S1 and S3 than in S2, S4 and S5. Butyric acid was detected in S1 (18 g/kg DM) and S3 (33 g/kg DM). The pH ranged from 4.73 in S1 to 6.00 in S4.

Table 11 Chemical composition and estimated contents of net energy for lactation (NEL) and metabolizable protein (MP) of the silages prepared from individual swards used for the preparation of the experimental diets (means of three independent samples each).

	Silage types					Maize		Soybean	
Item	S1	S2	S3	S4	S5	silage	Hay	meal	Wheat
Dry matter (DM) (g/kg wet weight)	352	548	426	528	535	323	933	882	878
Chemical composition (g/kg DM)									
Organic matter	896	906	889	893	897	974	954	930	978
Crude protein	124	123	194	220	127	66.4	78.6	520	141
Neutral detergent fiber	597	608	511	480	554	485	666	171	243
Acid detergent fiber	370	374	300	294	401	246	381	119	54.3
Acid detergent lignin	40.3	46.3	55.2	61.0	104	42.1	61.6	27.6	25.8
Hemicellulose	228	234	211	186	152	240	285	51.7	189
Cellulose	329	327	245	233	298	204	319	91.9	28.4
Non-fiber carbohydrates	131	137	129	136	176	387	190	223	575
Ether extract	42.7	37.7	55.0	56.0	39.3	35.0	19.0	15.0	19.0
Ca	33.5	41.2	86.6	76.4	107	16.0	33.8	26.7	4.25
P	39.9	33.3	39.6	35.6	40.6	22.0	17.1	65.2	47.1
Mg	10.4	11.8	22.7	22.6	19.4	11.3	12.1	30.3	13.4
K	31.1	28.1	32.6	31.9	28.0	53.2	11.1	20.3	43.8
Na	6.88	1.27	1.44	1.85	3.02	0.16	3.62	0.57	0.13
Phenols (g per kg DM)									
Extractable phenols	15.6	15.2	15.5	15.6	17.2	10.0	11.6	6.37	5.53
Non-tannin phenols	15.6	13.3	11.6	12.5	11.9	10.0	11.6	6.37	5.53
Total tannins	0.00	1.96	4.27	3.08	5.37	0.00	0.00	0.00	0.00
Condensed tannins	0.00	0.22	0.49	0.60	0.44	0.00	0.00	0.00	0.00
Hydrolysable tannins	0.00	1.74	3.78	2.40	4.93	0.00	0.00	0.00	0.00
Fatty acids (FA) methyl esters (g per kg total FA)									
C12:0	3.23	2.49	2.01	1.99	1.42	1.85	4.82	0.42	0.55
C14:0	5.49	3.94	4.80	4.54	7.20	2.05	13.6	0.99	0.90
C16:0	178	172	168	169	193	153	282	132	168
C18:0	17.5	16.2	19.3	20.8	21.5	19.9	29.9	37.4	13.1
C18:1 *n*-9	26.5	25.7	26.8	27.5	36.0	231	83.0	168	184
C18:2 *n*-6	165	186	177	163	197	496	204	561	555
C18:3 *n*-3	537	519	531	537	471	56.3	261	65.0	41.4
Σ Saturated FA	242	247	249	255	277	185	383	150	182
Σ Monounsaturated FA	37.1	37.4	35.7	37.2	45.9	249	106	194	208
Σ Polyunsaturated FA	721	716	715	708	677	566	512	656	608
Fermentation acids (g per kg DM)									
Lactic acid	64.1	14.1	45.8	3.65	2.57	44.7	–	–	–
Acetic acid	11.2	2.57	9.53	1.65	1.17	12.6	–	–	–
Butyric acid	18.0	<0.10	33.1	<0.10	<0.10	<0.10	–	–	–
Ammonia-N (mg per kg DM)	601	435	675	552	238	208	–	–	–
pH	4.73	5.57	4.90	6.00	5.63	3.80	–	–	–
Estimated values									
NEL (MJ per kg DM)	5.94	6.01	6.07	5.89	4.89	6.63	4.64	8.12	8.45
MP (g per kg DM)	12.7	13.4	16.2	17.1	13.0	10.1	9.90	27.8	12.5

4.4.2 Performance and nitrogen balance

Intakes of DM (DMI) and NEL were lower ($P < 0.05$) in G than in GCP and TMR, and intermediate with GC (Table 12). Cows fed G consumed less ($P < 0.05$) CP and MP compared to cows fed the other diets, while the CP intake was highest ($P < 0.05$) with GCP. The intake of NDF was similar between GCP, GC and TMR, but lower ($P < 0.05$) with G compared to GCP. Cows fed GCP ingested more ($P < 0.05$) ADF than cows fed the other diets. In GCP, cows had the highest ($P < 0.05$) TEP intake, followed by GC, and TEP intakes were lowest with TMR and G. Intakes of total SFA and 14:0 were higher ($P < 0.05$) with GCP than with the other diets. With TMR, cows consumed more ($P < 0.05$) C18:1 *n*-9 and LA than with the other diets. Ingestion of ALA was similar with GC and GCP, lower ($P < 0.05$) with G and lowest ($P < 0.05$) with TMR. Organic matter digestibility was lowest with GCP, intermediate with TMR and GC and highest with G ($P < 0.05$; not different from GC). Digestibility of NDF and ADF was higher ($P < 0.05$) with G and GC than with TMR and GCP.

Yield of milk, ECM and protein did not differ between TMR, GC and GCP but was lower ($P < 0.05$) with G compared to TMR and GCP (Table 12). Milk fat, protein and lactose contents did not differ between the diet types. The urea-N content was highest with GCP, intermediate with TMR and GC and lowest with G ($P < 0.05$). There were no diet type effects on feed conversion efficiency.

Nitrogen intake and excretion with feces and urine were highest with GCP, intermediate with TMR and GC and, for N intake, lowest with G ($P < 0.05$) (Table 13). Milk N excretion was higher with GCP compared to G and GC, and was higher for TMR compared with G ($P < 0.05$). Fecal N losses proportionate to N intake were lower ($P < 0.05$) with TMR compared with G and GCP. There was a tendency ($P = 0.09$) of a diet effect on urinary N proportion relative to N intake, where values were numerically highest with GCP. Cows fed G or GCP showed higher ($P < 0.05$) total excreta N proportions compared to those fed TMR. Utilization of dietary N for milk protein synthesis was highest in G, intermediate with TMR and lowest with GC and GCP ($P < 0.05$). Utilization of the apparently digested N for milk protein synthesis was higher ($P < 0.05$) with G compared with the other diets. Milk N relative to total excreta N was higher ($P < 0.05$) with TMR and G compared with GC and GCP. Urine N proportion of total excreta N did not differ between diet types.

Table 12 Intake, digestibility and performance.

Item	Treatment[1]				SEM	*P*-value
	GCP	GC	G	TMR		
Intake (kg per cow per day)						
Total dry matter (DM)	20.3[a]	17.2[ab]	14.0[b]	19.0[a]	0.65	0.01
Grass silage DM	17.4[a]	14.7[a]	11.1[b]	-	0.77	<0.001
Wheat DM	2.89	2.52	2.96	-	0.108	0.20
Net energy for lactation, MJ	118[a]	110[ab]	90.6[b]	127[a]	3.85	0.01
Metabolizable protein	2.99[a]	2.52[ab]	1.78[c]	2.42[b]	0.110	<0.001
Crude protein	3.54[a]	2.84[b]	1.79[c]	2.76[b]	0.143	<0.001
Organic matter	18.4[a]	15.6[ab]	12.8[b]	18.4[a]	0.62	<0.001
Neutral detergent fiber	9.62[a]	8.72[ab]	7.41[b]	8.62[ab]	0.277	0.05
Acid detergent fiber	6.11[a]	4.92[b]	4.27[b]	4.40[b]	0.199	<0.001
Extractable phenols (g)	301[a]	239[b]	188[c]	182[c]	11.57	<0.001
Fatty acid (FA) methyl esters (g)						
C12:0	0.58	0.62	0.61	0.65	0.019	0.55
C14:0	1.83[a]	1.19[b]	1.02[bc]	0.82[c]	0.090	<0.001
C16:0	64.9[a]	51.8[bc]	40.4[c]	56.2[ab]	2.35	<0.001
C18:0	7.23[a]	5.29[b]	3.82[b]	8.10[a]	0.396	<0.001
C18:1 *n*-9	19.0[b]	14.8[b]	14.4[b]	80.8[a]	6.25	<0.001
C18:2 *n*-6	83.8[b]	71.1[b]	58.1[b]	182[a]	11.07	<0.001
C18:3 *n*-3	162[a]	141[a]	98.2[b]	22.8[c]	11.98	<0.001
Σ Saturated FA	92.2[a]	73.2[b]	53.5[c]	67.8[bc]	3.49	<0.001
Σ Monounsaturated FA	23.4[b]	18.6[b]	17.7[b]	87.9[a]	6.59	<0.001
Σ Polyunsaturated FA	249[a]	215[a]	160[b]	211[ab]	8.6	0.01
Apparent total tract nutrient digestibility (proportion)						
Organic matter	681[c]	733[ab]	763[a]	720[b]	7.8	<0.001
Neutral detergent fiber	591[b]	704[a]	736[a]	574[b]	16.5	<0.001
Acid detergent fiber	564[b]	677[a]	744[a]	541[b]	19.0	<0.001
Milk yield (per cow per day)						
Absolute (kg)	25.5[a]	23.0[ab]	17.6[b]	25.7[a]	1.01	0.02
Energy corrected (kg)	27.1[a]	24.4[ab]	20.2[b]	27.8[a]	0.91	0.01
Protein (g)	910[a]	758[ab]	680[b]	911[a]	28.1	0.01
Milk composition (per 100 g)						
Fat (g)	4.46	4.59	5.17	4.64	0.120	0.22
Protein (g)	3.61	3.30	4.02	3.59	0.102	0.11
Lactose (g)	4.68	4.74	4.48	4.69	0.045	0.26
Urea-N (mg/dL)	14.2[a]	11.0[b]	8.22[c]	11.5[b]	0.562	<0.001
Feed conversion efficiency						
Energy-corrected milk (kg/kg DM intake)	1.35	1.43	1.44	1.46	0.031	0.78

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

[1] TMR = Total mixed ration composed of maize silage, soybean meal and hay, in a ratio of 0.76:0.15:0.09; G = Grass silage (type S1); GC = Grass and clover silage (1:1 of types S2 and S3; DM basis); GCP = Grass and clover and plantain silage (1:1 of types S4 and S5; DM basis).

Table 13 Balance and utilization of nitrogen (N).

Item	Treatment[1]				SEM	*P*-value
	GCP	GC	G	TMR		
N balance (g/day)						
N intake	566[a]	433[b]	286[c]	442[b]	23.3	<0.001
Fecal N	212[a]	147[b]	106[c]	142[bc]	9.6	<0.001
Urinary N	169[a]	103[b]	76[b]	100[b]	8.4	<0.001
Fecal and urinary N	381[a]	249[b]	182[c]	242[b]	16.9	<0.001
Milk N	141[a]	112[bc]	102[c]	139[ab]	4.7	0.01
N losses (g/kg of N intake)						
Fecal N	373[a]	338[ab]	368[a]	320[b]	6.9	0.01
Urinary N	300	236	269	229	11.9	0.09
Fecal and urinary N	673[a]	573[bc]	637[ab]	549[c]	13.4	<0.001
N utilization (g milk N per kg)						
N intake	251[c]	257[c]	357[a]	313[b]	10.5	<0.001
N apparently digested	400[b]	389[b]	566[a]	461[b]	17.0	<0.001
fecal and urinary N	372[b]	448[b]	565[a]	573[a]	21.5	<0.001
N loss (g/kg total fecal and urinary N loss)						
Urine N	44.5	41.1	41.5	41.4	1.23	0.71

[a-c]Means within a row with different superscripts differ at $P < 0.05$.
[1] TMR = Total mixed ration; G = Grass silage; GC = Grass and clover silage; GCP = Grass and clover and plantain silage.

4.4.3 Fatty acid composition of the milk fat

The milk FA profile, especially that of the long-chain FA, was strongly influenced by the diet types (Table 14). Among the short- and medium-chain FA, the proportions of C10:0 and C12:0 were higher ($P < 0.05$) with TMR compared to GC. The proportion of C16:0 was lower ($P < 0.05$) with GPC than with TMR, but similar to G and GC. The milk fat produced with TMR and G was lower ($P < 0.05$) in C18:0 proportion compared to that produced with GCP. The proportion of C18:1 *n*-9 was higher ($P < 0.05$) with GC than TMR. Within the group of the oleic acid isomers, there was a strong effect of GCP on the FA profile. The proportion of VA was higher ($P < 0.05$) in GCP compared to all other diets (2.1-, 3.3- and 3.4-fold compared to GC, G and TMR, respectively). This FA was also more concentrated ($P < 0.05$) in GC compared to G and TMR. The proportion of LA was lower ($P < 0.05$) with G than with TMR and GCP. The proportion of RA was highest ($P < 0.05$) with GCP (2.1-, 3.1- and 3.5-fold compared to GC, G and TMR, respectively), and it was higher ($P < 0.05$) with GC compared with TMR. The proportion of ALA was highest ($P < 0.05$) with GCP (1.5-, 2.8- and 6.2-fold compared to GC, G and TMR, respectively). Feeding grass silage enhanced ($P < 0.05$) the proportion of C18:3 *n*-6 compared to feeding TMR. A number of FA with chains lengths >18 C atoms were affected by diet type, but this was not the case with the most important *n*-3 FA (C20:4 *n*-3, C22:5 *n*-3 and C22:6 *n*-3). The proportion of total SFA was

higher with TMR compared with GC and GCP ($P < 0.05$). The MUFA proportion did not differ between diets. The proportions of total PUFA were highest with GCP and lowest with G and TMR ($P < 0.05$), while total *n*-3 FA were highest with GCP, followed by GC, G and lowest with TMR (all $P < 0.05$). Total *n*-6 FA were proportionally higher ($P < 0.05$) in TMR and GCP than in G and GC. The *n*-6 to *n*-3 FA ratio was highest ($P < 0.05$) in TMR, followed by G and lowest with GC and GCP ($P > 0.05$ for GCP compared with GC).

When related to dietary intake of LA, LA secretion with the milk was higher (P <0.001) with G, GC and GCP than with TMR (Table 15). Recovery of dietary ALA in milk was similar for TMR and GCP, but there was no difference between either GCP and GC or GC and G. The ratios of milk VA or RA outputs to LA plus ALA intakes were higher (P < 0.05) with GCP than with the other diets.

Table 14 Fatty acid (FA) composition of the milk fat (g/100 g fatty acid methyl esters).

	Treatment[1]					
Item	GCP	GC	G	TMR	SEM	*P*-value
Short- and medium-chain FA						
C4:0	14.8	14.7	14.3	15.7	0.26	0.28
C6:0	17.1^{ab}	16.2^{b}	16.1^{b}	18.0^{a}	0.25	0.02
C8:0	13.5^{ab}	12.4^{b}	12.8^{b}	14.8^{a}	0.28	0.01
C10:0	31.3^{ab}	28.0^{b}	29.2^{ab}	34.4^{a}	0.77	0.01
C10:1	3.45	3.46	3.59	3.76	0.098	0.68
C12:0	37.2^{ab}	33.6^{b}	36.3^{ab}	43.0^{a}	1.10	0.01
C13:0	1.16	1.02	1.31	1.41	0.055	0.06
C13:0 *iso*	1.54^{a}	1.10^{b}	1.42^{ab}	1.17^{b}	0.061	0.01
C14:0	125	122	124	136	2.4	0.14
C14:0 *anteiso*	5.78^{a}	5.40^{ab}	5.69^{ab}	4.70^{b}	0.162	0.04
C14:1	14.4	17.2	17.2	14.5	0.55	0.13
C15:0	14.5^{ab}	14.6^{ab}	16.0^{a}	12.1^{b}	0.44	0.01
C15:1	3.04	2.64	3.52	3.23	0.135	0.13
C16:0	329^{b}	355^{ab}	374^{ab}	387^{a}	7.1	0.02
C16:0 *anteiso*	2.99^{ab}	3.20^{ab}	3.68^{a}	2.93^{b}	0.113	0.04
C16:1	15.5	19.8	21.6	21.5	1.11	0.18
C17:0	7.55^{a}	8.03^{a}	7.18^{a}	4.52^{b}	0.319	<0.001
C17:0 *iso*	0.53^{c}	0.87^{b}	1.06^{a}	0.16^{d}	0.075	<0.001
C17:1	2.54^{b}	3.31^{a}	3.31^{a}	1.99^{b}	0.151	<0.001
Long-chain FA						
C18:0	83.5^{a}	75.4^{ab}	68.0^{b}	68.4^{b}	1.75	<0.001
C18:1 *n*-9	172^{ab}	188^{a}	179^{ab}	147^{b}	6.0	0.045
C18:1 *cis*-6-8 + C18:1 *trans*-C13-14	2.23^{a}	1.61^{b}	1.59^{b}	2.74^{a}	0.121	<0.001
C18:1 *cis*-10	0.62	0.56	0.46	0.66	0.045	0.52
C18:1 *cis*-11	6.12	6.10	4.93	5.12	0.320	0.46
C18:1 *cis*-12	1.18^{b}	0.89^{b}	0.89^{b}	2.23^{a}	0.131	<0.001
C18:1 *cis*-13	1.05	1.20	1.03	0.62	0.094	0.08
C18:1 *cis*-14 + C18:1 *trans*-16	1.6	1.34	1.21	1.27	0.067	0.13
C18:1 *trans*-6-8	1.65^{a}	0.97^{b}	0.66^{b}	2.17^{a}	0.148	<0.001

Table 14 continued.

Item	Treatment[1]				SEM	*P*-value
	GCP	GC	G	TMR		
C18:1 *trans*-9	1.66	1.51	1.55	1.60	0.087	0.95
C18:1 *trans*-10	1.88^{b}	1.31^{c}	1.00^{c}	2.77^{a}	0.157	<0.001
C18:1 *trans*-11 (VA)	18.4^{a}	8.93^{b}	5.59^{c}	5.40^{c}	1.190	<0.001
C18:1 *trans*-12	1.44	1.45	0.92	1.04	0.090	0.07
C18:2 *n*-6 (LA)	12.2^{a}	11.1^{ab}	9.4b	13.6^{a}	0.42	0.001
C18:2 *cis*-9, *cis*-11	0.53^{a}	0.43^{a}	0.38^{ab}	0.24^{b}	0.040	0.01
C18:2 *cis*-9, *cis*-15	1.59^{a}	1.43^{ab}	1.24^{bc}	1.12^{c}	0.044	<0.001
C18:2 *trans*-8, *cis*-9 + C18:2 *cis*-12, *trans*-13	2.59^{a}	1.86^{b}	1.67^{b}	1.70^{b}	0.111	0.01
C18:2, *cis*-9, *trans*-11 (RA)	7.49^{a}	3.65^{b}	2.45^{bc}	2.12^{c}	0.513	<0.001
C18:2, cis-9, *trans*-12	0.67	0.48	0.44	0.48	0.043	0.28
C18:2 *trans*-9, *trans*-11 + C18:2 *cis*-12, *cis*-15	7.49^{a}	3.37^{b}	2.24^{b}	0.83^{c}	0.556	<0.001
C18:2 *trans*-9, *trans*-11	0.71^{a}	0.36^{b}	0.44^{b}	0.11^{c}	0.053	<0.001
C18:3 *n*-3 (ALA)	11.1^{a}	7.21^{b}	3.94^{c}	1.79^{d}	0.790	<0.001
C18:3 *n*-6	1.01^{a}	1.11^{a}	1.11^{a}	0.70^{b}	0.040	<0.001
C20:0	0.04	0.03	0.04	0.04	0.004	0.92
C20:1 *trans*-1	0.11^{a}	0.08^{ab}	0.05^{b}	0.05^{b}	0.006	<0.001
C20:1 *cis*-5	1.13^{a}	0.42^{b}	0.28^{b}	0.23^{b}	0.084	<0.001
C20:1 *cis*-9	0.31^{a}	0.28^{a}	0.23^{a}	0.05^{b}	0.024	<0.001
C20:1 *cis*-11	1.20^{a}	1.22^{a}	1.26^{a}	0.93^{b}	0.034	<0.001
C20:2 *n*-6	0.74^{a}	0.54^{ab}	0.54^{ab}	0.45^{b}	0.035	0.002
C20:3 *n*-6	0.96^{a}	0.70^{ab}	0.54^{b}	0.91^{ab}	0.056	0.03
C20:4 *n*-6	0.91	0.77	0.83	0.92	0.039	0.48
C20:3 *n*-3	0.29^{a}	0.14^{ab}	0.09^{b}	0.05^{c}	0.029	<0.001
C22:0	0.62^{a}	0.53^{a}	0.58^{a}	0.30^{b}	0.030	<0.001
C20:4 *n*-3	0.85	0.70	0.48	0.71	0.071	0.44
C20:5 *n*-3	0.63^{a}	0.49^{ab}	0.39^{bc}	0.28^{c}	0.034	<0.001
C22:4 *n*-6	0.33^{a}	0.30^{a}	0.25^{b}	0.14^{c}	0.017	<0.001
C22:4 *n*-3 + 22:5 *n*-6	0.25	0.32	0.22	0.22	0.024	0.42
C22:5 *n*-3	0.59	0.53	0.54	0.50	0.020	0.44
C22:6 *n*-3	0.05	0.05	0.06	0.06	0.004	0.90
Groups of FA	0.04	0.03	0.04	0.04	0.004	0.92
Σ Saturated FA	690^{b}	695^{b}	716^{ab}	748^{a}	7.4	0.01
Σ Monounsaturated FA	257	268	256	224	6.3	0.07
Σ Polyunsaturated FA	53.7^{a}	36.8^{b}	28.2^{c}	27.8^{c}	2.41	<0.001
Σ *n*-3 FA	13.8^{a}	9.45^{b}	5.72^{c}	3.59^{d}	0.870	<0.001
Σ *n*-6 FA	20.5^{a}	16.7^{b}	14.3^{b}	19.8^{a}	0.64	<0.001
Σ *n*-6 FA/ Σ *n*-3 FA	15.0^{c}	17.9^{c}	25.2^{b}	56.6^{a}	3.72	<0.001

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

[1] TMR = Total mixed ration; G = Grass silage; GC = Grass and clover silage; GCP = Grass and clover and plantain silage.

Table 15 Milk fatty acid secreted relative to dietary fatty acids (g/kg fatty acids methyl esters ingested) C18:2 *n*-6 (LA) and C18:3 *n*-3 (ALA).

	Treatment[1]					
Item	GCP	GC	G	TMR	SEM	*P*-value
Relative to dietary LA						
C18:2 *n*-6 (LA)	0.201[a]	0.184[a]	0.159[a]	0.095[b]	0.0102	<0.001
Relative to dietary ALA						
C18:3 *n*-3 (ALA)	0.078[ab]	0.054[bc]	0.037[c]	0.094[a]	0.0054	<0.001
Relative to dietary LA and ALA						
C18:1 *trans*-11	0.084[a]	0.045[b]	0.032[b]	0.031[b]	0.0050	<0.001
C18:2 *cis*-9, *trans*-11	0.035[a]	0.018[b]	0.014[b]	0.012[b]	0.0024	<0.001

[a-c]Means within a row with different superscripts differ at $P < 0.05$.
[1] TMR = Total mixed ration; G = Grass silage; GC = Grass and clover silage; GCP = Grass and clover and plantain silage.

4.5 Discussion

4.5.1. Experimental silages and diets

The aim of the present study was to test whether or not, under the condition of excessive N supply, including plantain in a grass-silage based diet would favorably affect NUE and the FA profile of the milk of dairy cows. Plantain had established very well with a proportion in the sward of about 0.80 on a DM basis, which was probably promoted by the dry conditions during cultivation favoring its growth (Stewart, 1996). The advanced maturation stage of the plantain at harvest resulted in a large stem proportion, which was probably responsible for the fibrous and highly lignified silage. This would not have been the case at an earlier utilization (Pembleton et al., 2016). Accordingly, the plantain sward also had a low CP content as leaves get poorer in CP with maturation (Bowers and Stamp 1992). Therefore, the silage was mixed with that of a high-CP sward, but still diet digestibility and energy content was limited compared to the other silage types. Eventually, the grass silge diets differed in their CP content with 127, 165 and 174 g/kg DM for diets G, GC and GCP, respectively. It seems that the red clover in diets GC (1/4) and GCP (1/7) primarily contributed to these differences.

4.5.2 Performance

The cows fed diet G ingested the lowest amounts of feed, which was probably the result of the poor fermentation quality of the respective silage. Consistent with this, van Dorland et al. (2007b) found intake of red clover silage to be only higher compared to that from ryegrass silage in a choice situation when the ryegrass silage had a clearly lower quality than the red clover. The DMI of the cows fed GCP was numerically higher by 3.1 kg/d than that of cows fed GC although it contained more lignified fiber. This suggests a high palatability of the

ensiled plantain. Contrary to this, Gregorini et al. (2013) reported that forage DMI of cows grazing ryegrass and plantain at plantain proportions of 0, 0.20, 0.40 or 0.60 in the sward, respectively, did not differ between treatments. Minneé et al. (2017) fed fresh plantain at proportions of either 0.20 or 0.40 of total mixed with ryegrass and white clover to late lactating dairy cows and did not find differences in milk yield and milk composition compared to a ryegrass diet only. This is similar to our findings. Different from that, Totty et al. (2013), reported an increase in milk yield and a decrease in milk fat content when cows were switched from ryegrass-clover or high-sugar grass pastures to a plantain containing sward (0.184 of total on a DM basis, with additionally 0.256 of ryegrass, 0.039 of white clover and 0.361 of chicory). Pembleton et al. (2016) described an increase in milk protein yield of early to mid-lactation cows grazing ryegrass, white-clover and plantain compared to only ryegrass. The differences in effect between these studies might also be related to differences in the harvest stage of the plantain.

4.5.3 Nitrogen use efficiency

Dietary N supply with diets GCP, GC and G was mainly derived from forage N, whereas the main dietary N source of the TMR diet was soybean meal which generated a contrast between rather slowly ruminal degradable CP (diet TMR) and fast ruminal degradable CP (other diets) (Givens and Rulquin, 2004). Intake of CP was lowest in diet G both because of both the low CP content and the low DMI. The small among of N supplied to these cows obviously had to be used very efficiently. This resulted in the highest NUE (milk N per N intake), as milk protein yield was not depressed to the same level. In addition, it seems that the pure ryegrass diet was at the borderline to be deficient in RDP (with CP being below 130 g/kg DM). Therefore, despite the high NUE, diet G was considered of lesser quality and would have caused deficiencies in the cows in the longer term. The N intake was similar for cows fed GC compared to TMR, but the NUE was higher for the TMR fed cows. At the same organic matter digestibility, the substrates fermented presumably differed in the two treatments (less fiber and more non-fiber carbohydrates with TMR) which may have led to a more efficient removal of N by synthesis of microbial protein in the case of TMR.

The plantain in diet GCP was obviously not efficient in improving the NUE. Cows fed diet GCP had the highest CP intake and, consequently, the highest milk urea-N content and, together with diet GC, the numerically lowest NUE. Min et al. (2003) reviewed that levels of condensed tannins (CT) ranging between 20 to 45 g per kg DMI are beneficial in eliciting effect of the CT with regard to the formation of CT-protein complexes. In the present study,

in all experimental silages and feeds, CT were either not detected or levels were very low, even in the plantain silage. Different from that, literature reports on the CT content of plantain vary from 2.4 to 14 g/kg DM (Jeangros et al., 1994b; Al-Mamun et al., 2008). As ensiling was shown to decrease forage CT content (Huang et al., 2016), this might have reduced the CT content further. However, plantain contains bioactive compounds other than CT like the glycosides acteoside, aucubin, and catalpol (Al-Mamun et al., 2008). Navarrete et al. (2016) found *in vitro* that acteoside and aucubin may reduce ruminal ammonia concentrations. In addition, they found a seasonal increase and an annual variation in the concentrations of these two compounds in the plantain cultivar Ceres Tonic, a cultivar containing almost no catalpol. According to Navarrete et al. (2016), acteoside is fermented by the rumen microbes and therefore promoted their growth, whereas aucubin expresses an inhibitory effect on rumen microbes. Concerning N turnover, the lack of effect of plantain in the present study are in contrast to a number of results from other studies investigating diets containing plantain. Cheng et al. (2017) described that fresh plantain fed indoors to dairy heifers resulted in a reduced urinary N excretion when compared with feeding perennial ryegrass and white clover only. Grazing a diverse pasture including plantain was found to decrease urinary N concentration when compared to ryegrass-white clover or high-sugar ryegrass white clover pastures (Totty et al., 2013). Minneé et al. (2017) observed a reduction of rumen ammonia and urinary N concentrations in cows fed a plantain-ryegrass-white clover diet. However, both Totty et al. (2013) and Minneé et al. (2017) applied the urine spot sampling technique instead of a quantitative collection, and the level of water intake and excretion might have played a role in these experiments. Additionally, effects observed in the mixed pastures cannot be attributed to plantain solely. In the study of Totty et al. (2013), the chicory present in the plantain containing sward contains bioactive compounds, too (Jeangros et al., 1994b). Concerning red clover, the present study gave no indications that PPO improved NUE.

4.5.4 Fatty acid composition of the milk fat

Increasing the botanical diversity in the diet by stepwise adding a plant species from another functional group (grass, legume and herb) from diets G to GC to GCP in comparison to a diet primarily based on maize silage led to distinctly different patterns in the milk FA profile. As expected from other studies (Leiber et al., 2005), the FA profile of cows fed any of the grass-silage based diets was richer in unsaturated FA and had a lower ratio of *n*-6 to *n*-3 FA than that of the TMR-fed cows. An excessive dietary supply of *n*-6 relative to *n*-3 FA has been related to an increased risk to cardiovascular and coronary heart diseases (Barcelo-Coblijn

and Murphy, 2009). Petersen et al. (2011) showed that feeding a mixture containg herbs (at proportions of 0.430 chicory (*Cichorium intybus*), 0.210 plantain, 0.110 salad burnet (*Poterium sangusisorba*) and 0.250 other species may decrease the *n*-6 to *n*-3 ratio and simultaneously may increase the proportions of both *n*-3 and *n*-6 FA in the milk fat of cows when compared to diets based on either clover or TMR. Also red clover may increase the proportion of desired FA such as ALA compared to diets primarily composed of grass species (Adler et al., 2013). The authors attributed this to higher transfer rates and reduced biohydrogenation. Indeed, the proportion of ALA in GC was higher compared to G and TMR. However, the secretion of VA and RA relative to dietary intake of ALA and LA did not differ. It is therefore unlikely that, other than found earlier (Lee et al., 2010), the enzyme PPO which is specific for red clover (not analyzed in the present study) had expressed an inhibitory influence on ruminal biohydrogenation. Although plantain lipids have been described to contain PUFA up to 0.80 of total FA (Clapham et al., 2005), the plantain containing sward (S5) of the present study had even slightly lower PUFA and ALA proportions of the lipids compared to the grass-clover swards (S2, S3). Despite this, the milk fat of cows fed GCP compared to all other diets had clearly the highest proportions of *n*-3 FA, ALA, VA and RA. In addition, the highest ratios of RA and VA relative to the dietary intake of ALA and LA were found. It is, therefore, likely that the bioactive compounds provided by plantain with diet GCP caused a partial inhibition of ruminal biohydrogenation. There was a gradient in daily TEP intake from < 200 g with diets G and TMR to 240 g with diet GC and 300 g with diet GCP. Khiaosa-Ard et al. (2009) and Vasta et al. (2009a) showed that VA accumulates when the final step of biohydrogenation from VA to 18:0 is inhibited. In turn, RA accumulates in milk through endogenous synthesis from VA in the mammary gland (Griinari et al., 2000). However, in these studies the reduction of biohydrogenation was primarily attributed to dietary CT, which, however, were not elevated in GCP. Also the HT, which can affect biohydrogenation similarly effective as CT (Jayanegara et al., 2012) were found only in low levels in diet GCP leaving other bioactive compounds present in plantain as an explanation.

4.6 Conclusion

Feeding ensiled plantain (*P. lanceolata*) to dairy cows together with ensiled ryegrass and red clover at about 400 g/kg DM in the total forage improved the nutritional value of the milk fat by increasing proportions of ALA, VA and RA to 3- to 6-fold levels when compared to milk from cows fed a common total mixed ration based on maize silage and soybean meal. In this respect, this diet was clearly more efficient than a grass-clover and especially than a ryegrass only diet. An earlier stage of harvest might increase the value of plantain-containing silages less because of palatability reasons but to improve nutrient density and avoid excessive lignification and, possibly, enhance concentration of bioactive compounds. In that case, plantain might also contain enough (condensed) tannins to increase the NUE of dairy cows in a situation of excessive CP supply which was not accomplished in the present study. Future studies should focus on identification and quantification of individual active compounds in plantain or the mechanisms activated by including plantain in order to optimize its use for improving the fatty acid profile of the milk fat and NUE.

Chapter 5

General discussion and conclusions

Grassland is the major source of forage for dairy cows in temperate climates. Intensification in grassland utilization led to species poor swards primarily composed of grasses or legumes with fewer herbs. The forage produced is of high quality suitable for the nutrition of dairy cows with high milk yields. However, the forage protein of intensively utilized grassland swards is characterized by a high ruminal degradability and diets not supplemented with energy rich feeds result in a low NUE, with substantial losses of N to the environment. Among various approaches tested to increase the NUE of grassland swards fed to dairy cows, the use of plant secondary compounds which are particularly abundant in herbs, has been less studied. The purpose of this thesis was to contribute to the understanding of forage obtained from both semi-natural (Chapter 2, 3) or from artificially established grasslands (Chapter 4) with contrasting species richness on forage nutritive value and digestibility, N utilization and milk fatty acid profile in dairy cows. Species rich mountain swards with herbal proportions between 184 and 451 g kg DM^{-1} obtained from long-term mineral fertilization experiments located at different sites showed large variation in feeding value and content and composition of phenolic compounds between different fertilizer treatments (Chapter 2). Feeding flowering swards either from species rich mountain meadows (Chapter 3) or an artificially established ley containing plantain (Chapter 4) decreased *in vivo* measured digestibility in dairy cows. Neither feeding diets containing species rich swards from mountain meadows with elevated proportions of herbs (Chapter 3) nor a diet based on a ryegrass and red-clover sward containing plantain (Chapter 4) increased the NUE. However, there was a shift in the N excretion pattern from urinary N to fecal N when fed a sward from mountain origin (Chapter 3). Effects on the milk fatty acid profile varied between feeding experiments and were more pronounced when feeding a sward containing plantain (Chapter 4) than when feeding forage from mountain meadows containing various herbal species (Chapter 3).

5.1 Digestibility and palatability of forage from swards with contrasting species richness

During the last decades, intensification of grassland utilization has decreased forage plant species richness favoring species tolerant to frequent utilization and application of fertilizers (Dietl, 1995). Particularly in lowland areas, swards mainly composed of grass and legume species and fewer herbs have been established. Such swards are characterized by high forage DM production and digestibility (Armstrong et al., 1986; Nyfeler et al. 2009, 2011; van Dorland et al., 2007b).

Herbal species such as *Alchemilla xanthochlora* L. or *Crepis aurea* L. or forage from species rich swards of alpine origin (Khiaosa-Ard et al., 2012) may be as digestible as forage from lowland swards (Jayanegara et al., 2011b). Minnée et al. (2017) reported a digestibiltiy of platain of more than 700 g kg OM^{-1}, when utilized prior to flowering. When feeding ensiled buckwheat (*Fagopyrum esculentum* L.) or chicory (*Cichorium intybus* L.) at proportions of 460 and 730 g kg DM^{-1} (in total diets mixed with ensiled ryegrass and supplemented with concentrate) resulted in a comparably high digestibility of 720 and 781 g kg OM^{-1} respectively (Kälber et al., 2012) similar to values found with dairy cows fed forage from intensively utilized grasslands (van Dorland et al., 2007b).

In the current investigation, feeding herbs to dairy cows both in the form of species-rich swards with elevated proportions of herbs obtained from mountain grasslands (Chapter 3) or plantain in an artificially established sward (Chapter 4) decreased OM and fiber digestibility determined *in vivo* when compared to control diets (grass-based swards (Chapter 3) or TMR (Chapter 4)).

Several other authors found similar results when feeding species rich swards to dairy heifers or to dairy cows. Bruinenberg et al. (2006) found digestibility in dairy cows fed a species-rich sward prepared from semi-natural grassland when compared to an intensively managed ryegrass pure stand. Increasing plant species richness in artificially established swards decreased forage digestibility measured *in vivo* and comprised animal performance when compared to ryegrass pure stands, with the effects, however, related partially to swards management and maturity (Hammond et al., 2014).

The OM digestibility measured *in vitro* of species rich swards with elevated proportions of herbs as those obtained from the long-term mineral fertilization experiments varied largely (561 g kg OM^{-1} to 731 g kg OM^{-1}) and increased from the beginning to the end of the season (Chapter 2). The differences in digestibility were mainly related to sward management with respect to number and timing of harvests and consequently sward maturity. Consequently, feeding species rich swards like those obtained from the long-term mineral fertilization experiments during the second or third harvest (Chapter 2) would likely result in a similar forage digestibility of intensively managed species-poor swards. In the feeding experiment conducted in this thesis, herbs were fed in flowering stage. This stage is accompanied by larger proportions of leaves to stems in contrast to swards harvested during the vegetative stage when the proportion of leaves to stems is lower (Tilley and Terry, 1963) and consequently, the forage is more lignified. An earlier harvest of the swards harvested from species rich meadows (Chapter 3) or the sward containing either plantain (Chapter 2) or

harvest in the vegetative growth stage (second or further harvests) would therefore result in a higher digestibility as fiber contents and especially those of ADL would be lower. A higher forage digestibility is likely resulting in higher supply in net energy and consequently formation of milk in lactating dairy cows. Feeding diets containing herbs (Chapter 3 and 4) did, however, not affect milk yield or milk composition or excretion of fat and protein in both feeding experiments, which was primarily due to similar NEL intakes within diets, respectively.

Scehovic (1995b) demonstrated that plant secondary metabolites may reduce forage digestibility. Swards harvested from intensively utilized grasslands are generally low in content of phenolic compounds, except for swards containing tanniferous legume species (Jeangros et al. 1994a). Hydrolysable tannins (HT) are potentially toxic to ruminants (Reed, 1995; McSweeney et al., 2001) and may impair forage palatability (Pfister et al., 1997), as hypothesized for cows with reduced intake of herbal hay from mountain origin in this study (Chapter 3). Feeding species-rich swards is therefore challenging when swards may contain both herbs with beneficial and undesired traits (level of CT content, degree of digestibility, presence of toxic compounds). At the mineral fertilization experiment located at lowest site, particularly high concentrations of HT were measured and could be related to the herbal species *Geranium sylvaticum that* occurs at large proportions at this site. Feeding swards containing *G. sylvaticum* are therefore likely to be refused when fed to dairy cows or might elicit toxic effects.

5.2 Nitrogen utilization of dairy cows fed diets with contrasting species richness

There have been numerous feeding strategies in order to reduce excess formation of ruminal ammonia in order to decrease the proportion of urinary N, resulting potentially in an increased NUE. Among these are supplementation with concentrates rich in carbohydrates being highly effective (Keim and Anrique, 2011). Feeding of concentrates however, is restricted purposely in grassland based dairy production to reduce competition with feed components potentially edible (Wilkinson, 2011; Ertl et al., 2015). Therefore, the feeding experiment using hay swards was performed without supplementation of concentrates to evaluate effects of hay diets with elevated contents of phenolic compounds without interaction of concentrate feeds (Chapter 3).

Integration of herbal species in intensively utilized mixtures of grasses and legumes was one of the goals of this study (Chapter 4). Among various herbal species, plantain has been given attention in particularly pasture-based ruminant production in New Zealand and

Australia primarily with sheep and deer (Stewart, 1996) or more recently with cattle (Totty et al., 2013; Pembleton et al., 2016; Cheng et al., 2017; Minneé et al., 2017). Some studies reported increased milk yields, a reduction of urinary N or an increase in the NUE (Totty et al., 2013; Pembleton et al., 2016). A particular trait of plantain is its increased performance during conditions of summer drought compared to grass and legumes (Stewart, 1996). Ramírez-Restrepo and Barry (2005) reported CT contents in plantain as high as 14 g CT kg DM^{-1}. It was hypothesized that CT would be elevated in diets containing herbs and decrease the ruminal degradability of the forage protein and increase the supply of MP and eventually increase the NUE in dairy cows (Chapter 3 and 4). The contents of CT measured in the herbal hay fed to dairy cows in Chapter 3 were 3.4 g and 12.6 g CT kg DM^{-1} respectively, whereas in the swards containing plantain, the content of CT was below 1 g kg DM^{-1} (Chapter 4). Concentrations of 20 to 45 g CT kg DM^{-1} were shown to be effective in reducing ruminal degradation of forage protein, with higher levels causing adverse effects of CT on DM intake, palatability or animal performance (Min et al., 2003).

The diets containing swards rich in herbal species harvested from mountain meadows resulted in an intake of CT in dairy cows of 30.9 g and 115 g for diets HP^{-} and HP^{+}, respectively (Chapter 3). Neither feeding hay harvested from mountain swards rich in herbs (Chapter 3) nor feeding an ensiled mixture or ryegrass, red-clover and plantain from an artificially established sward did improve the NUE in dairy cows (Chapter 4), as contents of CT per kg DM were possibly too low. However, the diet richest in CT (HP^{+}) resulted in a decrease of the proportion of urinary N and increased the proportion of fecal N (Chapter 3). Reducing the pollution of the environment by N of dairy manure is a key element to increase the sustainability of particularly grassland based dairy production (Tamminga, 1992; 1996). Fecal N excretion is considered more favorable than urinary N excretion, as the degradation of fecal N is slower than the ammonia emission potential of urinary N (Powell et al., 2009). A shift from the excretion of N from the urinary route to the fecal route was also observed in other studies containing diets with elevated contents of CT (Carulla et al., 2005; Grosse Brinkhaus et al., 2016; Wang et al., 2018). In the study by Grosse Brinkhaus et al. (2016), the intakes of CT per day and cow of the diet supplemented with pelleted sainfoin or birdsfoot trefoil (*Lotus corniculatus*) were 754 g and 107 g CT, respectively. However, the source of the CT differed between the studies and were either from tanniferous legumes (sainfoin, *Onobrychis viciifolia*) (Grosse Brinkhaus et al., 2016) or shrubs (hazel leaves (*Corylus avellana*)) (Wang et al., 2018) in pelleted form each or in the form of an extract obtained from *Acacia mearnsii* (Carulla et al., 2005). Feeding diets containing ensiled herbs chicory

(*Cichorium intybus*) or buckwheat (*Fagopyrum esculentum*) compared to a reference diet composed of ryegrass improved the NUE and decreased urinary N excretion in the case of the diet containing buckwheat. This effect, however, was primarily related to the low content of CP in the buckwheat diet and its resource-effective utilization (Kälber et al., 2012).

Swards harvested from the highest located long-term mineral fertilization experiment (H) (Chapter 2) had particularly low CT contents (below 2.03 g kg DM^{-1}) and in the last harvest, were not detected at all. It is therefore unlikely if these swards were fed to ruminants, any effects on the NUE (or others) would be expected in contrast to swards harvested from other sites which had CT contents ranging from 2.15 to 11.6 g CT kg DM^{-1}. Highest CT contents were measured in forage from unfertilized swards or fertilized with PK at the lowest experimentation site (L), which was related to the presence of legumes species *Lotus corniculatus* and *Lathyrus pratensis*. Contents of CT are particularly concentrated when plants are in flowering stage (Iason et al., 1993) and were reported to decrease with increasing stage of maturiy (Fraisse et al., 2007). Other the the botanical composition or specific, forage plants, the season of harvest is therefore relevant when considering effects of CT on the NUE.

Besides seasonal variation in CT content of swards as reported in Chapter 2, the structure of CT and is chemical reactivity can be very diverse (McSweeney et al., 2001), as groups of phenolic compounds analyzed according to the protocol of Makkar (2003) each represent a large heterogenity in compounds. Although the content of CT was higher in diet HP^{+} than in diet HP^{-}, the chemical structure in which CT were present in the forage might have been more relevant than its concentration (McSweeney et al., 2001; Makkar, 2003; Min et al. 2003). Feeding ensiled plantain in a mixed diets was very low in CT content and intake and therefore very unlikely to increase the supply in MP to dairy cows. However, it cannot be excluded, as the CP content of this diet exceeded recommendations for lactating dairy cows. A supply in MP was therefore not limiting, which, however, might have limited the detection of an effect of CT or other plant secondary metabolites on the N metabolism of dairy cows fed ensiled plantain.

5.3 Milk fatty acids profile of dairy cows fed diets with contrasting species richness

Phenolic compounds affect a variety of fermentation processes among those are forage digestibility (Scehovic, 1995b), ruminal ammonia formation (Carulla et al., 2005), degradability of forage protein (Waghorn, 2008), formation of volatile FA's and production of methane (Jayanegara et al., 2011b; Hristov et al., 2013) as well as biohydrogenation of

polyunsaturated dietary FA (Khiaosa-Ard et al., 2009; Vasta et al., 2009b). Feeding of herbs in both experiments increased the proportions of beneficiary FA's secreted in the milk fat, however, to a different extent and with different mechanisms. Overall, the effects of feeding hay rich in herbs on the milk FA profile were lower than would have been expected when comparing to other studies of cows fed hay of mountain or alpine origin (Collomb et al., 2002b, Leiber et al., 2005). It was shown that the milk fat of cows fed species rich alpine pasture compared to the milk fat of dairy cows fed lowland ryegrass pasture had a higher proportion of ALA (Collomb et al., 2002a; Leiber et al., 2005). An increased transfer of ALA to the milk fat was hypothesized due to phenolic compounds reducing the ruminal biohydrogenation of PUFA (Leiber et al., 2005). Different phenolic fractions were attributed to inhibit different steps of the biohydrogenation cascade (refer to Figure 3, Chapter 1): Hydrolysable tannins were reported to inhibit the first step of biohydrogenation (Jayanegara et al., 2011a) and CT to reduce both the second (Jayanegara et al., 2011a) and the third step of biohydrogenation (Khiaosa-Ard et al., 2009, Vasta et al., 2009a,b).

The hay diet with the lower proportion of herbs (HP^{-}) showed an increase in the secretion of VA relative to the dietary intake of LA and ALA (Chapter 3). The accumulation of VA therefore indicated in inhibition by CT of the third step of biohydrogenation as reported by Khiaosa-Ard et al. (2009) and Vasta et al. (2009b). This diet however had a lower content of CT than did diet HP^{+}, which did not show an accumulation of VA, indicating that the structure and/or the chemical properties of the CT may have been more effective than the concentration of CT itself (McSweeney et al., 2001, Salminen et al., 2011). The plantain containing diet increased the proportion of VA and RA relative to the intake of LA and ALA in the milk fat of dairy cows and particularly proportions *n*-3 FA, when compared to diets fed without plantain (Chapter 4). Due to the negligible contents of CT in the plantain containing diet, however, it is unlikely that biohydrogenation was affected by CT. Besides phenols, other bioactive compounds may be relevant to ruminal fermentation too (Navarette et al., 2016) as glycosides found in plantain (Al-Mamun et al., 2008). These bioactive compounds were shown to act anti-bacterial (Ishiguro et al., 1982) and inhibitory to enzymes (Nishibe and Murai, 1995) relevant when feeding plantain described in Chapter 4. Bioactive compounds found in plantain other than CT include the glycosides acteoside, aucubin, and catalpol (Al-Mamun et al., 2008). According to Navarrete et al. (2016), acteoside is fermented by the rumen microbes, whereas aucubin expresses an inhibitory effect on rumen microbes. It is unknown, whether these compounds potentially affect the activity of the bacteria *B. fibrisolvens,* which was reported to synthesize RA (Kepler and Tove, 1967), although to a

lesser extent than is synthesized in the mammary gland (Griinari et al., 2000). It remains therefore unclear, what mechanism was relevant to decrease the ruminal biohydrogenation of ALA and RA respectively. Due to high content of CT in swards containing *L. corniculatus* and *L. pratensis* (Chapter 2), an accumulation of VA and potentially an increased formation of RA in the mammary gland could have been expected. In contrast, feeding swards dominated by *G. sylvaticum* rich in HT would have likely yielded a milk fat more concentrated in PUFA.

The milk FA profile of cows fed mountain swards rich in herbs did not affect the proportions of VA, total CLA, the sum of saturated (SFA) and monounsaturated FA or the ratio of *n*-6 to *n*-3 FA but increased those of ALA (Chapter 3). Collomb et al. (2002a) observed an increase in CLA in the milk fat of cows fed diets of mountain swards compared to those of lowland areas, which was related to an increase in the proportions of herbs from lowland grasslands to mountain grasslands. Particularly, RA is one of the major CLA (Collomb et al., 2002a). Conjugated linolenic acids have been related to inhibit carcinogenesis (Ha et al., 1987, Parodi, 2004, Lee and Lee, 2005), cause antidiabetic effects (Khanal, 2004) and lower body weight and are therefore desirable when present in increased proportions of the milk fat of dairy cows.

A low dietary supply of *n*-6 relative to *n*-3 FA's is considered beneficial to the health of humans and other mammalians (Barcelo-Coblijn and Murphy, 2009). The transfer efficiency of LA or ALA from feeds to milk is not only dependent by the proportion of herbs in the diet (Dewhurst et al., 2006), but also by the transfer efficiency, which is affected by type and composition of diet and related to the concentration of ALA in the forage (Hebeisen et al., 1993). In grassland based dairy production systems, there is potentially a higher supply in ALA to dairy cows through forage (Chapter 3) as to dairy cows receiving large proportions of corn or concentrate (Dewhurst et al., 2006), which are rich in LA (Chapter 4, in particular diet TMR mainly composed of corn silage). Consequently, dairy cows primarily fed with corn and concentrate results in a diet with a high ratio of *n*-6 relative to *n*-3 FA's is critical to the animal due to various physiological processes influenced by LA and ALA as reviewed by Barcelo-Coblijn and Murph (2009). Feeding the hay swards rich in herbs did not affect the ratio of *n*-6 relative to *n*-3 FA's when compared to the ryegrass diets (Chapter 3). Contrarily, feeding mixtures of either plantain, ryegrass and red-clover or ryegrass and red-clover was effective in decreasing the ratio of *n*-6 relative to *n*-3 FA's compared do diets based on ryegrass only or a TMR (Chapter 4), resulting in an improved composition of the milk fat for human consumption.

5.4 General conclusions and implications

In Switzerland, about 4/5 of a total of 1.6 million ha of agricultural land is grassland (including alpine areas suitable for agricultural use). Increasing the sustainability of grassland-based dairy production in terms of environmental, as well as societal, political and economic aspects is therefore of interest for the entire country. This thesis has increased the knowledge about forage from different grassland types characterized by high proportions of herbs being moderately to highly biodiverse with respect to forage production, nutrition and milk yield and milk quality of dairy cows. In the project, the characteristics and utilization of diets rich in herbs have been evaluated from forage obtained from semi-natural mountain meadows with a high species richness and from an artificially established sward of a mixture of ryegrass-red clover including plantain. Feeding forage with high proportions of herbs (both from semi-natural or artificially established grasslands) resulted in a lower digestibility when compared to forage primarily based on ryegrass optimized over decades for high nutrient density by plant breeding. However, it can be concluded from the present findings in the long-term mineral fertilization field experiments that, when both fertilization and cutting frequency are adjusted, the digestibility of swards rich in herbs can be appropriately high to cover the requirements of dairy cows. Particularly in the case of plantain, an earlier harvest might have improved the digestibility of the silage of this sward potentially resulting in higher contents of phenolic compounds and probably an increase of milk production. With the herbal diets tested in the experiment including plantain, the nitrogen use efficiency in dairy cows could not be improved. It is assumed that the concentrations of condensed tannins present in the forage were not sufficiently high to influence the nitrogen use efficiency in dairy cows. However, with a diet composed of approximately 400 g kg DM^{-1} of herbs of a species rich mountain meadow with a daily intake of about 115 g of condensed tannins and 400 g of total phenolic compounds resulting in a shift in the excretion of urinary nitrogen to fecal nitrogen was achieved. Feeding diets containing herbs with elevated contents of condensed tannins is therefore a suitable strategy to lower the nitrogen emission potential of dairy manure as losses of ammonia could be reduced. Future investigations should therefore aim to detect mountain herbs specifically rich in polyphenols without causing negative impacts on feed intake and palatability. Among those, especially tannins and thereof the condensed tannins have the potential to reduce the ruminal degradability of feed protein. As observed in swards obtained from the long-term mineral fertilization field experiment at the study site Eggenalp, limitation to that may, however, be that there could be times when no condensed tannins are present in

the plants like in the third harvest in late summer at the end of the vegetation period. As also some phenolic compounds such as hydrolysable tannins are potentially toxic to ruminants, the feeding of specific herbs is potentially further restricted to a certain level. Mixed swards might be helpful in this respect, and indeed no cows showed a substantial response to potentially toxic compounds other than having a reduced dry matter intake or milk yield but this possibly because of a limited palatability. Some legumes species were observed to essentially increase the content of condensed tannins of the experimental forages in swards of mountain origin investigated in this study. A combination of both herbal and tanniferous legume species may, therefore, be a promising strategy to increase the content of effective phenolic compounds in dairy rations. Forage mixtures of grass, legume and herbal species combining the effects of high forage digestibility, increased contents of phenolic compounds and particularly those of condensed tannins may therefore be the most promising type of forage in forage-based dairy cow nutrition. Feeding herbs, particularly in the case of plantain but also to some degree in the species-rich mountain forage, in addition was found to increase the content of polyunsaturated fatty acids in milk fat related to beneficiary effects on the human health. Both the proportions of vaccenic and rumenic acid, α-linolenic acid, the sum of total polyunsaturated fatty acids and omega-3 fatty acids were higher in the milk fat of cows fed plantain. Future investigations should therefore test the persistence of plantain in mixtures of ryegrass and red-clover over several seasons and aim to identify the compounds relevant for the decrease in biohydrogenation of polyunsaturated fatty acids observed with especially this forage plant mixture. Successful nutritional strategies with increased proportions of herbs should address a variety of other aspects, including the ability of forage mixtures with high proportions of herbs for different conservation methods and their methane mitigating potential. It remains to be clarified, which inclusion level of herbs with high contents of phenolic compounds is needed to have an effect on the nitrogen emission potential of manure from cows.

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Education

2014-2018	Doctoral candidate, Institute of Agricultural Sciences, Group of Animal Nutrition, ETH Zurich, Switzerland in collaboration with the Swiss College of Agriculture, Berne University of Applied Sciences HAFL, Switzerland
2010-2013	Master of Science ETH in Agricultural Sciences
2012-2013	Teaching Certificate ETH in Agricultural Sciences
2007-2010	Bachelor of Science ETH in Agricultural Sciences

Publications

Peer-Reviewed

Ineichen, S., Marquardt, S., Bracher, A., Kreuzer, M., Reidy, B., 2018. Feeding value of herbage from species-rich mountain grasslands subjected to zero, PK and NPK mineral fertilization for 40 years. In final preparation for submission to Grass and Forage Sci.

Ineichen, S., Kuenzler, A.D., Kreuzer, M., Marquardt, S., Reidy, B., 2018. Digestibility, nitrogen utilization and milk fatty acid profile of dairy cows fed hay from species rich mountainous grasslands with elevated herbal and phenolic contents. Submitted to Anim. Feed Sci. Technol.

Ineichen, S., Marquardt, S., Wettstein, H.-R., Kreuzer, M., Reidy, B., 2018. Milk fatty acid profile and nitrogen utilization of dairy cows fed ryegrass-red clover silage containing plantain (*Plantago lanceolata*). Submitted to Livest. Sci.

Conference Proceedings

Ineichen, S., Marquardt, S., Wettstein, H.-R., Kreuzer, M. and Reidy, B. (2017): Effect of species composition of grass silages on nitrogen use efficiency of dairy cows in comparison with a soybean meal and maize silage based diet (Einfluss der botanischen Zusammensetzung von Grassilage auf die N-Verwertung von Milchkühen im Vergleich mit einer Ration auf Basis Sojaextraktionsschrot und Maissilage). 71. Tagung vom 14. – 16. März 2017, Göttingen. Proceedings of the Society of Nutrition Physiology, Bd. 26, S. 128. Gesellschaft für Tierernährung (Hrsg.).

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