DISS. ETH NO. 25692

Potential of forages from temperate-climate woody and herbaceous plants to mitigate methane and ammonia in ruminants

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

presented by

MELISSA TERRANOVA
MSc Agricultural Sciences, University of Hohenheim
born on 3th October 1990
citizen of Germany

accepted on the recommendation of
Prof. Dr. Michael Kreuzer, examiner
Dr. Angela Schwarm, co-examiner
Prof. Dr. Karl-Heinz Südekum, co-examiner

2018

Table of Contents

List of Abbreviations

ADF	Acid detergent fibre
ADL	Acid detergent lignin
ADLI	Acid detergent lignin intake
AIC	Akaike information criterion
AOAC	Official methods of analysis
BW	Body weight
ΔBW	Body weight change
CH_4	Methane
CO_2	Carbon dioxide
CP	Crude protein
CT	Condensed tannins
CV	Coefficient of variation
DM	Dry matter
DMI	Dry matter intake
DIM	Days in milk
dOM	Digestible organic matter
ECM	Energy corrected milk
EE	Ether extract
GE	Gross energy
GHG	Greenhouse gases
H	Liner hazel leave proportion
H^2	Quadratic hazel leave proportion
HGT	Hohenheim gas test
HT	Hydrolysable tannins
H_2	Hydrogen
IVOMD	*In vitro* organic matter digestibility
N	Nitrogen
NDF	Neutral detergent fibre
NH_3	Ammonia
N_2O	Nitrous oxide
OM	Organic matter
O_2	Oxigen
PEG	Polyethylene glycol
PSM	Plant secondary metabolites
SCFA	Short chain fatty acids
SE	Standard error
SEM	Standard error of the mean
TP	Non-tannin phenols
TP	Total phenols
TPP	Test plant pellet
TT	Total tannins

List of Tables

List of Figures

Summary

The livestock sector, particularly the ruminant sector, contributes significantly to the total global warming effect. Greenhouse gases such as carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) play a central role in climate change. Especially CH_4 which is released during ruminal fermentation, has a global warming potential 23-times that of CO_2. Plant secondary metabolites play an important role in developing efficient feeding strategies to mitigate CH_4 and nitrogen emissions in ruminants, and they are prevalent in a number of woody and herbaceous temperate climate plant species.

The goal of the present doctoral thesis was to test and identify candidate plants to be fed as additives to a high quality diet to mitigate enteric CH_4 and nitrogen emissions in ruminant farming. The focus was put on forages from woody and herbaceous plants growing naturally in temperate climates. The selection of plants was determined by their availability in bulk, inexpensive procurement and lack of direct competition to human food production.

The first experiment of the doctoral project served to compare 18 plant parts deriving from 16 plant species *in vitro* for their potential to reduce CH_4 and ammonia (NH_3) emissions without negatively affecting the forage feeding value. The following plants were tested: leaves of bearberry (*Arctostaphylus uva-ursi*), silver birch (*Betula pendula*), sweet chestnut (*Castanea sativa*), hazel (*Corylus avellana*), aspen (*Populus tremula*), blackcurrant (*Ribes nigrum*), goat willow (*Salix caprea*) and grape vine (*Vitis vinifera* and *Vitis vinifera rubra*), aerial part of rosebay willow (*Epilobium angustifolium*), wood avens (*Geum urbanum*), birdsfoot trefoil (*Lotus corniculatus*) and comfrey (*Symphytum officinale*), fruit of horse chestnut (*Aesculus hippocastanum*) and blackthorn (*Prunus spinosa*; entire fruit, kernel, pulp). The plant material was added at 170 mg/g of total dry matter (DM) to a common total mixed ration and incubated with the *in vitro* Hohenheim gas test method. With any of the test materials the NH_3 concentration in the incubation liquid decreased by up to 35% compared to the unsupplemented mixed ration. Methane formation per unit of DM and per unit of digestible organic matter (dOM) was lowered by 13 of the 18 test materials by 12 to 28% and 5 to 20%, respectively. Net energy of lactation was only reduced with five plant additives, and utilizable crude protein at the duodenum was not affected by most of the plant materials, except blackcurrant, hazel and rosebay willow which increased it by up to 7%. The supplementation of the mixed ration with birch, blackcurrant, hazel, rosebay willow, vine and wood avens materials was particularly promising in terms of CH_4 and NH_3 mitigation without depressing *in vitro* digestibility and feeding value.

The second Hohenheim gas test experiment aimed to determine dose-response effects of the six most promising plants selected from the previous *in vitro* experiment. The plants were tested in dosages of 100, 200, 300, 400, 500 and 1000 mg/g DM, replacing increasing amounts of a mixed ration. All plants mitigated CH_4 and NH_3 formation, and this often linearly with the dosage. The level of effect differed between plants, and proportions of 100 (hazel, rosebay willow) to 400 mg/g DM of plant were necessary to achieve significant effects.

Subsequently, the same six plants with the highest CH_4 and NH_3 mitigation potential identified *in vitro* were tested for their short-term palatability in Experiment 3 in dairy cows. The plants were pelleted with lucerne at proportions to obtain the same total phenol content over all pellet types, but realised contents differed from expected contents. Pellets were provided separately from a mixed basal ration (0.4:0.6) in a randomized order for each cow for 3 days per plant. Four of the six plants were found to be as palatable as lucerne-only control pellets. Blackcurrant and birch pellets were eaten significantly less well. There was no substantial short-term effect on milk yield and composition, but all plants reduced milk urea content indicating their NH_3 mitigating potential in the rumen. The correlation analysis of the palatability results showed that the total phenol content was not most decisive for the acceptance of the plant pellets as the plant pellets with the highest total phenol content (rosebay willow) was among the most palatable plant pellets. The lignin content turned out to be more decisive in this respect, highest in blackcurrant and birch. Over all plants, hazel leaves were the most palatable test forage when provided pelleted with lucerne.

In Experiments 4 and 5 carried out with adult sheep and lactating cows, different hazel leave amounts (0 to 50%) were pelleted with lucerne in different ratios. Pellets were fed in addition to hay or a mixed basal diet in sheep and cows in ratios of 0.8:0.2 and 0.6:0.4, respectively. The high palatability of the hazel leaves was given even when fed during several weeks to cows and sheep. Diet digestibility and milk yield (only in dairy cows) declined with increasing hazel proportion. The feed supplementation with hazel leaves turned out to be very efficient in reducing CH_4/dOM emissions from sheep and cows, i.e. by up to 25% with the use of 500 g hazel leaves per kg DM intake. The level of the mitigating effect was very similar in the two experiments although the basal diets had been different. The relationship of hazel proportion in the diet with CH_4 yield and emission intensity (CH_4/dOM or CH_4/milk) had a non-linear component which demonstrated the presence of associative effects. However, these associative effects indicated that, different from expectations, high proportions of hazel leaves in the diet are needed to suppress CH_4 yield and emission. In both animal species, there was a clear shift

in nitrogen excretion from urine to faeces, which is to be considered highly favourable from an environmental perspective.

In conclusion, the experimental approach applied in the present thesis revealed that especially hazel leaves are a promising feed additive with a high palatability and a high potential to mitigate CH_4 and nitrogen emissions not only *in vitro,* but also *in vivo* in ruminants. Due to their palatability, the hazel leaves showed a great potential for their application in practical animal feeding. Further research should focus on the composition and biological activity of the phenolic compounds in the efficient woody plant leaves, which might be responsible for the changes found in rumen fermentation. Overall, the integration of woody plants such as the hazel shrub as diet component could have a great potential for a more sustainable and environmental friendly food production.

Zusammenfassung

Die landwirtschaftliche Nutztierhaltung, insbesondere die Wiederkäuerhaltung, trägt erheblich zur globalen Klimaerwärmung bei. Treibhausgase, wie Kohlendioxid (CO_2), Methan (CH_4) und Lachgas (N_2O) spielen eine zentrale Rolle beim Klimawandel. Insbesondere CH_4, das bei der Pansenfermentation freigesetzt wird, hat ein 23-fache höheres Treibhauspotential als CO_2. Sekundäre Pflanzeninhaltsstoffe, welche in einer Reihe von holzigen und krautigen Pflanzen des gemässigten Klimas zu finden sind, spielen eine wichtige Rolle bei der Entwicklung von wirksamen Fütterungsstrategien zur Minderung der CH_4- und Stickstoffemissionen aus der Wiederkäuerhaltung.

Das Ziel der vorliegenden Dissertation war es, potentiell in Frage kommende Pflanzen als Zusatz zu einem qualitativ hochwertigen Futter auf ihre ruminale CH_4 und Stickstoff reduzierende Wirkung bei Wiederkäuern zu testen und zu identifizieren. Speziell holzige und krautige Pflanzen, die in der gemässigten Klimazone natürlich vorkommen, standen im Fokus. Die Kriterien, welche die Auswahl bestimmten, waren die Verfügbarkeit in grossen Mengen, eine kostengünstige Beschaffung und sie durften nicht in Konkurrenz zur Produktion von menschlichen Nahrungsmitteln stehen.

Das erste Experiment hatte zum Ziel, von 18 verschiedenen Pflanzenteilen, von 16 verschiedenen Pflanzenspezies stammend, hinsichtlich ihres Potenzials zur Reduktion von CH_4- und Ammoniak(NH_3)-Emissionen ohne den Futterwert negativ zu beeinflussen *in vitro* zu testen. Die folgenden Pflanzen wurden getestet: Blätter der Bärentraube (*Arctostaphylus uva-ursi*), Hängebirke (*Betula pendula*), Edelkastanie (*Castanea sativa*), Gemeine Hasel (*Corylus avellana*), Zitterpappel (*Populus tremula*), Schwarze Johannisbeere (*Ribes nigrum*), Salweide (*Salix caprea*) und Weinrebe (*Vitis vinifera* und *Vitis vinifera rubra*), das Kraut des Schmalblättrigen Weidenröschens (*Epilobium angustifolium*), des Echten Nelkenwurz (*Geum urbanum*), des Gemeinen Hornklees (*Lotus corniculatus*) und des Gemeinen Beinwells (*Symphytum officinale*), die Frucht der Gemeinen Rosskastanie (*Aesculus hippocastanum*) und des Schlehdorns (*Prunus spinosa*; ganze Frucht, Kern, Fruchtfleisch). Das Pflanzenmaterial wurde in einer Dosierung von 170 mg/g Trockensubstanz (TS) einer gewöhnlichen Mischration für Kühe zugefügt und mit dem Hohenheimer Futterwerttest *in vitro* inkubiert. Jedes getestete Pflanzenmaterial reduzierte die NH_3-Konzentration im inkubierten Pansensaft um bis zu 35%, verglichen mit der Mischration ohne Pflanzenzusatz. Die CH_4-Produktion per Einheit TS und verdaulicher organischer Subastanz (vOS) wurde von 13 der 18 Pflanzenmaterialien zwischen 12 bis 28% bzw. 5 bis 20% reduziert. Die Netto-Energie-Laktation (NEL) wurde nur durch fünf

Pflanzenzusätze reduziert und das nutzbare Rohprotein (nXP) wurde durch die meisten Pflanzen nicht beeinflusst, nur die Schwarze Johannisbeere, Hasel und das Schmalblättrige Weidenröschen erhöhten das nXP um bis zu 7%. Die Supplementierung der Mischration mit Pflanzenmaterial der Birke, der Schwarzen Johannisbeere, der Hasel, des Schmalblättrigen Weidenröschens, der Weinrebe und des Echten Nelkenwurz war besonders effektiv in Bezug auf die CH_4 und NH_3-Reduktion, ohne die Verdaulichkeit und den Futterwert *in vitro* zu beeinträchtigen.

Im zweiten Hohenheim Futterwerttest-Experiment wurde die Dosis-Wirkungs-Beziehung der sechs vielversprechendsten Pflanzen des vorherigen Versuchs untersucht. Die Pflanzen wurden in den Dosierungen von 100, 200, 300, 400, 500 und 1000 mg/g TS getestet indem die respektive Menge an Mischration reduziert wurde. Alle Pflanzen reduzierten die CH_4 und NH_3-Emissionen meist in einem linearen Zusammenhang zu der Dosierung. Das Wirkungsniveau war unter den Pflanzen unterschiedlich und es waren Anteile von 100 (Hasel, Schmalblättriges Weidenröschen) bis zu 400 mg/g TS der Pflanzen nötig, um signifikante Effekte zu erzielen.

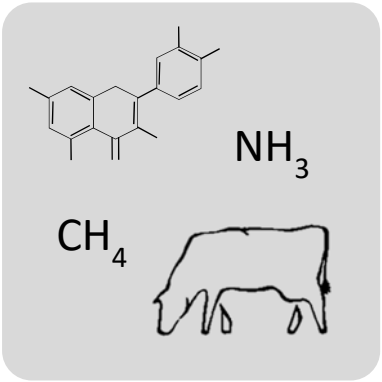
Im folgenden dritten Versuch, wurden die gleichen sechs Pflanzen, welche *in vitro* als die, mit dem höchsten CH_4- und NH_3-Reduktionspotenzial identifiziert wurden, auf ihre Kurzzeit-Schmackhaftigkeit bei Milchkühen getestet. Die Pflanzen wurden in verschiedenen Mengen mit Luzerne zu Pellets verarbeitet so, dass in allen Pflanzen-Pellets die gleiche Menge an totalen Phenolen sein sollte. Diese Phenolgehalte unterscheiden sich jedoch letztendlich von den erwarteten Werten. Die Pellets wurden separat von einer Mischration gefüttert (0.4:0.6). Die Kühe erhielten alle sechs Pflanzenpellets in randomisierter Reihenfolge für eine Dauer von je drei Tagen. Vier der sechs Pflanzen erwiesen sich als genauso schmackhaft wie die reinen Luzernepellets (Kontrollpellets). Die Pellets mit Blättern der Schwarzer Johannisbeere und Birke wurden deutlich schlechter gefressen, als die Kontrollpellets. Es gab keine erkennbare Auswirkung auf die Milchleistung und -zusammensetzung, aber alle Pflanzen reduzierten den Milchharnstoffgehalt, was auf ihr NH_3-Minderungspotenzial im Pansen hinweist. Die Ergebnisse der Korrelationsanalyse zeigten, dass der totale Phenolgehalt für die Schmackhaftigkeit der Pflanzenpellets nicht entscheidend war, da die Pflanzenpellets mit dem höchsten Phenolgehalt (Schmalblättriges Weidenröschen) zu den am besten gefressenen Pellets gehörten. Der Ligningehalt erwies sich als ausschlaggebend für den Verzehr, welcher am höchsten bei den Pflanzenpellets der Schwarzer Johannisbeere und der Birke waren. Von allen angebotenen Pflanzen pelletiert in Kombination mit Luzerne, zeigten die Haselblätter die beste Schmackhaftigkeit.

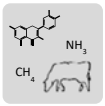
Die anschliessenden Versuche 4 und 5 wurden mit ausgewachsenen Schafen und Milchkühen durchgeführt, denen verschiedene Mengen an Haselblättern (0 bis 50% der Ration), pelletiert in verschiedenen Verhältnissen mit Luzerne, gefüttert wurden. Die Pellets wurden zusätzlich zu Heu (Schafe) oder einer Mischration (Milchkühe) im Verhältnis 0.8:0.2 bzw. 0.6:0.4 gefüttert. Die hohe Schmackhaftigkeit der Haselblätter konnte auch bei mehrwöchiger Fütterung an Kühe und Schafe bestätigt werden. Die Verdaulichkeit und Milchleistung (nur Kühe) nahmen mit steigenden Anteilen an Haselblättern in der Ration ab. Die Blätter erwiesen sich als sehr effizient bei der Reduktion des CH_4. Mit 500 g Haselblättern in der Futteraufnahme konnte das CH_4/vOS um bis zu 25% reduziert werden und das Niveau der CH_4-Reduktion war bei beiden Tierarten ähnlich, obwohl sie verschiedenes Grundfutter zu den Pellets bekamen. Das Verhältnis des Haselanteils im Futter zu der CH_4-Emission (CH_4/vOS oder CH_4/Milch) hatte eine nicht-lineare Komponente, welche das Vorhandensein von assoziativen Effekten zeigte. Diese assoziativen Effekte zeigten jedoch, dass, anders als erwartet, hohe Anteile an Haselblättern im Futter nötig waren, um die CH_4-Menge und Intensität zu reduzieren. Bei beiden Tierarten kam es zu einer deutlichen Verschiebung der Stickstoff-Ausscheidung vom Urin zum Kot, was aus Umweltaspekten vorteilhaft ist.

Zusammenfassend lässt sich sagen, dass insbesondere die Haselblätter ein vielversprechender Futterzusatz für Wiederkäuer sind, aufgrund ihrer guten Schmackhaftigkeit und dem hohen Potenzial zur Reduktion der CH_4- und NH_3-Emissionen sowohl *in vivo* als auch *in vitro*. Demzufolge zeigen sie ein grosses Anwendungspotenzial in der praktischen Tierernährung. Weitere Forschung sollte sich mit der Zusammensetzung und der biologischen Aktivität der Phenolverbindungen in den Blättern der vielversprechenden Pflanze, die für die Veränderung der Pansenfermentation verantwortlich sein könnten, befassen. Insgesamt könnte die Integration von Gehölzen, wie dem Haselnussstrauch als Futterkomponente, ein großes Potenzial für eine nachhaltigere und umweltfreundlichere Lebensmittelproduktion haben.

Chapter 1

General introduction





1. General Introduction

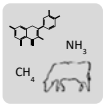
1.1 Emissions of noxious gases from the livestock sector

One of the greatest challenges facing mankind today is the continuous and progressing climate change. This change is dominated by human influence, and mediated through changes in atmospheric composition (Karl and Trenberth 2003). Many gases play a role in the composition of the atmosphere and these gases are responsible for the natural greenhouse effect that determines global temperature balance. Human activities over the last 50 years, particularly energy consumption and land-use changes, have caused increased anthropogenic emissions of greenhouse gases (GHG) such as carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) and this put the natural atmospheric system off balance (Karl and Trenberth 2003). CH_4 is particularly detrimental with a global warming effect 23-times that of CO_2 (Steinfeld et al. 2006). The livestock sector, particularly the ruminant sector, contributes up to 18% of the total global warming effect (Steinfeld et al. 2006) and is estimated to emit 7.1 Giga tons of CO_2-equivalents (Gerber et al. 2013). About 44% of livestock emissions are in the form of CH_4, 29% in the form of N_2O and 27% in the form of CO_2 (Gerber et al. 2013) In addition, this sector contributes about 9% of total CO_2 , 37% of total CH_4 and 65% of total N_2O anthropogenic emissions worldwide (Steinfeld et al. 2006). These emissions are continually increasing due to the rising demand of animal products (Rojas-Downing et al. 2017). Especially ruminants play a central role in GHG emission as CH_4 is formed from the degradation of feed by microbes present in the rumen. Cattle are the main emitters in the livestock sector, emitting 65% of the total emissions, corresponding to an amount of 4.6 Giga tons of CO_2-equivalents (Gerber et al. 2013). To maintain the anthropogenic global warming level below 2°C for this century (compared to the pre-industrial level) the United Nations ratified the Paris Agreement in 2015 (UN 2015) to help prevent climate change. This agreement requires all parties to reduce greenhouse gas (GHG) emissions as soon as possible. In the case of Switzerland, GHG emissions should be reduced by 50% until the year 2030 compared to the year 1990. In the livestock sector, there is a high potential to reduce GHG emissions. Gerber et al. (2013) estimated that the livestock sector has the potential to reduce GHG emissions by 30% within existing production systems. The largest mitigation potential of CO_2-equivalents is estimated in cattle production with a potential decrease of 65% (Figure 1).

Figure 1.1 Mitigation potential (%) of CO_2-equivalents of individual livestock species proportional to current emissions (modified from FAO 2006 with data from Gerber et al. 2013).

Ammonia (NH_3) is not a GHG but an air pollutant that was included in the Gothenburg Protocol in 1999. This protocol, aims to reduce acidification, eutrophication and ground-level ozone (UNECE 1999). In 2010, the total agricultural NH_3 emissions in Switzerland were 48 290 t N, representing 92% of total Swiss ammonia emissions. The main contributor of NH_3 emissions in agriculture is livestock and manure management with 43 489 t N (90% of total agricultural NH_3 emissions). Of this, the dairy sector produces 49% of all N emissions (Kupper et al. 2013). From 1990 to 2010 the NH_3 emissions have declined but the mitigation potential is still high. The formation and volatilization of NH_3 in animal manure is a process that begins immediately after excretion, primarily from urinary urea (Hristov et al. 2011). Many factors influence this volatilization including manure management, environmental temperature, manure composition and pH levels (Hristov et al. 2011).

Correspondingly, the development of strategies to mitigate methane and ammonia emissions from livestock production, particularly in ruminants, is one of today's major challenges for animal nutritionists.



1.2 Origin of emissions from ruminant production and mitigation strategies

During microbial fermentation in the rumen, dietary components are degraded under anaerobic conditions. The end products of this fermentation are acetate, propionate and butyrate. These substances then become readily available energy sources for both the microbes and the ruminant, and are absorbed directly in the rumen. During the microbial degradation process ruminal CH_4 is formed as a by-product. Hydrogen, released during microbial feed degradation, is used by methanogenic archaea to produce CH_4 from CO_2 (Moss et al 2000). This CH_4 cannot be used by the ruminant and its formation represents a loss of 2-12% of the feed energy for the animal (Goel and Makkar 2012) and has a detrimental environmental impact.

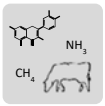
There are different strategies for CH_4 mitigation which can be divided into three main areas: rumen manipulation, breeding and management strategies (Cottle et al. 2011). Rumen manipulation includes, among others, the use of feed additives. Feeding strategies aim to reduce CH_4 and NH_3 emissions by manipulating ruminal fermentation. The application of specific feed additives and feeding strategies are two of the most promising ways to mitigate CH_4 formation during its synthesis in the rumen. Various feeding strategies, to reduce enteric methane production, have been described in reviews by Moss et al. (2000), Beauchemin et al. (2008), Martin et al. (2009) and Hristov et al. (2013). These include the use of plant secondary metabolites (PSM) naturally present in plants, or plant extracts, as well as lipids as feed additives. The advantages of using PSM is their mitigating effect on CH_4 and NH_3 emissions and they do not compete with human nutrition. One of the main goal in using feed additives and their PSM in animal feeding is the reduction of CH_4 emissions without affecting feed intake, digestibility and animal performance, or, if unavoidable, to a small extent. Mitigation of absolute CH_4 and NH_3 is only sustainable if the emission intensity (per unit of food produced) or at least yield (per intake of DM or digestible nutrients) is reduced.

For the NH_3 emissions, the main source is urea in the urine of ruminants (Hristov et al 2011). High amounts of rumen degradable protein in the diet lead to high amounts of NH_3 in the rumen causing increasing levels of urea in urine. The transformation of NH_3 to N_2 after excretion makes urinary N an important source of N_2O (Dijkstra et al. 2013). About 60 to 90% of urinary N from cattle originate from urea (Hristov et al 2011). Up to 40% of urinary N can be volatilized, this is in contrast to faecal N, where N volatilization is only between 1 and 13% (Hristov et al 2011). Therefore, a shift from urinary N to faecal N is desirable from an environmental point of view. Among others, feeding management to avoid protein contents exceeding animal requirements would help to reduce urinary N losses as well as including plants rich in PSM in the diet (Min and Solaiman 2018).

There have been numerous extensive *in vitro* screenings of plant supplements which tested their CH_4 and NH_3 mitigation potentials. The *in vitro* batch culture (e.g. Hohenheim Gas Test) is a well-suited instrument for screening large number of potential feed additives such as plants or plant extracts. For example, Banik et al. (2013) tested different plants from natural Australian grasslands for their CH_4 and NH_3 mitigating properties. The Rumen Up Project (Wallace 2008;*https://www.abdn.ac.uk/research/rumen-up/report*), screened a variety of 500 plants and plant extracts for their mitigating potential whereof 34 plant parts or extracts decreased the CH_4 by 15% to 91% when tested as a feed additive. The PSM play a central role in the development of the presented feeding strategies aiming to reduce CH_4 and NH_3 from ruminants (Jayanegara et al. 2012; Hristov et al. 2013) using plant additives. They have a high potential for application in livestock production (Acamovic and Brooker 2005) as they are natural plant extracts which are considered safer with regard to human health compared to chemical feed additives.

1.3 Plant secondary metabolites and their nutritional characteristics in ruminant feeding

The PSM are chemical compounds that are specific to different plant species or families. The PSM are not involved in primary metabolism or biochemical processes in plant growth (production of nutrients) or reproduction (Patra and Saxena 2010). The functions of PSM in plants are manifold as they contribute to specific odours, tastes and colours of plants. Additionally, they may have a defensive function against pathogens and be a deterrent for insects and herbivores (Bennett and Wallsgrove 1994). Therefore, secondary metabolites can have anti-nutritive or even toxic effects on herbivores, depending on their chemical structure and concentration in the plant. The concentration in plants is affected by genetic and environmental factors. The PSM composition and concentration may vary between seasons and habitats (Palo et al. 1985; Sauter and Wellencamp 1998), when grown in different soils, in varying climates and under different fertilization treatments (Tiemann et al. 2009 and 2010) or even when originating from different cultivars of the same species (Bekele et al. 2009). There are over 200 000 defined structures of PSM (Patra and Saxena 2010) and their differentiation into groups is based on their chemical structure and physiological properties. Groups that are of special interest in ruminant feeding comprise the phenols, saponins and essential oils. According to Makkar (2003) the phenolic compounds can be divided in total phenols (TP), non-tannin phenols (NTP) and total tannins (TT). The total tannins can be subdivided further into condensed tannins (CT) and hydrolysable tannins (HT) (Makkar 2003). The polyphenolic substances that are the most widely distributed are the tannins on which the present doctoral thesis focused on.



1.3.1 Feed intake and animal performance with tannins in ruminant feeding

It is well known that, depending on the dosage, PSM-rich plants can have a beneficial or adverse effect on animals (Makkar 2003). High levels of PSM can reduce the palatability of the feed and, concomitantly, feed intake and productivity of the animal. This may be the reason that promising plant additives, tested *in vitro*, are often less successful when tested *in vivo* (Goel and Makkar 2012). Low palatability, due to astringent tannins, can lead to reduced voluntary feed intake of PSM-rich plants or plant additives (Kumar and Singh 1984). Concentrations of 60-120 g/kg DM of CT were responsible for a depressed feed intake and reduced animal productivity (Aerts et al. 1999). Though, the same review study showed that a moderate dosage of CT (20-40 g/kg DM) can improve animal production efficiency. Studies have shown that feeding plants rich in CT, e.g. *L. corniculatus*, can increase milk yield, milk protein content and milk production efficiency (Wang et al. 1996, Woodward et al 2000). Tannins can also have health-promoting effects on animals. They are able to help control gastrointestinal parasites (Min and Hart 2003), promote wool growth and increase ovulation rates in ewes (Aerts et al. 1999) and can prevent bloat (Waghorn and McNabb 2003). Care should be taken when feeding high amounts of HT because of possible toxic effects. However, with a sufficient adaptation for the rumen microbes, this is also feasible (Waghorn 2008). Overall, animals preferences for certain feeds seem to be related to digestive consequences (Baumont 1996).

1.3.2 Effect of tannins on ruminal fermentation

Tannins can affect the feed quality and digestibility. The CT form complexes, primarily with proteins, in the rumen (pH 6.0 to 7.0). By this complexing, the proteins are protected from degradation by microbes in the rumen and can be transported to the duodenum. There, with a pH below 3.5, the complexes can be dissolved again (Kumar and Singh 1984). An increase of the utilisable crude protein (uCP) could be the direct consequence. Additionally a shift from urinary N to faecal N excretion can be observed when feeding diets supplemented with tannins (CT) to ruminants (Waghorn 2008, Gerlach et al. 2018), this is positive from an environmental point of view. This shift might be explained by the reduced protein degradation in the rumen and accordingly a reduced ruminal NH_3 production. Moreover, antimicrobial properties and a negative effect on ruminal fibre digestion because of decreased cellulolytic bacteria numbers or reduced enzyme activity were reported for tannins (Wallace 2004, Goel and Makkar 2012). Furthermore, tannins can differ in their influence on CH_4 formation in the rumen. The meta-analysis by Jayanegara et al. (2012) showed that at least 20 g of tannins per kg DM are needed *in vivo* and *in vitro* to obtain a clear methane mitigating effect. Effects of PSM such as phenols

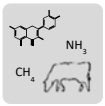
(including tannins) on CH_4 formation are not always unambiguous as not only concentration but also type and biological activity can have an influence (Waghorn and McNabb 2003). The combination of different tannin groups (HT and CT) showed a different mitigation potential than the use of only single groups (Bhatta et al. 2009). A decrease of protozoal numbers, a shift in the short chain fatty acid proportions or the aforementioned change in digestibility might be possible reasons for the modification in CH_4 emissions.

1.4 Woody and herbaceous plants as alternative and novel resources in ruminant feeding

In Europe the number of shrubs and trees in the agricultural landscape has decreased in the last decades due to the intensification and mechanisation of agriculture (Nerlich et al. 2013). Nowadays, the aforementioned study showed that agroforestry systems, including trees and shrubs in agricultural production systems, are regaining importance. In Switzerland for example, policies (Öko-Qualitätsverordnung, Bundesrat 2000) and agricultural programmes (IP-Suisse) promote the planting of shrubs as hedges. In southern Europe, woody plants play an important role in livestock feeding as a protein supplement, especially in dry or semi-dry Mediterranean areas to tide over feed shortage during inauspicious weather conditions (Makkar 2003; Papanastasis et al. 2008).

An important aspect to point out is that trees and shrubs do not compete with human food production. The increasing global demand for animal products evolving in the next decades will escalate the food-feed competition and novel feed resources will become more important (Makkar 2018). Additionally, trees, shrubs and herbaceous plants can positively affect the environment, as demonstrated in the review by Nair (2011). Benefits include natural habitats for numerous plants and animals and improved water and soil quality.

Trees, shrubs and herbaceous plants can be very rich in PSM contents (Frutos et al. 2004). Ben Salem (1994) remarked that shrubs and trees can be valuable feed resources in livestock feeding systems, but a good knowledge of their palatability and nutritive value is required. However the list of effective, indigenous plants in the temperate climate zone that have a high, or at least moderately high, feeding value is rather limited. Plants which are available in bulk are of particular interest because this allows an implementation in animal feeding practices. Several plants which can be found in natural grasslands are known to be rich in PSM and to have the potential of mitigating CH_4 and NH_3 formation in ruminants. Forages from woody plants could also be rich in PSM such as phenols and tannins (Makkar 2003; Frutos et al. 2004). Different studies have demonstrated that including woody plants as feed or additives into the rations of ruminants can be successful without having negative effects on the feed intake and performance



of the animal. In New Zealand temperate species like *Salix* spp. or *Populus* spp. are fed to sheep and cattle and in Belgium *Coryllus avellana* is given as a supplementary forage in ruminant production (Vandermeulen et al. 2018). Meier et al. 2014 showed that woody plants could be highly palatable for sheep, especially birch tree leaves (*Betula pendula*). Overall, the branches, leaves or the whole plant of shrubs can be used as fresh fodder, dried or even ensiled (Vandermeulen et al. 2018).

1.5 Experimental approach and scope of the doctoral thesis

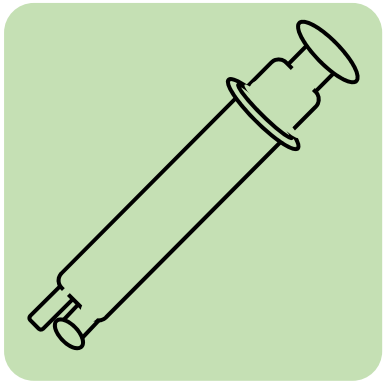
The overall aim of this doctoral project was to test and identify plant candidates to be fed as additives to a good quality ration common in ruminant farming. The focus was on forages from woody and herbaceous plants growing naturally in temperate climates. The selection of plants was determined by their availability in bulk, inexpensive procurement and no direct competition to human food production. To evaluate potential candidates, the plants were screened *in vitro* with the Hohenheim Gas test for their CH_4 and NH_3 mitigation potential. Furthermore, it was evaluated how the plants affect the digestibility and rumen fermentation when added to a common mixed basal diet in different dosages. The plants with the highest CH_4 and NH_3 mitigation potential, without negatively affecting digestibility, were chosen for a following palatability test in dairy cows. Finally, the most palatable plant with the highest CH_4 and NH_3 mitigation potential was applied as feed additive in sheep and dairy cows and evaluated for their *in vivo* effects.

In brief, the project was subdivided into the following five objectives:

1) Identification of plants with the highest CH_4 and NH_3 mitigation potential with negligible effects on the digestibility *in vitro* with the Hohenheim Gas test (Chapter 2)
2) Test the dose-response effect of the six most promising plants (from objective 1) *in vitro* (Chapter 3)
3) Test the palatability of the six most promising plants (from objective 1) as feed additive in dairy cows (Chapter 3)
4) Study the most palatable plant in low and high amounts with regard to the degree of CH_4 and NH_3 mitigation in sheep (Chapter 4)
5) Determine potential associative effects between woody plants as feed additive and high quality forage by combining increasing amounts of the most promising plant with high-quality lucerne to dairy cows and analysing the effects on animal performance and gas emissions (Chapter 5)

Chapter 2

In vitro screening of temperate climate forages from a variety of woody plants for their potential to mitigate ruminal methane and ammonia formation



This chapter is based on Terranova, M., Kreuzer, M., Braun, U. and Schwarm, A. 2018: *Journal of Agricultural Science*, 156, 929-941.

2.1 Summary

Feeding phenol-containing plants to ruminants has the potential to mitigate both, methane and ammonia formation. In the present study, mostly woody plants, such as the leaves of trees and shrubs, were tested for their influence on *in vitro* fermentation. Plants selected grow naturally under temperate climatic conditions, are usually available in bulk and do not directly compete with human food production. The detailed screening included whole plants or parts of different plant species reporting their effects on methane and/or ammonia formation. The plant materials were added at 167 mg/g of total dry matter to a common total mixed ration and incubated for 24 h with the Hohenheim gas test method. The results from the *in vitro* fermentation were also used to determine the net energy of lactation and the utilisable crude protein in the complete diets. Thirteen out of 18 test materials did not impair the organic matter digestibility of the diet. Ammonia concentrations decreased up to 35% ($P < 0{\cdot}05$) when adding any of the test materials. Methane formation per unit of feed dry matter and per unit of digestible organic matter was lowered ($P < 0{\cdot}001$) by 13 of the 18 test materials from 12 to 28% and 5 to 20%, respectively. In conclusion, a number of plant materials tested have the potential to mitigate ruminal ammonia and methane formation without adversely affecting digestibility. The leaves of *Betula pendula*, *Corylus avellana, Ribes nigrum, Vitis vinifera* and the aerial part of *Geum urbanum* were particularly promising in this respect.

2.2 Introduction

Methane (CH_4) is a greenhouse gas that substantially contributes to climate change. The livestock sector is estimated to contribute up to 18% of total global anthropogenic greenhouse gas emissions (Steinfeld et al. 2006). The application of specific feeds and feeding strategies are among the most promising ways to mitigate CH_4 formation during its synthesis in the rumen of ruminants. Depending on the dosage or the composition, plants can have positive or negative effects on digestive processes in herbivores. Plant secondary compounds (PSC), like saponins, phenols and essential oils play a central role in this respect (Waghorn 2008; Bodas et al. 2012). Apart from CH_4, there are other harmful emissions from animal excretions, particularly urinary N, as it is easily emitted as ammonia or nitrates. Urinary N excretion is closely related to ruminal ammonia concentration which, therefore, is indicative of the N emission potential in diets (Dijkstra et al. 2013).

There are some extensive *in vitro* screenings of plant supplements testing their CH_4 and ammonia mitigating properties, for example with plants from natural grasslands (Banik et al.

2013; Macheboeuf et al. 2014; Niderkorn et al. 2014). In the 'Rumen Up' project (Wallace 2008; *https://www.abdn.ac.uk/research/rumen-up/report*), 34 plants or plant extracts out of 500 were identified as being able to diminish CH_4 production by more than 15% *in vitro* when given as additives without negatively affecting ruminal fermentation. Individual results regarding digestibility, methane, ammonia, total gas or SCFA production were made available in peer-reviewed publications for only 14 of these 34 promising plants (Selje et al. 2007; Bodas et al. 2008).

In general, the list of effective and indigenous plants in the temperate climate zone, which at the same time, have a high or at least moderately high feeding value, is still rather limited. Of particular interest are those plants that are affordable and mostly available in bulk so that an implementation in animal feeding practice is feasible. Several plants growing naturally in grasslands, especially those in the legume family, are known to be rich in PSC and have the potential to mitigate CH_4 and ammonia (Tavendale et al. 2005; Williams et al. 2011). However, in terms of PSC contents, forages from woody plants like shrubs and trees might be more promising than herbaceous forages. In addition, these woody plant species typically do not directly compete with resources used for growing human food and have a particular ecological value as habitats for numerous animal and plant species. Nevertheless, forages from shrubs and trees can have higher lignification than herbaceous plants and thus offer a lower feeding value (Hummel et al. 2006). Besides, a potential drawback is that PSC content and composition as well as nutrient contents may vary between seasons and habitats (Palo et al. 1985; Sauter & Wellenkamp 1998).

Therefore, the aim of the present study was to screen a variety of woody plants for their magnitude of mitigation of ruminal CH_4 and ammonia if they are added to a good quality total mixed ration to a considerable extent. Besides describing the fermentation, the microbial count and indicators of the feeding value of the diets supplemented with the test plant additives (contents of net energy of lactation (NE_L) and the utilisable crude protein at the duodenum (uCP)), were evaluated. Two lots from each test plant material were tested separately to account for the variability of natural plant material in phenol and nutrient content and composition.

2.3 Materials and Methods

2.3.1 Test plant material

In the present study, 18 test plant materials derived from 16 plant species were chosen for *in vitro* incubation with the Hohenheim gas test (HGT) method (Table 2.1). Thirteen of the plants were selected from a list of 34 plant materials in the Rumen Up report (Result section 3.5, page 3.46). An over 15% decrease in CH_4 production *in vitro* was observed when these plant materials were given as additives and no negative effect on ruminal fermentation (digestibility, total gas and SCFA production) was reported. Our selection criteria were: (1) plants growing naturally under temperate climate conditions, (2) respective plant parts are inexpensive and available in bulk, and (3) plants do not directly compete with the production of human food.

The following plant materials were tested: leaves of *Arctostaphylus uva-ursi* (bearberry), *Betula pendula* (silver birch), *Castanea sativa* (sweet chestnut), *Corylus avellana* (hazel), *Populus tremula* (aspen), *Ribes nigrum* (blackcurrant), *Salix caprea* (goat willow), *Vitis vinifera* and *Vitis vinifera rubra* (grape vine), aerial part of *Epilobium angustifolium* (rosebay willow), *Geum urbanum* (wood avens), *Lotus corniculatus* (birdsfoot trefoil) and *Symphytum officinale* (comfrey), fruit of *Aesculus hippocastanum* (horse-chestnut) and *Prunus spinosa* (blackthorn; entire fruit, kernel and pulp) and root of *Paeonia alba* (peony). Leaves of *Castanea sativa* (sweet chestnut) were used as a positive control due to their demonstrated mitigating effect on methane and ammonia formation (Jayanegara et al. 2011). The aerial part of the *L. corniculatus* was tested because of contradicting reports in regard to CH_4 and ammonia mitigation potential (no effect: Macheboef et al. 2014 Niderkorn et al. 2014; effect: Tavendale et al. 2005; Williams et al. 2011). As summarized in Table 2.2, for nine test plant materials, no publications regarding effects on ruminal fermentation were available and for the remaining seven materials, investigations were incomplete in terms of the variables aimed at in the present study. The large variety of incubation methods used among the plant materials in the literature cited above and the missing results regarding variables of interest for the present study were reasons to still include these plants. Particularly the uCP and NEL contents of the plant materials were never reported in other studies, with the exception of uCP for *L. corniculatus* (Scharenberg et al. 2007). Thus, the results of the present study contribute considerably to the description of their feeding value. A total mixed ration without plant additive served as the negative control.

Table 2.1 Details of the experimental plant materials.

Plant species or variety plant part	Plant family	Lot/ supplier*	Collection site	Harvest time (where specified)
A. hippocastanum	Sapindaceae	1 / A	Poland	September
tree fruit		2 / B	Poland	September
A. uva-ursi	Ericaceae	1 / A	Russia	October,November
shrub leaves		2 / B	Russia	
B. pendula	Betulaceae	1 / A	Albania	August
tree leaves		2 / A	Romania	June
C. sativa	Fagaceae	1 / A	Albania	July
tree leaves		2 / B	Albania	
C. avellana	Betulaceae	1 / A	Albania	October
shrub leaves		2 / A	Albania	May
E. angustifolium	Onagraceae	1 / A	Poland	August
aerial part of herb		2 / A	Poland	June
G. urbanum	Rosaceae	1 / A	Poland	September
aerial part of herb		2 / B	Croatia	June
L. corniculatus	Fabaceae	1 / B	Switzerland	
aerial part of herb		2 / B	Switzerland	
P. alba	Paeoniaceae	1 / B	China	
shrub root		2 / B	China	
P. tremula	Salicaceae	1 / A	Serbia	June
tree leaves		2 / B	Serbia	June
P. spinosa	Rosaceae	1 / A	Poland	October
shrub fruit: entire; kernel; pulp		2 / A	Poland	October
R. nigrum	Grossulariaceae	1 / A	Poland	July
shrub leaves		2 / A	Poland	May - August
S. caprea	Salicaceae	1 / C	Germany	Autumn
tree leaves				
S. officinale	Boraginaceae	1 / B	Croatia	
aerial part of herb		2 / D	Croatia	
V. vinifera	Vitaceae	1 / A	Bulgaria	August,September
shrub leaves green		2 / A	Bulgaria	September
V. vinifera rubra	Vitaceae	1 / A	Serbia	November
shrub leaves red		2 / A	Spain	November

A, =Alfred Galke GmbH, Bad Grund, Germany; B, Berg pharmacy, Zurich, Switzerland; C, own collection; D, St. Peter pharmacy, Zurich, Switzerland.

The test plant material could be purchased from Alfred Galke GmbH (Bad Grund, Germany), Berg pharmacy (Zurich, Switzerland) and St. Peter pharmacy (Zurich, Switzerland), except for S. caprea which was not purchasable (Table 2.1). As stated by the suppliers, the two lots of each plant material were collected in the countries and during the seasons indicated in Table 2.2, dried conventionally at 35°C in the drying oven and cut to a size of 4 to 6 mm. Two lots per plant material were purchased from different suppliers, with the exception of *L. corniculatus, P. alba* and *P. spinosa*. The two lots each of *B. pendula*, *G. urbanum* and *V. vinifera rubra* were

grown in different countries. The two lots each of *C. avellana*, *E. angustifolium* and *R. nigrum* were grown in the same countries but harvested in different months of the year. The two lots of the other plant materials were grown in the same countries at unspecified harvest time or harvested in the same month of the year. Leaves of *S. caprea* were collected in the form of autumn foliage in the area of Baden-Baden, Germany, and dried at 50°C. For this plant only one lot was available.

2.3.2 Incubation mode

The HGT method was performed as outlined by Menke & Steingass (1988). For incubation, modified 100 ml glass syringes with two outlets, one for fluid and one for gas sampling, were used as described in Soliva & Hess (2007). All test materials were ground with a centrifugal mill (Model ZM1, Retsch GmbH, Haan, Germany) to pass through a 1 mm sieve. The material from the test plants was used in an amount of 40 mg dry matter (DM) and was added to 200 mg DM of a total mixed ration (167 mg test plant/g total substrate). This proportion was chosen as it was considered realistic for inclusion as supplement to a complete ruminant diet and because a comparable level has been tested in similar studies (e.g. Selje et al. 2007; Bodas et al. 2008), as well.

The total mixed ration (basal diet) was composed of maize silage, grass silage (mixed sward, ryegrass dominated), grass hay (mixed sward, balanced) and concentrate (0·50:0·25:0·10:0·15). The concentrate DM (g/kg) consisted of rapeseed cake, 500; soybean meal, 170; maize gluten, 150; wheat, 130; calcium phosphate, 30; limestone, 20. Rumen fluid was taken from a fistulated lactating Brown Swiss cow (Cantonal Veterinary Office approval number ZH 38/14) that was fed on second-cut grass hay and, additionally, a mineral supplement (Mineralsalz UFA 195; UFA AG, Herzogenbuchsee, Switzerland). On six occasions distributed across six weeks, rumen fluid was collected before morning feeding. It was transported in a preheated thermos flask to the laboratory. Within one hour after collection, rumen fluid was strained through four layers of gauze and added to a buffer solution in a 1:2 ratio according to the protocol of Menke & Steingass (1988).

In the next step, 30 ml of rumen fluid-buffer mixture were filled into syringes, which already contained the feed. Each of the two lots from the 17 plant materials was incubated in six HGT runs adding up to 12 observations (2 lots × 6 runs). For *S. caprea*, only 6 observations (1 lot × 6 runs) were available. Two syringes each per run were comprised of HGT standard hay, standard concentrate as well as HGT standard uCP (all material obtained from the Institute of

Animal Nutrition, University of Hohenheim, Stuttgart, Germany). Additionally, each run included a duplicate of blanks (syringes without feed) and two syringes with just the basal diet as the negative control adding up to 270 incubations in total.

2.3.3 Measurements, sampling and laboratory analysis

The incubation lasted for 24 h at 39°C in an incubator with integrated rotor. After 24 h, the fermentation gas volume was recorded from the calibrated scale printed onto the syringes, and the fermentation was terminated by removing the incubation fluid from the syringes while the gas phase remained inside. Measurements of pH and ammonia in the incubation fluid were performed with a potentiometer directly after collection (pH: model 632; ammonia: model 713; Metrohm, Herisau, Switzerland), which was equipped with appropriate glass electrodes (pH: 6.0204.100; ammonia: 6.0506.100; Metrohm, Herisau, Switzerland). Fermentation gas samples of 150 µl were taken from the incubation syringes and injected using a gas-tight Hamilton syringe (Hamilton AG, Bonaduz, Switzerland) into a gas chromatograph (6890N, Agilent Technologies, Wilmington, DE, USA) equipped with a thermal conductivity detector. Concentrations of CH_4 and carbon dioxide were analysed with this detector. Four millilitres of incubation fluid were centrifuged, and 2 ml of supernatant were conserved at -20°C for later short chain fatty acids (SCFA) analyses. This analysis was performed on a HPLC (La Chrom, L-7000 series, Hitachi Ltd., Tokyo, Japan) equipped with an UV detector. For protozoal counting, the incubation fluid was preserved with a 60 ml formaldehyde/l solution (Sigma-Aldrich, Buchs, Switzerland) in a ratio of 1:1. Samples for bacteria counting were diluted 1:100 in a 40 ml of formaldehyde/l solution. A Bürker counting chamber (Blau Brand, Wertheim, Germany) with depth of 0·1 mm was used to count protozoa, and a Neubauer improved counting chamber (Blau Brand) with a depth of 0·02 mm was used for bacterial counting.

The chemical composition of test plant materials and the basal diet were determined according to AOAC (1997). Dry matter (DM) and total ash (TA) were analysed by a TGA-701 furnace (Leco Corporation, St. Joseph, Michigan, USA; AOAC index no. 942.05), and organic matter (OM) was calculated as DM minus TA. The neutral detergent fibre (NDF) was assessed in a Fibertec System M 1020 Hot Extractor and a 1021 Cold Extractor (Tecator, Högamäs, Sweden) with the use of a heat-stable α-amylase, but without sodium sulphite (Van Soest et al. 1991). The acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined using the aforementioned Fibertec apparatus according to the protocol of Van Soest et al. (1991). The NDF and ADF values were expressed without residual ash.

For determining ether extract (EE), a Soxhlet extractor was used (Extraction System B-811, Büchi, Flawil, Switzerland; AOAC index no. 963.15). With a C/N analyser (TruMac CN, Leco Corporation, St. Josephs, Michigan, U.S.A) nitrogen (N) contents were measured (AOAC index no. 968.06). Crude protein (CP) was calculated as 6·25 × N. All samples were analysed in duplicate, except for ADF and ADL, which were analysed in triplicate. Analyses of phenolic fractions were performed according to Makkar (2003). The extraction for the subsequent measurements was done twice using a 700 ml acetone/l solution. Total phenols (TP) and non-tannin phenols (NTP) were analysed with the Folin-Ciocalteu method, but, in contrast to Makkar (2003), the results were expressed as gallic acid equivalents. This was because the calibration of the UV-spectrophotometer (Shimadzu UV-160A; Shimadzu Corporation, Kyoto, Japan) was done with a gallic acid solution. To calculate the total tannin (TT) contents of the samples, the NTP were subtracted from the TP. The determination of the condensed tannins (CT) was performed with the butanol-HCl-iron method (Makkar, 2003) and the contents were given as leucocyanidin equivalents. The hydrolysable tannins (HT) were calculated as the difference between TT and CT.

2.3.4 Calculations and statistical analysis

Total gas amounts produced by the treatment diets were calculated by the difference between the gas production from the blanks and the gas amount in the incubated samples. Afterwards, the average value of the gas amounts expected and produced by the standard hay (expected: 49·61 ml gas/200 mg DM; 24 h incubation) and concentrate (expected: 65·18 ml gas/200 mg DM; 24 h incubation) was used to adjust the gas formation data from each run separately before calculating the NE_L values. We accounted for the lower amount of fermentable OM provided by the basal diet alone (200 mg) when compared to the test diets (200 + 40 mg) by adjusting the basal diet values of all quantitative variables to an amount of 240 mg. We specified the values actually measured as 200 mg DM of basal diet in brackets in the tables. For the relationship of CH_4 to SCFA, the molar amount of CH_4 was calculated by assuming a 20 °C temperature and an atmospheric pressure of 960 Pa.

In vitro OM digestibility (IVOMD) and net energy of lactation (NE_L) were calculated according to the protocol of Menke & Steingass (1988) by:

IVOMD (mg/g) = 148·8 + 8·893 × GP (ml/200 mg DM) + 0·448 × CP (mg/g) + 0·651 × TA (mg/g);

NE_L (kJ/g DM) = 1·64 + 0·0269 GP (ml/200 mg DM) + 0·00078 GP^2 (ml/200 mg DM) + 0·0051 CP (mg/g) + 0·01325 EE (mg/g)

where GP = adjusted gas production after 24 h of incubation. The content of uCP was estimated according to Steingass & Südekum (2013) by:

uCP (mg/g DM) = (NH_3-N_{blank} (mg) + N_{sample} (mg) – NH_3-N_{sample} (mg)) / weight of sample (mg DM) × 6·25 × 1000

where N_{sample} = N originating from the incubated diet and NH_3-N_{blank} and NH_3-N_{sample} = ammonia-N measured in the blank and the incubated sample, respectively. The uCP values were corrected by the respective standard (183 mg uCP/g DM; 24 h incubation).

The data were analysed with the MIXED procedure of SAS version 9·4 (SAS Institute, Carry, NC, USA) with the Tukey-Kramer adjustment. The plant material (including the basal diet alone) was defined as fixed effect (n = 19), and incubation run (n = 6) was defined as random effect. The basal diet was replicated twice per run. Single incubations of each of the two different lots from the same plant material were repeated six times (runs). These six values per plant material were obtained over a period of 6 weeks where the presence of clear differences in the donor animal's rumen fluid were likely. Thus, the number of observations was n = 12 (2 lots × 6 runs) per test plant material, except for *S. caprea*, where only 6 observations (1 lot × runs) were available. For *B. pendula* and the basal diet, only 10 and 11 total observations were available, respectively. Calculations for uCP were only possible in four runs due to the lack of the uCP standard in the first run and values out of the standard range (183 ± 18 mg uCP/g DM) in the last run. Differences among the Tukey-Kramer adjusted means were considered to be significant at $P < 0·05$ and as trends at $0·05 \leq P < 0·10$. In a separate statistical evaluation, an indication of the presence or absence of lot differences in the data from the HGT experiment was tested as far as it was possible in the frame of the current set-up. This was accomplished by performing a simple Student's *t*-test for every fermentation variable and each individual lot of test plant material (except *S. caprea,* where only one lot was available).

Table 2.2 Overview of *in vitro* studies describing methane, ammonia, short-chain fatty acids (SCFA) or digestibility results with the plant species tested in the present study

Plant species or variety and plant part	CH_4	Ammonia, proteolysis	SCFA	Digestibility	uCP, NE_L	Plant inclusion level in g/kg DM (study number)	Incubation duration in h (study number)	Rumen fluid origin (study number)
A. hippocastanum fruit								
A. uva-ursi leaves		9				180 (9)	12 (9)	Cow (9)
B. pendula leaves								
C. sativa leaves	3	3	3	3		1000 (3)	24 (3)	Cow (3)
C. avellana leaves								
E. angustifolium aerial part	5,6	5,6	5,6	5,6		1000 (5,6)	24 (5,6)	Sheep (5,6)
G. urbanum aerial part								
L. corniculatus aerial part	1,5,6, 10,11	1,5,6,11, 12	1,5,6,10, 11,12	1,5,6,11,12	8	400 (11); 1000 (1,5,6,8,10,12)	24 (1,5,6,8,10); 48 (12); 216 (11)	Cow (8,11); Sheep (1,5,6,10,12)
P. alba root								
P. tremula leaves	2		2	2		91 (2)	24 (2)	Sheep (2)
P. spinosa fruit								
P. spinosa kernel								
P. spinosa pulp								
R. nigrum leaves								
S. caprea leaves	2		2	2		91 (2)	24 (2)	Sheep (2)
S. officinale aerial part								
V. vinifera leaves				4,7		1000 (4,7)	48 (4,7)	Cattle (7); Sheep (4)
V. vinifera rubra leaves								

Based on a search in Web of Science using 'plant name', 'ammonia', 'methane' and 'fermentation' as key words for the period from 1990 to 2018.
DM, dry matter; NE_L, net energy for lactation; uCP; utilizable crude protein.
1 = Banik et al., 2013; 2 = Bodas et al., 2008; 3 = Jayanegara et al., 2011; 4 = Kamalak 2005; 5 = Macheboeuf et al., 2014; 6 = Niderkorn and Macheboeuf 2014; 7 = Peiretti et al., 2017; 8 = Scharenberg et al., 2007; 9 = Selje et al., 2007; 10 = Tavendale et al., 2005; 11 = Williams et al., 2011; 12 = Chen et al., 2011.

2.4 Results

The content of OM (g/kg DM) for the test plant material lots ranged from 816 for the aerial part from *S. officinale* to 981 for the kernel from *P. spinosa* (Table 2.3). The variation was smaller in CP, with a difference of 145 g/kg DM between the highest (*R. nigrum* leaves) and the lowest (*P. spinosa* fruit pulp) values. Extremely small amounts of EE were found in both lots of *P. alba* roots, *L. corniculatus* aerial part and the fruit pulp of *P. spinosa.* In contrast, lots of *P. spinosa* kernels showed nearly ten times more EE (about 100 g/kg DM). The kernel of *P. spinosa* contained the most NDF (673 and 693 g/kg DM), and the root of *P. alba* contained the least NDF (183 and 164 g/kg DM). The aerial part of *E. angustifolium* showed the largest difference (71 g NDF/kg DM) between both lots. The contents of ADF (g/kg DM) were highest in the *S. caprea* leaves (627) and lowest in the *P. alba* root lots (99 and 106). The same plants were contrasting in ADL with on average 419 versus 1 to 5 g/kg DM, respectively. Among all plant materials, the leaves of *A. uva-ursi* clearly showed the highest contents of TP, NTP, TT and HT. All of them were > 80 g/kg DM. The kernel of *P. spinosa* had the lowest TP, TT and HT contents; all < 10 g/kg DM. The plant materials from *A. hippocastanum* (fruit)*, P. alba* (root)*, P. spinosa* (entire fruit, kernel, pulp) and *S. caprea* (leaves) were the only with < 10 g NTP/kg DM. In the leaves of *C. avellana,* the highest content (g/kg DM) of CT (65·4) was found. It was 500 and 260 times higher than the contents found in the basal diet (0·13) and in the aerial part of *S. officinale* (0·25), respectively.

The pH of the incubation fluid always ranged between 6·7 and 6·9. The addition of different test plant materials to the basal diet caused a large variation in the magnitude of effects compared to the incubation of the basal diet alone (Tables 2.4 and 2.5). All plant materials decreased the ammonia concentrations ($P < 0{\cdot}05$) by up to 36% compared to the basal diet as sole substrate. The bacteria count was affected ($P < 0{\cdot}05$) by some test plant materials. The count was high with the leaves of *V. vinifera rubra* and low with the leaves of *P. tremula*. Protozoa counts remained unaffected by the supplements compared to the basal diet (value adjusted to 240 mg DM incubated). All plant supplementations resulted in a decrease ($P < 0{\cdot}05$) in total SCFA concentration. The molar proportion of acetate in total SCFA was influenced by the plant additives ($P < 0{\cdot}05$), but only with the addition of the leaves of *B. pendula* and *C. sativa* the molar proportion was lower compared to the basal diet as sole substrate.

Propionate proportions of total SCFA in the incubation fluid were increased ($P < 0{\cdot}05$) by nine of the plant supplements compared to the control. The acetate-to-propionate ratio was highest at 3·74:1 for the *A. uva-ursi* leaves and lowest for the *C. sativa* leaves at 3·27:1. It was reduced ($P < 0{\cdot}05$) by nine of the plant supplements compared with the basal diet alone.

Butyrate proportion was slightly reduced ($P < 0{\cdot}05$) with the aerial part of *S. officinale,* and the *iso*-butyrate proportion was not influenced by any of the plant supplements. Molar proportions of valerate were decreased ($P < 0{\cdot}05$) by only the *B. pendula* leaves, and only the leaves of *B. pendula* and *P. tremula* increased the *iso*-valerate ($P < 0{\cdot}05$).

Compared to the basal diet alone, the total gas production of the incubated diets was increased ($P < 0{\cdot}05$) only by supplementing with the root of *P. alba* (Table 2.5). In contrast, with leaves of *P. tremula*, the aerial part of *E. angustifolium*, leaves of *C. sativa* and the kernel of *P. spinosa* the total gas amount was reduced ($P < 0{\cdot}05$) between 4 and 8 ml/24 h. The remaining 14 plant additives did not affect the total gas produced compared to the basal diet alone. Accordingly, IVOMD and the amount of OM digested in 24 h declined ($P < 0{\cdot}05$) with additions of *P. tremula, E. angustifolium, C. sativa* and kernels of *P. spinosa*. The amount of CH_4 produced per unit of dOM was reduced ($P < 0{\cdot}05$) by 13 of the plant additives compared to the control. This was to an extent of 0·05 to 0·20 of the basal diet alone.

The methane yield per unit of dietary DM and absolute CH_4 production in ml/24 h (adjusted to 240 mg of diet DM) showed similar results. *C. sativa* leaves exhibited the greatest CH_4 mitigating effect, and the kernels of *P. spinosa* exhibited the smallest effect. The carbon dioxide per unit of feed DM was 183 ml/g in the basal diet, and it was lowered ($P < 0{\cdot}05$) up to 25 ml/g DM by the aerial part of *E. angustifolium,* the leaves of *C. sativa,* and the kernels of *P. spinosa*. It was increased ($P < 0{\cdot}05$) by the root of *P. alba* (+20 ml/g DM).

The same plant supplements were similarly effective ($P < 0{\cdot}05$) in changing the amount of carbon dioxide per 24 h. The CH_4-to-carbon dioxide ratio was reduced ($P < 0{\cdot}05$) by ten of the plant supplements, with the highest decrease of 30 ml CH_4/l of carbon dioxide in the case of *C. sativa* leaves. Only a few test plant materials affected the CH_4-to-SCFA ratio. The ratio increased with the root of *P. alba* and the whole fruit of *P. spinosa* and decreased with the leaves of *C. sativa*. Adding five of the test plant materials reduced ($P < 0{\cdot}05$) the NE_L content of the diet to a degree ranging from 0·41 (*P. tremula* leaves) to 0·77 (*C. sativa* leaves) kJ/g of feed DM. Only one, the root of *P. alba*, increased ($P < 0{\cdot}05$) the NE_L content by 0·55 kJ/g of feed DM. The uCP content (g/kg DM) was reduced ($P < 0{\cdot}05$) by only one plant supplement, the kernel of *P. spinosa*. Adding the leaves of *R. nigrum, C. avellana* and the aerial part of *E. angustifolium* even increased the uCP content by about 12 g/kg DM.

The lot effect was only significant ($P < 0{\cdot}05$) with the kernel of *P. spinosa* for protozoal numbers and *iso*-valerate, with the pulp of *P. spinosa* for *iso*-valerate, with the leaves of *R. nigrum* for *iso*-butyrate, and with the leaves of *V. vinifera rubra* for total gas.

Table 2.3 Chemical composition (g/kg dry matter) of the basal diet and the experimental plant materials

Plant species	Lot	OM	CP	EE	NDF	ADF	ADL	TP	NTP	TT	CT	HT
Basal diet		927	146	31	426	275	36	16·5	14·9	1·60	0·13	1·47
A. hippocastanum	1	974	80	58	224	195	122	17·7	7·79	9·92	8·77	1·15
	2	974	77	52	213	208	106	20·2	7·45	12·7	7·24	5·48
A. uva ursi	1	973	40	55	230	190	121	236	87·5	149	6·74	142
	2	970	39	54	232	182	108	265	90·6	175	7·70	167
B. pendula	1	936	141	50	467	282	152	54·2	23·0	31·1	14·2	16·9
	2	953	138	42	408	231	116	72·2	51·0	21·3	16·2	5·04
C. sativa	1	956	106	56	406	260	94	148	23·0	125	3·92	121
	2	956	107	56	406	271	104	158	26·3	131	4·63	127
C. avellana	1	923	119	27	409	262	96	97·1	36·2	61·0	57·2	3·79
	2	927	104	30	418	289	136	113	40·5	73·0	65·4	7·59
E. angustifolium	1	927	121	15	473	406	108	72·8	13·7	59·2	0·95	58·2
	2	939	125	19	402	338	94	85·1	16·1	69·0	0·48	68·6
G. urbanum	1	908	129	17	327	272	57	89·1	21·4	67·7	8·78	59·0
	2	912	116	9	394	322	51	118	21·6	96·8	2·82	94·0
L. corniculatus	1	914	138	10	588	434	112	18·6	12·3	6·33	1·50	4·83
	2	912	135	7	570	460	104	20·1	13·8	6·35	1·84	4·51
P. alba	1	966	64	4	183	99	5	15·7	2·25	13·5	2·04	11·5
	2	968	63	7	164	106	1	15·7	1·76	13·9	1·92	12·0
P. tremula	1	916	111	38	387	309	122	88·8	34·9	53·9	28·8	25·1
	2	923	106	31	405	310	145	87·0	31·1	55·9	41·5	14·4
P. spinosa fruit	1	952	48	47	425	330	191	18·3	7·78	10·6	8·23	2·32
	2	927	46	40	430	360	229	10·3	6·06	4·22	3·58	0·64
P. spinosa kernel	1	980	75	95	693	551	295	7·20	5·05	2·13	1·22	0·91
	2	981	90	100	673	551	251	4·61	2·93	1·68	1·20	0·49
P. spinosa pulp	1	889	24	7	256	209	156	22·0	9·12	12·9	6·34	6·54
	2	880	26	5	241	203	125	13·0	5·70	7·28	4·1	3·18
R. nigrum	1	901	154	30	287	249	145	83·1	25·8	57·3	37·5	19·8
	2	899	169	33	295	270	154	73·6	33·2	40·4	29·7	10·7
S. caprea	1	915	68	28	660	627	419	10·7	5·64	5·06	1·68	3·38
S. officinale	1	816	139	11	315	298	141	33·5	21·0	12·4	0·15	12·3
	2	816	143	14	326	305	149	35·6	22·3	13·3	0·25	13·0
V. vinifera	1	911	113	35	312	202	87	85·9	14·2	71·7	19·6	52·1
	2	905	146	37	320	220	105	88·3	27·4	60·9	34·3	26·7
V. vinifera rubra	1	888	75	70	293	225	79	87·3	25·4	61·9	23·3	38·7
	2	892	101	48	287	252	118	58·3	21·0	37·2	10·8	26·4

Values are means of duplicate or triplicate analysis; OM, organic matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; TP, total phenols; NTP, non-tannin phenols, TT, total tannins; CT, condensed tannins; HT, hydrolysable tannins

Table 2.4 Fermentation characteristics as measured in the incubation fluid after incubation with the basal diet as sole substrate or supplemented with the experimental plants at a ratio of 5:1 (basal diet:plant)

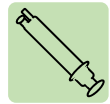
Plant species*	Ammonia (mmol/l)	Total bacteria (10^9/ml)	Total protozoa (10^4/ml)	Short chain fatty acids (mmol/l) and their molar proportions (mmol/mol))							
				Total	C_2	C_3	C_4	*iso* C_4	C_5	*iso* C_5	C_2/C_3 (x:1)
Basal diet†	12·6 (10·5)	6·19 (5·13)	4·01 (3·32)	97·4 (81·1)	676	185	108	8·0	9·9	12·3	3·66
A. hippocastanum	**8·60**	6·04	3·03	**88·9**	671	**196**	107	6·1	8·9	11·1	**3·45**
P. spinosa fruit pulp	**8·96**	5·60	3·65	**89·7**	666	**199**	108	5·7	10·3	11·0	**3·36**
P. alba	**8·07**	5·22	4·47	**92·9**	669	**203**	103	5·3	9·3	11·1	**3·31**
P. spinosa whole fruit	**9·36**	4·72	4·51	**88·1**	667	**195**	109	6·9	10·3	12·1	**3·44**
L. corniculatus	**10·1**	5·18	4·57	**88·5**	672	**191**	105	7·1	11·2	12·8	**3·52**
P. spinosa fruit kernel	**10·7**	**4·66**	3·66	**83·4**	669	**192**	109	7·0	10·3	13·1	**3·50**
V. vinifera rubra	**9·45**	6·22	3·55	**87·3**	681	187	106	5·9	9·3	11·0	3·67
S. caprea	**9·94**	5·26	3·14	**82·0**	676	186	109	6·6	9·7	12·5	3·64
B. pendula	**9·37**	5·06	3·64	**88·7**	**660**	**193**	114	7·5	**8·2**	**17·2**	**3·45**
V. vinifera	**8·90**	5·76	3·44	**87·8**	680	185	108	6·4	9·3	11·7	3·69
P. tremula	**9·22**	**4·49**	4·08	**87·8**	666	189	113	6·6	9·2	**16·1**	3·55
R. nigrum	**9·12**	5·40	3·60	**88·0**	676	188	107	7·2	9·3	12·1	3·60
G. urbanum	**8·78**	5·65	3·83	**86·7**	675	190	108	6·2	9·7	11·2	3·56
A. uva ursi	**8·29**	5·10	3·56	**89·7**	687	184	104	6·4	8·6	10·7	3·74
S. officinale	**9·38**	5·62	3·33	**86·6**	672	**198**	**102**	6·3	9·8	11·8	**3·40**
C. avellana	**8·45**	5·39	3·44	**85·4**	680	187	105	6·0	8·6	12·4	3·64
E. angustifolium	**8·61**	5·83	3·11	**84·8**	675	190	109	6·3	9·6	11·1	3·56
C. sativa	**8·54**	4·85	3·23	**84·5**	**660**	**203**	110	6·1	9·3	10·7	**3·27**
S.E.M.	0·741	1·062	0·814	4·39	3·6	4·6	4·1	0·76	0·31	0·90	0·084
D.F.	195	195	195	195	195	195	195	195	195	195	195
P value plant species effect	<0·001	0·002	0·199	<0·001	<0·001	<0·001	<0·001	0·292	<0·001	<0·001	<0·001

C_2, acetate; C_3, propionate; C_4, butyrate; C_5, valerate; C_2/C_3, acetate/propionate ratio.
Least-square means of six incubation runs × 2 plant material lots
Values in bold differ ($P < 0.05$) from basal diet as sole substrate.
* Plants ordered according to declining amount of CH_4 relative to dOM (cf. Table 2.5).
† Variables where two values are given: values outside of brackets are least-square means from data adjusted to 240 mg dry matter equivalent to the other treatments. Values in brackets give least-square means from data actually measured with 200 mg dry matter of the basal diet.

Table 2.5 Effects of the plant species incubated at a ratio of 1:5 with the basal diet on gas production, digestibility, as well as calculated contents of net energy for lactation (NE_L) and utilizable crude protein at the duodenum (uCP) in dry matter (DM).

Plant species*	Total gas (ml/24 h)	IVOMD (mg/g)	dOM (mg/24 h)	CH_4/DM (ml/g)	CO_2/DM (ml/g)	CH_4 (ml/24 h)	CO_2 (ml/24 h)	CH_4/dOM (ml/g)	CH_4/CO_2 (ml/l)	CH_4/SCFA (mmol/mol)	NE_L (kJ/g DM)	uCP (mg/g DM)
Basal diet†	52·7 (43·9)	660	158 (132)	33·2	183	7·97 (6·64)	43·8 (36·5)	50·4	182	121	5·14	161
A. hippocastanum	53·6	656	159	32·8	186	7·86	44·7	49·5	176	129	5·22	165
P.spinosa fruit pulp	53·3	661	159	32·8	184	7·86	44·2	49·4	178	128	5·18	152
P. alba	**58·1**	689	**167**	34·2	**203**	8·22	**48·6**	49·3	**169**	**133**	**5·69**	168
P.spinosa whole fruit	52·7	651	157	32·1	181	7·71	43·5	49·2	178	**130**	5·12	152
L. corniculatus	51·1	649	155	31·8	178	7·63	42·6	49·1	179	128	4·96	155
P. spinosa fruit kernel	**47·0**	**607**	**147**	**29·3**	**162**	**7·03**	**38·8**	**47·8**	181	124	**4·58**	**143**
V. vinifera rubra	50·3	641	153	**30·5**	175	**7·32**	42·1	**47·8**	174	123	4·89	158
S. caprea	48·0	**621**	150	**29·7**	167	**7·13**	40·0	**47·5**	179	128	**4·66**	148
B. pendula	49·5	634	152	**29·6**	174	**7·11**	41·7	**46·7**	**171**	118	4·82	167
V. vinifera	49·8	640	153	**29·8**	173	**7·14**	41·6	**46·6**	172	119	4·84	169
P. tremula	**48·7**	**628**	**150**	**29·1**	170	**6·97**	40·7	**46·4**	**172**	118	**4·73**	163
R. nigrum	50·7	650	155	**30·0**	177	**7·20**	42·5	**46·4**	**170**	121	4·94	**172**
G. urbanum	50·0	641	153	**29·4**	176	**7·10**	42·2	**46·0**	**167**	119	4·86	169
A. uva-ursi	51·2	635	153	**29·3**	179	**7·02**	42·8	**45·9**	**164**	115	4·97	161
S. officinale	50·1	654	154	**29·3**	175	**7·03**	42·0	**45·7**	**168**	121	4·87	167
C. avellana	49·0	631	151	**28·3**	171	**6·79**	41·0	**44·9**	**166**	118	4·77	**173**
E. angustifolium	**47·0**	**616**	**148**	**27·5**	**162**	**6·60**	**39·0**	**44·6**	**169**	115	**4·56**	**173**
C. sativa	**44·8**	**595**	**143**	**24·0**	**158**	**5·77**	**38·0**	**40·3**	**152**	**101**	**4·37**	170
S.E.M.	0·82	0·61	1·5	0·77	3·7	0·184	0·74	1·06	4·4	6·4	0·256	8·38
D.F.	195	195	195	195	195	195	195	195	195	195	195	125
P value plant species effect	<0·001	<0·001	<0·001	<0·001	<0·001	<0·001	<0·001	<0·001	<0·001	<0·001	<0·001	<0·001

dOM, digestible organic matter; CH_4, methane; CO_2, carbon dioxide; IVOMD, *in vitro* organic matter digestibility; SCFA, short-chain fatty acids.
Least-square means of six incubation runs × 2 plant material lots.
Values in bold differ ($P < 0.05$) from basal diet as sole substrate.
* Plants ordered according to declining amount of CH_4 relative to dOM.
† Variables where two values are given: values outside of brackets are least-square means from data adjusted to 240 mg dry matter equivalent to the other treatments.
Values in brackets give least-square means from data actually measured with 200 mg DM of the basal diet.



2.5 Discussion

2.5.1 Lot effects

The accuracy and repeatability of the results of plant screenings for feeding value and mitigation properties also depend on the recovery of the effects in other lots of the same plant material. Various factors may change the composition of woody plants and influence their digestibility. Palo et al. (1985) showed that the content of phenolic acids in fine twigs of *B. pendula* varies between April and July. At the same time, there were changes in the contents of CP (8-16%) and NDF (46-54%). Palo et al. (1985) and Sauter & Wellenkamp (1998) showed how the harvest period could have an effect on the composition of the plant material. Nour et al. (2014) noted clear differences in leaves of *R. nigrum* in phenolic and mineral content among different cultivars in a sampling period between June and August. In mid-June, they found the highest phenolic contents, followed by a decrease until August. Variability in woody plants seems to be particularly large in species that are very rich in PSC. Accordingly, tropical shrubs differed substantially in contents of nutrients and condensed tannins, as well as in their efficiency to affect ruminal fermentation and methanogenesis when grown on different soils, in varying climates and under different fertilisation treatments (Tiemann et al. 2009 and 2010) or when originating from different cultivars (Bekele et al. 2009). *In vitro* studies often used only one lot per plant material screened. In the present study, the two lots of every plant material differed in harvest time, country of harvest or supplier (with the exception of *L. corniculatus, P. alba* and *P. spinosa)*, as well as in nutrient and PSC contents. Significant lot effects were only found in five out of a total of 391 combinations investigated (17 test plant materials with two lots × 23 variables), the lot effects were considered negligible in the present set-up and plant material. The use of two lots per plant material cannot represent the entire natural variation of the plant material, but is nevertheless an approach to take it into account.

2.5.2 *In vitro* fermentation, feed nutritive value and potential side-effects

The use of materials from trees and shrubs as forage for ruminants is often limited by a high prevalence of PSC (Papanastasis et al. 2008). At the same time, woody plants are frequently underestimated for their nutritive value. The present *in vitro* screening provided several traits indicative of the feeding value of the test plant materials and the total diet. The high feeding value of the supplemented diets was indicated by variables demonstrating the presence of an intensive ruminal nutrient degradation like total gas production, high IVOMD and NEL contents.

All plant materials, except the kernels of *P. spinosa* and the leaves of *S. caprea*, increased total SCFA when compared to the value measured with 200 mg DM of the basal diet, resulting from the fermentation of the additional organic material (200 + 40 mg). When comparing the amount of total SCFA found with the basal diet (adjusted to 240 mg DM) and those with the added test plant material, findings indicated that the plant materials indeed were less fermentable than the basal diet. Nevertheless, IVOMD was similarly high for many of the plant additives compared to the basal diet as sole substrate. The plant materials causing the lowest total SCFA concentration also exhibited the lowest IVOMD and total gas production.

In the present study, the plant materials with the highest ADL content had the comparably lowest feeding value; these were the kernels of *P. spinosa* and the leaves of *S. caprea*. With these materials, the effect of the tannins on digestibility could have been superimposed by the effect of ADL. The leaves of *C. sativa* and *P. tremula* (the former rich in tannins) likewise showed a low feeding value. Corresponding with the latter, Jayanegara et al. (2012) described a decrease of total SCFA with an increasing dietary tannin level, and Tavendale et al. (2005) found inverse relationships of CT concentrations with SCFA. Adding these four plant materials to the basal diet also eventually resulted in low NE_L contents. The comparably low feeding value of *C. sativa* leaves was determined earlier (Jayanegara et al. 2011). The content of uCP was reduced only with the addition of the kernels of *P. spinosa.* This finding could have derived from the decrease in IVOMD and thus in fermentable OM needed for synthesis of microbial protein. Adding the plant materials which increased the uCP (leaves of *R. nigrum* and *C. avellana*, aerial part of *E. angustifolium*) compared to the basal diet was accompanied by a decrease in protein degradation to ammonia thus resulting in more rumen-bypass protein. The digestibility of *L. corniculatus* found in earlier studies ranged from 62% to 79% for DM (Yang et al. 2011; Banik 2013, incubated as plant alone), and it was 54% for OM (Williams et al. 2011) when including the plant at 400 g/kg DM. The leaves of *V. vinifera* were described by Kamalak (2005) and Peiretti et al. (2017) as plants with a high nutritive value for ruminant feeding. When the leaves of *V. vinifera* (green and red) were added to the basal diet in the present study, the IVOMD and uCP values were not affected. This supported the findings of the aforementioned studies.

The current results show that the majority of the plant materials chosen for the present screening study are quite well-suited as feed supplements from a net energy point of view. With the root of *P. alba,* the total gas amount and dOM were even increased. It seems that the root of *P. alba* even exceeded the feeding value of the basal diet. Bodas et al. (2008) tested six promising plants (i.e., *C. pycnocephalus*, *P. tremula*, *P. avium, Q. robur, R. nobile* and *S.*

caprea) from the original 500 samples in the Rumen Up project in more detail for their effect on digestibility, total gas, methane and SCFA production *in vitro* and concluded that leaves (and the little stem) of *P. tremula* were more promising than leaves (and the little stem) of *S. caprea*. This can be only partially confirmed by the present results of the leaves of *P. tremula* and *S. caprea*. In addition, woody plants may have favourable and unfavourable effects not obvious from *in vitro* rumen fermentation studies. Accordingly, incorporating woody plants or plants rich in PSC, including some of the plant materials tested in the present study, in animal diets might be advantageous to health and performance (Waghorn 2008). For example, extracts from woody plants (*Rubus fructicosus, Quercus robur* and *C. avellana*) had an inhibitory effect on worms of gastrointestinal nematodes *in vitro* (Paolini et al. 2004). The study by Marley et al. (2003) showed that faecal egg counts of helminth parasites were reduced in lambs grazing on *L. corniculatus.* In regard to performance, feeding *L. corniculatus* increased the milk yield in dairy cows (Woodward et al. 2000; 2004). Besides, feed with a high HT content can be toxic to the animal (Waghorn 2008). Therefore, the leaves of *A. uva-ursi* and *C. sativa* have to be used carefully in animal nutrition, although they may still be applicable after sufficient adaptation (Waghorn 2008). Pyrrolizidine alkaloids, present in *S. officinale* (Stickel and Seitz, 2000), and amygdalin, occurring in the kernel of *P. spinosa* (Kumarasamy et al. 2003), may also be toxic after ingestion. Great care is required when these materials are used in animal nutrition.

2.5.3 Ammonia mitigation potential of the test plant materials

Compared to the ammonia value from the basal diet alone, the reduction in ruminal ammonia by more than 3 mmol/l that was caused by 15 of the 18 plant supplements (value calculated for 240 mg DM incubated weight) is consistent with the numerical decrease in bacterial numbers. *A. hippocastanum* fruit, *A. uva-ursi* leaves, *C. avellana* leaves, *C. sativa* leaves, *E. angustifolium* aerial part and *P. alba* root were particularly effective, as they caused a decrease of at least 4 mmol ammonia/l. Bacteria are major producers of ammonia from dietary protein, and tannins can inhibit the growth of rumen bacteria by binding to bacterial cells (Molan et al. 2001). The tannins may reduce the export or activity of microbial enzymes or inhibit the separation of cells after division. Other studies found a significant decrease in bacterial growth with extracts from *C. avellana* leaves (Oliveira et al. 2007) and *E. angustifolium* herb (Rauha et al. 2000), which might be explained by the usage of extracts. Selje et al. (2007) tested eight promising plants of the original 500 samples from the Rumen Up project in more detail for their ruminal protein degradation effect *in vitro*. Among these, *A. uva-ursi* also inhibited proteolysis.

Macheboef et al. (2014) and Niderkorn et al. (2014) found ammonia-reducing effects with *E. angustifolium* as well. Besides their antimicrobial effects, tannins, especially CT, form non-soluble tannin-protein complexes, thereby preventing ruminal degradation of feed proteins by the microbes (Kumar & Singh 1984). The three plant materials (the aerial part of *L. corniculatus*, the kernel of *P. spinosa* and the leaves of *S. caprea*) with the lowest effect on ammonia formation (i.e., reduction of < 3 mmol/l) had CT and TT contents of < 2 and < 7 g/kg DM, respectively.

2.5.4 Methane mitigation potential of the test plant materials

Supplementing feed with plants rich in PSC is a promising strategy to reduce CH_4 emissions from ruminants (Jayanegara et al. 2012; Hristov et al. 2013). The mode of action of tannin and non-tannin phenols is their toxicity to some rumen microorganisms e.g. by inhibiting their growth as outlined in the preceding section. In the present study, 13 of the 18 plant materials decreased CH_4 per unit of digestible OM compared to the basal diet without supplements. Relating CH_4 to DM supply and CO_2 produced also pointed towards a similar result. All these measures consider that CH_4 mitigation is only useful in relation to nutrients and energy supplied to the animal. The CH_4 mitigating effect of the plant materials used in the present study was occasionally less pronounced than that indicated by Bodas et al. (2008) as part of the Rumen Up report, although these authors used a lower dosage (91 g/kg DM, Table 2.1). One reason might be the difference in the basal diet, because mainly forage (alfalfa hay) with a high CH_4 formation potential was used by Bodas et al. (2008) as part of the Rumen Up project. The present study used a mixed diet containing concentrate and maize silage. In the Rumen Up report, *S. caprea, P. tremula* and *C. avellana* were described to reduce CH_4 by more than 0·25 of total, and the root of *P. alba* even reduced it by 0·54 of total.

In the present study, such a reduction was not found for the root of *P. alba*; it even slightly increased the CH_4 formation per unit of DM or digestible OM when added to the basal diet. Variation in results of plant material between studies might be attributed to different collection periods, drying methods or storage durations. Also, the leaves of *S. caprea* only moderately reduced CH_4 when compared to the basal diet. In the present case, this material constituted fallen autumn leaves collected during winter. The biological degradation until collection obviously resulted in the highest contents of lignified fibre (ADF and ADL) of all plant materials investigated, whereas very few phenols were recovered. Among test plant materials, *C. sativa* leaves (positive control) were the most efficient in CH_4 mitigation, consistent with findings from Jayanegara et al. (2011) and Bhatta et al. (2009). However, *C. sativa* leaves also

suppressed total gas and CO_2 production, showing that a part of the mitigation effect originated in a general inhibition of ruminal fermentation and, consequently, the feeding value was low. The *C. sativa* leaves had the highest HT content of all plant materials. The addition of *C. avellana* leaves and *E. angustifolium* aerial part to the basal diet reduced CH_4 per digestible OM by > 5 ml/g. In the case of *C. avellana,* total gas and CO_2 formation was not affected. In the case of *E. angustifolium*, these indicators of fermentation intensity were depressed, like with *C. sativa*.

In a feeding experiment with sheep, Wang et al. (2018) demonstrated that *C. avellana* leaves also have a clear methane-mitigating effect *in vivo*. As a sole substrate, *L. corniculatus* has been reported to reduce CH_4 emissions *in vivo* (Pinares-Patiño et al. 2003; Woodward et al. 2004) and *in vitro* (Williams et al. 2011). However, in the present study, the addition of *L. corniculatus* aerial part at levels of 167 mg/g had no CH_4 mitigating effect *in vitro*. In the screenings of Macheboef et al. (2014) and Niderkorn et al. (2014), *L. corniculatus* was not effective in reducing CH_4.

Protozoa are involved in ruminal CH_4 formation. Because none of the plant materials decreased protozoal counts, the CH_4 mitigating effects most likely resulted from other influences on fermentation. In line with our findings, the meta-analysis of Jayanegara et al. (2012) revealed no relationship of dietary tannins and protozoal counts *in vitro*. In the Rumen Up report, *B. pendula*, *G. urbanum*, *P. tremula* and *S. officinale* were described to reduce the bacteriolytic activity of protozoa. Therefore, it can be speculated that the plant materials in the present study might have affected the activity of protozoa at unchanged total numbers.

In the present study, there was a shift towards a higher proportion of propionate with nine of the additives, while the acetate and butyrate proportions were not influenced by almost all of the plant additives. Consequently, the acetate-to-propionate ratio was decreased by these nine plant materials. In this variable, *C. sativa* leaves and *P. alba* root caused the highest decrease. However, among the materials decreasing the acetate-to-propionate ratio, unexpectedly only *B. pendula* (leaves), *C. sativa* (leaves), *P. spinosa* (kernel) and *S. officinale* (aerial part) also mitigated the CH_4 formation. Consequently, in the present study, the change in the SCFA profile was not the only reason for the observed decrease in CH_4 formation.

A higher potential to reduce methanogenesis in plant extracts containing both HT and CT than in those containing only HT was reported by Bhatta et al. (2009). However, this was not the case in the present study, as plant materials with high or low HT proportions were effective in mitigating CH_4 formation (highest ratios of HT to CT contents: 31:1 in *C. sativa* leaves and 1:15 in *C. avellana* leaves). There seems to be a clear general linear relationship between tannin

(CT, HT or CT+HT) content in feed and methanogenesis, as quantified by Jayanegara et al. (2012) in a meta-analysis. Accordingly, the plant materials in the present study, which had a notable total tannin content (≥ 40 g/kg), showed substantial CH_4 mitigation (i.e., the leaves of *C. avellana*, *C. sativa* and *R. nigrum* or the aerial part of *G. urbanum*). In the present study, even dietary tannin contents below the threshold (> 20 g tannins/kg, *in vitro* and *in vivo*) described in the meta-analysis by Jayanegara et al. (2012) were effective in CH_4 mitigation. The suggestion by Tavendale et al. (2005) that CT concentrations below about 80 g/kg DM can reduce CH_4 without inhibiting fermentation rate were also confirmed through the examples from the leaves of *B. pendula*, *C. avellana, R. nigrum* and *V. vinifera* and the aerial part of *G. urbanum* which caused no decrease in total gas production.

2.6 Conclusion

Methane formation per unit of digestible OM was reduced by 13 out of the 18 plant materials compared to the basal diet, and all materials decreased ammonia formation. The majority of the mostly woody plant supplements tested had no adverse effect on *in vitro* digestibility. Further *in vitro* studies should include the most promising and feasible plant materials as the leaves of *B. pendula*, *C. avellana, R. nigrum* and *V. vinifera* and the aerial part of *G. urbanum* at varying dosages and as sole substrates. Finally, experiments with live animals need to evaluate the palatability of the plant materials, confirm their mitigating effects and evaluate effects on production.

2.7 Acknowledgements

The authors are grateful to M. Wolter, C. Kunz, M. Mergani and P. Stirnemann for their contribution to the laboratory analyses.

The present study was funded by ETH Zurich Research Grant number ETH-49 15-1. The authors are grateful to M. Wolter, C. Kunz, M. Mergani and P. Stirnemann for their contribution to the laboratory analyses.

Chapter 3

Dose-response effect of forages from woody and herbaceous plants to *in vitro* ruminal methane and ammonia formation, and their short-term palatability in lactating cows

This chapter is based on Terranova, M., Wang, S., Eggerschwiler, L., Braun, U., Kreuzer, M. and Schwarm, A. Submitted to *Animal*, under review.

3.1 Abstract

Plant secondary compounds (PSC) are prevalent in a number of woody temperate climate species and play a central role in dietary attempts for methane mitigation in ruminants. However, their application requires sufficient palatability and feeding value. In the present study, leaves from silver birch (*Betula pendula*), hazel (*Corylus avellana*), blackcurrant (*Ribes nigrum*), green grape vine (*Vitis vinifera*) and the herbs rosebay willow (*Epilobium angustifolium*) and wood avens (*Geum urbanum*), were tested in various dosages with the Hohenheim gas test method *in vitro* and for their short-term palatability in dairy cows. For the latter, the plants were pelletd with lucerne at proportions to obtain the same phenol content, but realised contents differed from expected contents. The pellets were provided separately from a mixed basal ration (0.4:0.6) in randomized order to each cow for 3 days per plant. All plants mitigated *in vitro* methane and ammonia formation, and this often linearly with dosage. The level of effect differed between plants. Proportions of 100 (hazel, rosebay willow) to 400 g/kg of plant were necessary to reach significance in these variables. The test plants had a lower feeding value than the high-quality basal diet as indicated by *in vitro* organic matter digestibility, short-chain fatty acid formation and calculated contents of net energy of lactation. At the same time, the depression in ammonia formation showed that the plants may linearly enhance utilisable crude protein in the diet with increasing dosage. Four of the six plants were as palatable as lucerne alone, but blackcurrant and birch were eaten significantly less well. This aversion decreased on day 3 of offer. Rosebay willow pellets with the highest phenol content were not least palatable. This suggests that PSC are not the main factor of influence on palatability, and correlation analysis revealed that lignin content may be more decisive in this respect. There was no substantial difference between the plants in their short-term effect on milk yield and composition, and all plants substantially reduced milk urea content. Overall, the results suggest that hazel and vine leaves as well as rosebay willow and wood avens herbs are worthwhile to be tested for their *in vivo* mitigation potential concerning methane and N emission.

3.2 Implications

Plants rich in phenols as feed supplements in ruminant diets ideally help mitigating methane and nitrogen emissions at unchanged feed intake. Four of six plants tested in the present study, the leaves of hazel and green grape vine as well as the herb of rosebay willow and wood avens, dose-dependently reduced methane and ammonia formation *in vitro*. These plants were also

highly palatable in the short-term when fed as supplements to lactating dairy cows. Thus, their use might assist in mitigating environmentally harmful emissions from ruminant production and increase plant biodiversity at the same time.

3.3 Introduction

Plant secondary compounds (PSC), such as phenols, play an important role in the development of feeding strategies for ruminants aiming at abating methane and nitrogen emissions. Especially forage from trees, shrubs and herbaceous legumes may be rich in PSC (Frutos et al., 2004). Compared to tropical regions, however, temperate climate plants rich in effective PSC with a moderately high feeding value are rather rare. An extensive *in vitro* screening still revealed a number of woody plants which, in a common total mixed ration, mitigated ruminal methane and ammonia formation without affecting diet digestibility (Terranova et al., 2018). In addition, the PSC dosage has a high relevance in this context. Jayanegara et al. (2012) described in a meta-analysis, that >20 g tannins/kg diet DM are needed to effectively mitigate methane. According to Aerts et al. (1999), between 20 and 40 g condensed tannins (CT)/kg DM are required to improve protein utilization. However, elevated dietary PSC contents may impede intake and digestibility (Kumar and Singh, 1984; Beauchemin et al., 2008; Waghorn, 2008). Even 20 g tannins/kg DM were found to let grazing animals reject the feed (Donnelly and Anthony, 1969), and >50 g tannins/kg DM were frequently found to depress voluntary feed intake (Aerts et al., 1999; Barry and McNabb 1999). This illustrates that the range of the optimum tannin supply is small.

The aim of the present study was to quantify dose-response relationships *in vitro* and palatability in dairy cows for forages from six temperate climate woody plants which had turned out to be promising in an extensive screening (Terranova et al., 2018). These were leaves of birch (*Betula pendula*), hazel (*Corylus avellana*), blackcurrant (*Ribes nigrum*) and vine (*Vitis vinifera*), and the rosebay willow (*Epilobium angustifolium*) and wood avens (*Geum urbanum*) herb. To the knowledge of the authors, the palatability so far has only been tested in sheep and only for birch leaves (voluntary intake; Meier et al., 2014) and hazel leaves (level of refusals of pellets with different proportions; Wang et al., 2018). The highly tanniferous but low palatable (Meier et al., 2014) leaves of sweet chestnut (*Castanea sativa*) were included as an *in vitro* control as they are known to efficiently suppress ruminal methane formation (Jayanegara et al., 2011). The hypotheses tested were (1) plant dosages and ruminal fermentation variables are (linearly) related and (2) plant composition and their palatability are related.

3.4 Animals, materials and methods

3.4.1 Test plant material

For both experiments material from the seven test plants consisting of two production lots was obtained from Alfred Galke GmbH (Bad Grund, Germany), Dixa AG (St. Gallen, Switzerland), Ried pharmacy (Konstanz, Germany) and Herbathek Naturheilmittel (Berlin, Germany). All materials were available dried (at maximum 40°C) and cut (size of 4 to 6 mm). Test plant material was supplemented to mixed rations with (*in vivo*) or without (*in vitro*) lucerne as specified in the sections below.

3.4.2 Dose-response experiment (*in vitro*)

The *in vitro* model used was the Hohenheim Gas Test (HGT) operated according to Menke and Steingass (1988) and Soliva and Hess (2007). Rumen fluid was obtained after morning feeding from a cannulated Brown Swiss cow in mid lactation fed first and second cut grass hay and a mineral supplement. After being strained through four layers of cheese cloth (1 mm pore size), rumen fluid was added to the Menke buffer solution (ratio 1:2). The buffered rumen fluid (30 ml) and the substrate (200 mg DM, 1 mm size) were incubated in syringes with two outlets. All test plants (1:1 mixtures of the two lots each) were incubated in dosages of 100, 200, 300, 400, 500 and 1000 g/kg replacing increasing amounts of a mixed ration. The latter was composed of maize silage, grass silage (mixed sward, ryegrass dominated), grass hay (mixed sward, balanced) and concentrate (50:25:10:15). The concentrate consisted (g/kg DM) of rapeseed cake, 500; soybean meal 170; maize gluten, 150; wheat, 130; calcium phosphate, 30; calcium carbonate, 20. Each of the 42 treatments (seven plants × six dosages) was repeated in six independent HGT runs with fresh rumen fluid each across a period of 7 weeks. Additionally, each run included duplicates of no substrate (blank), basal diet only, standard hay, standard concentrate and utilizable crude protein (uCP) standard (obtained from the Institute of Animal Nutrition, University of Hohenheim, Stuttgart, Germany).

After 24 h of incubation at 39°C, fermentation gas volume was recorded. The incubation fluid was analysed for pH and ammonia with a potentiometer (pH: model 632; ammonia: model 713; Metrohm, Herisau, Switzerland). In fermentation gas, methane and CO_2 concentrations were measured on a gas chromatograph (6890N, Agilent Technologies, Wilmington, USA) equipped with a thermal conductivity detector. Microbial counts were obtained after fixation in formaldehyde using counting chambers (Blau Brand, Wertheim, Germany) with 0.1 and 0.02 mm depth for protozoa and bacteria, respectively. Short chain fatty acids (SCFA) were analysed

with HPLC (La Chrom, L-7000 series, Hitachi Ltd, Tokyo, Japan) equipped with an UV detector.

3.4.3 Palatability experiment (dairy cows)

Six late-lactating Brown Swiss cows, selected from the herd of the ETH Research Station Chamau (Hünenberg, Switzerland), were in first to fifth lactation and weighed 609 ± 37 kg. They were kept in single boxes to allow measuring individual feed intake and had permanent access to water.

Test plant pellets (TPP) were produced from the chopped (specified above) woody plants, lucerne (*Medicago sativa*; chopped to 3 mm particle size with a Sigma 5.2 hammer mill, Kuhn AG, Bottighofen, Switzerland) and 20 g molasses/kg DM. The ratios of test plants to lucerne (low in total phenols (TP), Table 3.1), were chosen in a way that all pellet types should contain about 60 g TP/kg DM based on the analyses of batches A and B (used *in vitro*). The lots C and D per plant were used to produce different batches of pellets. Control pellets consisted (g/kg DM) of lucerne 980 and molasses 20. Lucerne, plant materials and molasses were mixed with a Speedmix DFML-1000 (Bühler AG, Uzwil, Switzerland). Pellets (diameter of 4.5 mm) were produced with a Kahl 40P pellet press (Amandus Kahl GmbH, Reinbek, Germany) while adding steam (about 60°C, Installation Bühler AG, Uzwil, Switzerland).

The diets were composed of TPP (7.2 kg DM) and a mixed basal ration (MBR) in a proportion of 40:60. They were provided separately three times daily. The MBR consisted (g/kg DM) of grass silage (mixed sward, ryegrass dominated), 495; maize silage, 267; hay (mixed sward, ryegrass dominated), 91; soybean meal, 87; sugar beet pulp, 48; mineral supplement, 9; feed-grade urea, 3. Additionally, daily 100 g mineral-vitamin mix (KRONI; Kroni Locher, Altstätten, Switzerland) and 50 g salt (390 g NaCl/kg, Agrisal, Matile GmbH, Rubigen, Switzerland) were provided. Cows were adapted to lucerne pellet additions to their regular MBR for 5 days followed by 3 days of quantifying individual lucerne pellet intake as a reference value (control). Thereafter, all six types of TPP were fed in randomised order to each cow for 3 days each (see Appendices Table A3.1; each plant lot was fed to three cows). Intakes of TPP and MBR were measured daily. Separately, TPP intake during the first 5 h of feeding was recorded. Milk yield was recorded at each milking. Milk was sampled on day 3, conserved with Bronopol® and later analysed for fat, protein, lactose and urea content with a MilkoScan FT6000 (Foss, Hillerød, Denmark).

Table 3.1 Analysed composition (g/kg DM) of experimental plants and pellets.

Plant species	Lot[1]		OM	CP	EE	NDF	ADF	ADL	TP	NTP	TT	CT	HT
Plant material used *in vitro*													
Birch	A+B		944	140	45.7	438	257	134	63.2	37.0	26.2	15.2	11.0
Sweet chestnut	A+B		956	107	55.6	406	266	99	152.6	24.7	127.9	4.3	124.0
Hazel	A+B		925	112	28.6	414	276	116	105.3	38.4	67.0	61.3	5.7
Rosebay willow	A+B		933	123	17.2	438	372	101	79.0	14.9	64.1	0.7	63.4
Wood avens	A+B		910	123	12.6	361	297	54	103.8	21.5	82.3	5.8	76.5
Blackcurrant	A+B		900	162	31.6	291	260	150	78.4	29.5	48.9	33.6	15.3
Vine	A+B		908	130	35.8	316	211	96	87.1	20.8	66.3	27.0	39.4
Basal diet used *in vitro*			927	146	31.0	426	275	36	16.5	14.9	1.6	0.1	1.5
Feeds used *in vivo*		g/kg[2]											
Mixed basal ration			920	152	29.1	419	268	38	18.5	15.5	3.0	0.2	2.9
Lucerne pellets		980	858	198	17.3	432	334	87	13.4	11.7	1.7	0.1	1.5
Test plant pellets													
Birch	C	980	949	148	65.3	424	254	137	57.1	26.9	30.2	25.2	5.1
	D	980	955	152	65.9	408	241	128	67.6	25.1	42.5	30.3	12.1
Hazel	C	540	913	158	27.4	388	229	77	45.3	21.2	24.0	18.2	5.8
	D	540	900	183	20.0	426	158	102	50.4	22.8	27.6	21.7	5.9
Rosebay willow	C	800	916	159	23.5	326	242	68	97.7	16.2	81.6	3.4	78.1
	D	800	916	139	25.0	302	251	64	92.6	15.6	76.9	0.4	76.5
Wood avens	C	600	893	160	17.0	365	274	79	76.5	15.9	60.5	8.1	52.5
	D	600	903	178	30.3	337	222	58	62.1	12.6	49.5	4.3	45.3
Blackcurrant	C	800	887	183	34.3	320	260	138	67.2	28.6	38.7	34.7	4.0
	D	800	891	178	38.2	272	240	118	72.4	27.8	44.6	37.0	7.7
Vine	C	720	897	144	37.7	333	214	91	70.2	15.9	54.3	17.5	36.8
	D	720	897	164	35.1	361	248	114	67.8	19.2	48.7	32.2	16.4

ADF = acid detergent fibre; ADL = acid detergent lignin; CP = crude protein; CT = condensed tannins; EE = ether extract; NDF = neutral detergent fibre; HT = hydrolysed tannins; NTP = non tannin phenols; OM = organic matter; TP = total phenols; TT = total tannins.
[1]Average values of lots A and B depicted because 1:1 mixtures incubated *in vitro*.
[2]Values indicate proportion of plant material in pellet. Difference to 1000 g/kg consisted of lucerne and molasses (always 20 g/kg).

3.4.4 Laboratory analyses

Diet components were analysed for contents of DM, ash, nitrogen, ether extract, neutral and acid detergent fibre, and acid detergent lignin according to standard procedures (AOAC, 1995). Concentrations of TP, non-tannin phenols (NTP) and CT were determined according to Makkar (2003) and total tannins (TT) and hydrolysable tannins (HT) were calculated from these results. Details on procedures and equipment used are described by Wang et al. (2018).

3.4.5 Calculations and statistical analysis

In HGT, total gas amounts produced, *in vitro* organic matter digestibility (IVOMD) and net energy of lactation (NEL) contents were calculated after adjustment of gas production by the blanks and the hay and concentrate standards, respectively, applying the equations by Menke and Steingass (1988). The uCP content was calculated as outlined by Edmunds et al. (2012) The uCP values were adjusted by the 183 mg uCP/g DM Hohenheim standard. Palatability *in vivo* was determined according to Ben Salem et al. (1994), with MBR or lucerne pellet intake as reference for TPP intake by the palatability index (PAL). Accordingly, $PAL_{72\ h}$ (%) = (test plant pellet intake (kg)/test plant pellet offered (kg))/(MBR or lucerne pellet intake (kg)/(MBR or lucerne pellet offered (kg)) × 100. In addition, PAL was calculated individually for each day in relation to lucerne-only pellets.

Data were analysed with the MIXED procedure of SAS (version 9.4, SAS Institute, Carry NC, USA) with Tukey-Kramer adjustment. Model 1, which included 42 treatments (seven plants × six dosages), considered plant, dosage and interaction as fixed and run as random factor. Model 2 compared the plant effect within dosage considering run as random factor. Treatments and non-supplemented control were compared with treatment as fixed and run as random effect. Linear and non-linear effects of dosage within plants were evaluated by orthogonal polynomial contrasts. Model 3 (*in vivo* data) considered TPP and lactation stage as fixed and lactation number and animal as random factor. Individual animal data recorded during control feeding were used as covariate for the analysis of milk yield and composition. Significance level was set to $P < 0.05$. Pearson correlation coefficients between variables and their significance were calculated using the CORR procedure.

Table 3.2 In vitro organic matter digestibility (IVOMD), total short-chain fatty acids (SCFA) and proportions of acetate (C_2) and propionate (C_3) in incubation fluid as affected by experimental plants (further results on individual SCFA in Appendices Tables A3.2 and A3.3).

Variable	Dosage (g/kg DM)	Basal diet	Birch	Sweet chestnut	Hazel	Rose-bay willow	Wood avens	Black-currant	Vine	SEM	*P*-value	*P*-value		
												Plant	Dosage	Plant × dosage
IVOMD	0	67.7								0.99		<0.001	<0.001	<0.001
(%)	100		66.7	65.7	65.7	64.8	64.4	64.7	65.6		0.815			
	200		62.8*^{b}	61.8*^{b}	63.4^{ab}	62.6*^{b}	64.3^{ab}	66.2^{a}	65.1^{ab}		0.004			
	300		61.6*^{a}	56.9*^{b}	58.9*^{ab}	60.2*^{ab}	62.9*^{a}	62.5*^{a}	61.5*^{a}		0.001			
	400		59.8*^{abc}	49.0*^{d}	57.2*^{bc}	56.0*^{c}	61.9*^{a}	62.0*^{a}	60.5*^{ab}		<0.001			
	500		56.7*^{bc}	46.0*^{d}	53.9*^{c}	55.1*^{c}	60.7*^{a}	60.1*^{ab}	59.2*^{ab}		<0.001			
	1000		43.1*^{c}	32.9*^{e}	39.6*^{d}	45.9*^{c}	57.0*^{a}	53.3*^{b}	53.6*^{b}		<0.001			
	Contrast[1]		L	L Q	L	L	L	L	L					
Total	0	72.5								2.78		<0.001	<0.001	<0.001
SCFA	100		74.9	73.9	73.4	73.8	72.9	72.5	73.1		0.318			
(mmol/l)	200		71.4^{a}	69.9^{ab}	70.4^{ab}	68.9^{b}	70.8^{ab}	70.2^{ab}	71.1^{ab}		0.031			
	300		71.4^{ab}	64.7*^{c}	66.7*^{bc}	67.0*^{bc}	69.8^{a}	66.7*^{bc}	68.2*^{ab}		<0.001			
	400		66.8*^{ab}	61.5*^{b}	65.3*^{ab}	66.7*^{ab}	70.1^{a}	66.4*^{ab}	69.5^{a}		0.002			
	500		66.6*^{bc}	57.8*^{e}	63.2*^{d}	64.7*^{cd}	68.9^{a}	66.0*^{bc}	67.2*^{ab}		<0.001			
	1000		54.7*^{c}	49.2*^{d}	50.0*^{d}	54.9*^{c}	65.7*^{a}	59.5*^{b}	61.9*^{b}		<0.001			
	Contrast		L	L Q	L	L	L	L	L					
C_2	0	66.7								0.36		<0.001	<0.001	<0.001
(mmol/	100		65.8^{cd}	65.7^{d}	66.3^{ab}	66.1^{bc}	66.1^{bc}	66.3^{ab}	66.5^{a}		<0.001			
mol	200		66.5^{b}	65.9^{b}	67.3^{a}	67.5^{a}	67.4^{a}	67.5^{a}	67.9^{a}		<0.001			
SCFA)	300		67.6^{b}	65.3*^{c}	68.3*^{ab}	68.5*^{ab}	68.6*^{ab}	68.9*^{a}	69.4*^{a}		<0.001			
	400		68.7*^{ab}	65.0*^{c}	69.6*^{a}	68.2*^{ab}	67.8^{b}	68.0*^{b}	69.0*^{ab}		<0.001			
	500		67.3^{c}	64.0*^{d}	69.2*^{ab}	69.3*^{ab}	68.6*^{b}	69.0*^{b}	70.1*^{a}		<0.001			
	1000		69.3*^{d}	66.7^{e}	73.8*^{b}	74.6*^{ab}	71.3*^{c}	73.6*^{b}	74.9*^{a}		<0.001			
	Contrast		L	L	L	L	L	L	L					
C_3	0	18.9								0.40		<0.001	<0.001	<0.001
(mmol/	100		19.3^{ab}	19.3^{a}	18.9^{c}	19.0^{c}	19.0^{bc}	18.8^{cd}	18.6^{d}		<0.001			
mol	200		18.8^{b}	19.2^{c}	18.3^{c}	18.2^{c}	18.4^{bc}	18.2^{c}	17.8*^{d}		<0.001			
SCFA)	300		18.5*^{b}	19.4^{a}	17.8*^{bc}	17.6*^{cd}	18.0*^{bc}	17.7*^{cd}	17.1*^{d}		<0.001			
	400		18.0^{b}	19.5^{a}	17.6*^{bc}	17.5*^{bc}	18.1*^{b}	17.8*^{bc}	17.0*^{c}		<0.001			
	500		19.0^{b}	19.6^{a}	17.4*^{cd}	17.1*^{d}	17.7*	17.3*^{cd}	16.4*^{e}		<0.001			
	1000		19.8*^{a}	19.7^{a}	16.1*^{b}	14.6*^{c}	16.3*^{b}	15.6*^{b}	14.4*^{c}		<0.001			
	Contrast		L Q	L	L	L	L	L	L Q					

[a-e]Least-square means within a row with no common superscript differ ($P < 0.05$).

*Values differ ($P < 0.05$) from those of basal diet alone.

[1]Significant ($P < 0.05$) linear (L) or quadratic (Q) contrasts of the response to incremental doses (from 0 to 500 g/kg) of each plant.

Table 3.3 Calculated contents of net energy for lactation (NEL) and utilisable crude protein (uCP) per unit of DM as well as measured ammonia concentration in incubation fluid as affected by experimental plants.

Variable	Dosage (g/kg)	Basal diet	Birch	Sweet chest-nut	Hazel	Rose-bay willow	Wood avens	Black-currant	Vine	SEM	*P*-value	*P*-value		
												Plant	Dosage	Plant × dosage
NEL	0	5.23								0.115		<0.001	<0.001	<0.001
(kJ/g DM)	100		5.14	5.05	5.01	4.90	4.81	4.83	4.97		0.677			
	200		4.66*	4.59*	4.72	4.61*	4.80	4.99	4.89		0.059			
	300		4.53*^{ab}	4.06*^{b}	4.19*^{ab}	4.34*^{ab}	4.62*^{a}	4.48*^{ab}	4.42*^{ab}		0.022			
	400		4.42*^{a}	3.35*^{d}	4.01*^{bc}	3.87*^{c}	4.49*^{a}	4.38*^{a}	4.29*^{ab}		<0.001			
	500		4.00*^{abc}	3.05*^{d}	3.66*^{c}	3.79*^{bc}	4.33*^{a}	4.14*^{ab}	4.14*^{ab}		<0.001			
	1000		2.77*^{cd}	2.23*^{e}	2.47*^{de}	2.95*^{c}	3.87*^{a}	3.28*^{b}	3.49*^{b}		<0.001			
	Contrast[1]		L	L	L	L	L	L	L					
uCP	0	162								7.5		<0.001	0.002	<0.001
(mg/	100		171^{b}	182*^{a}	180^{ab}	183.5*^{a}	177^{ab}	180^{ab}	178^{ab}		0.024			
g DM)	200		177^{ab}	183*^{ab}	184*^{a}	175^{ab}	172^{ab}	181^{ab}	168^{b}		0.025			
	300		180^{b}	182*^{ab}	181^{ab}	177^{b}	179^{b}	195*^{a}	185*^{ab}		0.010			
	400		182*^{b}	174^{b}	183*^{b}	179^{b}	185*^{b}	200*^{a}	186*^{b}		<0.001			
	500		189*^{ab}	177^{c}	180^{bc}	168^{c}	179^{bc}	194*^{a}	175^{c}		<0.001			
	1000		197*^{b}	172^{c}	175^{c}	169^{c}	175^{c}	216*^{a}	190*^{b}		<0.001			
	Contrast		L	Q	L Q	Q	L	L Q	L Q					
NH_3	0	12.8								0.57		<0.001	<0.001	0.226
(mmol/L)	100		11.9^{a}	10.8*^{b}	11.1*^{b}	10.9*^{b}	11.5^{ab}	11.4^{ab}	11.4^{ab}		<0.001			
	200		11.7^{ab}	10.7*^{b}	10.7*^{b}	11.5^{ab}	11.9^{a}	11.9^{a}	12.1^{a}		<0.001			
	300		11.8	10.7*	11.0*	11.2*	11.1*	10.8*	10.8*		0.076			
	400		11.0*	10.4*	10.0*	10.9*	10.4*	10.5*	10.5*		0.087			
	500		10.4*^{abc}	10.1*^{bc}	9.8*^{c}	11.1*^{a}	10.4*^{abc}	10.7*^{ab}	10.9*^{ab}		<0.001			
	1000		9.4*^{bc}	9.1*^{c}	9.1*^{c}	10.4*^{a}	10.0*^{ab}	10.0*^{ab}	9.4*^{bc}		<0.001			
	Contrast		L	L Q	L	L Q	L	L Q	L					

[a-d]Least-square means within a row with no common superscript differ ($P < 0.05$).

*Values differ ($P < 0.05$) from those of basal diet alone.

[1]Significant ($P < 0.05$) linear (L) or quadratic (Q) contrasts of the response to incremental doses (from 0 to 500 g/kg) of each plant.

Table 3.4 Methane formation per unit of DM supply, CO_2, short-chain fatty acids (SCFA) and digestible organic matter (dOM) as affected by experimental plants (further results on gas production in Appendices Tables A3.4 and A3.5).

Variable	Dosage (g/kg)	Basal diet	Birch	Sweet chest-nut	Hazel	Rose-bay willow	Wood avens	Black-currant	Vine	SEM	*P*-value	*P*-value		
												Plant	Dosage	Plant × dosage
CH_4/DM	0	34.2								0.80		<0.001	<0.001	<0.001
(ml/g)	100		33.3	31.4	31.6	30.8*	31.0	31.2	31.8		0.258			
	200		29.6*^{a}	26.4*^{b}	29.0*^{a}	28.7*^{a}	29.6*^{a}	30.7*^{a}	30.3*^{a}		<0.001			
	300		27.9*^{a}	18.6*^{b}	25.0*^{a}	26.8*^{a}	24.7*^{a}	27.9*^{a}	27.9*^{a}		<0.001			
	400		25.8*^{a}	11.7*^{d}	23.1*^{c}	23.2*^{bc}	26.7*^{a}	26.2*^{a}	25.6*^{ab}		<0.001			
	500		22.8*^{abc}	6.1*^{d}	20.4*^{c}	22.1*^{bc}	25.2*^{a}	24.1*^{ab}	24.7*^{a}		<0.001			
	1000		9.5*^{d}	1.2*^{e}	8.3*^{d}	13.7*^{c}	20.2*^{a}	16.3*^{b}	16.7*^{b}		<0.001			
	Contrast[1]		L	L Q	L	L	L Q	L	L					
CH_4/CO_2	0	179								4.5		<0.001	<0.001	<0.001
(ml/l)	100		176	170	172	174	175	177	174		0.511			
	200		175^{a}	155*^{b}	167^{ab}	168^{ab}	169^{a}	167^{ab}	170^{a}		0.004			
	300		167^{a}	122*^{b}	165^{a}	167^{a}	158*^{a}	169^{a}	172^{a}		<0.001			
	400		158*^{a}	94.8*^{b}	156*^{a}	166^{a}	163^{a}	162^{a}	164		<0.001			
	500		155*^{a}	56.6*^{b}	153*^{a}	161*^{a}	156*^{a}	159*^{a}	162^{a}		<0.001			
	1000		109*^{c}	22.0*^{d}	121*^{bc}	139*^{a}	140*^{a}	145*^{a}	132*^{ab}		<0.001			
	Contrast		L	L Q	L	L	L	L	L					
CH_4/	0	137								4.9		<0.001	<0.001	<0.001
SCFA	100		133^{a}	126^{ab}	125^{ab}	122^{b}	126^{ab}	131^{ab}	127^{ab}		0.043			
(mmol/	200		123^{a}	111*^{b}	121*^{a}	122^{a}	122^{a}	128^{a}	123^{a}		<0.001			
mol)	300		119*^{a}	84*^{b}	110*^{a}	116*^{a}	104*^{ab}	122^{a}	118*^{a}		<0.001			
	400		113*^{a}	56*^{b}	105*^{a}	102*^{a}	113*^{a}	116*^{a}	108*^{a}		<0.001			
	500		99*^{a}	30*^{b}	94*^{a}	98*^{a}	106*^{a}	106*^{a}	105*^{a}		<0.001			
	1000		50*^{c}	7*^{d}	48*^{c}	72*^{b}	90*^{a}	81*^{ab}	78*^{b}		<0.001			
	Contrast		L	L Q	L	L	L	L	L					
CH_4/dOM	0	50.6								1.08		<0.001	<0.001	<0.001
(ml/g)	100		49.5^{a}	47.3^{b}	47.8^{ab}	47.3^{b}	47.9^{ab}	48.1^{ab}	48.3^{ab}		0.012			
	200		47.0^{a}	42.5*^{b}	45.5*^{a}	45.5*^{a}	45.9*^{a}	46.3^{a}	46.5^{a}		<0.001			
	300		45.0*^{a}	32.4*^{b}	42.1*^{a}	44.2*^{a}	39.6*^{ab}	44.8*^{a}	45.2*^{a}		<0.001			
	400		42.7*^{a}	23.3*^{b}	40.2*^{a}	41.2*^{a}	43.1*^{a}	42.6*^{a}	42.3*^{a}		<0.001			
	500		39.6*^{ab}	12.9*^{c}	37.5*^{b}	39.6*^{ab}	41.5*^{a}	40.6*^{ab}	41.6*^{a}		<0.001			
	1000		21.6*^{c}	3.42*^{d}	20.8*^{c}	29.4*^{b}	35.6*^{a}	31.3*^{b}	31.1*^{b}		<0.001			
	Contrast		L Q	L Q	L	L	L	L	L					

[a-e]Least-square means within a row with no common superscript differ ($P < 0.05$).

*Values differ ($P < 0.05$) from those of basal diet alone.

[1]Significant ($P < 0.05$) linear (L) or quadratic (Q) contrasts of the response to incremental doses (from 0 to 500 g/kg) of each plant.

Table 3.5 Intake, palatability, milk yield and performance in cows fed mixed basal ration and lucerne (control) or test plant pellets.

Variable	Lucerne[1]	Birch	Hazel	Rosebay willow	Wood avens	Blackcurrant	Vine	SEM	*P*-value
Intake (kg DM)									
Total (per day)	17.3[a]	14.3[b]	18.0[a]	17.5[a]	17.8[a]	15.2[b]	17.5[a]	0.31	<0.001
Mixed basal ration (per day)	10.8	10.7	10.8	10.8	10.8	10.8	10.8	0.06	0.459
Pellets (per day)	6.58[a]	3.58[b]	7.19[a]	6.68[a]	6.95[a]	4.34[b]	6.64[a]	0.31	<0.001
Pellets (first 5 h)	–	1.67	2.88	2.55	2.53	1.72	2.60	0.47	0.366
Palatability index (%)									
Mixed basal ration[2]	92.6[a]	50.8[b]	100.0[a]	92.7[a]	96.5[a]	60.3[b]	92.5[a]	4.30	<0.001
Pellets[3]	–	54.4[b]	110.5[a]	102.5[a]	106.9[a]	66.6[b]	102.0[a]	4.92	<0.001
Day 1[3]	–	62.3[b]	110.8[a]	102.3[a]	110.4[a]	72.6[b]	106.0[a]	4.77	<0.001
Day 2[3]	–	30.6[b]	110.1[a]	106.4[a]	110.6[a]	54.6[b]	102.7[a]	8.10	<0.001
Day 3[3]	–	70.4	110.5	98.9	99.8	72.6	97.2	11.50	0.115
Milk yield									
kg/day	19.2[a]	16.1[d]	17.1[cd]	17.7[bc]	18.5[ab]	17.1[cd]	17.7[bc]	0.28	<0.001
kg/kg DM intake	1.11[ab]	1.16[a]	0.95[c]	1.01[bc]	1.04[abc]	1.15[a]	1.02[bc]	0.028	<0.001
Milk fat (%)	4.71	4.76	4.47	4.50	4.71	4.73	4.82	0.178	0.754
Milk protein (%)	4.07	4.05	4.06	3.97	4.00	3.98	4.02	0.056	0.812
Milk lactose (%)	4.59	4.48	4.60	4.62	4.56	4.59	4.59	0.042	0.296
Milk urea (mg/dl)	27.5[a]	21.5[b]	16.4[bc]	14.4[c]	17.1[bc]	17.8[bc]	17.6[bc]	1.17	<0.001

[a-d]Least-square means within a row with no common superscript differ ($P < 0.05$).

[1]Lucerne pellets without plant additives were fed during control feeding (Appendices Table A3.1).

[2]Test pellet intake related to mixed basal ration intake

[3]Test plant pellet intake related to average lucerne pellet intake (control feeding; Appendices Table A3.1).

Table 3.6 Pearson correlation coefficients between variables describing intake or palatability and composition of the test plant pellets (n = 6).

Variable	OM	CP	EE	NDF	ADF	ADL	TP	NTP	TT	CT	HT
Intake (DM)											
Total	-0.353***	0.136	-0.602***	0.073	-0.029	-0.533***	-0.099	-0.510***	0.027	-0.450***	0.248**
MBR	-0.108	0.011	-0.149†	-0.115	-0.050	-0.0916	0.047	-0.038	0.058	-0.042	0.071
Pellets	-0.344***	0.137	-0.591***	0.088	-0.023	-0.529***	-0.106	-0.513***	0.021	-0.451***	0.242**
Pellets first 5 h	-0.136	0.061	-0.272	0.042	-0.323†	-0.322†	-0.033	-0.318†	0.070	-0.267	0.167
Palatability index											
MBR	-0.345***	0.144	-0.585***	0.102	-0.012	-0.521***	-0.119	-0.514***	0.008	-0.451***	0.231**
Pellets	-0.393**	0.097	-0. 651***	0.007	-0.316*	-0.616***	-0.013	-0.629***	0.181	-0.540***	0.364**
Day 1	-0.370*	0.098	-0.606***	-0.022	-0.348*	-0.615***	0.031	-0.633***	0.219	-0.487**	0.364*
Day 2	-0.462**	0.079	-0.728***	-0.054	-0.250	-0.652***	0.062	-0.666***	0.255	-0.579***	0.430**
Day 3	-0.214	0.140	-0.374*	0.152	-0.062	-0.362*	-0.178	-0.357*	-0.093	-0.351*	0.014

ADF = acid detergent fibre; ADL = acid detergent lignin; CP = crude protein; CT = condensed tannins; EE = ether extract; HT = hydrolysed tannins; MBR = mixed basal ration; NDF = neutral detergent fibre; NTP = non tannin phenols; OM = organic matter; TP = total phenols; TT = total tannins.
$^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$; $^{\dagger}P < 0.10$.

3.5 Results

3.5.1 Chemical composition of experimental plants and feeds

Organic matter (OM) contents varied between 858 and 956 g/kg DM but were very similar in the different lots of one plant species (Table 3.1). The CP content was highest in lucerne pellets. Birch and rosebay willow were highest and blackcurrant TPP (lot D) were lowest in NDF. The ADF was >210 g/kg DM in all materials, except hazel (lot D). The ADL was lowest in the basal diet and highest in blackcurrant. All five phenol fractions were low in basal diet, lucerne pellets and MBR. Chestnut leaves were richest in TP, TT and HT. Among the TPP, rosebay willow pellets contained most TP, TT and HT. The highest NTP and CT contents were found in hazel leaves. Birch, hazel and blackcurrant TPP contained >20 g NTP/kg DM. The CT content differed from 0.4 (rosebay willow TPP, lot D) to 37 g/kg DM (blackcurrant TPP, lot D).

3.5.2 In vitro experiment

After 24 h of fermentation, the incubation fluid pH ranged between 6.8 and 6.9 (data not shown). All plants reduced the IVOMD linearly ($P < 0.05$; Table 3.2). Values became significantly different from 0 g/kg at either 200 or 300 g/kg. With increasing dosage of sweet chestnut, the decline became larger (quadratic contrast, $P < 0.05$). The different response to the plants resulted in an interaction ($P < 0.001$) between plant and dosage in IVOMD. Total SCFA concentration in the incubation fluid responded similarly to IVOMD with respect to plant and dosage. Acetate proportion increased linearly ($P < 0.05$) with each plant, except sweet chestnut where acetate decreased ($P < 0.05$). The proportions of the other SCFA declined with most plants depending on the dosage (Table 3.2, Appendices Table A3.2). This also provoked large changes in the ratio of acetate to propionate (Appendices Table A3.3). There were interactions ($P < 0.001$) between plant and dosage in all SCFA related variables.

The NEL contents decreased linearly ($P < 0.05$) with each plant material and differed ($P < 0.05$) from the basal diet alone with proportions of at least 200 to 300 g/kg (Table 3.3). In comparison to the non-supplemented control, the calculated uCP content increased ($P < 0.05$) with each plant either linearly (birch and wood avens), non-linearly (sweet chestnut and rosebay willow) or with linear and non-linear components (hazel, blackcurrant and vine). In both variables, NEL and uCP content, there was an interaction ($P < 0.001$) of plant and dosage. The NH_3 concentration linearly decreased ($P < 0.05$) with all plants, but the effective dosages differed between plants. There was no interaction between the two factors.

The amount of methane produced per unit of DM, CO_2, SCFA and digestible OM (dOM) decreased linearly ($P < 0.05$) with increasing levels of all plants (Table 3.4). The decrease found

with sweet chestnut and in one variable each with birch and wood avens also had a non-linear component ($P < 0.05$). The magnitude of the dosage effects was higher in relation to unit DM than to CO_2, SCFA and dOM. The level of absolute (Appendices Table A3.4) and relative methane mitigation largely differed between plants and dosages and there were generally interactions ($P < 0.001$). Among plants, sweet chestnut was most efficient (declines of 88 to 96% of control) , followed by hazel (32-76%), birch (39-72%), rosebay willow (22-60%), blackcurrant (19-52%), vine (26-51%) and wood avens (22-41%).

3.5.3 Dairy cow experiment

During control feeding, 91% of the lucerne pellets offered (7.2 kg DM) were consumed. The MBR offered (~11 kg DM) was always completely consumed by each animal (Table 3.5). Hazel, rosebay willow, wood avens and vine TPP (7.2 kg offered) were consumed equally to the lucerne control pellets. Therefore their PAL did not differ in the first 5 h and across all 3 days. However, TPP intake and PAL were reduced ($P < 0.05$) with birch and blackcurrant on the first and second day but not on the third day. Milk yield was lower ($P < 0.05$) with any TPP, except wood avens, than with lucerne pellets. The milk yield per kg DM intake was reduced ($P < 0.05$) with the hazel pellets but not with the other TPP. Contents of milk fat, protein and lactose did not differ between TTP and lucerne pellets. Milk urea concentration was reduced ($P <0.05$) by all TPP, thereby most with rosebay willow, least with birch.

There were negative correlations ($P < 0.05$ to < 0.001) between total intake, daily pellet intake and all PAL variables with concentrations of NTP, CT, ADL, OM (except PAL on Day 3) and EE (Table 3.6). Positive relationships ($P < 0.05$ to < 0.01) were found between HT and most of intake and PAL parameters. Weak correlations occurred between ADF content and intake related to PAL variables, and none for NDF, TP, TT and CP concentrations.

3.6 Discussion

3.6.1 Dose-response relationship of test plants and ruminal methane and ammonia mitigation

Following the selection of the six most promising woody plants from the forages when investigated at a single dosage (167 mg/g; Terranova et al., 2018a), the dose-response relationship and the optimal dose were determined in the present study. Compared to the basal diet, methane yields (CH_4/DM) were reduced by 33 to 96% with the positive control (sweet chestnut) and by 10 to 76 % with the other plants. Higher dosages enhanced the effect with all plants. The even more relevant relationship, CH_4/dOM decreased with all plants when included

at ≥300 g/kg DM. Even then, sweet chestnut was most efficient, although IVOMD declined to half when incubate alone. Jayanegara et al. (2012) showed that ≥20 g tannins/kg diet are needed for a clear methane mitigation. In the present study, hazel, wood avens and rosebay willow were effective at lower tannin contents, but NTP or other compounds may have been bioactive, too (Beauchemin et al., 2008; Jayanegara et al., 2012). Consistent with the current *in vitro* results, CH_4/dOM was found to be reduced by 25% in sheep with 50% hazel leaves in the diet (Wang et al. 2018). In the present study, the level of methane mitigation at 200 mg plant/g DM was similar to that found with 167 mg/g by Terranova et al. (2018a). Typically, methane mitigation is associated with a lower acetate-to-propionate ratio. The opposite was the case in the present study, with the exception of sweet chestnut, which indicates that the methane decline was not primarily caused by a depressed fibre degradation.

All plants (dose-dependently) reduced ruminal NH_3 formation *in vitro* and likely also in the cows as shown by the sharp decline in milk urea content. Consistent with that Wang et al. (2018) found in sheep a linear and substantial decrease in urinary nitrogen excretion with increasing hazel proportion in the diet. Birch, hazel and vine pellets, all containing ≥18 g CT/kg DM, were most efficient in the present experiment. The capacity of PSC, especially tannins, to form undegradable complexes with proteins is known since long (Waghorn et al., 1994). Comparing similar dosages, the effects of the plants on ruminal NH_3 concentration were less pronounced than previous results suggested (Terranova et al. 2018a), indicating the presence of plant lot differences.

3.6.2 Nutritional value of the test plants

Feeds that are suitable for reducing methane and nitrogen should have the highest possible feeding value as this is a common constraint in such feeds. In the present study, the nutritional value of the plants was obvious from their effects on IVOMD, NEL and uCP contents and on short-term milk yield and composition. Both, IVOMD and NEL are largely determined by fermentation gas production, which decreased linearly, like the SCFA, with increasing plant proportions, showing that their energy content was lower than that of the high-quality basal diet. This coincides with the decline in digestibility reported for higher proportions of hazel leaves in sheep (Wang et al., 2018). Among plants, hazel, blackcurrant, vine and wood avens were superior to birch, rosebay willow and, as expected from the previous screening (Terranova et al., 2018a), sweet chestnut. However, in that study birch had no such low energy content. The NEL content of the plants was probably limited by two factors, fibre lignification (high in birch) and depression of digestibility by PSC (high in chestnut). The increase in estimated uCP

content by all plants was likely related to the capacity of plant PSC to bind feed proteins and preserve them from ruminal degradation. However, it remains to be demonstrated, that the PSC-protein complexes are cleaved at the low abomasal pH allowing postruminal amino acid absorption to take place (Waghorn et al., 1994; O'Connell and Fox 2001).

Along with the limited NEL contents estimated *in vitro*, milk yield declined with all plants (except wood avens) compared to the high-quality forage lucerne. This gap might be less pronounced when compared with a forage control of moderate quality. Milk yield is known to respond quickly after dietary changes. Still, the 3 days of measurement are quite limited to show the full effects of feeding the plants on milk yield and milk composition. A response in milk protein (variation in NEL and uCP) and fat content (variation and SCFA profile) could thus be expected in the longer term.

3.6.3 Palatability of the plants

Many forages from woody plants rich in PSC are not well palatable. However, a high palatability is a prerequisite for the implementation of methane- and ammonia-reducing feeds by farmers. Our intention was to compare palatability at the same TP content. However, phenol contents in lots A+B used *in vitro* largely differed from lots C and D used *in vivo*. This indicates the presence of natural variation in plant chemical composition as influenced by season, region and soil type (Palo et al., 1985, Tiemann et al., 2009). Still, the least palatable plants, birch and blackcurrant, both had average TP contents, and there was no relationship between palatability and TP contents suggesting that further factors influenced palatability. Palatability seems to differ also between grazing livestock species. Meier et al. (2014) reported that sheep prefer birch leaves out of six woody plants from which they could choose. Our cows least preferred birch leaves and the lots used were rich in lignified fibre, another factor which influenced palatability according to the results of the correlation analysis. Unexpectedly, four of the TPP were as palatable as the high-quality forage lucerne alone. This included rosebay willow with the highest TP and HT contents in pellets and a high (80%) proportion of plant in pellet. The pellets including rosebay willow and wood avens (lot C) had HT contents >50 g/kg pellet DM, but were as palatable as the control pellets. Nevertheless, all TPP-containing diets had CT and HT contents far below 50 g/kg dietary DM each, the thresholds for decreasing voluntary feed intake in ruminants due to astringency and decelerated digestion (Waghorn et al., 1994; O'Connell and Fox, 2001; Frutos et al., 2004). The high palatability of the hazel pellets coincides with findings of Wang et al. (2018), where sheep consumed a diet consisting half of hazel leave pellets equally well as a control diet over 18 days. The reduced palatability of birch

and blackcurrant TPP was not yet significant after 5 h of offering and became less pronounced on day 3. Astringency may need up to 4 h to develop after ingestion (O'Connell and Fox, 2001). The results also support Ben Salem et al. (1994) claiming that 1-day intake measurements are not sufficient to predict acceptance in the following 15 days. Accordingly, the cows of the present study may have become accustomed to birch and blackcurrant over time.

3.7 Conclusion

This study revealed that forages from four woody plants growing in temperate climates, hazel, rosebay willow, wood avens and vine, have the potential to mitigate methane and nitrogen emissions from livestock, while being palatable. However, their nutritional value is limited in terms of net energy, especially at dietary dosages that are needed for a substantial mitigation effect. It is important to note that the four promising plants mitigated methane formation also per unit dOM and SCFA, despite the lower ruminal fermentability compared to the basal diet, which reflects a genuine and truly beneficial suppression of methanogenesis. Further studies need to demonstrate the mitigation potential of the four plants in live ruminants.

3.8 Acknowledgements

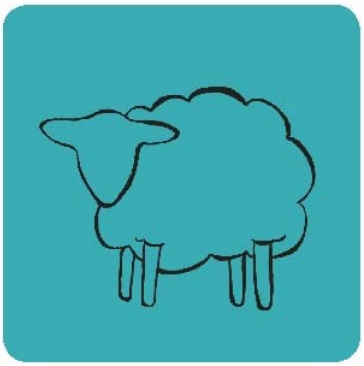
Special thanks to Carmen Kunz, Muna Mergani, Rebecca Müller, Andreas Simonett, Tina Stiefel, Peter Stirnemann. The project was funded by ETH Zurich Research Grant number ETH-4915-1 and the China Scholarship Council.

Ethics statement

The experimental protocol complied with the Swiss legislation for Animal Welfare and was approved by the Committee on Animal Experimentation of the Cantonal Veterinary Offices Zurich (ZH 38/14, rumen fluid collection) and Zug (ZG 93716, palatability experiment).

Chapter 4

Supplementation of hazel (*Corylus avellana*) leaves decreases methane and urinary nitrogen emissions by sheep at unchanged forage intake



This chapter is based on based on Wang, S., Terranova, M., Kreuzer, M., Marquardt, S., Eggerschwiler, L. and Schwarm, A. 2018: *Scientific Reports* 8, 5427, doi: 10.1038/s41598-018-23572-3. This paper is included in the doctoral thesis of S. Wang (Diss. ETH N°24899).

Classifying contribution:

Study conception: S. Wang, M. Terranova, S. Marquardt, A. Schwarm and M. Kreuzer
Plant in vitro: M. Terranova
Experimental feed production: M. Terranova and L. Eggerschwiler
Performing of the experiment, statistical analysis and manuscript preparation: S. Wang
Critical review of the manuscript: M. Terranova, M. Kreuzer, S. Marquardt, L. Eggerschwiler and A. Schwarm

4.1 Abstract

This study is the first to quantify the effects of hazel (*Corylus avellana*) leaves on methane and urinary nitrogen emissions, digestibility, nitrogen and the energy balance of ruminants. Four experimental pellets were produced with 0, 30% and 60% hazel leaves, the latter also with 4% polyethylene glycol. Hazel leaves gradually replaced lucerne. The diet was composed of the pellets and grass hay (80%: 20%). Six adult sheep were allocated to all four treatments in a 6 × 4 crossover design. Including hazel leaves did not affect the feed intake, but it decreased the apparent digestibility of organic matter and fibre, especially at the high level. Methane emission was reduced by up to 25 to 33% per day, per unit of intake and per unit of organic matter digested. Urinary nitrogen excretion decreased by 33 to 72% with increasing levels of hazel leaves. The treatment with polyethylene glycol demonstrated that tannins in hazel leaves caused significant shares of the effects. In conclusion, the current results indicated a significant potential of hazel leaves as forage for ruminants to mitigate methane and urinary nitrogen emissions. Even high dietary hazel leave proportions were palatable. The lower digestibility needs to be compensated with easily digestible diet ingredients.

4.2 Introduction

Methane (CH_4) from the livestock sector was calculated to account for 28% of the total global anthropogenic emissions, and is predicted to rise further due to an increasing worldwide demand for meat, milk and other animal-source products (Beauchemin et al. 2008; Shi et al. 2014). Manipulating the rumen fermentation by nutritional approaches with the aim of reducing CH_4 and nitrogen (N) emissions from ruminant husbandry at concomitantly unchanged or even improved feed intake and digestibility are among the most important goals of current animal nutrition research. Various nutritional attempts to mitigate CH_4 emission from ruminants have been undertaken, as reviewed by Beauchemin et al. (2008) and Hristov et al. (2013). Special attention has been given to plants or extracts rich in plant secondary compounds (PSC), such as tannins, essential oils or saponins. A number of screenings provided promising plants of at least moderate nutritional value that are effective in mitigating noxious emissions (Bodas et al. 2008; Soliva et al. 2008; Wang et al. 2017). Different from synthetic compounds, the public's growing concern about food safety is met by PSC-based forages (Jayanegara et al. 2015).

Tannins are a complex group of polyphenolic PSC with widely varying molecular weights that are found in many representatives of the plant kingdom (McSweeney et al. 2001). When provided at high dosages, tannins were predominantly found to be antinutritional because of their adverse effects on feed intake and nutrient utilisation[9]. In recent years, however, they have

been recognised as useful phytochemicals when provided at moderate dosages. Tannins can be beneficial modulators of rumen microbial fermentation promoting metabolic protein supply, animal productivity and animal health (Muller-Harvey 2006; Patra et al. 2012). The feeding of tannin-containing plants or extracts from such plants to ruminants was found at times, but not always, to substantially reduce enteric CH_4 emissions (Beauchemin et al. 2007; Animut et al. 2008; Maia et al. 2016). The effectiveness varies depending on the source, structure and supplemented level of the tannins (Bodas et al. 2008). In addition, tannins bind to proteins and thus protect at least part of the dietary protein from being degraded in the rumen. These bonds are cleaved in the abomasum, and thus the protein becomes at least partially digestible in the small intestine or, otherwise, will be excreted with the faeces (Waghorn et al. 1987; Beauchemin et al. 2007). Feeding diets containing tannins, therefore, is an efficient means to reduce the amount of dietary N to be excreted via urine and with that the N emission potential of the manure (Carulla et al. 2005; Ebert et al. 2017).

The shrub hazel (*Corylus avellana*) grows wild in Europe and western Asia, but it is also cultivated for its nuts. Its leaves are considered a potential means of antioxidant and antibacterial effects because of their richness in phenolic compounds (Oliveira et al. 2007; Riethmüller et al. 2013). Besides, among the plant materials tested in the extensive EU screening project "Rumen Up" (Wallace 2008), hazel leaves were found *in vitro* to depress CH_4 production (mmol/g dry matter incubated) by 25%. Another recent *in vitro* screening confirmed the CH_4 mitigation potential of hazel leaves and indicated their concomitantly favourable forage potential (Terranova et al. 2018a). Hazel leaves were also found to inhibit the proteolytic activity in rumen fluid (Wallace 2008; *https://www.abdn.ac.uk/research/rumen-up/report*). However, the effect of replacing part of the diet by hazel leaves on intake, total tract digestibility and mitigation of CH_4 and urinary N losses in live ruminant livestock has not yet been determined. In addition, it is unclear yet whether the active ingredients of the hazel leaves are indeed represented by the tannins or also by other PSC.

The objective of the present study was to determine the quantitative effects of replacing lucerne (*Medicago sativa*) with hazel leaves at different proportions in forage-only diets fed to adult sheep. The following predictions were made (i) Hazel leaves contain PSC which affect digestion in a way that CH_4 and urinary N formation are mitigated in a dose-dependent way. (ii) These changes happen without major adverse effects on intake, nutrient and energy utilisation. (iii) The main active ingredients in hazel leaves responsible for the observed effect are their tannins, rather than other PSC like flavonoids. For this purpose, polyethylene glycol

(PEG), which binds to tannins and inactivates them (Silanikove et al. 1996; Degen et al. 1998), was added to one of the diets.

4.3 Material and Methods

4.3.1 Experimental diets

Diets were composed of hay (late cut, ryegrass-dominated), dehydrated hazel leaves and lucerne. Hazel leaves were purchased from Alfred Galke GmbH (Bad Grund, Germany) and lucerne (*Medicago sativa*) from Landi (Sense-Düdingen, Switzerland). Hazel leaves and lucerne were harvested in Albania in 2015 and in France (Marne) in 2016, respectively. Four types of experimental pellets were produced from lucerne and hazel leaves containing 0%, 30% and 60% hazel leaves, the latter also with the addition of 3.8% PEG (molecular weight of 6000; Sigma, St. Louis, MO, USA) on a DM basis (Table 4.1). The PEG replaced lucerne in the pellets to maintain the dietary content of phenolic compounds from hazel. The realised dietary PEG : CT and PEG : TT ratios were 1.6:1 and 0.8:1, respectively. Hazel leaves as purchased had a cutting size of 4 to 6 mm. Lucerne was chopped to a size of 3 mm using a hammer mill (Sigma 5.2, Kuhn AG, Bottighofen, Switzerland). Chopped hazel leaves and lucerne were mixed with a batch mixer (Speedmix DFML-1000, Bühler AG, Uzwil, Switzerland) and subsequently pelleted (Kahl 40PS, Amandus Kahl GmbH & Co, Reinbek, Germany) with the use of steam (about 60 °C; Installation Bühler AG, Uzwil, Switzerland). Pellets had a diameter of 4.5 mm. The complete diets consisted of hay and experimental pellets at a ratio of 20% : 80% in DM, resulting in hazel leave proportions of approximately 0, 25 and 50% in the total diet (realised: 0, 23.4 and 46.8%). The amounts of diets offered provided 1.6 times the recommended DM supply for the maintenance requirements of adult female sheep (Arrigo and Frioud 2016). The pellets were not balanced for CP because the CP content in the complete diets (≥ 13.2% CP in DM) was always higher than the threshold where the RDP supply is assumed to become critical[31], although this did not consider possible RDP declines by tannin-protein bonds. Half of the daily portion of the experimental pellets was fed at 08:00 h and the other half at 15:00 h. Around 30 min later, the corresponding proportion of the hay was offered, a time when most to all of the pellets had been consumed. The animals had free access to water.

4.3.2 Animals and experimental design

The experimental protocol complied with the Swiss legislation for Animal Welfare and was approved (ZH 25/16) by the Committee on Animal Experimentation of the Cantonal Veterinary Office Zurich. Six female non-lactating Swiss Black-Brown Mountain sheep with an initial age

of 18 ± 1.7 months and an average BW of 71 ± 5.7 kg were used from August 2016 to January 2017. No worm treatment was performed, as the faecal egg count showed that the animals were free of worms. Animals were fed lucerne-only pellets and hay during 8 days before the main experiment started. The six sheep were allocated to different sequences of four experimental diets in four subsequent 18-day periods in a 6 × 4 crossover design. The animals were weighed after the morning feeding on Days 1, 12 and 19, using data from Day 12 as the reference value for defining daily DM supply on Days 12 to 18. To resume social grouping behaviour and to minimize the carry-over effects of the previous treatment to the following treatment, periods were separated by 2 days of washout period when the animals were group-housed and fed the lucerne-only pellets and hay but no lucerne+hazel leaves pellets. Each period was divided into 11 days (Days 1 to 11) of adaptation to diets in individual pens (size of 1.25 m × 2.5 m, floor covered with sawdust) and 7 days (Days 12 to 18) of complete collection of faeces and urine, including 2 days of individual gaseous exchange measurements performed subsequently with two sheep in parallel. For the 7 days of sample collection and gaseous exchange measurements, the sheep were transferred into metabolic crates (floor area 1.9 m^2) with transparent acrylic glass side panels allowing visual contact. The animals were tethered at the front end. and the floor was covered with a rubber mattress. The front part was non-perforated, and the rear part was grated (column width 20 mm / beam width 40 mm) allowing faeces and urine to fall into the funnel beneath. A screen (beam width 4 mm) in the funnel retained faeces, but not urine. The daily intake of hay and experimental pellets was recorded by weighing amounts offered and refused.

Between Days 12 and 18, individual hay leftovers were sampled and water consumption and the amounts of faeces and urine excreted were recorded. A proportion of 10% of total faeces was collected daily and frozen at -20 °C. The urine was ducted into two containers, one for untreated urine and the other containing 7.1 mol/l sulphuric acid to inhibit N evaporation. Urine from each container was collected daily in proportions of 0.5% of the amounts excreted and stored at -20 °C. Later all types of excreta samples were composited per animal per period. Part of the faeces was dried at 60 °C to a constant weight. The hay was sampled four times and the pellets two times. Hay leftovers were pooled across animals per period.

For the gaseous exchange measurements, two sheep at a time were transferred to two open-circuit respiration chambers (8.3 m^3 each) (Grandl et al. 2016b). For the feeding, the chambers were entered via an airlock. From the total stay of 48 h in the chambers, data of 1 to 1.7 h had to be interpolated from adjacent values, as the measurement was then interrupted for cleaning purpose. The chambers were air-conditioned, and the temperature, relative humidity, air

pressure and airflow were set to 18°C, 70%, -60 Pa and 400 l/min (Promethion FG-1000 flow generators, Sable Systems Europe GmbH, Berlin, Germany), respectively. Lighting conditions were diurnal (7 h lights on, 19 h lights off). The concentrations of CH_4, CO_2 and O_2 were measured in each respiration chamber every 3 min for 1 min with a gas analyser (Promethion GA-4, Sable Systems). Prior to gaseous exchange measurement, the gas analyser was calibrated automatically using pure N_2 (99.999%) and a mixed gas (19.8% O_2, 1.0% CO_2, 0.1% CH_4, in N_2 as carrier). Recovery was tested before each experimental period by burning propane gas. The mean recovery was 95%.

4.3.3 Laboratory analyses

Feed pellets and dried faeces were ground with a centrifugal mill (1-mm screen) and hay with a cutting mill (1-mm screen). Feeds, refusals and dried faeces were analysed for DM, total ash (TGA 701, Leco Corporation, St Joseph, MI, USA; AOAC index no. 942.05), NDF, ADF and acid detergent lignin (Fibertec System M 1020 Hot Extractor and 1021 Cold Extractor; Tecator, Höganäs, Sweden). Fibre data were corrected for ash content, and heat stable α-amylase was used for the NDF analysis, but no sodium sulphite was added (Van Soest et al. 1991). Acid detergent lignin was determined sequentially after the ADF analysis by digestion in sulphuric acid (72%) for 3 h. The content of GE was determined using a Calorimeter (C7000, IKA-Werke GmbH & Co. KG, Staufen, Germany). The N content of feeds, refusals, non-dried faeces and acidified urine was analysed with a C/N analyser (TruMac CN, Leco Corporation, St. Joseph, Michigan, USA; AOAC index no. 968.06). Crude protein (CP) was calculated as 6.25 × N. The carbon content of the non-acidified urine was determined with the same analyser. The feeds were analysed for ether extract (extraction System B-811, Büchi, Flawil, Switzerland; AOAC index no. 963.15). Total phenols in feed items were extracted by 70% acetone based on Makkar[44] with modifications described in Jayanegara et al. (2011). In brief, a modified Folin-Ciocalteu method was applied for TP and non-tannin phenols and data were expressed as gallic acid equivalents (Sigma, St. Louis, MO, USA). The CT were measured using the butanol-hydrochloride-iron method, and the values were expressed as leucocyanidin equivalents (Makkar 2003; Jayanegara et al. 2011). The contents of total tannins and HT were calculated as the difference between TP and non-tannin phenols, and between total tannins and CT, respectively.

4.3.4 Calculations and Statistical Analyses

The equations used to calculate the energy-balance related variables are specified in the footnote to Table 4.5. Individual feed intake, digestibility, N and energy balance were calculated based on the data combined across the 7 day collection periods and, in the case of gas exchange data, across the 2 days of respiration chamber measurements. The BW is given as the average of Days 1 and 19 of each period. To relate variables to $BW^{0.75}$, BW means of measurements made on Days 12 and 19 of each period were used. Data were subjected to analysis of variance with the Mixed procedure of SAS (version 9.4, SAS Institute, Cary, NC), considering the treatment and the period as fixed effects and animals as the random effect. Pairwise multiple comparisons among the least-square means were performed by the Tukey-Kramer test. Linear and quadratic effects of the level of hazel leaves (0%, 25% and 50%) were evaluated by orthogonal polynomial contrasts. Differences were considered statistically significant at $P < 0.05$ and trends at $0.05 \leq P < 0.10$.

Table 4.1 Ingredients and chemical composition of the experimental diets[4]

	Hay[1]	**Hazel leaves**	**Experimental pellets**[1]			
Hazel leaves (% of pellets)			**0**[2]	**30**	**60**	**60+PEG**[3]
Pellet ingredients (% of dry matter (DM))						
Lucerne (% of total pellets)			100	70.8	41.5	37.9
Hazel leaves (% of total pellets)			0	29.2	58.5	58.3
Polyethylene glycol (PEG, % of total pellets)			0	0	0	3.8
Analysed composition (% of DM)						
Organic matter	92.7	92.9	85.6	88.1	90.0	90.8
Crude protein	7.0	12.4	20.2	17.9	15.7	14.8
Ether extract	1.4	1.1	1.9	1.9	1.6	1.5
Neutral detergent fibre	68.9	48.5	44.2	42.9	44.4	44.9
Acid detergent fibre	41.4	30.7	33.1	31.1	30.1	32.5
Acid detergent lignin	7.4	12.2	9.5	11.0	11.5	14.5
Gross energy (MJ/kg DM)	17.5	18.3	16.9	17.4	17.8	18.2
Total phenols	1.43	8.16	1.72	4.14	6.55	5.94
Non-tannin phenols	0.82	1.95	0.96	1.33	1.75	1.58
Total tannins	0.61	6.21	0.76	2.82	4.80	4.36
Condensed tannins	0.02	3.39	0.01	1.11	2.43	1.36
Hydrolysable tannins	0.59	2.82	0.74	1.71	2.37	3.00

[1]The ratio of hay to pellet was 20% : 80% in total dietary DM. [2]Equivalent to the composition of lucerne. [3]Analysis, especially of the phenols, might have been compromised by the presence of PEG. [4]Each animal received additionally 30 g/day of a commercial mineral-vitamin mixture (Kroni 461 Ovipress, Kroni Locher, Altstätten, Switzerland) consisting, per kg, of Ca, 140 g; P, 70 g; Mg, 40 g; Na, 50 g; Zn, 7 g; Mn, 3.5 g; Fe, 2.65 g; I, 60 mg; Co, 30 mg; Se, 40 mg; vitamin A, 400,000 IU; vitamin D_3, 80,000 IU; vitamin E, 2 g; vitamin B_1, 0.4 g and biotine, 0.1 g.

Table 4.2 Effect of hazel leaves on intake, nutrient digestibility and body weight of the sheep (n =6).

	Experimental diets				SEM	*P* values		
Hazel leaves (% of diet)	**0**	**25**	**50**	**50+PEG**		**Diet**	**L[1]**	**Q[1]**
Dry matter intake (g/day)								
Total	2182	2147	2174	2170	45.5	0.64	0.71	0.56
Pellets	1794	1762	1780	1792	36.4	0.73	0.52	0.59
Hay	388	385	394	378	14.5	0.50	0.48	0.88
Daily intake (g/kg body weight$^{0.75}$)								
Drinking water	217^{a}	187^{ab}	157^{b}	162^{ab}	7.8	0.037	0.024	0.95
Dry matter	85.5	85.3	85.9	85.0	0.80	0.66	0.84	0.79
Organic matter	74.1^{b}	75.9^{ab}	77.7^{a}	77.4^{a}	0.77	0.006	<0.001	0.78
Crude protein	15.3^{a}	13.6^{b}	12.2^{c}	11.4^{d}	0.33	<0.001	<0.001	0.33
Neutral detergent fibre	41.5	40.5	41.8	41.6	0.47	0.19	0.30	0.02
Acid detergent fibre	29.5^{a}	28.0^{bc}	27.5^{c}	28.9^{ab}	0.34	<0.001	<0.001	0.091
Gross energy (MJ)	1.45^{c}	1.48^{bc}	1.52^{ab}	1.54^{a}	0.016	0.001	<0.001	0.98
Apparent digestibility (%)								
Organic matter	58.2^{a}	55.5^{a}	48.6^{c}	51.5^{b}	0.85	<0.001	<0.001	0.046
Neutral detergent fibre	49.0^{a}	41.6^{b}	31.2^{c}	39.8^{b}	1.46	<0.001	<0.001	0.32
Acid detergent fibre	45.2^{a}	30.7^{b}	11.8^{d}	23.4^{c}	2.60	<0.001	<0.001	0.15
Body weight (kg)	74.5	73.6	73.8	74.9	1.32	0.62	0.22	0.84

L, linear effect of hazel leave proportion; PEG, polyethylene glycol; Q, quadratic effect of hazel leave proportion; SEM, standard error of mean. Means carrying no common superscript are different at $P < 0.05$.
[1]For this analysis, only diets 0, 25 and 50 were compared.

4.4 Results

4.4.1 Chemical composition of the diet ingredients

The hazel leaves contained slightly more organic matter (OM) than lucerne (treatment 0) but clearly less crude protein (CP) (Table 4.1). The hay was even lower in CP content. Both pellet ingredients (hazel leaves and lucerne) were similar in contents of neutral detergent fibre (NDF) and acid detergent fibre (ADF), whereas the hay contained much higher levels of NDF and ADF. The lignification of the fibre was low in the hay, intermediate in the lucerne and high in the hazel leaves. The hazel leaves contained about 8% of total phenols (TP), whereof 76% were tannins, with almost equal proportions of condensed tannins (CT) and hydrolysable tannins (HT). Both lucerne and hay contained much less TP and were almost free of CT. In the experimental pellets, a variation in TP contents from 2 to 7% was achieved, the range for the CT was from 0 to 2% and for the HT from 1 to 3%.

4.4.2 Intake and digestibility

The total DM intake of the sheep, expressed per day or per kg of metabolic body weight ($BW^{0.75}$), did not differ among treatments. The same was true for the intake of the two diet components, pellets and hay. However, a small but significant increase in OM intake per unit of $BW^{0.75}$ was observed with the diet containing 50% hazel leaves with or without PEG

compared to the control with no hazel leaves (Table 4.2). Water consumption linearly ($P < 0.05$) declined with an increasing hazel leave proportion. Following the differences in the composition of the pellets, the CP and ADF intake per unit of $BW^{0.75}$ largely differed, becoming linearly ($P < 0.001$) smaller with increasing levels of hazel leaves. The apparent digestibility of OM, NDF and ADF was linearly ($P < 0.001$) declining; in the case of OM digestibility, the decline became larger (quadratic contrast at $P < 0.05$) with a higher hazel leave proportion, where the means also differed ($P < 0.001$) from the other diets. The addition of PEG to the diets containing 50% hazel leaves improved ($P < 0.001$) apparent digestibility to values intermediate between the diets with 25% and 50% hazel leaves or even to the level of the 25% diet (NDF digestibility). No difference ($P > 0.10$) between treatments was observed in the body weight (BW) of the sheep and NDF intake per unit of $BW^{0.75}$.

4.4.3 Methane emission

The average daily amount of CH_4 produced by the sheep decreased ($P < 0.001$) from 31 g without hazel leaves to 22 g per sheep fed with 50% hazel leaves in their diet. The decrease had linear ($P < 0.001$) and non-linear components ($P < 0.05$), where the reduction was particularly pronounced with 50% hazel leaves (Table 4.3). With the diet containing 50% hazel leaves together with PEG, the level of CH_4 produced, though numerically higher, was not different from 50% hazel leaves without PEG, but different ($P < 0.001$) from the treatments with 25% hazel leaves and lucerne alone. The same patterns were found when relating CH_4 to intakes of DM, OM and NDF. When scaling CH_4 to $BW^{0.75}$, to digestible DM, and to digestible OM, the effects of hazel leaves without PEG were similar, but the effect with the PEG-treated diet was no longer significantly different from the treatment with 25% hazel leaves. The CH_4 release per unit of NDF digested did not differ between treatments, but a trend ($P = 0.07$) for a non-linear dosage effect was observed.

4.4.4 Nitrogen balance

The daily N intake linearly ($P < 0.001$) declined with an increasing proportion of hazel leaves (Table 4.4). At the same time, the absolute faecal N excretion linearly ($P < 0.001$) increased. This was accompanied by large and linear ($P < 0.001$) declines in the excretion of absolute urinary N and the proportion of urinary N of either total N ingested or total N excreted. The latter two variables also included a trend ($P = 0.09$) or a significant ($P < 0.05$) non-linear component where the 25% dosage of hazel leaves was proportionately less effective than the extra 25% hazel leaves in the diet with 50% hazel leaves. As a consequence of the increase in

faecal N losses, the apparent N digestibility decreased along with the increased proportion of hazel leaves compared to lucerne alone in the pellets. This happened in a linear ($P < 0.001$) manner with a non-linear component ($P < 0.05$). Increasing the hazel leave proportion from 25% to 50% in the diet decreased the body N retention and the utilisation of dietary N for the body N retention ($P < 0.001$). The addition of PEG to the diet with 50% hazel leaves further decreased ($P < 0.001$) the daily N intake, faecal N and the losses of faecal and urinary N, but increased ($P < 0.001$) urinary N compared to the non-PEG 50% diet. Adding PEG prevented ($P < 0.001$) the extra reduction in relative N losses from urine (per total N intake or total N excreted) and apparent N digestibility when increasing the hazel leave proportion from 25% to 50%. The adverse effect on the relative loss of faecal N as well as faecal and urinary N, and the relative body N retention per total N intake was alleviated ($P < 0.05$) by the addition of PEG.

4.4.5 Energy balance

The supply with digestible energy and metabolisable energy (ME) decreased linearly ($P < 0.001$) with increasing proportions of hazel leaves (Table 4.5), although relative and absolute GE intake differed only marginally between treatments (Table 4.2, 4.5). This decrease was slightly more pronounced with the high compared with the low hazel leave proportion (trends for non-linear effects) (Table 4.5). The faecal energy losses were increasing and those with urine and CH_4 were declining with increasing hazel leaves proportion, all in a linear manner ($P < 0.01$) with a certain quadratic effect ($P < 0.05$) in the latter two. Heat energy losses were not affected by the treatments. For total energy losses, the overriding effects were faecal losses resulting in linearly ($P < 0.01$) increasing total losses. However, body energy retention also linearly declined ($P < 0.05$) with increasing hazel leave proportions. As GE intakes did not differ much, the diet effects for relative energy turnover (per unit of GE intake) were similar to absolute energy losses. There was a decline by 11 percentage units in the apparent digestibility of GE when the hazel leave proportion was increased from 0 to 50%. The decline had linear ($P < 0.001$) and by tendency non-linear components ($P = 0.051$). The decline in metabolisability was less pronounced in magnitude because part of the energy losses in faeces were counterbalanced by lower urinary and CH_4 energy losses. The addition of PEG to the diet with 50% hazel leaves increased ($P < 0.001$) the supply with digestible energy and ME. Adding PEG prevented ($P < 0.001$) part of the faecal energy loss per GE intake and the considerable decline in digestibility when increasing the hazel leave proportion from 25% to 50%. No difference ($P > 0.10$) was observed between treatments in the absolute and relative heat energy losses of the sheep.

Table 4.3 Effect of hazel leaves on enteric methane emission of the sheep (n =6).

	Experimental diets				SEM	*P* values		
Hazel leaves (% of diet)	**0**	**25**	**50**	**50+PEG**		**Diet**	**L[1]**	**Q[1]**
Methane per day								
g/sheep	30.8[a]	29.4[a]	21.6[b]	24.9[b]	1.35	<0.001	<0.001	0.023
g/kg body weight[0.75]	1.20[a]	1.13[ab]	0.85[c]	0.97[bc]	0.045	<0.001	<0.001	0.054
Methane (g/kg intake of)								
Dry matter	15.6[a]	14.4[a]	10.5[b]	12.5[b]	0.50	<0.001	<0.001	0.003
Organic matter	17.9[a]	16.3[a]	11.6[b]	13.7[b]	0.60	<0.001	<0.001	0.003
Neutral detergent fibre (NDF)	32.0[a]	30.9[a]	21.6[b]	25.4[b]	1.02	<0.001	<0.001	0.002
Digestible dry matter	28.0[a]	26.7[a]	21.6[b]	25.3[ab]	0.85	0.007	0.004	0.085
Digestible organic matter	30.3[a]	28.5[a]	22.8[b]	26.5[ab]	0.92	0.002	<0.001	0.064
Digestible NDF	64.3	71.1	63.4	63.0	2.00	0.098	0.57	0.069

L, linear effect of hazel leave proportion; PEG, polyethylene glycol; Q, quadratic effect of hazel leave proportion; SEM, standard error of mean. Means carrying no common superscript are different at $P < 0.05$. [1]For this analysis, only diets 0, 25 and 50 were compared.

Table 4.4 Effect of hazel leaves on nitrogen balance of the sheep (n =6).

	Experimental diets				SEM	*P* values		
Hazel leaves (% of diet)	**0**	**25**	**50**	**50+PEG**		**Diet**	**L[1]**	**Q[1]**
g/day								
Nitrogen (N) intake	62.4[a]	54.8[b]	49.3[c]	46.7[d]	1.69	<0.001	<0.001	0.44
Faecal N	24.9[bc]	28.7[b]	37.5[a]	23.9[c]	1.36	<0.001	<0.001	0.16
Urinary N	25.8[a]	17.0[b]	7.0[c]	14.8[b]	1.49	<0.001	<0.001	0.47
Faecal and urinary N	50.6[a]	45.7[b]	44.5[b]	38.1[c]	1.44	<0.001	0.009	0.29
Body N retention[2]	11.7[a]	9.1[ab]	4.9[c]	7.9[bc]	0.65	<0.001	<0.001	0.40
% of N intake								
Faecal N	39.9[c]	52.3[b]	75.7[a]	51.3[b]	2.80	<0.001	<0.001	0.022
Urinary N	41.3[a]	30.9[b]	14.0[c]	31.7[b]	2.12	<0.001	<0.001	0.091
Faecal and urinary N	81.2[b]	83.2[b]	89.7[a]	83.0[b]	1.04	0.010	0.007	0.31
Body N retention[2]	18.8[a]	16.8[a]	10.3[b]	17.0[a]	1.04	0.010	0.007	0.31
Apparent N digestibility (%)	60.1[a]	47.7[b]	24.3[c]	48.7[b]	2.80	<0.001	<0.001	0.022
Urinary N (% of N excreted)	50.9[a]	37.1[b]	15.6[c]	38.2[b]	2.71	<0.001	<0.001	0.040

L, linear effect of hazel leave proportion; PEG, polyethylene glycol; Q, quadratic effect of hazel leave proportion; SEM, standard error of mean. Means carrying no common superscript are different at $P < 0.05$. [1]For this analysis, only diets 0, 25 and 50 were compared. [2]Including N in wool grown during the periods.

Table 4.5 Effect of hazel leaves on energy balance of sheep (n =6).

	Experimental diets				SEM	*P* values		
Hazel leaves (% of diet)	**0**	**25**	**50**	**50+PEG**		**Diet**	**L[1]**	**Q[1]**
Energy intake (MJ/day)								
Gross energy (GE)	37.1^{b}	37.4^{ab}	38.6^{ab}	39.2^{a}	0.81	0.03	0.009	0.68
Digestible energy	20.6^{a}	19.6^{ab}	17.2^{c}	18.8^{b}	0.47	<0.001	<0.001	0.058
Metabolisable energy[2] (ME)	17.4^{a}	16.8^{ab}	14.8^{c}	16.0^{b}	0.38	<0.001	<0.001	0.078
Energy loss (MJ/day)								
Faeces	16.4^{b}	17.7^{b}	21.4^{a}	20.4^{a}	0.63	<0.001	<0.001	0.14
Urine[3]	1.55^{a}	1.20^{b}	1.22^{b}	1.46^{ab}	0.056	0.007	0.005	0.047
Methane[4]	1.70^{a}	1.63^{a}	1.19^{b}	1.37^{b}	0.075	<0.001	<0.001	0.023
Heat[5]	10.7	10.4	9.9	10.8	0.33	0.72	0.56	0.69
Total energy loss	30.3^{b}	30.9^{b}	33.7^{a}	34.1^{a}	0.84	0.001	0.003	0.33
Body energy retention[6]	6.74$^{(a)}$	6.42$^{(ab)}$	4.90$^{(b)}$	5.20$^{(ab)}$	0.334	0.055	0.041	0.44
Energy turnover (% of GE intake)								
Faeces	44.2^{d}	47.5^{c}	55.3^{a}	52.0^{b}	0.96	<0.001	<0.001	0.051
Urine	4.16^{a}	3.22^{b}	3.15^{b}	3.75^{ab}	0.127	0.005	0.002	0.040
Methane	4.59^{a}	4.35^{a}	3.10^{b}	3.48^{b}	0.178	<0.001	<0.001	0.039
Heat	28.7	27.7	25.6	27.5	0.68	0.67	0.26	0.71
Total energy loss	81.7^{b}	82.8^{ab}	87.2^{a}	86.8^{ab}	0.92	0.032	0.025	0.47
Body energy retention	18.3^{a}	17.2^{ab}	12.8^{b}	13.2^{ab}	0.92	0.032	0.025	0.47
Heat energy (% of ME intake)	61.6	61.7	66.7	67.6	1.71	0.31	0.13	0.60
Apparent digestibility (% of GE)	55.8^{a}	52.5^{b}	44.7^{d}	48.0^{c}	0.96	<0.001	<0.001	0.051
Metabolisability[7] (% of GE)	47.0^{a}	44.9^{a}	38.5^{b}	40.8^{b}	0.80	<0.001	<0.001	0.083

L, linear effect of hazel leave proportion; PEG, polyethylene glycol; Q, quadratic effect of hazel leave proportion; SEM, standard error of mean. Means carrying no common superscript without or with brackets are different at $P < 0.05$ and $P < 0.10$, respectively. [1]For this analysis, only diets 0, 25 and 50 were compared. [2]Metabolisable energy (ME; MJ/day) = GE intake (MJ/day) – faecal energy loss (MJ/day) – CH_4 energy loss (MJ/day) – urinary energy loss (MJ/day). [3]Urinary energy (Hoffmann and Klein 1980)(MJ/day) = 0.0348 × urinary carbon (g/day) + 0.009 × urinary N (g/day). [4]CH_4 energy (Brouwer 1965) (MJ/day) = CH_4 (l/day) × 0.03957. [5]Heat energy (Chwalibog et al. 1996) (MJ/day; corrected for assumed CO_2 production from microbial fermentation) = 0.01618 × O_2 (l/day) + 0.00502 × [CO_2 (l/day) – 3 × CH_4 (l/day)] – 0.00217 × CH_4 (l/day) – 0.00599 × urinary N (g/day). [6]Body energy retention (MJ/day) = ME (MJ/day) – heat energy loss (MJ/day). [7]Metabolisability = ME intake (MJ/day) / GE intake (MJ/day).

4.5 Discussion

4. 5.1 Forage value of hazel leaves

Little information is available regarding the forage value of hazel leaves as feed for ruminant livestock. To our knowledge, the current experiment is the first to provide data on the feed intake, nutrient digestibility, as well as the N and energy balance of sheep fed hazel leaves in comparison with a known high quality forage. The amounts of 25 and 50% hazel leaves included in the diets were substantial and were intended to provoke the expression of adverse effects on nutrient intake and digestibility if there were any.

Compared to the lucerne (treatment 0), the hazel leaves were characterised by 39% lower CP contents (being excessive for ruminants in the lucerne), quite similar in fibre contents, and 28% higher lignin contents. The addition of PEG to the pellets should not have caused a decline in the content of phenolic compounds due to maintaining the hazel leave proportion. However, according to the analysis, the pellets with PEG appeared to contain fewer TP, total tannins and CT than the corresponding pellets without PEG. As the difference in CT content was particularly large, stable bonds between CT and PEG likely compromised the CT extraction applied in the laboratory analysis. Therefore, for the PEG-containing pellets, the ratios of PEG : CT and PEG : total tannins were calculated based on the CT and total tannin contents measured in the PEG-free pellets.

One important constraint in employing tanniferous feeds in ruminant nutrition is their often low palatability, which may either limit the intake of these feeds below the levels intended or, especially when mixed with the rest of the diet, impair the overall feed intake. This was not the case in the present study, even when feeding the hazel leaves in a dietary proportion of 50%. It was reported that moderate amounts of tannins (depending on the type of tannins) may improve digestion and animal performance without affecting the voluntary feed intake (Patra et al. 2012). When exceeding a CT concentration of 5 to 6% in dietary DM, a reduction in feed palatability and thus feed intake is commonly observed (Tiemann et al. 2008; Peng et al. 2016). In the present experiment, the dietary CT contents of about 2% in DM remained far below this threshold. However, the restricted access to the diet and mixing the hazel leaves with lucerne might have also prevented differences in DM intake, which could have occurred under conditions of either *ad libitum* access or separate presentation of the hazel leaves. A second constraint in feeding tanniferous feeds often involves adverse effects on nutrient digestibility. Only in cases of dietary CP, a decreased ruminal digestion is favourable, provided the tannin-CP bonds are later cleaved and the protein can be digested in the lower gut. In the present study, the apparent total tract digestibility of OM, and especially of NDF, ADF and N, was reduced

when the sheep consumed pellets with increasing proportions of hazel leaves. As lucerne is a high-quality forage, the effect on digestibility might in part be the consequence of the comparably lower digestibility of the hazel leaves, an assumption supported by the higher lignin content of the hazel leaves compared to lucerne (12.2 vs. 9.5% of DM). Lignin is the diet constituent most adverse to fibre digestion by the ruminant (Jung 1989). However, the difference in lignin content was not sufficiently high to serve as the only explanation. The tannins present in the hazel leaves may have additionally impaired digestibility. Tannins not only bind to protein but also to carbohydrates although to a lesser extent (Patra et al. 2012; Makkar 2003), thus slowing down their ruminal degradation. Tannins are also known to lower the activity of fibrolytic enzymes, an effect depending on the dose and type of tannins (Bhatta et al. 2005; Patra et al. 2012; Al-Kindi et al. 2017). Accordingly, approximately 4% of tannins in the diet are sufficient to lower digestibility of OM, NDF and ADF, as Carulla et al. (2017) found in growing lambs when using *Acacia mearnsii* extract and Al-Kindi et al. (2017) identified in goats when using quebracho tannin extract. Indeed, the addition of PEG to the diet containing 50% hazel leaves alleviated the adverse effect of the hazel leaves on digestibility, but this only partially and not fully. A PEG : CT ratio of 1.6:1 in the diet should have been sufficiently high to prevent the bioactivity of the CT in the rumen (Waghorn 2008). However, still not all dietary tannins might have been inactivated by the PEG because, when also considering the hydrolysable tannins which made up half of the total tannins, the PEG : total tannin ratio was only 0.8:1. It has been described in the literature (Degen et al. 1998) that PEG can also form complexes with HT. In addition, it cannot be excluded that other active ingredients impaired digestibility, such as the non-tannin phenols that made up to 28% of dietary TP. Finally, the low fibre digestibility with the high proportion of hazel leaves may have been indirectly caused by the inactivation of dietary protein in the rumen by the CT. This might have resulted in a deficiency of the supply of rumen-degradable protein (RDP). A deficiency of RDP is especially impairing to the activity of the fibre degrading rumen microbes (Soliva et al. 2015). However, as the decline with increasing hazel leave proportions was mostly linear for OM and NDF digestion and, therefore, also occurred with the lower level of leaves, a RDP deficiency with the high leave proportion seems unlikely. Nevertheless, further studies are needed to determine whether the hazel leave effects are independent from the level of supply with RDP. It also must be stated that according to Makkar et al. (1995) the analysis of the fibre digestibility of tanniferous feeds using the detergent extraction techniques, as applied in the present study, is not always accurate in the presence of tannins and PEG.

Consistent with the lower digestibility, the addition of the hazel leaves also resulted in lower energy and protein retention in the body of the sheep and, again, part of this effect was compensated for by adding PEG. The results agree well with those of a previous study by Tiemann et al. (2008) in which digestible energy and metabolisable energy were adversely affected by increasing the proportion of the CT-rich foliage of *Calliandra calothyrsus* and *Flemingia macrophylla* in the diet of sheep, where also fibre digestibility was reduced. Nevertheless, the efficiency of energy utilization in the metabolism was obviously not impaired, as the treatments had no significant effect on heat energy loss in relation to GE intake. That means that the substrates absorbed from the gut with the four experimental diets appear to have been similarly valuable to the animal. The use of a tanninferous forage like the hazel leaves could be advantageous for metabolic protein utilisation by the animals because of the proposed increase in ruminal bypass feed protein (Beauchemin et al. 2007) and in the absorption of essential amino acids from the intestine in the presence of tannins (reviewed by Min et al. 2003). till, body N (protein) retention declined with increasing hazel leave proportions from about 12 to 5 g/day, consistent with the negative effect on the apparent total tract N digestibility found in the present study and elsewhere (Carulla et al. 2005; Al-Kindi et al. 2017). This indicates that the cleavage of the tannin-protein bonds in the lower gut was not efficient enough to maintain or improve the metabolic supply with protein and amino acids. Whether or not the tannin-protein complexes protected from ruminal degradation are cleaved depends on the type of tannin, its chemical structure and structural flexibility (Knowles et al. 2017) as well as on the pH of the gut environment (Patra et al. 2012). The presence of tannins may even enhance endogenous N losses, leaving the extent to which the increased faecal N losses are really caused by a decreased true N digestibility unclear (Carulla et al. 2005).

4.5.2 Effects of hazel leaves on methane emission

The addition of hazel leaves to the diet clearly depressed CH_4 emissions. This included both the absolute CH_4 emission per head per day and the relative CH_4 emission per intake as well as per $BW^{0.75}$. The present study, with levels of CH_4 mitigation ranging from 25% (per unit of digestible OM) to 35% (per unit of OM intake) in live animals, confirms previous *in vitro* results (Wallace 2008; Terranova et al. 2018a). The level of CH_4 mitigation per unit of digestible OM of 6% found in the present study at a dietary tannin content of 2.4% was exactly as predicted by the equation of Jayanegara et al. (2012) for in vivo studies, but for 4% dietary tannins the mitigation level found was far higher with 25% than the predicted 11%. Similarly, Archimède et al. (2016) observed a decline by 29% in the CH_4 emission per unit of digestible OM of sheep

when adding cassava (*Manihot esculenta*) leaves to a grass-based diet at a level equivalent to 4% tannins in dietary DM. Carulla et al. (2005) found that 4% tannins from *Acacia mearnsii* could decrease methanogenesis per unit of digestible OM by 7% from sheep. As with digestibility, the addition of PEG counterbalanced part of the effect, indicating that hazel leave tannins directly or indirectly lowered methanogenesis. The mode of action by which tannins mitigate CH_4 emission from ruminants is not entirely understood. Among the most accepted explanations is the concept of substrate deprivation, such as the reduction in fibre digestion, which decreases H_2 production being available for reducing carbon dioxide (CO_2) to CH_4, and the direct inhibition of the growth of the methanogens (Jayanegara et al. 2015). In the present study, the CH_4 produced per unit of NDF digested was not affected by the supplementation with hazel leaves, at least not in a linear manner. This lack of response is in agreement with observations made by Carulla et al. (2005). However, a direct reducing effect of hazel leaves on either methanogens or other ruminal microorganisms, such as the protozoa, cannot be fully excluded. In addition, when employing intact plant parts as diet components, other ingredients apart from the tannins, such as lipids, saponins and essential oils, may be bioactive (Jayanegara et al. 2012). This was not examined in the present study.

4.5.3 Effects of hazel leaves on urinary nitrogen excretion

The traits affected most intensively by the dietary treatments were those related to urinary N excretion. Urinary N is the main way of removal of excessive metabolic N from the body of ruminants. However, it is also the major source of ammonia emission from stored manure, leading to air and water pollution, soil acidity and fine particulate matter formation (Lee et al. 2011; Montes et al. 2013) and of nitrous oxide emission from manure-amended soil (Montes et al. 2013). In the present study, replacing lucerne with hazel leaves was effective to reduce the metabolic N load in two ways. First, due to the lower N content of the hazel leaves, the excessive N intake from the lucerne was substantially reduced. Lucerne protein is highly ruminally degradable and promotes absorption through the rumen wall as ammonia and disposed via urine (Patra et al. 2012). Accordingly, along with a decreasing proportion of lucerne in the pellet (from 100 to 38% in the PEG-supplemented pellet), and thus decreasing N intake, the urinary N excretion per unit of N intake decreased, whereas the faecal N excretion per unit of N intake increased. This modified the excretory pattern (urinary N:faecal N) from about 1:1 to 0.6:1. Second, the tannins present in the leaves depressed the net N absorption from the gut. It was not possible to separate these two factors of influence in the present study, even when relating urinary N excretion to N intake. Still, the present results reflect the current feeding practice

where a high quality, highly fertilised forage grass or legume is complemented by tanniferous plant material like the hazel leaves. In combination, the absolute urinary N excretion was reduced by up to 73%, and the reduction relative to N intake was still as great as 27 percentage units. This caused a significant shift of N excretion from urine to faeces, which was clearly less when PEG was added in the present study. When stored as slurry, gaseous N emissions would have been drastically reduced by this feeding measure (Külling et al. 2003). Therefore, the substantial shift in N excretion from urine to faeces when feeding hazel leaves is highly favourable from an environmental perspective, as gaseous N emission and N leaching potentials are considerably reduced. The lower amount of N to be disposed via urine also led to a decrease in water consumption (-28%) and urine volume (2.3 vs. 1.2 kg per day; data not shown in the table) for the diet with 50% compared to that with 0% hazel leaves, respectively. Tannin supplementation was previously found to be effective in shifting urinary N to faecal N, as in the study of Carulla et al. (2005) where 4% *Acacia mearnsii* tannin extract in DM decreased urinary N and increased the faecal N excretion per N intake by 14% and 30%, respectively.

An application of hazel leaves in ruminant nutrition on a broad scale would require ample amounts of hazel leaves. This would require the joint impetus of farmers, consumers and politicians to produce and consume climate-friendly foods from livestock. In addition, an extended cultivation of this woody plant would increase biodiversity, also by providing a habitat for numerous species.

4.6 Conclusion

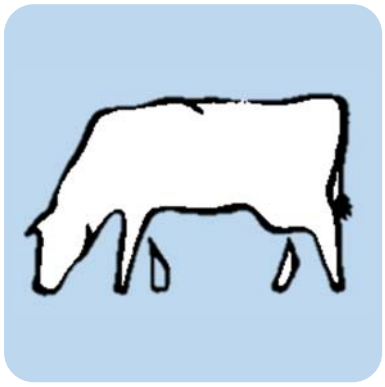
Hazel leaves, found promising in mitigating CH_4 emission from *in vitro* rumen fermentation screenings, indeed turned out to be very efficient in live animals receiving high hazel levels. To be implemented in feeding practice, the forage value in terms of intake (or palatability) and digestibility may not be compromised. In the present study, dietary intake was sustained, even with high levels of hazel leaves replacing the high-quality forage lucerne. However, organic matter digestibility and body energy and N retention was lower with diets containing high levels of hazel leaves. This limitation is theoretically easy to overcome, for instance by adding diet ingredients rich in rumen fermentable energy, providing both metabolisable energy and protein (from microbes). However, this needs to be ascertained in further experiments as rumen fermentation and CH_4 emission may be affected by this dietary modification as well. The unchanged intake indicating a high palatability of hazel leave supplements provides an advantage over many other plant-based supplements.

4.7 Acknowledgements

The authors are grateful to C. Kunz, P. Stirnemann, and M. Mergani (ETH Zurich) for their assistance in the lab, and to H. Renfer, R. Sonderegger, M. Hunziker, and K. Luthiger (Strickhof), S. Amelchanka and R. Stoz (AgroVet Strickhof) and H. R. Wettstein and I. Widmer (ETH Research Station Chamau) for their assistance with the animal experiment. This study was supported by the China Scholarship Council and by the ETH Zurich Research Grant (ETH-49 15-1).

Chapter 5

Associative effects of combining tanniferous hazel leaves with a high quality forage lucerne on methane, urine N, digestion, energy and protein utilization



This chapter is based on Terranova M., Eggerschwiler, L., Clauss, M., Ortmann, S., Kreuzer, M. and Schwarm, A. *Journal of Dairy Science,* in preparation to submission.

5.1 Abstract

Various feeds have been identified which help mitigating the important greenhouse gas methane from ruminants. However, even when there is success in suppressing absolute methane emissions, often intake, digestibility and performance are concomitantly declined. The search has to concentrate on dietary levels of effective feeds where methane already declines and performance-related variables not yet. Such positive associative effects have been demonstrated *in vitro* by combining a high quality forage and phenol rich plants. In the present study this was tested in a dairy cow experiment where a high quality forage (dried alfalfa) was gradually exchanged from 0 to 82% by the phenol rich leaves of hazel (*Corylus avellana*) in pellets. Total hazel proportion of the diet, consisting also of a mixed basal ration and few concentrate, reached some 40%. The experiment was conducted with 20 dairy cows with a mean body weight of 711 ± 49.7 kg and 151 to 310 days in milk. After 14 days of adaptation, 8 days were used for intensive sampling of feces (labelled with markers for determining passage rate) and urine as well as letting cows stay for 2 days in open circuit respiration chambers. Like in former experiments, hazel leaves did not impair feed intake. However, likely due to the lower feeding value due to a high lignin content, digestibility and milk yield were declining with increasing hazel proportion. In the case of milk yield this was unexpectedly most pronounced at low hazel proportions whereas high proportions from about 25% in diet did not impair yield further. Methane emission decreased clearly absolute and per unit of DM intake or body weight. When related to intake of digestible organic matter and especially with milk yield, low hazel levels were ineffective or even promoted methane emission. This demonstrated that there were associative effects but not on the desired direction. Increasing hazel proportions substantially shifted N excretion as anticipated from urinary (decline from about 30 to 10% of intake) to fecal N (from <40 to 60%). This could also be concluded from the sharp decline in milk urea content (from about 30 to 10 mg/dl). The decline in urinary N was mostly linear, but associative effects in relation to milk yield occurred. In conclusion, hazel leaves again showed a great potential as palatable feed supplement for dairy cows to mitigate methane and urinary nitrogen emissions, but positive associative effects seem not to exist when combining with alfalfa.

5.2 Introduction

Various attempts are made to mitigate the emission of the greenhouse gas methane (CH_4) from ruminant husbandry due to its large share of total greenhouse gas emission. (Steinfeld et al. 2006). Feeding strategies have a high potential to mitigate CH_4 emissions from enteric fermentation in livestock (Hristov et al., 2013). Some nutritional mitigation strategies based on feed supplements are ready to be applied in the field (Martin et al. 2010) and have the greatest immediate impact of all mitigation measures (Cottle et al., 2011). Among the most promising dietary attempts for CH_4 mitigation is the strategic use of plants rich in secondary metabolites (PSM) including phenols and, within phenols, tannins as an important group (Hristov et al., 2013). Furthermore, as a result of the increasing food-feed competition when potential food is used in animal nutrition, alternative feeds are becoming increasingly important (Makkar 2018). In this respect, shrub and tree leaves gain interest as they often are rich in PSM (Frutos et al., 2004). Accordingly, some of such leaves were shown to have potential to mitigate formation of CH_4 and, in the case tannins are prevalent, of ammonia in the rumen (Bodas et al., 2008, Jayanegara et al., 2011). Drawbacks of leaves from woody plants may be a low palatability and adverse effects on nutrient digestibility (Moss et al., 2000). The ruminal and total tract passage time of the feed could be affected as well by its phenol content (Baker and Hobbs, 1987; Melaku et al., 2005). Often mitigation of absolute methane or ammonia emission is reported from feeds tested, but sustainable effects are only given when also the emission intensity (per unit of food produced) or at least yield (per intake of dry matter (DM) or digestible nutrients) is reduced. Especially the latter would not be given in case diet digestibility is hampered by supplementation of PSC rich feeds. It could, however, be that the adverse effects on digestibility are at high supplementation levels whereas methane and ammonia mitigation starts at lower dosages. The opposite is also possible. Its occurrence would need non-linearity of either digestibility or methane and ammonia formation with increasing PSC dosage resulting in so called associative effects, a term introduced by Niderkorn and Baumont (2009) for a situation where one dietary item affects the digestion of another item either positively or negatively. In general, dietary associative effects are considered to be very common and cause over- or underestimations of the nutritional value of supplements (Van Soest 1994). In the present context, combinations of high quality feeds with phenol rich plants which suppress ruminal methane and ammonia formation at still high total diet digestion are particularly of interest. For methane and ammonia this was indeed demonstrated before *in vitro* when incubating the high quality forage papaya leaves with several other PSM rich plants (Jayanegara et al., 2013). This phenomenon has not yet been studied by a specifically designed *in vivo* experiment.

Therefore, the aim of the present study was to determine whether similarly favorable results can also be achieved in dairy cows. This needed a plant rich in PSM which is highly palatable to exclude confounding effects between intake and digestion and which has a clear methane mitigating effect. The leaves of the shrub hazel (*Corylus avellana*) were selected for this purpose, which are rich in phenols, especially the flavanols myricetin 3-rhamnoside and quercetin 3-rhamnoside from the group of the condensed tannins (CT) (Amaral et al., 2005). In previous *in vitro* studies leaves of the hazel were effective in reducing CH_4 and ammonia emissions without affecting the ruminal digestibility, and were found to be high palatable to dairy cows (Terranova et al., 2018a,b). In addition, the leaves were palatable in sheep where it effectively suppressed CH_4 formation (Wang et al., 2018). There were indications of dosage dependent effects of the hazel leaves *in vitro* and *in vivo.* In order to test the associative effects on methane and nitrogen excretion of dairy cows in relation to yield and emission intensity, 20 diets with a gradient in hazel leaves exchanging increasing amounts of dried alfalfa as a model for a high quality forage were designed and evaluated by multiple regression.

5.3 Materials and methods

5.3.1 Animals, diets and experimental design

The experiment, taking place in autumn 2017 at AgroVet Strickhof (Lindau, Switzerland), was approved by the Cantonal Veterinary Office of Zurich (licence no. ZH271/16). Twelve Brown Swiss and eight Holstein cows were selected. At the start of the experiment, cows were between the 2nd and 7th lactation, between 151 to 310 days in milk and weighed 711 ± 49.7 kg. The selection aimed to include animals with a similar and rather low milk yield and excluded heifers.

The experimental diets consisted of a mixed basal ration and experimental pellets (0.4:0.6), and additional energy and protein supplements (Table 5.1). The mixed basal ration was composed of 55% corn silage, 38% grass silage and 2% of hay (both from ryegrass dominated swards), and 5% of dairy concentrate (UFA 250). The pellets were composed of hazel leaves, alfalfa (*Medicago sativa*) and molasses (Table 5.2). Hazel leaves were provided from Alfred Galke GmbH (Bad Grund, Germany) dried and cut to a 4 to 6 mm size. They had been harvested in 2015 and 2016 in Albany. The alfalfa, purchased from Landi (Sense-Düdingen, Switzerland), had been harvested in 2016 in France. Twenty different types of experimental pellets were produced with increasing proportion of hazel leaves (0 to 80%) and decreasing levels of alfalfa (17 to 97%) and each with 3% of molasses on a DM basis (Table 5.2). Alfalfa was chopped to 3 mm particle length with a Sigma 5.2 hammer mill (Kuhn AG, Bottighofen, Switzerland).

Chopped hazel leaves, alfalfa and molasses were mixed with a batch mixer (Speedmix DEML-1000, Bühler AG, Uzwil, Switzerland) and afterwards pelleted to 4.5 mm diameter (Kahl 40 PS, Amandus Kahl GmbH & Co, Reinbeck, Germany) using steam of max. 60°C (Bühler AG, Uzwil, Switzerland). Each cow got a different pellet type and thus amount of hazel leaves. In addition, each experimental diet was adapted by a soybean meal and wheat flakes mixture to meet the animals' requirements for protein and energy by considering pellet composition and milk yield. In addition each cow received 80 g/day of a mineral-vitamin mix and 40 g/day of NaCl.

The experiment was conducted in ten subsequent runs with two animals each being subjected to the experimental procedures in parallel. Diets were allocated to these pairs in a randomized way. Each animal concluded 22 days of experiment. At first animals were moved from loose housing to tied stalls and were allowed to adapt to housing and diets for 14 days. In that time they also had a first visit to the respiration chambers for 3 h. After adaptation, 8-days of sampling followed. On days 7 and 8, the cow pairs stayed in the two respiration chambers for 50 h.

5.3.2 Data recording and sampling

The individual tied stalls were equipped with a swing-over milking system, which recorded milk yield, and separated weighing plates developed by Mettler-Toledo (Greifensee, Switzerland) for the purpose of determining individual feed intake. Cows were milked at 0530 and 1630 h. On the first adaptation day and during each milking event in the sampling period, milk samples were taken and conserved with Bronopol. Feed intake was recorded daily across all 22 experiment days. The mixed basal ration, the soybean meal, wheat flakes and mineral supplement was offered on the weighing plates. Leftovers were recorded and removed before the morning feeding. The pellets were fed in separate troughs and leftovers were manually weighed before morning feeding. During the stay of the cows in the respiration chambers the pellets were also offered separately and all feed leftovers were recorded. Feeding was done at the time of the morning and evening milkings and, additionally, at 1300 h. Mineral and NaCl supplements were given a one daily portion during the evening milking. The animals had permanent free access to water. Samples of the mixed basal ration and of grass and corn silage were taken weekly resulting in eight samples per feed. Hay samples were taken three times, concentrate, soybean meal and wheat flakes samples two times in total. Samples of each pellet type were taken on days 1, 15 and 22 of the individual animal's experimental period. The cows

were weighed on the first day of adaptation and on the first and last day of the sampling period with a truck load scale (Waagen Döhrn GmbH & Co. KG, Wesel, Germany).

During the sampling period, the entire feces were collected in steel trays located below a grid at the end of the tie stall. Urine was collected separately from the feces with urinals which were attached around the vulva of the cows. The urine was led into a container and a small subsample was diverted into a canister containing 30 g of 5 M sulfuric acid to prevent gaseous N losses. Acidified and non-acidified urine samples of 50 ml were taken daily during the sampling period and frozen at -20°C for later analysis. The total daily amount of feces and urine was weighed and recorded once per day. A proportion of 0.5% of the feces was frozen at -20°C. Feces and urine were later pooled to one sample per cow. For determination of the passage time, Co-EDTA was used as solute marker and fiber from hay cut to 2, 5 and 8 mm length and mordanted with Cr, La and Ce was used as particle marker. Details on the production of the markers are described in Grandl et al. (2018). On day 1 within the sampling period, the animals received a dosage of approximately 0.1 g of each particle marker and 0.01 g Co-EDTA per kg of body weight. The frozen boli were applied to the animals with a commercially bolus applicator for cows. About 250 g of fecal samples were taken on the day before the marker application (3 samples) 4, 8, 12, 18, 22, 26, 30, 36, 42, 46, 52, 58, 66, 74, 82, 90, 98, 106, 114, 126, 138 and 150 h after application. These samples were dried at 100°C to constant weight and ground through a 1-mm screen with a centrifugal mill (Model ZM1, Retsch GmbH, Haan, Germany). The same procedure was applied for part of the feces samples pooled across the entire sampling period.

5.3.3 Respiration chambers measurements

Two open-circuit respiration chambers with a volume of 22.4 m^3 (cf. Grandl et al., 2016b) were used for measurement of the gaseous exchange of the animals. For milking and feeding, the chambers were entered through an airlock. The chambers were air-conditioned to maintain 17°C ambient temperature, a relative humidity of 60%, and an air pressure of -60 Pa. Airflow was set so 800 L/min (Promethion FG-1000 flow generators, Sable Systems, Las Vegas, NV). Concentrations of CH_4, CO_2 and O_2 were measured in each respiration chamber every 3 min for 1 min with a gas analyzer (Promethion GA-4, Sable Systems). Before starting the measurement, the gas analyzer was calibrated with pure N_2 (99.999%) and a mixed gas (19.8% O_2, 1.0% CO_2, 0.1% CH_4, in N_2 as carrier). Recovery was tested three times in the experiment by burning of propane gas. The mean recovery rate was 94.0%. Data from 48 h were used for

gaseous exchange evaluation. The individual CH_4, CO_2 and O_2 amounts were calculated as averages of the 2 days measured.

5.3.4 Laboratory analyses

Feed items and excreta were analyzed according to standard procedures (AOAC, 1995). Dry matter (DM) and total ash (TA) were analyzed with a TGA-701 furnace (Leco Corporation, St. Joseph, Michigan, USA; AOAC index no. 942.05). Organic matter (OM) was calculated as DM minus TA. Nitrogen (N) contents in feed items, non-dried feces and acidified urine were quantified with a C/N analyzer (TruMac CN, Leco Corporation; AOAC index no. 968.06). Crude protein (CP) was calculated as 6.25 × N. Carbon (C) content of the non-acidified urine was determined with the same device. Contents of ether extract (EE) were determined with a Soxhlet extractor (Extraction System B-811, Büchi, Flawil, Switzerland; AOAC index no. 963.15). Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were assessed in a Fibertherm system FT 12 (Gerhardt GmbH & Co. KG, Koenigswinter, Germany). Heat-stable α-amylase (Sigma-Aldrich, St. Luis, USA) was used for the NDF analysis by methods 6.5.1 and 6.5.2, respectively of VDLUFA (2012). The ADF was analyzed by method 6.5.2. Determination of ADL was sequentially after the ADF analysis by incubation in sulfuric acid (72%) for 3 h. The gross energy (GE) content was measured in feed items and feces with a bomb calorimeter (C7000, IKA-Werke GmbH & Co. KG, Staufen, Germany). Analysis of total phenols and phenol fractions was performed according to Makkar (2003). Extracts from the feed items were twice with a 700ml acetone/l solution. Contents of total phenols (TP) and non-tannin phenols (NTP) were expressed as gallic acid equivalents. Total tannin (TT) contents were calculated by the difference of TP and NTP. Condensed tannins (CT) were given as leucocyanidin equivalents and hydrolysable tannins (HT) were the result of the difference between TT and CT. The phenol fractions were quantified with a double beam spectrophotometer (UV-6300PC, VWR, Leuven, Belgium).

The marker concentrations were analyzed after wet ashing in the mordanted hay particles and in the feces using inductively coupled plasma optical emission spectrometry (Optima 8000, Pekin Elmer, Rodgau, Germany). The mordanted particles contained, per kg DM, 32.8 g Cr, 49.5 g La, 41.5 g Ce and 151 g Co. The baseline concentrations measured before the marker application were used to correct for fecal background levels in each individual animal.

The Bronopol conserved milk was analyzed for fat, lactose, protein and urea contents with a MilkoScan FT6000 (Foss, Hillerød, Denmark) at SuisseLab AG (Zollikofen, Switzerland).

5.3.5 Calculations and statistical analysis

Energy corrected milk was calculated as ECM (kg) = milk (kg) × (0.38 × fat (%) + 0.24 × protein (%) + 0.17 × lactose (%))/3.14 (Agroscope, 2017).

Mean retention time (MRT) in the gastrointestinal tract (GIT) was computed for each marker according to Thielemans et al. (1978) as MRT GIT = (Σ C_i x t_{i-1} x dt_i) / (Σ C_i x dt_i), where t_{i-1} = mean time (h) after application of markers of two subsequent samplings i-1 and i calculated as t_{i-1} + (t_i-t_{i-1}) / 2, C_i = marker content in the fecal sample voided in the interval represented by time t_i and t_{i-1}, and dt_i = sampling interval [h] of the respective sample calculated as ((t_{i-1} –t_i) +(t_i –t_{i-1}))/2. The MRT of the solute marker Co-EDTA in the reticulorumen (RR) was calculated according to Grovum and Williams (1973). The MRT of the particles in the RR was calculated according to Huhtanen and Kukkonen (1995) as MRT RR particles = MRT GIT particles – (MRT GIT solute – MRT RR solute).

The following equations were applied for calculating digestibility and energy-balance related variables:

(1) Apparent digestibility = (intake (g or MJ/day) – fecal loss (g or MJ/day)) / intake (g or MJ/day);

(2) CH_4 energy (MJ/day) = CH_4 (l/day) × 0.03957 (Brouwer, 1965);

(3) Urine energy (MJ/day) = 0.0331 × urine C (g/day) + 0.0092 × urine N (g/day) (Hoffmann und Klein, 1980);

(4) Heat energy (MJ/day) = 0.01618 × O_2 (l/day) + 0.00502 × (CO_2 (l/day) - 3 × CH_4 (l/day)) – 0.00217 × CH_4 (l/day) – 0.00599 × urine N (g/day) (Chwalibog et al., 1996);

(5) Body energy retention (MJ/day) = GE intake (MJ/day) – fecal energy loss (MJ/day) – CH_4 energy loss (MJ/day) – urine energy loss (MJ/day) – heat energy loss (MJ/day);

(6) Metabolizability (% of GE) = ME intake (MJ/day) / GE intake (MJ/day) × 100 (GfE, 2001).

Data analysis was performed with SAS (version 9.4, SAS Institute, Carry NC, USA). To determine the relationship between the hazel leave proportion and the dependent variables, regression analysis was performed with procedure REG. The full model applied was:

$$Y_{ijklmn} = \mu + \beta_i H + \beta_j H^2 + \beta_k ECM + \beta_l DIM + \beta_m \Delta BW + \beta_n ADLI + \varepsilon_{ijklmn}$$

where Y_{ijklmn} is the individual observation of the respective variable, μ is the overall mean, β_{ijklmn} is the regression coefficient of the fixed effects of hazel leave proportion linear (H) and quadratic (H^2), and (to obtain adjusted effects of hazel leave proportion) of the covariates ECM yield (ECM), days in milk (DIM), body weight change (ΔBW) during the experimental days, and ADL intake (ADLI). For milk data and in methane emission intensity ECM yield or ΔBW

or both covariates were excluded from the full regression model to avoid confounding. The latter served as additional covariate to correct for individual differences in intake of indigestible fiber fractions. Including the hazel leave proportion in a linear and quadratic term allowed modelling linear and nonlinear relationships of the variables with hazel leave proportion. For the passage time the model was modified with ΔBW being excluded and DIM being replaced by lactation number as it is known that animal age can influence passage time (Grandl et al. 2016a). The models with the lowest Akaike information criterion were chosen which comprised H or H^2 or both. The parameter estimates are given with the unadjusted R^2, standard errors (SE), the coefficient of variation (CV) and the model specific significance as well as the significance levels for individual parameters. The plots were drawn with SigmaPlot 13. The regression analysis was applied to 18 of the 20 experimental animals. One animal was excluded because of diarrhea during the sampling period, the other because it consumed hazel pellets only at 60% of total for most of the time. When substantial hazel leave proportion effects were occurring, results were illustrated in graphs showing observations and regression lines which did not always fully coincide due to the effects of the covariates. For all variables evaluated, regression coefficients are given in full in the appendix.

5.4 Results

5.4.1 Composition of the experimental feeds

Phenol contents were low in all feed items except the hazel leaves (Table 5.1). Most of the hazel phenols were CT. Alfalfa contained 5.5% more CP than the hazel leaves, slightly more NDF and ADF, but clearly less ADL. The ratio between hazel leaves and alfalfa in the experimental pellets was varied in a gradient in the 20 pellet types from 0 to 1 to 0.82 to 0.18 (Table 5.2). Along with that, the hazel proportion in the total diet was ranging from 0 to some 40%, and contents of TP, NTP, TT, CT and HT in the pellets were increased from very low levels to 9, 2, 5-6, 5 and 1%. This increased the respective contents in the total diet to 5, 1, 4, 3, and 1% (data not shown in table). Concomitantly, CP content declined and ADL content increased in the pellets with increasing hazel inclusion.

Table 5.1 Diet composition and analyzed chemical composition of the mixed basal ration, the energy and protein compensatory feed and the main ingredients of the pellet raw material.

	Ingredients of the experimental diets						Main ingredients of the pellets	
	Mixed basal ration (MBR)				Compensatory feeds[1]			
	Grass silage	Corn silage	Hay	Concentrate	Soybean meal	Wheat flakes	Hazel leaves	Alfalfa
% of mixed basal ration DM	38.0	55.0	2.0	5.0				
Composition, % of DM								
DM, % of wet weight	35.5	35.4	87.4	97.0	88.6	88.9		
Organic matter	87.8	96.7	88.7	81.0	92.7	98.2	92.3	89.3
Crude protein	17.1	7.27	12.4	32.2	54.7	13.0	12.2	17.8
Ether extract	2.79	3.43	1.65	2.06	1.32	1.85	1.66	1.78
NDF	60.3	48.8	43.9	14.7	13.7	53.7	45.7	48.9
ADF	38.7	29.9	34.4	11.1	9.32	12.9	40.8	44.7
ADL	7.39	5.00	6.45	5.87	1.72	6.25	23.8	14.7
Total phenols	1.38	0.879	1.28	0.455	0.355	0.121	10.3	0.986
Non-tannin phenols	1.15	0.730	0.993	0.420	0.304	0.084	2.18	0.859
Total tannins	0.223	0.150	0.289	0.035	0.050	0.037	8.14	0.127
Condensed tannins	0.031	0.008	0.031	0.000	0.000	0.000	7.93	0.009
Hydrolysable tannins	0.191	0.141	0.258	0.035	0.050	0.037	0.21	0.118
GE (MJ/kg DM)	18.0	18.4	14.7	14.7	19.7	18.3	19.1	18.2

DM, dry matter; GE, gross energy.

[1]Offered in a ratio of 1:1 and used to balance the energy and protein content of the different pellets and to account for different milk yields; each cow received 80 g/day of a mineral-vitamin mix and 40 g/day of NaCl.

Table 5.2 Ingredient composition and analyzed chemical composition of the individual experimental pellets.

Hazel, % of pellet	0	9	18	27	36	45	48	51	53	56	58	61	64	66	69	72	74	77	80	82
Lucerne, % of pellet	100	91	82	73	64	55	52	49	47	44	42	39	36	34	31	28	26	23	20	18
Hazel, % of DM[1]	0	5.1	9.7	15.2	19.3	24.9	24.8	27.9	26.9	30.6	31.3	33.0	34.5	33.0	34.1	38.6	39.1	33.3	41.4	41.3
Pellet ingredients, % of DM																				
Hazel	0.0	8.8	17.5	26.3	35.1	43.8	46.4	49.0	51.6	54.2	56.7	59.3	61.9	64.5	67.1	69.7	72.2	74.8	77.4	80.0
Lucerne	97.0	88.2	79.5	70.7	61.9	53.2	50.6	48.0	45.4	42.8	40.3	37.7	35.1	32.5	29.9	27.3	24.8	22.2	19.6	17.0
Molasses	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Pellet composition, % of DM																				
Organic matter	88.4	89.2	89.3	89.6	90.1	90.3	90.6	90.6	90.5	91.0	91.2	91.4	91.4	91.3	90.9	91.6	91.7	89.6	91.8	92.0
Crude protein	19.0	17.1	16.9	16.3	16.3	15.1	15.1	14.8	15.1	14.5	14.4	14.3	14.3	13.6	13.4	13.6	13.3	13.5	13.2	12.8
Ether extract	2.24	1.78	1.83	1.68	1.95	1.83	1.91	1.84	1.78	1.84	1.94	2.09	2.15	2.01	1.97	2.21	2.16	1.99	2.10	1.94
NDF	40.7	48.6	48.1	46.6	48.3	48.8	48.3	47.5	52.3	51.2	52.2	48.6	51.2	50.5	49.8	50.0	51.1	49.2	48.6	48.1
ADF	38.3	42.8	39.8	40.0	41.7	42.5	47.4	41.9	41.3	39.7	49.1	39.8	47.2	40.5	37.1	39.1	49.9	41.6	42.3	41.1
ADL	9.1	14.8	13.8	16.6	22.9	16.8	17.5	17.1	18.3	17.5	22.7	17.3	19.4	17.2	18.0	18.1	22.4	19.9	19.4	19.1
Total phenols	0.52	1.83	2.36	3.32	4.85	4.64	4.95	5.72	6.29	6.62	6.70	6.81	6.94	7.36	7.72	8.34	8.38	8.16	8.57	8.62
Non-tannin phenols	0.45	1.04	1.11	1.31	1.67	1.42	1.44	1.49	1.61	1.71	1.80	1.69	1.68	1.75	1.86	1.99	1.89	1.85	1.92	1.95
Total tannins	0.08	0.80	1.25	2.00	3.18	3.22	3.51	4.22	4.51	4.92	4.56	4.88	5.27	5.61	5.87	6.35	6.49	6.31	6.65	6.49
Condensed tannins	0.03	0.40	0.76	1.41	2.42	2.49	2.71	3.29	3.72	3.50	3.44	3.73	4.15	4.76	4.94	4.66	4.89	4.91	4.92	4.93
Hydrolyz. tannins	0.05	0.40	0.49	0.59	0.76	0.73	0.81	0.93	0.70	0.66	1.12	0.95	1.12	0.55	0.92	0.63	1.61	1.41	1.94	1.55
GE (MJ/kg DM)	17.9	17.6	17.7	18.0	18.1	18.2	18.4	17.9	18.0	17.7	18.7	17.9	18.4	18.2	18.2	18.4	18.4	18.3	18.5	18.5

DM, dry matter DM; Hazel, hazel leaves.

[1]Certain variation in dietary pellet proportion due to cow-specific amounts of compensatory feeds.

5.4.2 Feed intake, milk yield and composition

The regression analysis showed that DMI absolutely and per kg of metabolic BW were not significantly affected by hazel leave proportion in the diet when the covariates were included in the regression model (Appendices Table A5.1). There was a curvilinear negative relationship between hazel proportion and ECM yield absolute and per DMI in a way that after a clear decline at lower proportions no further decline happened at high hazel levels ($P < 0.01$ for linear and non-linear coefficients; Figure 5.1). The decline in ECM yield, (values compared to yield three days before the first experimental day), was linear when considered separately. Milk fat and protein contents increased ($P < 0.05$) with increasing hazel proportion, this linear with milk fat and curvilinear ($P < 0.05$) with milk protein where a plateau was reached at around 300 g hazel/kg DM (Figure 5.1). Lactose content remained unaffected. Milk urea sharply and linearly ($P < 0.001$) declined with increasing hazel proportion from over 30 mg/dl to almost 10 mg/dl.

5.4.3 Digesta passage and digestibility

Hazel proportion had only weak effects on mean retention times of particles and solute in the reticula rumen (RR) and in the total digestive tract (GIT) which all pointed towards and increase with hazel proportion (Figure 5.2; Appendices Table A5.2). Especially the passage in the GIT of solute matter ($P < 0.05$) and that of the medium sized particles ($P < 0.10$) showed this trend. In other cases the model was significant but there was no clear hazel effect. The digestibility coefficients of DM, OM and NDF linearly declined ($P < 0,05$ to 0.001) with increasing hazel proportion, but the decline was more pronounced in fiber digestibility than that in DM and OM (Figure 2; Appendices Table A5.1).

5.4.4 Energy and nitrogen balance

The regression models for almost all variables determining energy balance was significant, but hazel proportion only affected some of the variables (Figure 5.3; Appendices Table A5.3). Fecal energy losses linearly increased with hazel proportion both in absolute amounts ($P < 0.01$) and as a proportion of gross energy intake ($P < 0.001$). This resulted in a linear decline of energy digestibility ($P < 0.001$). Different from that, energy loss through CH_4 linearly declined both absolute and relative to gross energy intake with increasing hazel proportion ($P < 0.001$). Overall this decline was not sufficient to fully compensate the fecal losses causing metabolizability of energy to decline linearly as well ($P < 0.01$).

With increasing hazel proportion fecal N increased and the urinary N decreased linearly ($P < 0.001$) both when expressed in absolute levels and per unit of N intake (Figure 5.3; Appendices Table A5.3). This caused an opposite relationship with apparent N digestibility which, however, also included a non-linear component ($P < 0.05$) where low hazel proportions even increased this variable (data not shown in figure). In the case of urinary N in proportion of N intake the decline also had a trend for a non-linear component ($P < 0.10$), letting this variable decrease more than proportionate at higher hazel proportion. Excretion of milk N absolute and relative to N intake showed a curvilinear effect with linear and non-linear components ($P < 0.05$) where the decline got less pronounced at higher hazel proportions. Body N retention also responded in a curvilinear manner to hazel proportion both in absolute terms and per unit of N intake (regression components mostly $P < 0.05$) and also urine N relative to milk N showed a curvilinear shape.

5.4.5 Methane emissions

All CH_4 variables analysed were related ($P < 0.10$ to 0.001) to hazel proportion leaves in the diet (Figure 5.4; Appendices Table A5.4). In detail, the CH_4 emission absolute and per units of DMI, GE (Ym), BW and metabolic BW was linearly ($P < 0.001$) and substantially declining with increasing hazel proportion. When related to intake of digestible OM and ECM, CH_4 emissions per kg and per kg ECM did not decline (digestible OM) or even increase (ECM) at low hazel proportions (quadratic term, $P < 0.001$ and $P < 0.05$ for digestible OM intake and ECM, respectively). Hazel proportions exceeding 250 g/DM, CH_4 emissions relative to digestible OM intake and ECM were clearly decreasing. There was a trend ($P < 0.10$) for a weak increase trend of CH_4 emission per unit of digestible NDF consumed with increasing hazel proportion.

5.5 Discussion

5.5.1 Pellet composition, feed intake and performance

The variation achieved with the exchange of the high quality forage alfalfa by the PSC rich forage hazel leaves in the pellets was large, and as the pellets made up 55% of the diet, also the individual diets varied extensively in nutrient composition. The main exchange was a decrease in CP content by up to 33% and an increase in phenol and lignin content in the experimental pellets by up to 94 and 60%. The latter was likely associated with a substantial decline in net energy content. It is well known that phenol contents and their composition may vary due to

cultivar and environmental conditions (Palo et al., 1985; Wam et al., 2017). The level of TP in the batch of hazel leaves used was 10.3 g/kg which was similar as that investigated *in vitro* earlier Terranova et al. (2018a) whereas it was higher than in the batch used by Wang et al. (2018) where also equal proportions of CT and HT in the TT were found whereas in the present and the previous study (Terranova et al., 2018a) TT mostly consisted of CT concentrations than HT.

Despite the lower feeding value and the high concentrations of phenols and lignin, high hazel proportions in the pellets did cause significant refusals only in one cow which received pellets with 330 g hazel/kg pellet. Highly tanniferous feeds are often of low feed palatability in ruminants (Frutos et al. 2004, Waghorn 2008) and, as known for long, also those high in lignin content. In a recent preference study (Terranova et al., 2018b) hazel was the most palatable out of six tannin-rich plants when processed with alfalfa to pellets. In that study it turned out that the ADL content had a greater effect on the palatability than the tannin content, and hazel leaves were among the plants with low lignin content. Also adult sheep were found to consume hazel leaves pelleted with alfalfa (0.6:0.4) well (Wang et al., 2018). Besides, Vandermeulen et al. (2016) showed heifers giving the opportunity to browse hazel on pasture, this plant was one of the most ingested species. As expected, energy and nutrient, especially fiber, digestibility of the diet declined with increasing hazel proportion like also found by Wang et al. (2018). This was mostly linear. This was contradictory to the associative effects described by Van Soest (1994) when integrating a lower-quality feed into high-quality diet (Van Soest 1994). Hazel leave effect on total diet digestibility was quite extensive in the present study, with declines from 50 to 27% in NDF digestibility at 0 and 41% hazel leave in the diet. Wang et al. (2018) found a decrease from 49 to 31% in NDF digestibility in adult sheep receiving 0 or 50% of hazel leaves in the diet. Compared to fiber digestibility, OM digestibility was only weakly affected by the hazel leaves fed. The lignin fraction in the diet has the ability to decelerate physical and microbial feed degradation in the rumen because of the elastic covalent binding to hemicellulose and cellulose (Waghorn 2003). This would likely also increase mean retention time especially of particles in the RR and in the entire GIT. However, the increase with increasing hazel amounts was rather weak. Baker and Hobbs (1987) found in mountain sheep and elk that ingested browse increased the passage time of grass hay, facilitating fiber digestion. Changes in digestion may also be explained by a longer MRT of particles than solutes, a phenomenon described by their ratio which is named selectivity factor. A high selectivity factor is indicative of a particularly high fluid throughput, facilitated by particularly short retention times of solutes (fluid) due to a high saliva flow. Melaku et al. (2005), found a faster passage

rate of the particulate matter with the addition of multipurpose trees to tef straw. However, in the present study, variables of MRT and selectivity factors were only weakly changing with hazel proportion in the diet.

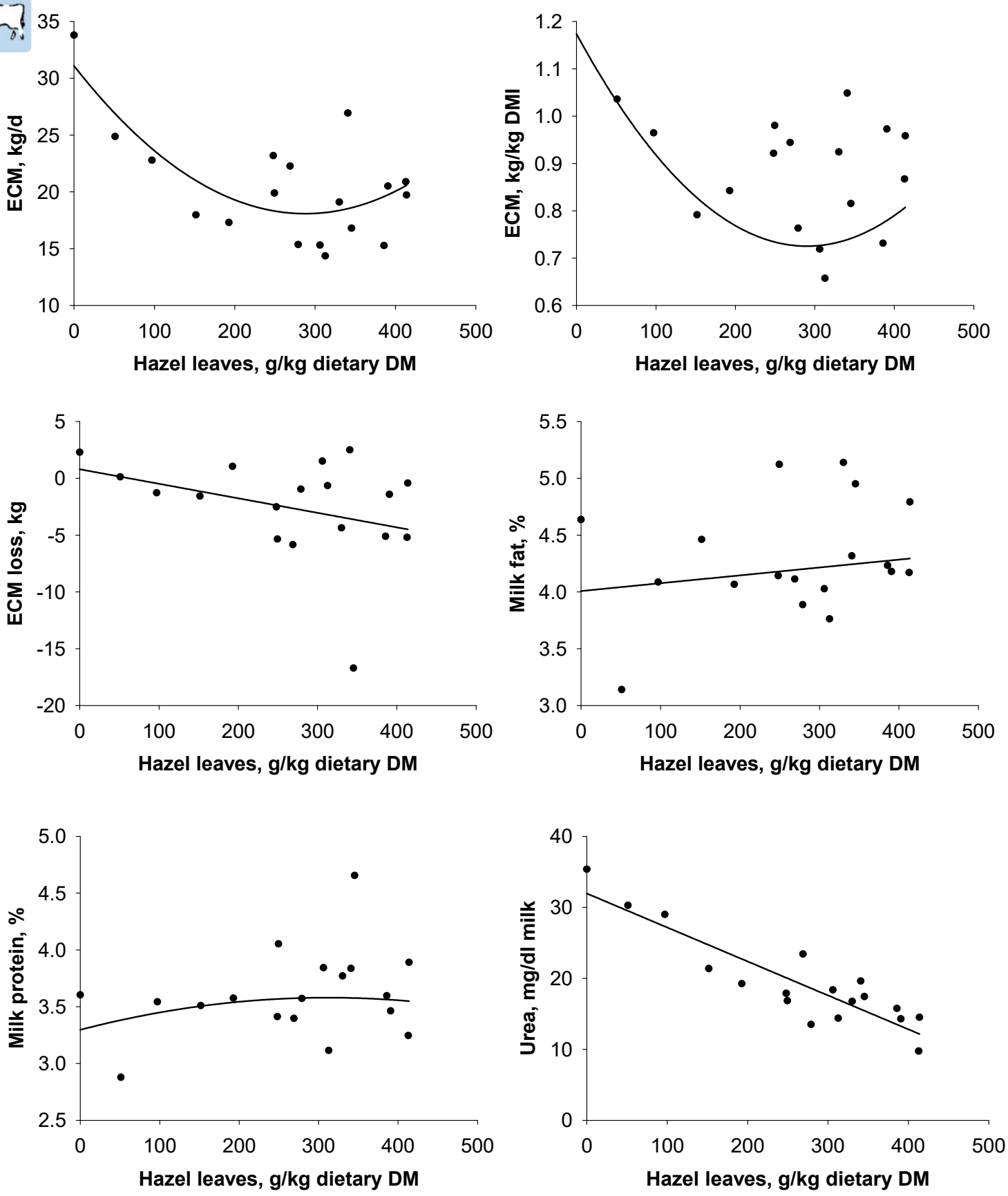
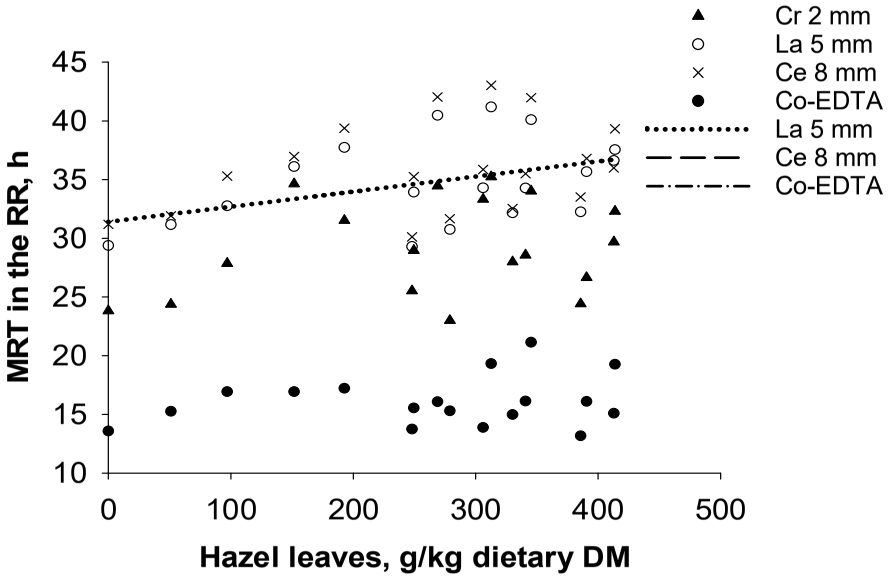
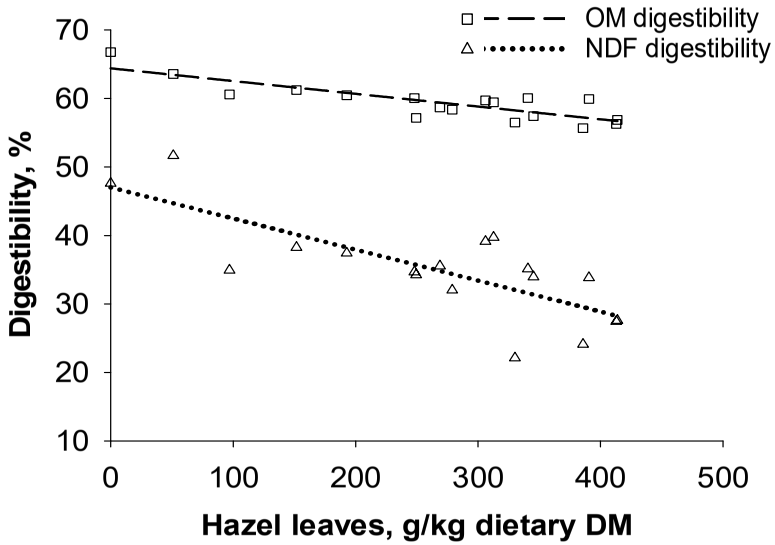


Figure 5.1 Values of variables describing milk yield and composition observed in cows receiving diets with different hazel leave proportions and the corresponding regression lines (for regression coefficients see Appendices Table A5.1).





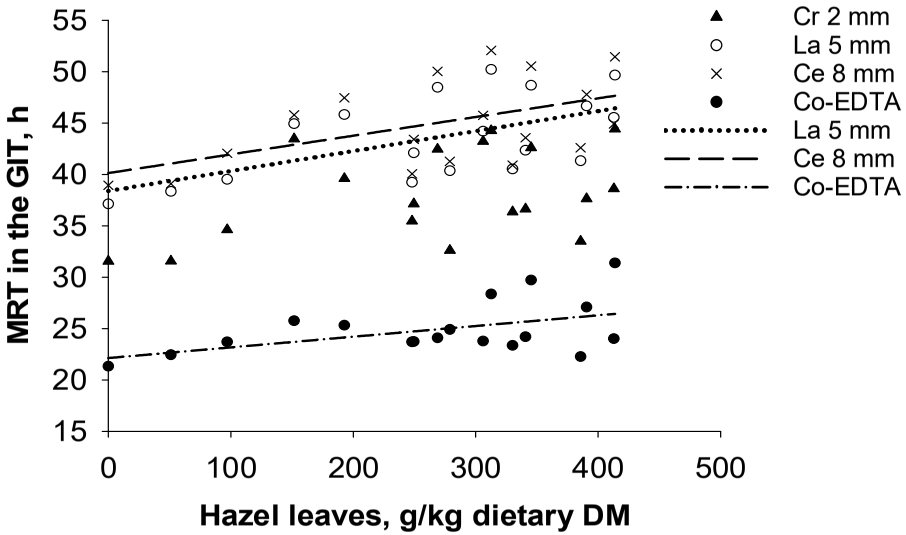


Figure 5.2 Values of variables describing digesta passage (MRT, mean retention time; GIT, gastrointestinal tract; RR, reticulorumen) and digestibility observed in cows receiving diets with different hazel leave proportions and the corresponding regression lines (for regression coefficients see Appendices Tables A 5.1 and A 5.2). Prediction line was only drawn when the model P value was $P < 0.05$.

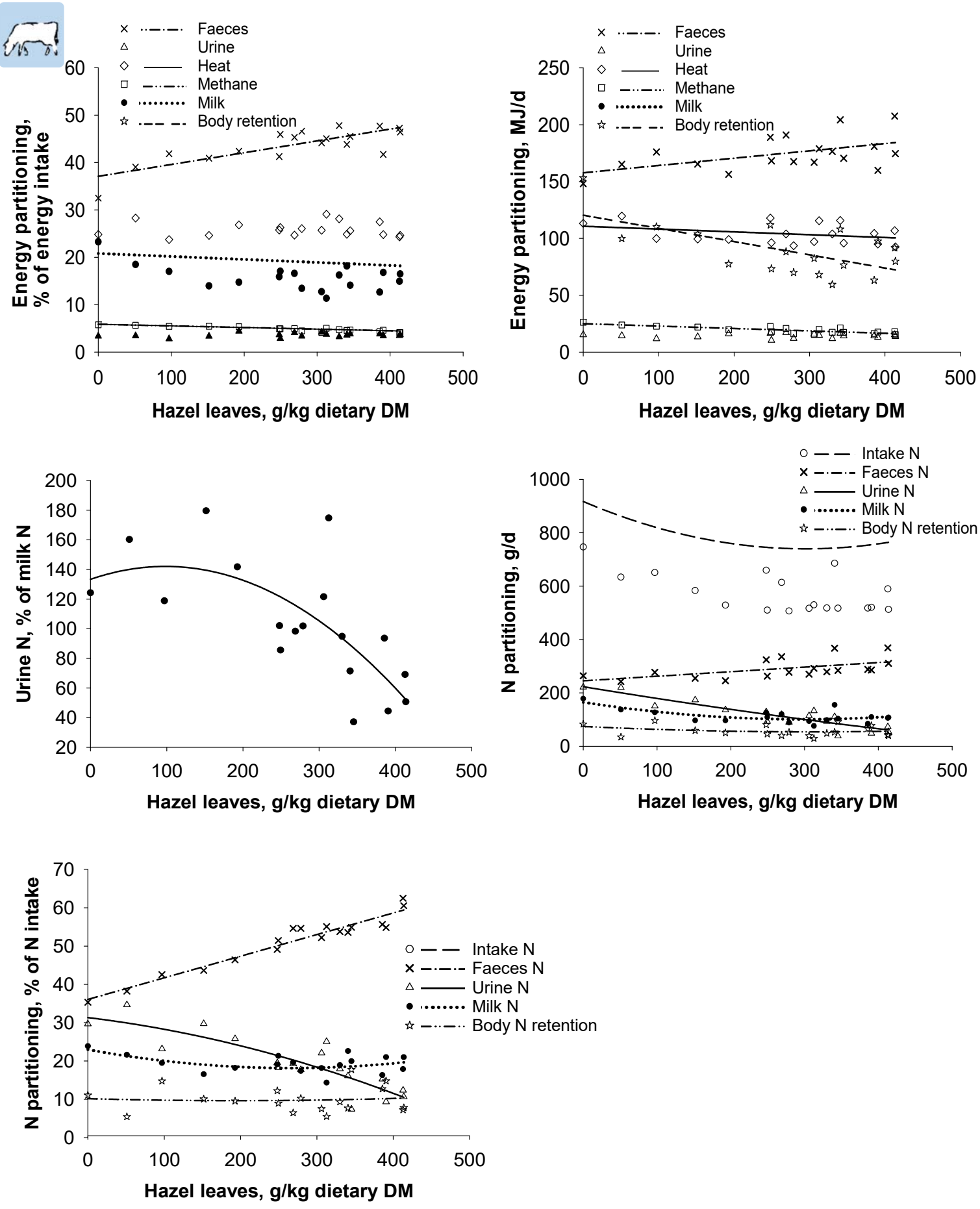


Figure 5.3 Values of variables describing energy and nitrogen balance observed in cows receiving diets with different hazel leave proportions and the corresponding regression lines (for regression coefficients see Appendices Table A5.3). Prediction line was only drawn when the model P value was $P < 0.05$.

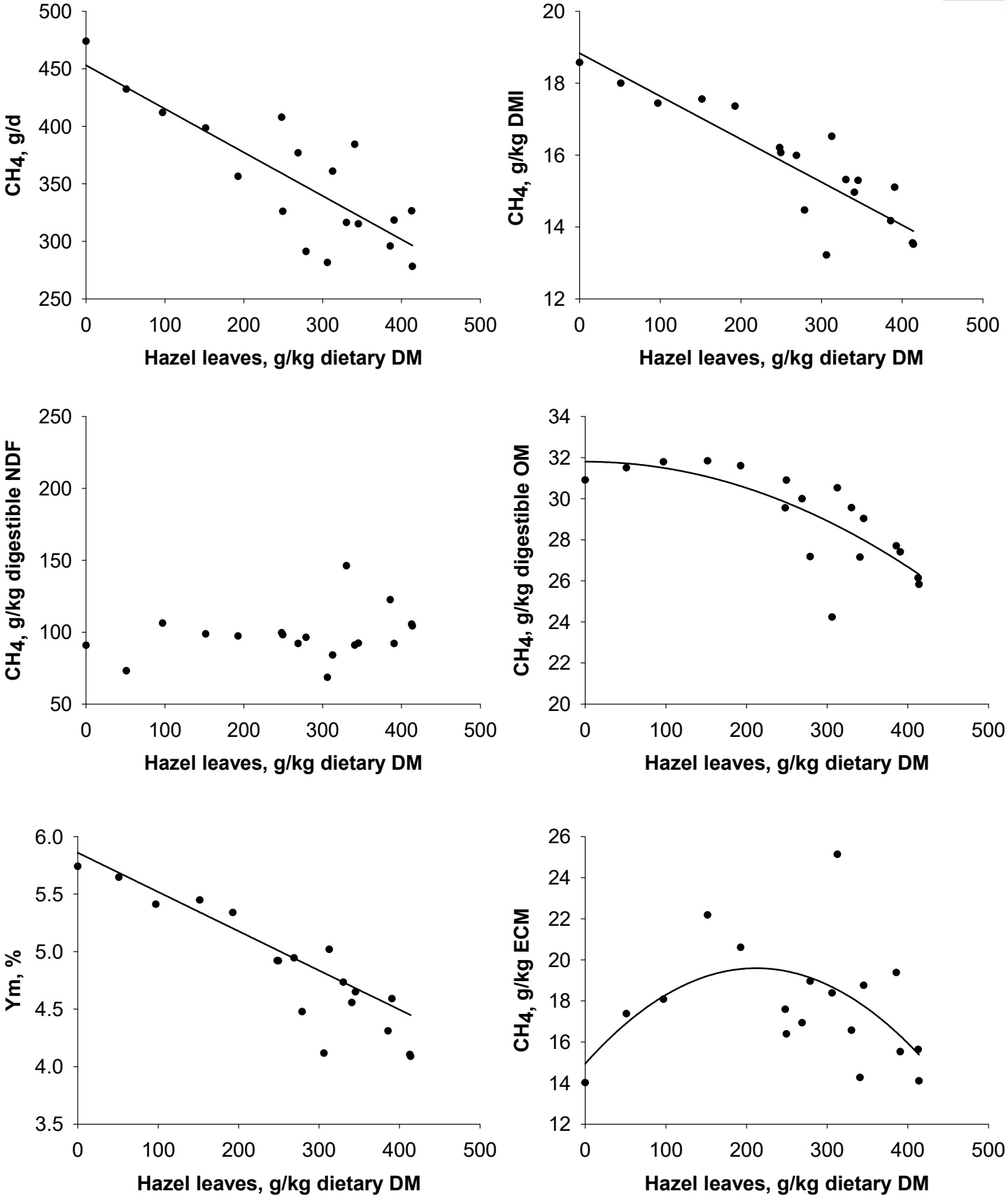


Figure 5.4 Values of variables describing methane emission observed in cows receiving diets with different hazel leave proportions and the corresponding regression lines (for regression coefficients see Appendices Table A5.4). Prediction line was only drawn when the model P value was $P < 0.05$.

As digestibility was declining in a linear manner with increasing hazel proportion, a linear decline or even a lack of response (owing to initial reserve mobilization of the cow) in milk yield would have expected at the lower hazel proportions. However, the opposite was the case and, similarly unexpectedly, the cows did not further decline milk yield from medium to high hazel proportions. Similar shapes (although not always significant) were observed with energy and N excretion with the milk. Concomitantly, body N retention declined in a curvilinear way with increasing hazel proportion, but not body energy retention and BW.

In the present study, the heat energy was not affected by the tannin rich hazel leaves in the diet. The digestion of shrub leaves rich in secondary compounds, like phenols and tannins, may require a detoxification which costs the animal additional energy (White and Lawler, 2002). As a consequence, the changes in heat production should show the extent of this cost comparing tannin rich diets to a tannin-low diet. The lack of such an effect can be explained by the high proportion of the indigestible CT in total hazel phenols.

5.5.2 Methane mitigation effect

In the present study, the CH_4 emissions were clearly and substantially declining with increasing hazel leave proportion. The level of decline was similar as that reported by Wang et al. (2018) with similar dietary hazel proportions. The major phenolic compounds of hazel leaves are the flavanols myricetin 3-rhamnoside and quercetin 3-rhamnoside, two CT (Amaral et al., 2005). The CT in a number of plants have been shown to have the potential to mitigate enteric CH_4 (reviewed Beauchemin et al., 2008; Martin et al., 2009). Jayanegara et al. (2012) described by a meta-analysis that there is a general linear relationship between dietary tannin content and methanogenesis like the one found in the present study. The decline found in the present study of 17% CH_4/dOM with 41% hazel leaves (dietary TT content of 3.6%) was higher as the 10% calculated by Jayanegara et al. (2012). It is well known that a reduced fiber digestion reduces H_2 availability in the rumen and this will inhibit the activity of the methanogens. In the present study, the reduction in fiber digestibility might as well be part of the CH_4 mitigating effect.

Mitigation of enteric CH_4 is only useful if it is related to a constant or only less than proportionate decline in intake, digestibility and milk yield. Methane yield per unit of DM intake declined by a similar magnitude as absolute CH_4 because intake was not affected. However, when related to intake of digestible OM and ECM yield, the efficiency of CH_4 reduction was less effective due to the adverse effects on both variables. In addition, the slope was no longer linear thus indicating that there are associative effects. Niderkorn and Baumont (2009) found that, when animals are fed with a mixture of forages, digestive interactions can

occur between the substrates contained in the plants resulting in associative effects. Indeed, Jayanegara et al. (2013) found *in vitro* with the combination of different phenol-rich plants higher mitigation for all CH_4-related parameters than expected from the single plant analysis. On the contrary, according to Jayanegara et al (2012), a tannin level over 2% is needed to reduce CH_4 effectively and clearly. In the present study, the cows exceeded this threshold by a diet containing 250 g hazel leaves/kg DM. This is the point where the CH_4 per unit of digested OM began to decrease rapidly and where a decrease of CH_4 per unit of ECM was about to start. Still these findings are puzzling, less so for the linear decrease of absolute CH_4 but more the kind of response of ECM yield to the increasing hazel proportion.

5.5.3 Effects on urinary nitrogen losses

Nitrogen emissions from manure are a further environmental problem. Although not directly measured in the present study, urine N excretion is a good marker for the potential N emissions from manure as it is easily volatile (Dijkstra et al. 2013) Tannins, especially CT, have the capacity to bind through hydrogen bonds forage proteins in a pH-reversible manner. These tannin-protein complexes are stable in a pH range from 3.5 to approximately 8 and therefore under ruminal (Frutos et al. 2004). In the present experiment, the apparent N digestibility increased in a curvilinear way with increasing hazel levels showing that only high levels of tannins were able to bind most dietary protein. With very high hazel dosages, the protein availability in the rumen was so low that very low milk urea contents were found. The observed substantial shift from urinary N to fecal N with increasing hazel proportion is to be considered highly favorable from the environmental point of view as the gaseous N emissions from urine are consequently reduced (Hristov et al., 2011). In the present study, a number of indicators of the efficiency of the gradually increasing proportions of hazel leaves were evaluated. These included absolute urine N excretion, urine N excretion relative to intake (‘urine N yield’), urine N excretion relative to milk N formation (‘urine N emission intensity), and milk urea N losses. Also in urinary N-related traits associative (non-linear) effects of exchanging alfalfa by hazel could be expected. Indeed, even with urine N yield a curve with a similar shape, although less pronounced, as with methane emission intensity was found, i.e. a greater efficiency of higher hazel leave proportions to reduce urine N in proportion of N intake than small hazel proportions. Concerning urine N emission intensity (urine N, % of milk N) curve shape was similar to them in methane due to the decrease not only in urine N but as well in ECM yield with consequently a decrease in milk N excretion. Milk protein yield was less adversely affected by hazel due to a counterbalancing response of milk protein content.

5.6 Conclusions

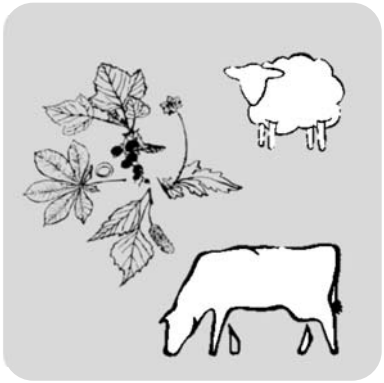
In the present experiment, hazel leaves turned out to be a highly effective and palatable feed for mitigating CH_4 and urine N losses in dairy cows complementing first findings in adult sheep. The main goal of the study had been to test with this model, whether there are positive associative effects allowing to feed a mitigating forage to a high quality diet without greater performance losses up to a certain dosage. However, the associative effects found for both emission sources were of an opposite direction. This was highly unexpected from the previous in vitro evidence and would indeed render small dosages of such forages useless. Further studies have to confirm the absence of such positive associative effects in livestock. To alleviate adverse effects of on the feeding value of the diet, hazel leaves might be harvested at an early growth stage where lower ADL contents could be anticipated. The extensive use of the hazel in animal nutrition requires an extension of cultivation which could have other positive environmental effects like a higher biodiversity in agriculture.

5.7 Acknowledgments

The authors are grateful to C. Kunz, P. Bucher, M. Mergani, E. Manzocchi, J. Welteroth, T. Denninger (ETH Zurich), H. Barleben (IZW Berlin), A. Simonett, T. Stiefel, M. Hunziker and H. Renfer (Strickhof), as well as R. Stoz and S. Amelchanka (AgroVet-Strickhof) for their assistance during animal experiment and laboratory analyses. The project was funded by ETH Zurich Research Grant no. ETH-49 15-1.

Chapter 6

General discussion and conclusion



6.1. Palatability of woody and herbaceous plants

The palatability of a feed is determined by its taste, smell, appearance, temperature and texture (Baumont 1996). High amounts of PSM, like phenols and tannins can impede the feed intake due to their astringent effect (Kumar and Singh, 1984). Trees, shrubs and herbaceous leguminous plants can be rich in PSM contents (Frutos et al. 2004). Aerts et al. (1999) stated that tannin contents greater than 5% in the diet might negatively affect feed intake. However, there are African plant species with >10% of CT in the foliage which are still palatable to browsing ruminants as the russet bushwillow (*Combretum hereroense)* or spike-thorn (*Maytenus heterophylla)* (Owen-Smith, 1987). Phenols seem to have a higher biological activity (protein precipitation capacity, PPC) under harsh environmental conditions with a limited availability of water which cause higher levels of phenols, at least partially because they are not diluted as much by nutrients (Makkar 2003). Therefore, plant materials from temperate climatic conditions might have a higher potential for use as feed in ruminal feeding.

The immediate feed intake can be a good parameter to evaluate the sensory response induced by a feed (Baumont 1996). The study of Scherer et al. (2018) showed that the first 3 min of intake of ensiled forages are decisive for the feed acceptance in goats in the following 3 h in a free-choice situation. Kaitho et al. (1997) observed that the best prediction of feed acceptance of wilted and dried multipurpose tree leaves over a 12-day feeding period in sheep and goats is with the average intake between day 5 and 8 and they discouraged a palatability test over a period shorter than 5 days. In the present thesis, the palatability was determined during a 3-days feeding period and, with that, in the first 5 h of offering the new feed (Chapter 3). The hazel leaves which resulted after 3 days of feeding to be most palatable out of the six fed plants, showed the same high acceptance in sheep and dairy cows in the following feeding experiments over 21-days (Chapter 4 and 5). Consequently, the predicted high palatability could be confirmed in both species when calculated over a 3-day feeding period.

The combination of the experimental plants with lucerne in the animal experiments seemed to be very useful from the palatability aspect. The acceptance of lucerne hay is very high in sheep and cows (de Vega et al. 2000; Horadagoda et al. 2009). The pelleting process obviously had no negative effect on the acceptance of most of the experimental plants in the present studies. Only the palatability of the birch leaves may have been negatively affected by pelleting, as the dried plant leaves (not pelleted) were high palatable in sheep (Meier et al. 2014),

In the palatability study from the present thesis (Chapter 3), most of the offered woody and herbaceous plants showed a high short-term palatability in the dairy cows despite their TP and TT content above 45 and 24 g/kg DM, respectively. There was no relationship found between

the palatability and the TP contents. In addition, when the most palatable plant, hazel leaves, were fed over several weeks in the following experiments (Chapter 4 and 5) to sheep and dairy cows in amounts up to 50% of the diet, with TP contents of 65 and 66 g/kg DM in the pellets, they did not cause any refusals in almost all animals. The palatability difference found in birch leaves between sheep and cows did not occur for the hazel leave pellets. Additionally, further factors have an effect on the palatability of a plant. The corresponding experiment (Chapter 3) revealed that the palatability was highly correlated to the ADL content. However, ADL contents of hazel leave pellets fed to sheep (Chapter 4) and cows (Chapter 5) reached 178 and g 194/kg DM, which were higher than the ADL content in the less palatable plants (birch and blackcurrant) from the short-term palatability experiment (Chapter 3). The NTP and CT contents showed a correlation with the palatability as well. Therefore, probably not the ADL contents alone, but the combination of ADL, NTP and CT can explain the palatability of a plant. Resistance to grinding which is related to the fibre content and the amount of stems in the forage can reduce palatability as well (Baumont 1996). Horadagoda et al. (2009) found that water-soluble carbohydrates (positive effect) and nitrate-nitrogen (negative effect) contents in pasture plant species are important factors for the palatability in cows as well, but this was beyond the scope of this thesis.

6.2. The potential of woody and herbaceous plants to mitigate methane emissions

Methane is a potent GHG with global warming effect 23-times of that of CO_2 (Steinfeld et al. 2006). Ruminant livestock is a major contributor to anthropogenic CH_4 production and therefore, the reduction of CH_4 emissions from ruminant production is of great interest. The studies performed in the present thesis showed the high potential of plants growing naturally in the temperate climate to mitigate the formation of enteric CH_4. The leaves of the birch, blackcurrant, hazel and vine as well as the herb of rosebay willow and wood avens decreased CH_4 emissions significantly *in vitro* (Chapter 2 and 3). With up to 300 g/kg DM all plant additives decreased the CH_4/dOM. Bhatta et al. (2009) stated that the combination of different tannin groups, like the CT and HT, shows different mitigation potentials than their single use. Therefore, plants rich in both tannin groups might be more effective in mitigating CH_4 emissions. However, the plants in the present thesis were not always rich in both, CT and HT, when used *in vitro* (Chapter 2 and 3) and in the experiment with cows (Chapter 5), and showed nonetheless a CH_4 mitigation. Therefore, the TP and NTP content in the plants might play a central role as well. The phenolic contents analysed in the pellets were very close to the values expected from the raw material. Thus, the process of pelleting seems not to have influenced the

phenol and tannin content of the used plants. Additionally, the hazel leaves showed clear mitigating effect when added as sole plant material to a mixed ration *in vitro* (Chapter 2 and 3), and also when pelleted with the lucerne for *in vivo* experiments indicating that pelleting also did not impair bioactivity.

The hazel leaves were the most promising feed additive to reduce the CH_4 with only slightly affecting nutrient digestibility *in vitro* and *in vivo*. The dosage, which was needed to achieve a significant decrease of CH_4/dOM *in vitro*, was 200 g/kg DM in the diet. The decrease was linear *in vitro* and non-linear *in vivo* (Figure 6.1), i.e. associative effects were observed *in vivo*. The CH_4 mitigation effect in sheep and cow was very similar although the basal diets were different: in sheep only hay was fed beside the hazel pellets and in cows the basal diet was composed of grass silage, maize silage, hay and concentrate. With the use of 250 g of hazel leaves per kg DM, the CH_4/dOM decreased 6% and with the use of 500 g/kg it decreased 25% in sheep and cows *in vivo* (calculated for the cows with the regression parameters estimated) (Figure 6.1). The *in vitro* effect of 200 g hazel leaves per kg DM was slightly higher, with 10% less CH_4/dOM produced compared to the basal diet without supplementation, than those of 250 g used *in vivo*. The relationship found in the literature between *in vivo* and *in vitro* CH_4 production was describes as very poor with $R^2 = 0.26$ (Moss and Givens 1997). Therefore the relationship found in the present thesis *in vivo* is very close to the *in vitro* prediction.

The mode of action by which tannins mitigate CH_4 emissions from ruminants is not yet clear. A suppression of protozoa by the tannins as they form symbiosis with methanogenic bacteria in the rumen could be one explanation (Goel and Makkar, 2012). Oliveira et al. (2007) found inhibiting effects on bacterial growth of hazel leaves. However, the bacterial and protozoal counts were not affected with most of the plant supplementation *in vitro* (Chapter 2 and 3). Probably their activity may have changed with the addition of the plant leaves but this was not analysed in the *in vitro* or *in vivo* experiments. A reduced ruminal nutrient degradation is an indirect effect of tannins on the CH_4 emission. The *in vivo* feeding experiments with sheep and cows (Chapter 4 and 5) showed a decreased fibre and OM digestibility with increasing hazel amount in the diet. With the reduced fibre digestion also the H_2 production decreases, which is needed for reduction of CO_2 to CH_4 (Moss et al. 2000), and therefore CH_4 decreases. Tannin contents greater than 5% might impede the nutrient utilization (Waghorn 2008). However, some studies also showed no effect on digestibility in association with a CH_4 reduction (reviewed by Patra and Saxena 2010). Nevertheless, the effect of the tannins on the digestibility *in vivo* as well as *in vitro*, could have been superimposed by the effect of high ADL contents in most of the plants used, as the ADL fraction in the diet has the ability to decrease the physical and

microbial feed degradation in the rumen (Waghorn 2003). Compared to the hazel leaves used *in vitro*, the hazel leave batch used *in vivo* in cows contained 122 g more ADL per kg DM (116 vs. 238 g/kg DM). Different harvest modes, e.g. harvesting only the leaves or harvesting the leaves with the branches could explain these huge differences, but this information from the suppliers is not given. The time of harvest, i.e. the season, could also have had an influence.

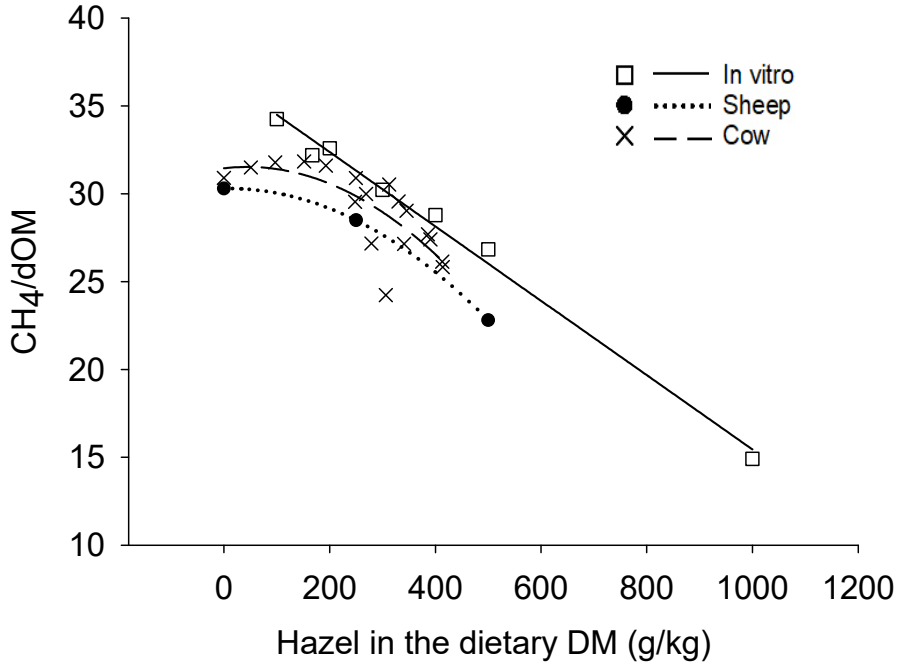


Figure 6.1 Methane (CH_4) production per unit of digestible organic matter (dOM) consumed in relation to the hazel leave proportion in the in vitro experiments (Chapter 2 and 3) and the in vivo experiment in sheep (Chapter 4) and cows (Chapter 5).

6.3. Effects of woody and herbaceous plants on nitrogen turnover

The formation and volatilization of NH_3 in animal manure is a process that begins immediately after excretion, primarily from urinary urea (Hristov et al. 2011). Tannins are known to form complexes with forage proteins in the rumen (Kumar and Singh 1984; Min et al. 2003) which can reduce their ruminal degradation. This bond is reversible, depending on the chemical structure of the tannin, with a pH below 3.5, consequently in the lower gut, where the protein could be absorbed (McSweeney, 2001). With a permanent, non-dissolving bond, the tannin-protein complexes are excreted in faeces. The *in vitro* experiment described in Chapter 3 demonstrated that with the supplementation of all six most promising plants (from Chapter 2) the uCP content, as estimated according to Steingass and Südekum (2013), increased and the NH_3 concentrations in the buffered rumen fluid decreased. These effects were underlined by

the sharp urea decline in cow's milk (Chaper 3 and 5). Across all experiments in the present thesis, plants with significant effect on the N turnover had TT contents above 60 g/kg DM. Min et al. (2003) showed that the non-ammonia-N outflow from the rumen increased with increasing CT content in the diet while the microbial protein outflow was not affected. Beside the complex forming capacity of dietary CT they could additionally reduce the activity of the proteolytic bacteria and consequently reduce the NH_3 production in the rumen (Min et al. 2003). However, plants with low CT and high HT contents (wood avens and rosebay willow) showed also a significant decrease in NH_3 concentrations *in vitro*. In both animal species i*n vivo* a substantial shift from urinary N towards an increased faecal N was found with increasing hazel proportions as a consequence of the affected protein metabolism. Urinary N excretion was similarly although the different basal diets and decreased in sheep (Chapter 4) and cows (Chapter 5) by up to 72% and 67%, respectively. Nitrogen compounds emitted from manure are air pollutants. From the environmental point of view, the shift towards an increased faecal N is therefore advantageous. When faeces from tannin-fed ruminants are used as manure, with the presence of tannin-bound-N in the faeces it is expected that the N release in the soil is slower, which may be beneficial for the long term to pastures and crops (Makkar 2003), but its release at a time when the plants have no requirement could also be problematic.

6.4. Integration of woody and herbaceous plants in ruminant feeding

In the preindustrial century, trees and shrubs were part of the forages for sheep and cows (Rahmann 2004; Vandermeulen et al. 2018). Ben Salem (1994) stated that shrubs and trees can be a valuable feed resource in livestock feeding systems with a good knowledge of their palatability and nutritive value. With the Hohenheim gas test, the feeding value of the selected plants in combination with the basal diet was estimated from their total gas production and chemical composition (Chapter 2 and 3). The six most promising plants birch, blackcurrant, hazel, rosebay willow, vine and wood avens used in the second *in vitro* experiment (Chapter 3) showed no negative effects on the IVOMD and the NEL content of the forages with the lowest dosages (100-200 g/kg DM). Tannins are known to form complexes not only with protein but also with cellulose, hemicellulose and pectin and inhibit cellulolytic bacteria and fungi which could reduce the ruminal fibre degradation (McSweeney, 2001). Dosages over 300 g/kg DM resulted in a reduced IVOMD and NEL in all six plants described in Chapter 3, compared to the basal diet without supplementation. With increasing amounts of hazel leaves *in vivo*, the NDF and ADF digestibility decreased for sheep as well as for cows. This shows that high amounts of tannins in the diets reduced the digestibility. Using plants rich in HT, like the wood

vavens and rosebay willow, could be challenging as HT are considered to be toxic to the animal (Waghorn 2008). However, they could be still applicable after sufficient adaptation due to adaptive strategies of ruminal microbes and metabolism (Waghorn 2008; McSweeney et al. 2001). Nevertheless, feeding phenol and tannin rich plants as feed additive can have positive effects on the ruminant beyond CH_4 and NH_3 mitigation. An improvement of milk yield was observed by feeding CT-rich plants with birdsfood trefoil by Waghorn (2008) and Woodward et al. (2000), but not in the present thesis. Additionally, tannins may have a promoting effect on the immune system and the health of the ruminant because of their antioxidant activity as shown for example for the hazel leaves (Oliveira et al. 2007). In addition, Paolini et al. (2003) found inhibitory effects of hazel leave tannin extracts on gastrointestinal nematodes *in vitro*.

An application of the most promising of all plants, the hazel leaves, in ruminal nutrition on a broad scale would require huge amounts of hazel leaves. Using an effective dosage of 250 g/kg DM, this would result in a daily amount of 4.8 kg DM per cow and 0.5 kg DM per sheep at a daily total intake of 19 kg DM and 2kg DM, respectively. Therefore, an extension of the hazel plant cultivation would be necessary. However, such an expansion could also be an opportunity to increase biodiversity in agriculture (Vandermeulen et al. 2018). Woody plants provide habitats for a species-rich flora and fauna (Vandermeulen et al. 2018) and their integration into agricultural systems could improve soil fertility by a higher N-fixation and a better utilization of nutrients from deeper layers (Nair 2011). In addition, woody plants contribute to soil erosion management and increase carbon sequestration on farmlands (Vandermeulen et al. 2018). The ongoing climate change as well as the land degradation and water shortage will increase the demand for sustainable food production systems, which do not compete with human food (Makkar 2018). A system that combines sustainable and alternative feeds such as combining woody plants with ruminant production is the silvopastoralism, one of the most ancient agroforestry systems (Etienne 1996). The silvopastoral system integrates woody forage resources into grazing systems (Vandermeulen et al. 2018). Vandermeulen et al. (2016) showed that heifers browsed woody plants throughout the entire grazing season if they had unrestricted access and that the hazel plant was one of the most consumed shrub. In the present thesis, the palatability was high even without choice feeding. The in-depth investigation on the feeding value and the mitigation potential in CH_4 and nitrogen emissions of this plant revealed the high potential of the plant to be integrated into existing agricultural systems with beneficial effects on animals and environment.

6.5. General conclusion and outlook

The experimental approach applied in the present thesis identified stepwise the plants with the highest potential to affect the rumen fermentation positively. The birch, blackcurrant, hazel, rosebay willow, vine and wood avens had the ability to decrease CH_4 emissions significantly and reduce the enteric NH_3 formation *in vitro* when added as supplement to a high-quality basal diet and the hazel leaves showed the highest palatability *in vivo*. The hazel leaves showed a great potential to mitigate CH_4 and N losses in sheep and dairy cows as well *in vivo*. The dosage of leaves in the diet with the least adverse side effects seemed to be at 250 g/kg DM as with this dosage significant CH_4 mitigation occurred and the NH_3 turnover was affected positively. The high acceptance as diet components found in both ruminant species, sheep and cows, makes this plant a particularly suitable feed additive.

Further research should focus on the composition and biological activity of the phenolic compounds in the woody plant leaves, which might be responsible for the changes in rumen fermentation. Especially when positive associative effects are found, the combination of two or more plants with high CH_4 mitigation potential might provide an additional opportunity to decrease CH_4 emissions with lower amounts of the individual plant and a lower risk of palatability problems. Further investigations should also focus on the combination of the hazel leaves with different basal diets in order to find a combination that does not reduce the nutrient digestibility that much. An application of hazel leaves in the diet could probably be also found in ruminant fattening. Organic production systems might be suited for the plant supplement use. The hazel leaves showed a great potential for their use in practical animal feeding. However, an implementation of plant supplements in diets to reduce noxious emissions from ruminant production can only be achieved if farmers are encouraged to use them. The plant supplements will cause additional costs, which must be rewarded in some way. An extra subsidy for environmental friendly produced meat or milk may offer an opportunity. This can be either achieved by creating tailor-made political framework conditions or upon private labelling initiatives by retailers like those currently under way for feeding of linseed to mitigate methane and improve fatty acid profile of the milk. Overall, the integration of woody plants such as the hazel shrub as diet component could have a great potential for more sustainable and environmental friendly food production.

Appendices

Appendices Chapter 3

Table A3.1 Feeding plan to determine the short-term palatability of the test plant pellets.

Duration	Animals					
(days)	Cow 1	Cow 2	Cow 3	Cow 4	Cow 5	Cow 6
5	Adaptation (lucerne pellets[1])					
3	Control feeding (lucerne pellets[1])					
3	Rosebay willow D	Wood avens C	Black-currant C	Vine D	Hazel C	Birch D
3	Birch C	Rosebay willow D	Wood avens D	Black-currant D	Vine D	Hazel C
3	Hazel D	Birch D	Rosebay willow C	Wood avens C	Black-currant D	Vine C
3	Vine C	Hazel C	Birch D	Rosebay willow C	Wood avens D	Black-currant C
3	Black-currant D	Vine D	Hazel D	Birch C	Rosebay willow C	Wood avens D
3	Wood avens C	Black-currant C	Vine C	Hazel D	Birch C	Rosebay willow D

C, D = batch C and batch D, respectively
[1]Consisted (g/kg DM) of lucerne 980 (*Medicago sativa*; harvested in France 2016, purchased from Landi Sense Düdingen, Switzerland) and molasses 20.

Table A3.2 Proportions of butyrate (C_4) and valerate (C_5) of total short-chain fatty acids (SCFA) in incubation fluid as affected by experimental plants.

Variable	Dosage (g/kg)	Basal diet	Birch	Sweet chest-nut	Hazel	Rose-bay willow	Wood avens	Black-currant	Vine	SEM	*P*-value	*P*-value		
												Plant	Dosage	Plant × dosage
C_4	0	11.0								0.28		<0.001	<0.001	<0.001
(mmol/	100		11.1	11.2	11.1	11.3	11.2	11.2	11.1		0.521			
mol	200		11.1	11.6	11.1	11.1	11.0	11.0	11.0		0.151			
SCFA)	300		10.8^{b}	12.4^{*a}	11.1^{b}	11.2^{b}	10.8^{b}	10.7^{b}	10.8^{b}		<0.001			
	400		10.7^{b}	12.7^{*a}	10.1^{*b}	11.0^{b}	10.8^{b}	10.7^{b}	10.7^{b}		<0.001			
	500		10.4^{b}	13.4^{*a}	10.6^{b}	10.8^{b}	10.6^{b}	10.7^{b}	10.5^{b}		<0.001			
	1000		8.7^{*bc}	11.3^{a}	8.3^{*c}	9.0^{*b}	9.0^{*b}	9.0^{*b}	8.8^{*bc}		<0.001			
	Contrast[1]		L Q	L	L		L	L	L					
iso C_4	0	0.686								0.058		<0.001	<0.001	<0.001
(mmol/	100		1.04^{*a}	1.00^{*abc}	0.98^{*bc}	0.94^{*c}	0.95^{*c}	0.96^{*bc}	1.01^{*ab}		<0.001			
mol	200		0.92^{*a}	0.86^{ab}	0.82^{bc}	0.78^{c}	0.69^{d}	0.80^{bc}	0.81^{bc}		<0.001			
SCFA)	300		0.67^{a}	0.58^{ab}	0.54^{abc}	0.52^{bc}	0.44^{*c}	0.50^{bc}	0.54^{bc}		<0.001			
	400		0.40^{*b}	0.48^{*b}	0.49^{b}	0.84^{a}	0.90^{*a}	0.87^{a}	0.82^{a}		<0.001			
	500		0.74^{b}	0.54^{c}	0.63^{bc}	0.66^{bc}	0.93^{*a}	0.70^{b}	0.68^{b}		<0.001			
	1000		0.34^{*b}	0.25^{*b}	0.24^{*b}	0.34^{*b}	1.75^{*a}	0.28^{*b}	0.32^{*b}		<0.001			
	Contrast		L Q	L Q	L	L	L Q		L					
C_5	0	1.06								0.039		<0.001	<0.001	<0.001
(mmol/	100		1.10	1.10	1.09	1.12	1.14	1.07	1.11		0.592			
mol	200		1.07^{a}	1.07^{a}	1.00^{ab}	1.03^{ab}	0.99^{b}	1.01^{ab}	1.03^{ab}		0.019			
SCFA)	300		0.95^{ab}	0.98^{a}	0.85^{*b}	0.89^{*ab}	0.93^{ab}	0.89^{*ab}	0.91^{ab}		0.044			
	400		0.87^{*ab}	0.94^{a}	0.74^{*b}	0.97^{a}	0.98^{a}	0.92^{a}	0.97^{a}		<0.001			
	500		0.88^{*ab}	0.94^{a}	0.84^{*ab}	0.88^{*ab}	0.89^{*ab}	0.79^{*b}	0.89^{*ab}		0.013			
	1000		0.53^{*bc}	0.92^{a}	0.47^{*c}	0.60^{*bc}	0.69^{*b}	0.53^{*bc}	0.65^{*bc}		<0.001			
	Contrast		L	L	L	L	L	L	L					
iso C_5	0	1.59								0.099		<0.001	<0.001	<0.001
(mmol/	100		1.66^{ab}	1.66^{ab}	1.67^{a}	1.59^{b}	1.64^{ab}	1.68^{a}	1.68^{a}		0.012			
mol	200		1.67^{a}	1.49^{bc}	1.54^{b}	1.46^{bc}	1.43^{c}	1.54^{b}	1.49^{bc}		<0.001			
SCFA)	300		1.47^{b}	1.36^{*ab}	1.38^{*ab}	1.28^{*bc}	1.21^{*c}	1.32^{*bc}	1.32^{*bc}		<0.001			
	400		1.24^{*b}	1.28^{*b}	1.50^{a}	1.50^{a}	1.52^{a}	1.69^{a}	1.53^{a}		<0.001			
	500		1.71^{a}	1.49^{b}	1.33^{*bc}	1.37^{*bc}	1.30^{*c}	1.50^{b}	1.38^{*bc}		<0.001			
	1000		1.30^{*a}	1.13^{*ab}	1.05^{*ab}	0.92^{*b}	0.911^{*b}	1.02^{*ab}	0.97^{*b}		0.001			
	Contrast		L Q	L Q	L	L Q	L Q	L Q	L					

[a-d]Least-square means within a row with no common superscript differ ($P < 0.05$).

*Values differ ($P < 0.05$) from those of basal diet alone.

[1]Significant ($P < 0.05$) linear (L) or quadratic (Q) contrasts of the response to incremental doses (from 0 to 500 g/kg) of each plant material.

Table A3.3 Acetate/propionate ratio (C_2/C_3 ratio) as well as microbial counts in incubation fluid as affected by experimental plants.

Variable	Dosage (g/kg)	Basal diet	Birch	Sweet chest-nut	Hazel	Rose-bay willow	Wood avens	Black-currant	Vine	SEM	*P*-value	*P*-value		
												Plant	Dosage	Plant × dosage
C_2/C_3	0	3.53								0.089		<0.001	<0.001	<0.001
(x:1)	100		3.43^{c}	3.42^{c}	3.52^{b}	3.49^{b}	3.49^{b}	3.53^{b}	3.59^{a}		<0.001			
	200		3.55^{c}	3.45^{d}	3.69^{b}	3.72^{*b}	3.67^{b}	3.72^{*b}	3.83^{*a}		<0.001			
	300		3.67^{c}	3.40^{d}	3.84^{*b}	3.89^{*b}	3.82^{*bc}	3.90^{*b}	4.07^{*a}		<0.001			
	400		3.8^{*b}	3.35^{*e}	3.96^{*ab}	3.90^{*ab}	3.76^{*b}	3.84^{*b}	4.07^{*a}		<0.001			
	500		3.56^{d}	3.27^{*e}	3.99^{*bc}	4.06^{*b}	3.89^{*c}	3.99^{*bc}	4.28^{*a}		<0.001			
	1000		3.52^{d}	3.40^{d}	4.59^{*b}	5.10^{*a}	4.37^{*c}	4.71^{*b}	5.21^{*a}		<0.001			
	Contrast[1]		L	L	L	L	L	L	L					
Bacteria	0	9.35								0.457		0.005	0.147	0.158
(10^9/ml)	100		8.68	8.84	8.99	8.80	8.65	8.93	8.72		0.993			
	200		8.53	9.08	8.91	9.68	9.24	9.96	8.83		0.169			
	300		9.45^{ab}	9.34^{ab}	8.26^{b}	8.61^{ab}	8.81^{ab}	9.34^{ab}	9.73^{a}		0.013			
	400		9.00	8.68	8.77	9.23	9.93	9.64	9.11		0.474			
	500		9.18	8.63	9.36	8.96	9.34	9.72	9.18		0.391			
	1000		8.59^{bc}	8.33^{c}	8.98^{abc}	10.3^{a}	9.54^{abc}	9.97^{ab}	9.82^{abc}		0.003			
	Contrast													
Protozoa	0	5.65								0.675		0.322	<0.001	0.758
(10^4/ml)	100		5.04	4.41	3.74	4.74	4.30	4.44	4.89		0.698			
	200		5.56	4.93	5.15	5.96	4.19	5.30	4.19		0.567			
	300		4.44	3.89	4.04	4.30	5.11	5.78	4.59		0.224			
	400		5.07	4.93	5.19	4.85	5.30	5.93	5.04		0.783			
	500		5.00	4.19	4.59	5.22	4.48	4.04	3.67		0.081			
	1000		3.52	3.78	3.70	4.22	3.93	3.74	3.74		0.991			
	Contrast								L					

[a-e]Least-square means within a row with no common superscript differ ($P < 0.05$).

*Values differ ($P < 0.05$) from those of basal diet alone.

[1]Significant ($P < 0.05$) linear (L) or quadratic (Q) contrasts of the response to incremental doses (from 0 to 500 g/kg) of each plant material.

Table A3.4 Production of fermentation total gas and individual fermentation gases as affected by experimental plants.

Variable	Dosage (g/kg)	Basal diet	Birch	Sweet chest-nut	Hazel	Rose-bay willow	Wood avens	Black-currant	Vine	SEM	*P*-value	*P*-value		
												Plant	Dosage	Plant × dosage
Total gas	0	45.8								1.09		<0.001	<0.001	<0.001
(ml/24h)	100		44.8	44.0	43.7	42.7	42.0	42.1	43.3		0.681			
	200		40.6*	40.0*	41.2	40.3*	41.9	43.5	42.7		0.066			
	300		39.5*^{ab}	35.0*^{b}	36.4*^{ab}	37.9*^{ab}	40.4*^{a}	39.0*^{ab}	38.5*^{ab}		0.020			
	400		38.4*^{a}	27.4*^{d}	34.6*^{bc}	33.2*^{c}	33.2*^{a}	38.1*^{a}	37.3*^{ab}		<0.001			
	500		34.3*^{abc}	23.6*^{d}	31.1*^{c}	32.4*^{bc}	32.4*^{a}	35.7*^{ab}	35.8*^{ab}		<0.001			
	1000		19.8*^{d}	11.0*^{f}	15.8*^{e}	22.9*^{cd}	33.5*^{a}	26.4*^{bc}	28.9*^{b}		<0.001			
	Contrast[1]		L	L Q	L	L	L	L	L					
CH_4	0	6.84								0.161		<0.001	<0.001	<0.001
(ml/24h)	100		6.66	6.29	6.32	6.17*	6.20	6.25	6.37		0.258			
	200		5.93*^{a}	5.29*^{b}	5.80*^{a}	5.74*^{a}	5.92*^{a}	6.14*^{a}	6.06*^{a}		<0.001			
	300		5.59*^{a}	3.73*^{b}	5.00*^{a}	5.37*^{a}	4.95*^{a}	5.59*^{a}	5.58*^{a}		<0.001			
	400		5.15*^{a}	2.35*^{d}	4.62*^{c}	4.64*^{bc}	5.33*^{a}	5.25*^{a}	5.13*^{ab}		<0.001			
	500		4.56*^{abc}	1.22*^{d}	4.08*^{c}	4.42*^{bc}	5.05*^{a}	4.83*^{ab}	4.94*^{a}		<0.001			
	1000		1.90*^{d}	0.23*^{e}	1.66*^{d}	2.74*^{c}	4.04*^{a}	3.26*^{b}	3.34*^{b}		<0.001			
	Contrast		L	L Q	L	L	L Q	L	L					
CO_2	0	38.4								1.06		<0.001	<0.001	<0.001
(ml/24h)	100		37.8	36.9	36.8	35.7	35.5	35.5	36.6		0.716			
	200		34.0	34.2	34.8	34.2	35.1	36.8	35.7		0.316			
	300		33.4*	30.7*	30.3*	32.2*	31.1*	33.1*	32.6*		0.476			
	400		32.6*^{a}	24.6*^{c}	29.6*^{ab}	28.0*^{b}	32.7*^{a}	32.4*^{a}	31.2*^{a}		<0.001			
	500		29.5*^{ab}	21.5*^{c}	26.7*^{b}	27.7*^{b}	32.4*^{a}	30.5*^{ab}	30.5*^{ab}		<0.001			
	1000		17.5*^{d}	10.6*^{e}	13.7*^{e}	19.7*^{cd}	29.0*^{a}	22.7*^{bc}	25.3*^{ab}		<0.001			
	Contrast		L	L Q	L	L	L	L	L					

[a-e]Least-square means within a row with no common superscript differ ($P < 0.05$).

*Values differ ($P < 0.05$) from those of basal diet alone.

[1]Significant ($P < 0.05$) linear (L) or quadratic (Q) contrasts of the response to incremental doses (from 0 to 500 g/kg) of each plant material.

Table A3.5 Production of carbon dioxide in relation to supply of dry matter (DM) and digestible organic matter (dOM) as affected by experimental plants.

	Dosage (g/kg)	Basal diet	Birch	Sweet chest-nut	Hazel	Rose-bay willow	Wood avens	Black-currant	Vine	SEM	*P*-value	*P*-value		
												Plant	Dosage	Plant × dosage
CO_2/DM	0	192								5.3		<0.001	<0.001	<0.001
(ml/g)	100		189	185	184	179	177	177	183		0.716			
	200		170	171	174	171	175	184	178		0.316			
	300		167	154*	152*	161*	155*	166*	163*		0.476			
	400		163*^{a}	123*^{c}	148*^{ab}	140*^{b}	164*^{a}	162*^{a}	156*^{a}		<0.001			
	500		148*^{ab}	108*^{c}	134*^{b}	138*^{b}	162*^{a}	153*^{ab}	153*^{ab}		<0.001			
	1000		87*^{d}	53*^{e}	69*^{e}	98*^{cd}	145*^{a}	114*^{bc}	127*^{ab}		<0.001			
	Contrast[1]		L	L Q	L	L	L	L	L					
CO_2/dOM	0	283								5.4		<0.001	<0.001	<0.001
(ml/g)	100		281	278	279	273	274	273	278		0.684			
	200		269	275	273	271	272	278	274		0.803			
	300		269	267	255*	265	248*	265	264		0.539			
	400		271^{a}	248*^{b}	258^{ab}	248*^{b}	265^{a}	263^{a}	258^{ab}		<0.001			
	500		256*^{ab}	228*^{c}	245*^{bc}	247*^{abc}	266^{a}	256*^{ab}	256*^{ab}		<0.001			
	1000		199*^{c}	156*^{d}	172*^{d}	211*^{c}	255*^{a}	217*^{bc}	236*^{ab}		<0.001			
	Contrast		L	L Q	L	L	L	L	L					

[a-e]Least-square means within a row with no common superscript differ ($P < 0.05$).

*Values differ ($P < 0.05$) from those of basal diet alone.

[1]Significant ($P < 0.05$) linear (L) or quadratic (Q) contrasts of the response to incremental doses (from 0 to 500 g/kg) of each plant material.

Appendices Chapter 5

Table A5.1 Regression coefficients and their statistical significance for the dry matter intake (DMI), milk related parameters and digestibility (n=18).

				Covariate				R^2	SE	CV	*P*-value
	Intercept	Hazel	Hazel^2	ECM	DIM	ΔBW	ADLI				
DMI, kg/day	13.8**	-0.003	-	0.373***	-0.016†	0.013	2.24*	0.821	0.985	4.41	<0.001
DMI, kg/kg BW$^{0.75}$ per day	124.6**	-	-6.30×10^{-5}	3.05***	-0.186*	0.102	11.0	0.839	8.21	5.08	<0.001
BW change	-66.4**	0.313†	-	excluded	-	excluded	-	0.196	33.7	-106.6	0.066
Milk yield											
Milk kg/day	29.5**	-0.109**	1.97×10^{-4}**	excluded	-0.037	-0.040†	3.80	0.760	2.89	14.7	0.002
ECM, kg/day	27.4***	-0.085**	1.62×10^{-4}**	excluded	-	-0.058*	-	0.669	3.09	15.2	0.001
ECM loss, kg/day	-3.65	-0.004	-	excluded	-	-0.067	-	0.377	3.74	-153.7	0.029
ECM / DMI	1.11***	-0.003**	5.42×10^{-6}**	excluded	-	-0.001*	-	0.682	0.096	10.6	<0.001
Milk composition											
Fat %	6.27***	0.002*	-	-	-	excluded	-0.954*	0.368	0.424	9.88	0.032
Lactose, %	3.91***	-	1.13×10^{-6}	0.027*	-	excluded	-	0.276	0.209	4.59	0.089
Protein, %	2.10†	0.010**	-1.83×10^{-5}*	0.035	0.007*	excluded	-0.779**	0.658	0.269	7.45	0.014
Urea, mg/d	21.2***	-0.039***	-	0.413*	-	excluded	-	0.869	2.52	13.0	<0.001
Digestibility, %											
DM	56.7***	-0.044*	5.56×10^{-5}	0.156	-	-	2.39	0.809	1.55	2.60	<0.001
OM	56.9***	-0.036*	3.78×10^{-5}	0.116	-	-	2.40†	0.833	1.28	2.15	<0.001
NDF	28.7**	-0.056***	-	-	-	-	7.73†	0.678	4.40	12.6	<0.001

*** P< 0.001 ** P< 0.01 * P < 0.05 $^{\dagger}P$ < 0.10; ADLI, intake of acid detergent lignin; CV = coefficient of variation; ΔBW, BW change; DIM, days in milk; hazel, hazel leave proportion in dietary DM; SE, standard error.

Table A5.2 Regression coefficients and their statistical significance for mean retention time (h) in the gastrointestinal tract (GIT) and in the reticulorumen (RR) (n=18).

				Covariate						
Passage time	Intercept	Hazel	Hazel^2	ECM	DIM	ADLI	R^2	SE	CV	P value
GIT Cr 2 mm	22.2*	0.008	-	-	-	4.95	0.290	4.00	10.5	0.077
GIT La 5 mm	31.8***	0.012†	-	-	-0.900†	4.27	0.594	2.87	6.58	0.005
GIT Ce 8 mm	33.4**	0.011	-	-	-0.816	4.19	0.473	3.40	7.58	0.026
GIT Co	25.2***	0.009*	-	-	-0.784*	-	0.449	2.08	8.35	0.011
RR Cr 2 mm	14.4†	0.002	-	-	-	5.27	0.204	3.90	13.3	0.181
RR La 5 mm	22.5**	0.005	-	-	-0.674	4.87†	0.446	3.02	8.70	0.036
RR Ce 8 mm	20.0*	0.003	-	-	-	5.52†	0.285	3.51	9.74	0.081
RR Co	17.4***	0.003	-	-	-0.584†	-	0.218	2.01	12.5	0.159
GIT Cr / Co	1.42***	0.001	3.10×10^{-6}	-	-	-	0.126	0.129	8.46	0.365
RR Cr / Co	0.861†	0.003	5.69×10^{-6}	0.034*	-	-	0.369	0.185	9.91	0.125

*** P< 0.001 ** P< 0.01 * P < 0.05 $^{\dagger}P < 0.10$; ADLI, intake of acid detergent lignin; CV = coefficient of variation; ΔBW, BW change; DIM, days in milk; hazel, hazel leave proportion in dietary DM; SE, standard error.

Table A5.3 Regression coefficients and their statistical significance for the energy balance (n=18).

				Covariate							
	Intercept	Hazel	Hazel^2	ECM	DIM	ΔBW	ADLI	R^2	SE	CV	*P*-value
Gross energy (GE) intake, MJ/day	209.3*	-	-4.68×10^{-5}	7.24***	-0.249	0.252	43.8*	0.810	18.2	4.52	<0.001
Energy partitioning, MJ/day											
Feces	181.3***	0.100**	-	1.088	-0.221*	-	-	0.557	11.4	6.51	0.008
Urine	5.94	-	1.59×10^{-6}	0.163†	-0.017	-	3.37*	0.469	1.59	11.2	0.066
Methane	10.2**	-0.020***	-	0.380***	-0.011	0.020**	3.71***	0.959	0.756	3.88	<0.001
Heat	50.0*	-0.023	-	1.01*	-	-	14.5*	0.481	7.12	6.85	0.024
Total energy loss	221.4**	0.047	-	2.67*	-0.251†	-	31.8*	0.564	16.8	5.37	0.021
Body energy retention	30.1	-0.042	-	3.47***	-	-	-	0.781	11.4	12.7	<0.001
Energy partitioning, % of GE intake											
Feces	58.0***	0.022***	-	-0.354**	-0.026†	-	-2.35	0.828	1.80	4.12	<0.001
Urine	1.92	1.16×10^{-4}	-	-	-	-	0.588	0.240	0.385	10.8	0.128
Methane	4.84***	-0.004***	-	-	-	-	0.430*	0.882	0.192	3.98	<0.001
Heat	30.1***	-0.003	-	-0.165†	-	-	-	0.193	1.47	5.69	0.201
Total energy loss	85.1***	0.011†	-	-0.499**	-	-	-	0.674	2.52	3.23	<0.001
Milk	7.13*	0.001	-	0.475***	0.010	-0.011	-1.52*	0.944	0.772	4.90	<0.001
Body energy retention	-0.158	-0.014	-	-	-	-	-	0.254	3.04	-78.7	0.033
Apparent digestibility, % of GE	42.0***	-0.022***	-	0.354**	0.026†	-	2.35	0.828	1.80	3.19	<0.001
Metabolizability, % of GE	38.7***	-0.016**	-	0.352**	0.025†	-	-	0.805	1.70	3.54	<0.001
Nitrogen (N) intake, g/day	302*	-	-3.60×10^{-4}*	14.6***	0.393	0.499†	43.4†	0.905	27.1	4.71	<0.001
N partitioning, g/day											
Fecal N	150.2*	0.273***	-	5.20***	-0.411*	-	23.6	0.819	18.2	6.25	<0.001
Urinary N	231.3**	-0.680**	5.99×10^{-4}	-	-0.322†	-	34.4†	0.902	18.8	16.3	<0.001
Fecal and urinary N	340.7**	-0.122	-	5.94**	-0.643*	-	50.1†	0.779	27.1	6.69	<0.001
Milk N	146.3***	-0.403**	7.48×10^{-4}*	excluded	-	-0.290*	-	0.608	17.7	15.8	0.004
Body N retention	-103.5†	0.292†	-7.24×10^{-4}*	4.51**	0.279*	0.527***	-	0.752	12.1	21.2	0.002
N partitioning, % of N intake											
Fecal N	42.9***	0.058***	-	-	-0.029†	-	-	0.949	1.74	3.40	<0.001
Urinary N	55.6**	-0.129**	1.41×10^{-4} †	-0.826*	-0.052†	-0.052†	5.26†	0.887	3.12	15.8	<0.001
Fecal and urinary N	30.0***	0.027***	-	-	-0.014*	-	-	0.955	0.747	2.22	<0.001
Milk N	20.8***	-0.033*	7.09×10^{-5}*	excluded	-	-0.034*	-	0.488	1.88	9.75	0.022
Body N retention	-11.1	0.062*	-1.37×10^{-4}*	0.574**	0.056**	0.087***	-2.46	0.783	1.98	19.8	0.004
Urine N / Milk N (%)	45.9	-	-6.25×10^{-4}***	excluded	-	-	40.6	0.581	28.9	27.9	0.002
Apparent N digestibility, %	104.2***	-0.074*	1.66×10^{-4}*	-1.23***	-0.086**	-0.085**	2.03†	0.789	2.49	3.52	0.003

*** P< 0.001 ** P< 0.01 * P < 0.05 $^{\dagger}P < 0.10$; ADLI, intake of acid detergent lignin; CV = coefficient of variation; ΔBW, BW change; DIM, days in milk; hazel, hazel leave proportion in dietary DM; SE, standard error.

Table A5.4 Regression coefficients and their statistical significance for the enteric methane emission (n=18).

				Covariate							
Methane	Intercept	Hazel	Hazel^2	ECM	DIM	ΔBW	ADLI	R^2	SE	CV	*P*-value
Methane production, g/day	184.6**	-0.359***	-	6.88***	-0.196	0.358**	67.1***	0.959	13.7	3.88	<0.001
Methane yield											
g/kg of DMI	15.3***	-0.014***	-	-	-	-	1.49*	0.846	0.676	4.29	<0.001
g/kg of digestible OM intake	31.8***	-	-3.20×10^{-5}***	-	-	-	-	0.594	1.53	5.26	<0.001
g/kg of digestible NDF	139.6***	0.070†	-	-	-	-	-22.1	0.219	16.1	16.5	0.156
% of gross energy (Ym)	4.84***	-0.004***	-	-	-	-	0.430*	0.882	0.192	3.98	<0.001
Methane emission intensity											
g/kg ECM	10.1*	0.024	-8.28×10^{-5}*	excluded	-	0.045*	3.58†	0.632	2.00	11.2	0.008
g/kg of BW	0.405*	-5.04×10^{-4}***	-	0.009**	$-5.19*10^{-4}$	excluded	0.064†	0.885	0.037	7.42	<0.001
g/kg of $BW^{0.75}$	1.91**	-0.003***	-	0.044***	-0.002	excluded	0.362*	0.909	0.161	6.29	<0.001

*** P< 0.001 ** P< 0.01 * P < 0.05 $^{\dagger}P < 0.10$; ADLI, intake of acid detergent lignin; CV = coefficient of variation; ΔBW, BW change; DIM, days in milk; hazel, hazel leave proportion in dietary DM; SE, standard error.

References

Agroscope 2017. Feeding recommendations and nutrient tables for ruminants (in German). Online version. Accessed November 2018. http://www.agroscope.admin.ch/futtermittel datenbank/04834/.

AOAC 1997. Official methods of analysis. Association of Official Analytical Chemists, Arlington, VA, USA .

Acamovic, T., Brooker, J. 2005. Biochemistry of plant secondary metabolites and their effects in animals. Proc. Nutr. Soc. 64, 403-412.

Aerts, R.J., Barry, T.N., McNabb, W.C. 1999. Polyphenols and agriculture: beneficial effects of proanthocyanidins in forages. Agr. Ecosyst. & Environ. 75, 1-12.

Al-Kindi, A., Schiborra, A., Buerkert, A., Schlecht, E. 2017. Effects of quebracho tannin extract and activated charcoal on nutrient digestibility, digesta passage and faeces composition in goats. J. Anim. Physiol. Anim. Nutr. 101, 576-588.

Amaral, J.S., Ferreres, F., Andrade, P.B., Valentao, P., Pinheiro, C., Santos, A., Seabra, R. 2005. Phenolic profile of hazelnut (Corylus avellana L.) leaves cultivars grown in Portugal. Nat. Prod. Res. 19, 157-163.

Animut, G., Puchala, R., Goetsch, A.L., Patra, A.K., Sahlu, T., Varel, V.H., Wells, J. 2008. Methane emission by goats consuming different sources of condensed tannins. Anim. Feed Sci. Tech. 144, 228-241.

Archimède, H., Rira, M., Barde, D.J., Labirin, F., Marie-Magdeleine, C., Calif, B., Periacarpin, F., Fleury, J., Rochette, Y., Morgavi, D.P., Doreau, M. 2016. Potential of tannin-rich plants, Leucaena leucocephala, Glyricidia sepium and Manihot esculenta, to reduce enteric methane emissions in sheep. J. Anim. Physiol. Anim. Nutr. 100, 1149-1158.

Arrigo, Y., Frioud, E. 2016. Feeding recommedations for sheep, in Agroscope, Posieux (Ed.) Feeding recommendations for ruminants (Green Book) (in German), Chapter 11, Network https://www.agroscope.admin.ch.

Baker, D.L., Hobbs, N.T. 1987. Strategies of digestion: digestive efficiency and retention time of forage diets in montane ungulates. Can. J. Zool. 65, 1978-1984.

Banik, B., Durmic, Z., Erskine, W., Ghamkhar, K., Revell, C. 2013. In vitro ruminal fermentation characteristics and methane production differ in selected key pasture species in Australia. Crop Pasture Sci. 64, 935-942.

Barry, T., McNabb, W. 1999. The implications of condensed tannins on the nutritive value of temperate forages fed to ruminants. Brit. J. Nutr. 81, 263-272.

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

Beauchemin, K.A., McGinn, S.M., Martinez, T.F., McAllister, T.A. 2007. Use of condensed tannin extract from quebracho trees to reduce methane emissions from cattle. J. Anim. Sci. 85, 1990-1996.

Beauchemin, K.A., Kreuzer, M., O'Mara, F., McAllister, T.A. 2008. Nutritional management for enteric methane abatement: a review. Aust. J. Exp. Agric. 48, 21-27.

Bekele, A., Clément, C., Kreuzer, M., Soliva, C.R. 2009. Efficiency of Sesbania sesban and Acacia angustissima in limiting methanogenesis and increasing ruminally available nitrogen in a tropical grass-based diet depends on accession. Anim. Prod. Sci. 49, 145-153.

Bennett, R.N., Wallsgrove, R.M. 1994. Secondary metabolites in plant defence mechanisms. New Phytol. 127, 617-633.

Bhatta, R., Uyeno, Y., Tajima, K., Takenaka, A., Yabumoto, Y., Nonaka, I., Enishi, O., Kurihara, M. 2009. Difference in the nature of tannins on in vitro ruminal methane and volatile fatty acid production and on methanogenic archaea and protozoal populations. J. Dairy Sci. 92, 5512-5522.

Bhatta, R., Vaithiyanathan, S., Shinde, A.K., Jakhmola, R.C. 2005. Effect of feeding complete feed block containing Prosopis cineraria leaves and polyethylene glycol (PEG)-6000 on nutrient intake, its utilization, rumen fermentation pattern and rumen enzyme profile in kids. J. Sci. Food Agric. 85, 1788-1794.

Bodas, R., López, S., Fernández, M., García-González, R., Rodríguez, A.B., Wallace, R.J., González, J.S. 2008. In vitro screening of the potential of numerous plant species as antimethanogenic feed additives for ruminants. Anim. Feed Sci. Tech. 145, 245-258.

Bodas, R., Prieto, N., García-González, R., Andrés, S., Giráldez, F., López, S. 2012. Manipulation of rumen fermentation and methane production with plant secondary metabolites. Anim. Feed Sci. Tech. 176, 78-93.

Brouwer, E. 1965. Report of sub-committee on constants and factors. Proceedings of the 3rd symposium on energy metabolism of farm animals, 1965. European association for animal production, Publ. no. 11, 441-443.

Bundesrat, S. 2000. Öko-Qualitätsverordnung. Bern.

Carulla, J.E. Kreuzer, M., Machmüller, A., Hess, H.D. 2005. Supplementation of Acacia mearnsii tannins decreases methanogenesis and urinary nitrogen in forage-fed sheep. Aust. J. Agric. Res. 56, 961-970.

Chwalibog, A., Jensen, K., Thorbek, G. 1996. Oxidation of nutrients in bull calves treated with ß-adrenergic agonists. Arch. Anim. Nutr. 49, 255-261.

Cottle, D., Nolan, J., Wiedemann, S. 2011. Ruminant enteric methane mitigation: a review. Anim. Prod. Sci. 51, 491-514.

Degen, A.A., Mishorr, T., Makkar, H.P.S., Kam, M., Benjamin, R., Becker, K., Schwartz, H. 1998. Effect of Acacia saligna with and without administration of polyethylene glycol on dietary intake in desert sheep. Anim. Sci. 67, 491-498.

Dijkstra, J., Oenema, O., Van Groenigen, J., Spek, J., Van Vuuren, A., Bannink, A. 2013. Diet effects on urine composition of cattle and N 2 O emissions. Animal 7, 292-302.

Ebert, P.J., Bailey, E.A., Shreck, A. L., Jennings, J.S., Cole, N.A. 2017. Effect of condensed tannin extract supplementation on growth performance, nitrogen balance, gas emissions, and energetic losses of beef steers. J. Anim. Sci. 95, 1345-1355.

Etienne, M., Rapey, H. 1999. Simulating integration of agroforestry into livestock farmers' projects in France. Agroforestry for Sustainable Land-Use Fundamental Research and Modelling with Emphasis on Temperate and Mediterranean Applications. Springer, pp. 257-272.

Frutos, P., Hervas, G., Giráldez, F., Mantecón, A. 2004. Tannins and ruminant nutrition. Spanish J. Agric. Res. 2, 191-202.

Gerber, P.J., Steinfeld, H., Henderson, B., Mottet, A., Opio, C., Dijkman, J., Falcucci, A., Tempio, G. 2013. Tackling climate change through livestock: a global assessment of emissions and mitigation opportunities. Food and Agriculture Organization of the United Nations (FAO).

Gerlach, K., Pries, M., Südekum, K.-H. 2018. Effect of condensed tannin supplementation on *in vivo* nutrient digestibilities and energy values of concentrates in sheep. Small Ruminant Res. 161, 57-62.

GfE 2001. Recommendations for the energy and nutrient supply of dairy cows and heifers (in German). DLG-Verlag, Frankfurt, Germany 8th rev. Ed

Goel, G., Makkar, H.P. 2012. Methane mitigation from ruminants using tannins and saponins. Trop. Anim. Health Pro. 44, 729-739.

Gothenburg Protocol 1999. Protocol to the convention on long-range transboundary air pollution to abate acidification, eutrophication and ground-level ozone. United Nations Economic Commission for Europe, Geneva.

Grandl, F., Luzi S.P., Furger, M., Zeitz, J. O., Leiber F., Ortmann S., Clauss M., Kreuzer M., Schwarm, A. 2016a. Biological implications of longevity in dairy cows: 1. Changes in feed intake, feeding behaviour, and digestion with age. J. Dairy Sci. 99:3457-3471.

Grandl, F., Amelchanka, S.L., Furger, M., Clauss, M., Zeitz, J.O., Kreuzer, M., Schwarm, A. 2016b. Biological implications of longevity in dairy cows: 2. Changes in methane emissions and efficiency with age. J. Dairy Sci. 99, 3472-3485.

Grandl, F., Schwarm, A., Ortmann, S., Furger, M., Kreuzer, M., Clauss, M. 2018. Kinetics of solutes and particles of different size in the digestive tract of cattle of 0.5–10 years of age, and relationships with methane production. J. Anim. Physiol. Anim. Nutr. 102, 639-651.

Grovum, W., Williams, V. 1973. Rate of passage of digesta in sheep: 4.Passage of marker through the alimentary tract and the biological relevance of rate-constants derived from the changes in concentration of marker in faeces. Brit. J. Nutr. 30, 313-329.

Hoffmann, L., Klein, M. 1980. Die Abhängigkeit der Harnenergie vom Kohlenstoff-und Stickstoffgehalt im Harn bei Rindern, Schafen, Schweinen und Ratten. Arch. Anim. Nutr. 30, 743-750 (in German).

Horadagoda, A., Fulkerson, W.J., Nandra, K.S., Barchia, I.M. 2009. Grazing preferences by dairy cows for 14 forage species. Anim. Prod. Sci. 49, 586-594.

Hristov, A.N., Hanigan, M., Cole, A., Todd, R., McAllister, T.A., Ndegwa, P.M., Rotz, A. 2011. Ammonia emissions from dairy farms and beef feedlots. Can. J. Anim. Sci. 91, 1-35.

Hristov, A.N., Oh, J., Firkins, J.D., Dijkstra, J., Kebreab, E., Waghorn, G., Makkar, H.P.S., Adesogan, A.T., Yang, W., Lee, C., Gerber, P.J., Henderson B., Tricarico, J.M. 2013. Special topics—Mitigation of methane and nitrous oxide emissions from animal operations: I. A review of enteric methane mitigation options. J. Anim. Sci. 91, 5045-5069.

Huhtanen, P., Kukkonen, U. 1995. Comparison of methods, markers, sampling sites and models for estimating digesta passage kinetics in cattle fed at two levels of intake. Anim. Feed Sci. Tech. 52, 141-158.

Hummel, J., Südekum, K.H., Streich, W.J., Clauss, M. 2006. Forage fermentation patterns and their implications for herbivore ingesta retention times. Funct. Ecol. 20, 989-1002.

Jayanegara, A., Goel, G., Makkar, H.P.S., Becker, K. 2015. Divergence between purified hydrolysable and condensed tannin effects on methane emission, rumen fermentation and microbial population in vitro. Anim. Feed Sci. Tech. 209, 60-68.

Jayanegara, A., Leiber, F., Kreuzer, M. 2012. Meta-analysis of the relationship between dietary tannin level and methane formation in ruminants from in vivo and in vitro experiments. J. Anim.l Physiol. Anim. Nutr. 96, 365-375.

Jayanegara, A., Marquardt, S., Kreuzer, M., Leiber, F. 2011. Nutrient and energy content, in vitro ruminal fermentation characteristics and methanogenic potential of alpine forage plant species during early summer. J. Sci. Food Agric. 91, 1863-1870.

Jayanegara, A., Marquardt, S., Wina, E., Kreuzer, M., Leiber, F. 2013. In vitro indications for favourable non-additive effects on ruminal methane mitigation between high-phenolic and high-quality forages. Brit. J. Nutr. 109, 615-622.

Jung, H.G. 1989. Forage lignins and their effects on fibre digestibility. Agron. J. 81, 33-38.

Kaitho, R.J., Umunna, N.N., Nsahlai, I.V., Tamminga, S., van Bruchem, J., Hanson, J. 1997. Palatability of wilted and dried multipurpose tree species fed to sheep and goats. Anim. Feed Sci. Tech. 65, 151-163.

Kamalak, A. 2005. Chemical composition and in vitro dry matter digestibility of leaves of *Vitis vinifera*. Livestock Research for Rural Development 17, Article #3.

Karl, T.R., Trenberth, K.E. 2003. Modern global climate change. Science 302, 1719-1723.

Knowles, M.M., Pabón, M.L., Hess, H.D., Carulla, J.E. 2017. Changes in in vitro ruminal and post-ruminal degradation of tropical tannin-rich legumes due to varying levels of polyethylene glycol. J. Anim. Physiol. Anim. Nutr. 101, 641-648.

Külling, D.R., Menzi, H., Sutter, F., Lischer, P., Kreuzer, M. 2003. Ammonia, nitrous oxide and methane emissions from differently stored dairy manure derived from grass- and hay-based rations. Nutr. Cycl. Agroecosys. 65, 13-22.

Kumar, R., Singh, M. 1984. Tannins: their adverse role in ruminant nutrition. J. Agr. Food Chem. 32, 447-453.

Kumar, R., Vaithiyanathan, S. 1990. Occurrence, nutritional significance and effect on animal productivity of tannins in tree leaves. Anim. Feed Sci. Tech. 30, 21-38.

Kumarasamy, Y., Cox, P., Jaspars, M., Nahar, L., Sarker, S. 2003. Cyanogenic glycosids from Prunus spinosa (Rosaceae). Biochem. Syst. Ecol. 31, 1063-1065.

Kupper, T., Bonjour, C., Menzi, H. 2015. Evolution of farm and manure management and their influence on ammonia emissions from agriculture in Switzerland between 1990 and 2010. Atmos. Environ. 103, 215-221.

Lee, C., Hristov, A.N., Cassidy, T., Heyler, K. 2011. Nitrogen Isotope Fractionation and Origin of Ammonia Nitrogen Volatilized from Cattle Manure in Simulated Storage. Atmosphere 2, 256.

Macheboeuf, D., Coudert, L., Bergeault, R., Lalière, G., Niderkorn, V. 2014. Screening of plants from diversified natural grasslands for their potential to combine high digestibility, and low methane and ammonia production. Animal 8, 1797-1806.

Maia, M.R.G., Fonseca, A.J.M., Oliveira, H.M., Mendonça, C., Cabrita, A.R.J. 2016. The potential role of seaweeds in the natural manipulation of rumen fermentation and methane production. Sci. Rep. 6, 32321.

Makkar, H.P.S. 2018. Feed demand landscape and implications of food-not feed strategy for food security and climate change. Animal 12, 1744-1754.

Makkar, H.P.S., Borowy, N.K., Becker, K., Degen, A. 1995. Some problems in fiber determination of a tannin-rich forage (Acacia saligna leaves) and their implications in in vivo studies. Anim. Feed Sci. Tech. 55, 67-76.

Makkar, H.P.S. 2003. Quantification of tannins in tree and shrub foliage: a laboratory manual. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Martin, C., Morgavi, D.P., Doreau, M. 2009. Methane mitigation in ruminants: from microbe to the farm scale. Animal 4, 351-365.

McSweeney, C.S., Palmer, B., McNeill, D.M., Krause, D.O. 2001. Microbial interactions with tannins: nutritional consequences for ruminants. Anim. Feed Sci. Tech. 91, 83-93.

Meier, J., Liesegang, A., Louhaichi, M., Hilali, M., Rischkowsky, B., Kreuzer, M., Marquardt, S. 2014. Intake pattern and nutrient supply of lactating sheep selecting dried forage from woody plants and straw offered in binary or multiple choice. Anim. Feed Sci. Tech. 188, 1-12.

Melaku, S., Peters, K., Tegegne, A. 2005. Intake, digestibility and passage rate in Menz sheep fed tef (Eragrostis tef) straw supplemented with dried leaves of selected multipurpose trees, their mixtures or wheat bran. Small Ruminant Res. 56, 139-149.

Menke, K.H., Steingass, H. 1988. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. Anim. Res. Dev 28, 7-55.

Min, B.R., Barry, T.N., Attwood, G.T., McNabb, W.C. 2003. The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: a review. Anim. Feed Sci. Tech. 106, 3-19.

Min, B.R., Hart, S.P. 2003. Tannins for suppression of internal parasites. J. Anim. Sci. 81, 102-109.

Min, B.R., Solaiman, S. 2018. Comparative aspects of plant tannins on digestive physiology, nutrition and microbial community changes in sheep and goats: A review. J. Anim. Physiol. Anim. Nutr. 102, 1181-1193.

Molan, A.L., Attwood, G.T., Min, B.R., McNabb, W.C. 2001. The effect of condensed tannins from Lotus pedunculatus and Lotus corniculatus on the growth of proteolytic rumen bacteria in vitro and their possible mode of action. Can. J. Microbiol. 47, 626-633.

Montes, F., Meinen, R., Dell, C., Rotz, A., Hristov, A.N., Oh, J., Waghorn, G., Gerber, P.J., Henderson, B., Makkar, H.P.S., Dijkstra, J. 2013. Mitigation of methane and nitrous oxide emissions from animal operations: II. A review of manure management mitigation options. J. Anim. Sci. 91, 5070-5094

Moss, A.R., Givens, D.I. 1997. Methane production from a range of feedstuff as determined in vitro using the cumulative gas production technique and compared with that measured *in vivo*. Proc. Brit. Soc. Anim. Sci., p. 194.

Moss, A.R., Jouany, J.-P., Newbold, J. 2000. Methane production by ruminants: its contribution to global warming. Ann. Zootech. 49, 231-253.

Mueller-Harvey, I. 2006. Unravelling the conundrum of tannins in animal nutrition and health. J. Sci. Food Agr. 86, 2010-2037.

Nair, P. 2011. Agroforestry systems and environmental quality: introduction. J. Environ. Qual. 40, 784-790.

Nerlich, K., Graeff-Hönninger, S., Claupein, W. 2013. Agroforestry in Europe: a review of the disappearance of traditional systems and development of modern agroforestry practices, with emphasis on experiences in Germany. Agroforest. Syst. 87, 475-492.

Niderkorn, V., Baumont, R. 2009. Associative effects between forages on feed intake and digestion in ruminants. Animal 3, 951-960.

Niderkorn, V., Macheboeuf, D. 2014. Identification of bioactive grassland plants for reducing enteric methane production and rumen proteolysis using an *in vitro* screening assay. Anim. Prod. Sci. 54, 1805-1809.

Nour, V., Trandafir, I., Cosmulescu, S. 2014. Antioxidant capacity, phenolic compounds and minerals content of blackcurrant (Ribes nigrum L.) leaves as influenced by harvesting date and extraction method. Ind. Crop. Prod. 53, 133-139.

O'Connell, J.E., Fox, P.F. 2001. Significance and applications of phenolic compounds in the production and quality of milk and dairy products: a review. Int. Dairy J. 11, 103-120.

Oliveira, I., Sousa, A., Valentão, P., Andrade, P.B., Ferreira, I.C.F.R., Ferreres, F., Bento, A., Seabra, R., Estevinho, L., Pereira, J.A. 2007. Hazel (Corylus avellana L.) leaves as source of antimicrobial and antioxidative compounds. Food Chem. 105, 1018-1025.

Owen-Smith, N., Cooper, S.M. 1987. Palatability of Woody Plants to Browsing Ruminants in a South African Savanna. Ecology 68, 319-331.

Palo, R.T., Sunnerheim, K., Theander, O. 1985. Seasonal variation of phenols, crude protein and cell wall content of birch (Betula pendula Roth.) in relation to ruminant in vitro digestibility. Oecologia 65, 314-318.

Paolini, V., Dorchies, P., Hoste, H. 2003. Effects of sainfoin hay on gastrointestinal infection with nematodes in goats. Vet. Rec. 152, 600-601.

Paolini, V., Fouraste, I., Hoste, H. 2004. In vitro effects of three woody plant and sainfoin extracts on 3rd-stage larvae and adult worms of three gastrointestinal nematodes. Parasitology 129, 69-77.

Papanastasis, V.P., Yiakoulaki, M.D., Decandia, M., Dini-Papanastasi, O. 2008. Integrating woody species into livestock feeding in the Mediterranean areas of Europe. Anim. Feed Sci. Tech. 140, 1-17.

Patra, A.K., Min, B.-R., Saxena, J. 2012. Dietary tannins on microbial ecology of the gastrointestinal tract in ruminants, in Dietary phytochemicals and microbes (ed. Patra, A.K.). 237-262, Springer.

Patra, A.K., Saxena, J. 2010. A new perspective on the use of plant secondary metabolites to inhibit methanogenesis in the rumen. Phytochemistry 71, 1198-1222.

Peiretti, P., Masoero, G., Tassone, S. 2017. Comparison of the nutritive value and fatty acid profile of the green pruning residues of six grapevine (Vitis vinifera L.) cultivars. Livestock Research for Rural Development 29, Article # 194.

Peng, K., Shirley, D.C., Xu, Z., Huang, Q., McAllister, T.A., Chaves, A.V., Acharya, S., Liu, C., Wang, S., Wang, Y. 2016. Effect of purple prairie clover (Dalea purpurea Vent.) hay and

its condensed tannins on growth performance, wool growth, nutrient digestibility, blood metabolites and ruminal fermentation in lambs fed total mixed rations. Anim. Feed Sci. Tech. 222, 100-110.

Pinares-Patino, C.S., Ulyatt, M.J., Waghorn, G.C., Lassey, K.R., Barry, T.N., Holmes, C.W., Johnson, D.E. 2003. Methane emission by alpaca and sheep fed on lucerne hay or grazed on pastures of perennial ryegrass/white clover or birdsfoot trefoil. J. Agr. Sci. 140, 215-226.

Rahmann, G. 2004. Gehölzfutter-eine neue Quelle für die ökologische Tierernährung (German) p. 29-42, Naturschutz als Aufgabe des Ökologischen Landbaus, Bundesforschungsanstalt für Landwirtschaft (FAL) (in German).

Riethmüller, E., Alberti, À., Tóth, G., Béni, S., Ortolano, F., Kéry, Á. 2013. Characterisation of diarylheptanoid- and flavanoid-type phenolics in *Corylus avellana L*. leaves and bark by HPLC/DAD-ESI/MS. Phytochem. Analysis 24, 493-503.

Rojas-Downing, M.M., Nejadhashemi, A.P., Harrigan, T., Woznicki, S.A. 2017. Climate change and livestock: Impacts, adaptation, and mitigation. Clim. Risk Manag. 16, 145-163.

Salem, H.B., Nefzaoui, A., Abdouli, H. 1994. Palatability of shrubs and fodder trees measured on sheep and dromedaries: 1. Methodological approach. Anim. Feed Sci. Tech. 46, 143-153.

Sauter, J.J., Wellenkamp, S. 1998. Seasonal changes in content of starch, protein and sugars in the twig wood of *Salix caprea* L. Holzforschung-International J. Biol. Chem. Phys. Tech. Wood 52, 255-262.

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

Scherer, R., Gerlach, K., Südekum, K.-H. 2018. Decision-making of goats when exposed to choice feeding: Triggered by taste or smell? Appl. Anim. Behav. Sci. online, in press

Selje, N., Hoffmann, E.M., Muetzel, S., Ningrat, R., Wallace, R.J., Becker, K. 2007. Results of a screening programme to identify plants or plant extracts that inhibit ruminal protein degradation. Brit. J. Nutr. 98, 45-53.

Shi, W., Moon, C.D., Leahy, S.C., Kang, D., Froula, J., Kittelmann, S., Fan, C., Deutsch, S., Gagic, D., Seedorf, H. 2014. Methane yield phenotypes linked to differential gene expression in the sheep rumen microbiome. Genome Res. 24, 1517-1525.

Silanikove, N., Shinder, D., Gilboa, N., Eyal, M., Nitsan, Z. 1996. Binding of poly (ethylene glycol) to samples of forage plants as an assay of tannins and their negative effects on ruminal degradation. J. Agr. Food Chem. 44, 3230-3234.

Soliva, C.R., Hess, H.D. 2007. Measuring methane emission of ruminants by *in vitro* and *in vivo* techniques. Measuring methane production from ruminants. 15-31, Springer.

Soliva, C.R., Amelchanka, S.L., Kreuzer, M. 2015. The requirements for rumen-degradable protein per unit of fermentable organic matter differ between fibrous feed sources. Front. Microbiol. 6, 715.

Soliva, C.R., Zeleke, A.B., Clément, C., Hess, H.D., Fievez, V., Kreuzer, M. 2008. In vitro screening of various tropical foliages, seeds, fruits and medicinal plants for low methane and high ammonia generating potentials in the rumen. Anim. Feed Sci. Tech. 147, 53-71.

Steinfeld, H., Gerber, P., Wassenaar, T.D., Castel, V., Rosales, M., de Haan, C. 2006. Livestock's Long Shadow: Environmental Issues and Options. Food and Agriculture Organization of the United Nations. FAO, Rome, Italy.

Steingass, H., Südekum, K.-H. 2013. Proteinbewertung beim Wiederkäuer–Grundlagen, analytische Entwicklungen und Perspektiven. Übersichten zur Tierernährung 41, 51-73 (in German).

Stickel, F., Seitz, H.K 2000. The efficacy and safety of comfrey. Pub. Health Nutr. 3, 501-508.

Tavendale, M.H., Meagher, L.P., Park-Ng, Z.A., Waghorn, G.C., Attwood, G.T. 2005. Methane production from in vitro incubation of kikuyu grass, lucerne and forages containing condensed tannins. Proc. N. Z. Grassl. Assoc. 67, 147-153.

Terranova, M., Kreuzer, M., Braun, U., Schwarm, A. 2018a. *In vitro* screening of temperate climate forages from a variety of woody plants for their potential to mitigate ruminal methane and ammonia formation. J. Agric. Sci. 156, 929-941.

Terranova, M., Wang, S., Kreuzer, M., Eggerschwiler, L., Schwarm, A. 2018b. Intake potential by cows of different woody and herbaceous plants rich in phenols. Proc. Soc. Nutr. Physiol. 26, 130.

Thielemans, M.-F., Francois, E., Bodart, C., Thewis, A., Dupont, F., Lallemand, L., Malburny, C. 1978. Mesure du transit gastrointestinal chez le porc a l'aide des radiolanthanides. Comparaison avec le mouton. Ann. Biol. Anim. Biochim. Biophys. 18, 237-247.

Tiemann, T.T., Franco, L.H., Peters, M., Frossard, E., Kreuzer, M., Lascano, C.E., Hess, H.-D. 2009. Effect of season, soil type and fertilizer on the biomass production and chemical composition of five tropical shrub legumes with forage potential. Grass Forage Sci. 64, 255-265.

Tiemann, T.T., Franco, L.H., Ramírez, G., Kreuzer, M., Lascano, C.E., Hess, H.-D. 2010. Influence of cultivation site and fertilisation on the properties of condensed tannins and in vitro ruminal nutrient degradation of Calliandra calothyrsus, Flemingia macrophylla and Leucaena leucocephala. Anim. Feed Sci. Tech. 157, 30-40.

Tiemann, T.T., Lascano, C.E., Wettstein, H.-R., Mayer, A.C., Kreuzer, M., Hess, H.D. 2008. Effect of the tropical tannin-rich shrub legumes Calliandra calothyrsus and Flemingia macrophylla on methane emission and nitrogen and energy balance in growing lambs. Animal 2, 790-799.

UN 2015. Paris Agreement, United nations framework convention on climate change: Paris, France.

Van Soest, P.J. 1994. Nutritional ecology of the ruminant. Cornell University Press, Ithaca, USA.

Van Soest, P.J., Robertson, J., Lewis, B. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583-3597.

Vandermeulen, S., Ramírez-Restrepo, C.A., Beckers, Y., Claessens, H., Bindelle, J. 2018. Agroforestry for ruminants: a review of trees and shrubs as fodder in silvopastoral temperate and tropical production systems. Anim. Prod. Sci. 58, 767-777.

Vandermeulen, S., Ramírez-Restrepo, C.A., Marche, C., Decruyenaere, V., Beckers, Y., Bindelle, J. 2016. Behaviour and browse species selectivity of heifers grazing in a temperate silvopastoral system. Agroforest. Syst. 92, 705-716.

VDLUFA, 2012. Methodenbuch, Bd. III, Die chemische Untersuchung von Futtermitteln.Verband deutscher landwirtschaftlicher Untersuchungs-und Forschungsanstalten, Speyer, Germany (in German).

de Vega, A. de, Gasa, J., Guada, J.A., Castrillo, C. 2000. Frequency of feeding and form of lucerne hay as factors affecting voluntary intake, digestibility, feeding behaviour, and marker kinetics in ewes. Aust. J. Agr. Res. 51, 801-809.

Waghorn, G.C. 2008. Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production—Progress and challenges. Anim. Feed Sci. Tech. 147, 116-139.

Waghorn, G.C., Ulyatt, M.J., John, A., Fisher, M.T. 1987. The effect of condensed tannins on the site of digestion of amino acids and other nutrients in sheep fed on *Lotus corniculatus L*. Br. J. Nutr. 57, 115-126.

Waghorn, G.C., McNabb, W.C. 2003. Consequences of plant phenolic compounds for productivity and health of ruminants. Proc. Nutr. Soc. 62, 383-392.

Waghorn, G.C., Shelton, I.D., McNabb, W.C., McCutcheon, S.N. 1994. Effects of condensed tannins in Lotus pedunculatus on its nutritive value for sheep. 2. Nitrogenous aspects. J. Agr. Sci.123, 109-119.

Wallace, R.J. 2008. Rumen-up. Final reports. https://www.abdn.ac.uk/research//rumen-up/report/ Accessed on March 2016.

Wallace, R.J. 2004. Antimicrobial properties of plant secondary metabolites. Proc. Nutr. Soc. 63, 621-629.

Wam, H.K., Stolter, C., Nybakken, L. 2017. Compositional Changes in Foliage Phenolics with Plant Age, a Natural Experiment in Boreal Forests. J. Chem. Ecol. 43, 920-928.

Wang, S., Müller, A., Hilfiker, D., Marquardt, S., Kreuzer, M., Braun, U., Schwarm, A. 2017. Effect of individual Ayurveda plants and mixtures thereof on in vitro ruminal fermentation, methane production and nutrient degradability. Anim. Prod. Sci. 58, 2258-2268.

Wang, S., Terranova, M., Kreuzer, M., Marquardt, S., Eggerschwiler, L., Schwarm, A. 2018. Supplementation of Pelleted Hazel (Corylus avellana) Leaves Decreases Methane and Urinary Nitrogen Emissions by Sheep at Unchanged Forage Intake. Sci. Rep. 8, 5427.

Wang, Y., Douglas, G., Waghorn, G., Barry, T., Foote, A. 1996. Effect of condensed tannins in Lotus corniculatus upon lactation performance in ewes. J. Agr. Sci. 126, 353-362.

White, R.G., Lawler, J.P. 2002. Can methane suppression during digestion of woody and leafy browse compensate for energy costs of detoxification of plant secondary compounds? A test with muskoxen fed willows and birch. Comp. Biochem. Phys. 133, 849-859.

Williams, C.M., Eun, J.S., MacAdam, J.W., Young, A.J., Fellner, V., Min, B.R. 2011. Effects of forage legumes containing condensed tannins on methane and ammonia production in continuous cultures of mixed ruminal microorganisms. Anim. Feed Sci. Tech. 166-167, 364-372.

Woodward, S.L., Waghorn, G.C., Laboyre, P. 2004. Condensed tannins in birdsfoot trefoil (*Lotus corniculatus*) reduce methane emissions from dairy cows. Proc. N.Z. Soc. Anim. Prod. 64, 160-164.

Woodward, S.L., Laboyrie, P.J., Jansen, E.B.L. 2000. *Lotus corniculatus* and condensed tannins-effects on milk production by dairy cows. Asian-Austr. J. Anim. Sci 13, 521-525.

Yang, B., Abula, R., Xu, X.-L., Aniwaer, A. 2011. Chemical composition and in vitro ruminal fermentation characteristics of tetraploid black locust (Robinia pseudoacacia L.). Asian J. Anim. Vet. Adv. 6, 706-714.

Source of cover pictures

Thomé, O. W., 1885. Flora von Deutschland, Österrech und der Schweiz.

Käusel, R., Merxmüller, H., Nothdurft, H., 1957. Mitteleuropäische Pflanzenwelt: Kräuter und Stauden: Eine Auswahl unter Berücksichtigung der in Deutschland geschützten Arten, Sammlung naturkundlicher Tafeln. Kronen-Verlag, Hamburg, Germany.

Sturm, J., Sturm, J. G., 1796. Deutschlands Flora in Abbildungen. Tafeln. www. BioLib.de / accessed on December 2018

Viala, P., Vermorel, V., Bacon, A., 1901. Traité géenéral de viticulture: Ampélographie. Paris :Masson.

Acknowledgement

Zu aller erst möchte ich mich bei Prof. Dr. Michael Kreuzer für seine stetige Unterstützung während des gesamten Doktorats bedanken. Danke, für die Möglichkeit mich mit dem Wiederkäuer und seinen Geheimnissen tiefgründig beschäftigen zu dürfen. Es war ein spannendes und zielführendes Doktoratsprojekt. Ausserdem vielen Dank für die unheimlich schnellen, konstruktiven Korrekturen der zahlreichen Manuskipte, die manchmal doch ziemlich kurz vor knapp erst auf deinem Schreibtisch landeten.

Dr. Angela Schwarm, der betreuenden Oberassistentin, danke ich für die Unterstützung und Hilfe. Du hattest immer einen guten Tipp parat und hast mir besonders beim Verfassen der Publikationen sehr geholfen, vor allem durch das kritische Hinterfragen so mancher Textpassagen.

Prof. Dr. Karl-Heinz Südekum danke ich recht herzlich für die Übernahme des Koreferats.

Dieses Doktoratsprojekt wurde dankenswerterweise von einem ETH Research Grant der ETH Zürich unterstützt.

Ein grosses Dankeschön gilt auch dem Team der ehemaligen Forschungsstation Chamau und des AgroVet-Strickhof.
Im Besonderen möchte ich Andreas Simonett danken. Deine Unterstützung und Hilfsbereitschaft während der Versuche, sowohl auf der Chamau als auch am AgroVet-Strickhof waren enorm. Für jedes Problem hast du immer eine schnelle kreative Lösung gefunden, ein «geht nicht» gab es nicht für dich! Vor allem in der Anfangszeit im neuen Stoffwechselstall warst du immer zur Stelle, wenn Not am Mann war.
Auch Tina Stiefel soll hier nicht vergessen sein - Danke dir Tina, für deine tolle Unterstützung während des Bilanzversuchs am AgroVet-Strickhof. Durch dich konnte ich ein paar Stunden mehr schlafen, wenn du die 23-Uhr-Passageprobe übernommen hast ☺. Durch deine Erfahrung aus verschiedenen Versuchen konnte ich vieles für meinen eigenen Versuch übernehmen und optimieren.
Ein grosses Dankeschön gilt auch Hanspeter Renfer, Rudolf Stoz, Dr. Sergej Amelchanka und Mirjam Hunziker für die Hilfe bei der Organisation und Durchführung des Versuchs am AgroVet-Strickhof. Danke lieber Ruedi, dass du die Respirationskammern immer zum Laufen

bekommen hast und auch die unterhaltsame Aufarbeitung der zahlreichen Probleme zwischendurch.

Dem Laborteam mit Carmen, Muna und Pascal möchte ich danken. Danke für eure stetige Unterstützung und Bereitschaft für jedes Problem eine Lösung zu finden und für die lustigen Zeiten im Labor. Auch danke ich Tiziana und Ruth, denn die vielen scheinbar unwichtigen Dinge des Alltags, tragen zum Erfolg jedes einzelnen Doktorats bei. Vielen Dank Tiziana für deine stetige Hilfe bei Computerproblemen. Danke liebe Ruth für deine Hilfe bei jeglichem Papierkram und all den Rechnungen.

Danke an all die Kollegen und Freunde, die die drei Jahre zu einer tollen Zeit gemacht haben. Susanne, Susi, Christina, Simon, Fränzi, Thomas, Elisa und all die anderen, die ich hier nicht namentlich genannt habe, danke für die lustigen Zeiten im Büro, im Stall oder in der Kaffeepause.

Ganz besonders möchte ich meiner Familie danken. Meinen Eltern, die mich bei meiner Ausbildung und meinen Entscheidungen immer unterstützt haben. Danke, dass ihr immer für mich da seid und ich immer auf euren Rückhalt in jeder Lebenssituation zählen kann. Jochen, dir möchte ich dafür danken, dass du mir den «Agrar-Floh» schon während der Schulzeit ins Ohr gesetzt hast, welcher mich nun bis zu meiner Doktorarbeit gebracht hat. Meiner Schwester Milena danke ich sehr für die tollen Kapitel-Symbole.

Ein ganz besonderer Dank gilt meinem Mann Jan. Du hast immer an mich geglaubt und warst auch in anstrengenden Zeiten für mich da. So einige Wochenenden hast du mit mir im Stall verbracht. Ob beim Bolis pressen, Füttern oder Kot eintüten, du warst eine grosse Stütze und immer für mich da. Danke, dass du mich immer geduldig ertragen hast.

Ausserdem bedanke ich mich bei Baronin, Bambi, Ibiza, Fleur und den anderen 20 Kühen der Chamau und AgroVet-Strickhof, die den Versuch so kooperativ mitgemacht haben. Wir hatten eine gute Zeit im Stall. ☺

Curriculum Vitae

Personal information

Name	Melissa Terranova
Date of birth:	03.10.1990
Place of birth:	Karlsruhe, Germany
Email address:	meterranova@msn.com

Education

2015.12 – 2018.12	ETH Zurich, Zurich, Switzerland Doctoral candidate, Animal Nutrition group, Institute of Agricultural Sciences
2013.10 – 2015.11	University of Hohenheim, Stuttgart, Germany Master of Agricultural Science with profile Animal science
2010.10 – 2013.09	University of Hohenheim, Stuttgart, Germany Bachelor of Agricultural Science with profile Animal science

List of publications

Journal Articles

Terranova, M., Kreuzer, M., Braun, U. and Schwarm, A. 2018. *In vitro* screening of temperate climate forages from a variety of woody plants for their potential to mitigate ruminal methane and ammonia formation. Journal of Agricultural Science 156, 929-941.

Wang, S., Terranova, M., Kreuzer, M., Marquardt, S., Eggerschwiler, L., Schwarm, A. 2018. Supplementation of pelleted hazel (Corylus avellana) leaves decreases methane and urinary nitrogen emissions by sheep at unchanged forage intake. Scientific Reports 8, 5427.

Gomez, Y., Terranova, M., Zähner, M., Hillmann, E. and Savary, P. 2017. Effects of milking stall dimensions on behavior of dairy cows during milking in different milking parlor types. Journal of Dairy Science 100, 1331-1339.

Conference Proceedings

2018

Terranova, M., Wang, S., Eggerschwiler, L., Kreuzer, M. and Schwarm, A. 2018. Hazel leaves as environmentally friendly novel feed for ruminants. World Food System Center Research Symposium 2018, ETH Zurich, Zurich, Switzerland, Poster No. 20.

Terranova, M., Wang, S., Kreuzer, M., Eggerschwiler, L. and Schwarm, A. 2018. Palatability of plants rich in phenols and their effect on milk yield and composition in cows. Proceedings of the 10th International Symposium on the Nutrition of Herbivores 9, 490.

Terranova, M., Kreuzer, M., Eggerschwiler, L. and Schwarm, A. 2018. Dosis-Wirkungsbeziehung zwischen dem Anteil von Haselnussblättern im Futter und der Methanemission von Milchkühen. In: Zukunft der Tierernährung zwischen Effizienz und Konsumentenansprüchen (Kreuzer, M., Lanzini, T., Liesegang, A., Bruckmaier, R., Hess, H.D. und Ulbrich, S.E., Hrsg.). ETH- Schriftenreihe zur Tierernährung, Bd. 41, 180-182.

Wang, S., Terranova, M., Kreuzer, M., Marquardt, S., Ulbrich, S.E., Eggerschwiler, L. and Schwarm, A. 2018. Effect of hazel (Corylus avellana) leaves on methane emission and cellular immune response in sheep. In: Zukunft der Tierernährung zwischen Effizienz und

Konsumentenansprüchen (Kreuzer, M., Lanzini, T., Liesegang, A., Bruckmaier, R., Hess, H.D. und Ulbrich, S.E., Hrsg.). ETH- Schriftenreihe zur Tierernährung, Bd. 41, 183-185.

Terranova, M., Wang, S., Kreuzer, M., Eggerschwiler, L., and Schwarm, A. 2018. Intake potential by cows of different woody and herbaceous plants rich in phenols. Proceedings of the Society of Nutrition Physiology 27, 175.

Wang, S., Terranova, M., Kreuzer, M., Marquardt, S., Eggerschwiler, L. and Schwarm, A. 2018. Effect of hazel (Corylus avellana) leaves on methane and urinary nitrogen emissions by sheep. Proceedings of the Society of Nutrition Physiology 27, 67.

2017

Terranova, M., Kreuzer, M., Eggerschwiler, L. and Schwarm, A. 2017.Auswahl potenzieller Methansenker – welche phenolreiche Pflanze schmeckt den Milchkühen am besten? In: Klimawandel und Nutztiere: eine wechselseitige Beeinflussung (Kreuzer, M., Lanzini, T., Liesegang, A., Bruckmaier, R., Hess, H.D. und Ulbrich, S.E., Hrsg.). ETH- Schriftenreihe zur Tierernährung, Bd. 40, 96-99.

Terranova, M., Kreuzer, M., Eggerschwiler, L and Schwarm, A. 2017. Einfluss phenolreicher Pflanzen auf die Milchinhaltsstoffe bei Kühen. Forschung für die Milchproduktion. Profi-Lait-Forschungtag. HAFL, Zollikofen, 21.11.2017.

Terranova, M., Wolter, M., Kreuzer, M., Braun, U. and Schwarm, A. 2017. Search for European plants containing bioactive compounds to lower *in vitro* methane formation from ruminally fermentable organic matter. Proceedings of the Society of Nutrition Physiology 26, 130.

2016

Terranova, M., Wolter, M., Kreuzer, M., Braun, U. and Schwarm, A. 2016. *In vitro*-Screening von wirkstoffreichen europäischen Pflanzen hinsichtlich positiver Effekte auf die Pansenfermentation. In: Umdenken in der Eiweissversorgung der Nutztiere (Kreuzer, M., Lanzini, T., Liesegang, A., Bruckmaier, R., Hess, H.D. und Ulbrich, S.E., Hrsg.). ETH-Schriftenreihe zur Tierernährung, Bd. 39, 116-118.

Terranova, M., Schwarm, A. and Kreuzer, M. 2016. Identification of the presence of positive associative effects in dairy cattle: Environmental and animal health effects of suitable plant species combinations exceed adverse side-effects on digestion and metabolism. In: Abstr. Symposium for new PhD students. Institute of Agricultural Sciences (Ulbrich, S.E., Kühne, R. and Ravelhofer, E., eds), p. 44.

Terranova, M., Kreuzer, M. and Schwarm, A. 2016. *In vitro*-screening of European plants for their potential to mitigate methane from ruminal fermentation. Cost Methagene Annual Meeting. University of Padova, Padova, Italy, 12.-14.10.2016.

Gómez, Y., Terranova, M., Zähner, M., Hillmann, E. and Savary, P. 2016. Influence of milking-parlour size on the behaviour of dairy cows. In: Proc. 50th Congress. International Society for Applied Ethology 2016, p. 219.

Gómez, Y., Terranova, M., Zähner, M., Hillmann, E. and Savary, P. 2016. Influence of milking parlour size on the behaviour of dairy cows. In: Recent Advances in Animal Welfare Science V: p. 55.