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**EXAMINING THE INTER- AND INTRASPECIES VARIABILITY
IN THE POLYPHENOLIC PROFILE AND *IN VITRO*
ANTIMETHANOGENIC POTENTIAL OF FORAGE SPECIES**

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submitted by

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Abbreviations

ADF	Acid Detergent Fiber
AIC	Akaike Information Criterion
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
C	Carbon
CH ₄	Methane gas
CO ₂	Carbon dioxide gas
CP	Crude Protein
CT	Condensed Tannins
DDG	Distillers' Dried Grains
DHHP	Dehydrohexahydroxydiphenyl
DM	Dry Matter
DMI	Dry Matter Intake
DMI	Dry Matter Intake
DP	Degree of Polymerisation
E-PAs	Extractable PAs
ESI	Electrospray Ionisation
ETs	Ellagitannins
EU	European Union
F-PAs	Fiber-Bound PAs
GEI	Gross Energy Intake
GHG	Greenhouse Gas Emissions
GP	Gas Production
GTs	Gallotannins
GWP	Global Warming Potential
H ₂	Hydrogen gas
HGT	Hohenheim Gas Test
HHDP	Hexahydroxyphenolyl
HTs	Hydrolysable Tannins
<i>m/z</i>	Mass-To Charge Ratio
MAE	Microwave-Assisted Extraction
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight
mDP	Mean Degree of Polymerisation
MP	Methane Proportion in Total Gas
MRM	Multiple Reaction Monitoring
MW	Molecular Weight
N	Nitrogen
N ₂	Nitrogen gas
N ₂ O	Nitrous Oxide gas
NDF	Neutral Detergent Fiber
NH ₃	Ammonia gas
NO ₃	Nitrate

N-PAs	Non-Extractable PAs
OH	Hydroxyl group
OM	Organic matter
PA	Proanthocyanidin
PC	Procyanidin
PD	Prodelphinidin
PD%	Prodelphinidin Percentage
PEG	Polyethylene Glycol
P-PAs	Protein-Bound PAs
PPC	Protein Precipitation Capacity
PSMs	Plant Secondary Metabolites
PTFE	Polytetrafluoroethylene
SEM	Standard Error of The Mean
TF	Total Flavanoids
TMR	Total Mixed Ration
TOF	Time-of-Flight
TP	Total Phenols
TP	Total Polyphenols
TRFs	Tannin Rich Forages
TT	Total Tannins
uCP	Undegraded Crude Protein
UPLC	Ultra High-Performance Liquid Chromatography
UV	Ultraviolet
VFA	Volatile Fatty Acids
WSC	Water Soluble Carbohydrates

Zusammenfassung

Die Viehzucht spielt für die Ernährungssicherheit eine wichtige Rolle, da sich der Bedarf an Lebensmitteln in den nächsten zwei bis drei Jahrzehnten voraussichtlich verdoppeln wird. Gleichzeitig trägt die Viehwirtschaft jedoch in hohem Maße zu den anthropogenen Treibhausgasemissionen (THG) bei, da beispielsweise die Methanemissionen (CH_4) von Wiederkäuern bereits rund 39 % der weltweiten landwirtschaftlichen Emissionen ausmachen. Bei den derzeitigen Trends werden diese Emissionen bis 2050 voraussichtlich um weitere 30 % ansteigen. Die Nutzung von tanninreichen Futtermitteln (TRF) in der Ration ist eine der vielversprechenden Strategie zur Verringerung der CH_4 -Emissionen bei Wiederkäuern. Da die enterischen CH_4 -Emissionen bei Rindern einen Energieverlust von 6 bis 12 % darstellen, bietet die Verringerung von CH_4 einen zusätzlichen Vorteil in Form einer verbesserten Futterverwertung. Folglich können TRF mit Tanninen wie den Proanthocyanidinen (PAs, *syn.* kondensierte Tannine) potenziell die Lebendgewichtszunahme und die Wollproduktion bei Wiederkäuern verbessern und sogar die ernährungsphysiologische Qualität von tierischen Erzeugnissen wie Milch und Fleisch steigern. Darüber hinaus können PAs in der Ration die Tiergesundheit verbessern indem sie das Blähen bei Rindern verringern und die parasitäre Belastung durch gastrointestinale Nematoden senken. Schließlich kann insbesondere die Beweidung von TRFs den ökologischen Fußabdruck weidebasierter Produktionssysteme im Vergleich zu konventionellen Tierproduktionssystemen weiter reduzieren, indem auch die Lachgasemissionen verringert werden. Trotz der langjährigen Forschung über die Verwendung von TRF zur Verringerung der CH_4 -Emissionen ohne Beeinträchtigung der Tiergesundheit und -Leistungsfähigkeit sind die Ergebnisse der verschiedenen Studien bisher widersprüchlich, was ihre praktische Anwendung erschwert. Diese widersprüchlichen Ergebnisse können auf verschiedene Faktoren zurückzuführen sein, unter anderem auf unterschiedliche Analysetechniken zur Quantifizierung der Tannine und auf die unterschiedliche Bioaktivität der Tannine, die von der Pflanzenart, sowie der Tanninkonzentration und den strukturellen Merkmalen der Tannine abhängt. Derzeit werden beispielsweise strukturelle Merkmale von PAs mit ihrer Bioaktivität in Verbindung gebracht, aber sie werden in Studien zur Tierernährung nur selten berücksichtigt. Daher bestand das übergeordnete Ziel dieser Dissertation darin, ein umfassendes Verständnis der beobachteten Variabilität der gemessenen Auswirkungen PA-haltiger Futtermittel auf die CH_4 -Produktion bei Wiederkäuern zu vermitteln und die Zusammenhänge zwischen den strukturellen Merkmalen von PA und ihrem antimethanogenen Potenzial zu bewerten.

Kapitel 2 beschreibt die derzeitigen Wissenslücken in der Forschung zur Bioaktivität von Tanninen und liefert ein besseres Verständnis des antimethanogenen Potenzials von TRF. Es wird veranschaulicht, wie die Zusammensetzung und Struktur der Tannine zu den widersprüchlichen Ergebnissen in Bezug auf ihre Bioaktivität führen. Außerdem wird ein kurzer Überblick gegeben über die für die Tanninanalyse verwendeten Analysetechniken und wie diese optimiert werden können, um reproduzierbare Ergebnisse zu erzielen. Dies ist eine notwendige Voraussetzung für die Auswahl (und längerfristig für die Züchtung) der idealen Futterarten, die sowohl eine hohe Futterqualität besitzen um eine hohe Produktivität aufrechtzuerhalten, als auch die Umweltauswirkungen der Wiederkäuersysteme verringern. Es ist bekannt, dass es sowohl zwischen den Futterarten als auch innerhalb der Arten große Unterschiede in der polyphenolischen Zusammensetzung und den strukturellen Merkmalen der Tannine in den Pflanzen gibt, jedoch wurde diese Variabilität innerhalb der Arten für Sorten/Varietäten nur für wenige Arten festgestellt. Dementsprechend ist eine Verallgemeinerung der Ergebnisse sehr schwierig. Ausgehend davon wurde ein Gewächshausversuch mit 17 Sorten aus 8 Futterarten durchgeführt. Diese wurden unter identischen Umweltbedingungen angebaut und im gleichen phänologischen Stadium geerntet (Kapitel 3). Die Blattproben dieser Sorten wurden auf ihre polyphenolische Zusammensetzung und ihre PA-Strukturmerkmale untersucht. Wie erwartet, unterschied sich das polyphenolische Profil der Blattextrakte deutlich zwischen den verschiedenen Arten. Die höchste Konzentration an Polyphenolen wies der kleine Wiesenknopf auf, der jedoch hauptsächlich hydrolysierbare Tanninen experimentiert. Die Reihenfolge der PA-haltigen Futtermittel in unserer Studie lautete: Sulla > Sumpfhornklee > Esparsette > Hornklee. Sowohl Zichorie als auch Spitzwegerich wiesen eine niedrige Polyphenolkonzentration auf, und in der Luzerne wurden keinerlei Polyphenole gefunden. Im Gegensatz zur Variation zwischen den Arten war die Variabilität zwischen den Sorten derselben Art sowohl bei der PA-Konzentration als auch bei den strukturellen Merkmalen wesentlich geringer. Darüber hinaus wurde die Verteilung der PAs auf die verschiedenen oberirdischen Pflanzenorgane der Sorten über drei aufeinanderfolgende Ernten hinweg bestimmt. Hier war der PA-Gehalt in den Blättern von allen Pflanzenorganen am höchsten, was auf die Bedeutung des Blattanteils in der Pflanze hinweist. Diese Ergebnisse waren vielversprechend, da die allgemein viel geringere Variabilität innerhalb der Arten als zwischen den Arten darauf hindeutet, dass eine Analyse auf Artniveau repräsentative Ergebnisse liefern kann.

Um zu beurteilen, ob sich die beobachtete Variabilität bei den Polyphenolen und Tanninen auf die Bioaktivität dieser Futtermittel auswirkt, wurde der Hohenheimer Gastest durchgeführt mit dem Ziel, das CH₄-Reduktionspotenzial der ausgewählten Arten im Vergleich zur Luzerne als tanninfreier Negativkontrolle zu bestimmen (Kapitel 4). Mit Ausnahme der Hornkleesorten reduzierten alle Arten die CH₄-Bildung im Vergleich zu Luzerne um 1 bis 48%. Es zeigte sich jedoch, dass sowohl die CH₄- als auch die Gasproduktion signifikant von der Sorte abhingen. Da nicht nur die Tanninkonzentration und -zusammensetzung, sondern auch die Futterqualität einen starken Einfluss auf die Methanemissionen und die Verdaulichkeit haben, wurde auch eine Behandlung mit Polyethylenglykol (PEG), einem Tannin-inaktivierenden Mittel, einbezogen. Hier wies das Pflanzenmaterial mit inaktivierten Tanninen im Vergleich zum normalen Pflanzenmaterial höhere Methanbildungsraten auf, was darauf hindeutet, dass die Tannine tatsächlich CH₄ reduzieren. Die einzige Ausnahme bildeten die Hornklee-Sorten (Bull und Lotar). Schließlich wurde die Beziehung zwischen der CH₄-Produktion und den Parametern der Futterqualität sowie der PA-Konzentration und den PA-Strukturmerkmalen untersucht. Es wurde festgestellt, dass der mittlere Polymerisationsgrad (mDP), die PA-Konzentration und die Rohproteinkonzentration eine wichtige Rolle bei der Bestimmung des antimethanogenen Potenzials von PA-haltigem Futter spielen. Dennoch war das Modell nicht in der Lage, das antimethanogene Potenzial allein aus diesen Parametern vorherzusagen. Außerdem ging die Abnahme der CH₄-Bildung der tanninhaltigen Arten mit einer Abnahme der Verdaulichkeit des Futters einher. Unter den getesteten Arten waren also die Tannin freien Arten Spitzwegerich und Zichorie die vielversprechendsten Kandidaten, da sie die CH₄-Produktion verringerten, ohne die Verdaulichkeit des Futters zu beeinträchtigen.

Zusammenfassend ist zu konstatieren lässt, dass die Forschungsergebnisse der vorliegenden Studie zeigen, dass die Variabilität der Pflanzen zwischen und innerhalb der Arten in Bezug auf die Polyphenol- und Tanninkonzentration in den Blättern auf ihr antimethanogenes Potenzial zurückzuführen ist. Künftige Forschungsarbeiten sollten sich auf die Optimierung von Futtermitteln konzentrieren, die die CH₄-Produktion verringern, ohne die Verdaulichkeit in vivo zu beeinträchtigen. Außerdem sollten die Auswirkungen von Mischungen aus Tanninexprimierenden und Tanninfreien Futtermitteln auf die CH₄-Produktion weiter untersucht werden. Schließlich sind Züchtungsprogramme, die das agronomische potenzial bioaktiver Futtermittel verbessern, für ihre Nutzung im landwirtschaftlichen Maßstab erforderlich.

Summary

Livestock production plays a crucial role in achieving food security as the demand for food is projected to double in the next 2 to 3 decades. However, at the same time, the livestock sector contributes heavily to anthropogenic greenhouse gas (GHG) emissions, for example, methane (CH₄) emissions from ruminants contribute to around 39 % of the global agricultural emissions. Under current policies and trends, these emissions are projected to increase by 20% until 2050. The inclusion of tannin rich forages (TRFs) in the ruminant diet is one of the most promising strategies to reduce CH₄ emissions in ruminants. Also, as enteric CH₄ emissions represent an energy loss of 6 to 12 % in cattle hence, the reduction of CH₄ provides an added benefit of improved feed utilisation efficiency. Consequently, TRFs including proanthocyanidins (PAs, syn. condensed tannins) can potentially improve live weight gains, wool production and even enhance the nutritional quality of animal products such as milk and meat. Additionally, dietary PAs can improve animal health by reducing bloating incidences in cattle and lowering the parasitic burdens of gastrointestinal nematodes. Finally, particularly grazing of TRFs can reduce the environmental footprint of the pasture-based production systems further compared to conventional livestock production systems by also reducing nitrous oxide emissions. Despite the research focus of using TRFs to reduce CH₄ emissions without compromising animal health and productivity, results so far have been inconsistent across different studies, making their practical application difficult. These contradicting results can arise from varying factors including but not limited to incongruencies in analytical techniques used to quantify tannins, and the variation in tannin bioactivity arising from tannin source, type, structural features, and dose. For instance, nowadays structural features of PAs have been linked to their bioactivity, but they are rarely considered in animal nutrition studies. Thus, the overall objective of this dissertation was to provide a comprehensive understanding of the observed variability in the measured effects of PA containing forages on CH₄ production in ruminants, and assess the links between PA structural characteristics and their antimethanogenic potential.

Chapter 2 describes the current knowledge gaps which exist in the research on tannin bioactivity, and provides a better understanding of the antimethanogenic potential of TRFs. It is illustrated how the tannins' composition and structure lead to the contradictory results with regards to their bioactivity. Also, we provided a brief overview of the analytical techniques used for tannin analysis and how these could be optimised to produce repeatable results. This is a prerequisite for the selection (and in the longer term, breeding) of the ideal forages,

which are both nutritious enough to maintain high productivity, yet reduce the environmental impact of ruminant agriculture. It is well known that large differences exist across forage species as well as within the species in terms of polyphenolic composition and tannin structural features in plants, however, this variability within the cultivars has been established only for few species, and generalization of results is very difficult. Taking this as a starting point, a greenhouse experiment was conducted using 17 cultivars from eight species. These were grown under identical environmental conditions and harvested at the same phenological stage (Chapter 3). Leaf samples for these cultivars were analysed for their polyphenolic composition and PA structural features. As expected, the polyphenolic profile of the leaf extracts differed significantly among different species. Salad burnet was found to contain the highest concentration of polyphenols, yet comprised mainly hydrolysable tannins. PA containing forages in our study ordered according to their polyphenol concentration in leaves were sulla > big trefoil > sainfoin > birdsfoot trefoil. Both chicory and plantain had low polyphenol concentration, and no polyphenols were found in lucerne. Contrary to the variation among the species, the variability among cultivars of the same species was much less for both PA concentration and structural traits. Furthermore, the distribution of PAs among different aboveground plant organs for the cultivars was determined across three progressive harvests. Here, PA yields from the leaves were highest among all the plant organs, thus indicating the importance of the leaf share in the plant. These results were promising as the generally much lower variability within species than across species indicates that analysis at the species level can generate representative findings.

To assess whether the variability observed in terms of polyphenols and tannins is translated to the bioactivity of these forages, the Hohenheim Gas Test was performed to determine the CH₄ reduction potential of the selected species compared to Lucerne as a tannin free negative control (Chapter 4). Except for birdsfoot trefoil cultivars, all the species reduced CH₄ formation ranging from 1 to 48% compared to lucerne. Here, however, both, CH₄ and gas production were found to be significantly dependent on the type of cultivar. As not only the tannin concentration and composition, but also the forage quality has a strong effect on CH₄ emissions and digestibility, a treatment with polyethylene glycol (PEG) was also included, which is a tannin inactivating agent. Here, the plant material with inactivated tannins had higher CH₄ formation rates compared to the normal plant material, thus indicating that indeed tannins reduce CH₄. The only exception was birdsfoot trefoil cultivars (Bull and Lotar). Finally, the relationship between CH₄ production and the forage quality

parameters, as well as the PA concentration, and PA structural features, was also explored. It was found that the mean degree of polymerisation (mDP), PA concentration and crude protein concentration play an important role in determining the antimethanogenic potential of PA containing forages. Still, the model was unable to predict the antimethanogenic potential from these parameters alone. Also, the decrease in CH₄ from tannin containing species was accompanied by a decrease in the digestibility of the forage. Thus, among the tested species, the tannin-free species, i.e., plantain and chicory, were the most promising candidates, as they decreased CH₄ production without affecting the digestibility of the forage.

In summary, the research results of the present study highlighted that the inter- and intraspecies variability of plants in terms of polyphenol and tannin concentrations in the leaves was translated to their antimethanogenic potential. Future research should focus on optimising the forages that reduce CH₄ production without affecting the rate and extent of nutrient digestion *in vivo*. Additionally, the effect from mixtures of tannin-containing and tannin-free forages on the CH₄ production should be further evaluated. Finally, breeding schemes that enhance the agronomic potential of bioactive forages are required for their exploitation at a farm scale.

Chapter 1

General Introduction

1.1 The hurdles to sustainable livestock production and near future effects on the environment

The unprecedented rate at which anthropogenic climate change is happening, is causing an increase in the incidences of heat waves, floods, droughts, and wildfires, both at regional and global scale (Gibson and Newman, 2019). This poses new challenges for agricultural productivity, and threatens food security worldwide. Furthermore, the accompanied widespread species extinctions and changes in native species compositions, will impact the stability of ecosystem services and affect food security globally (Ghahramani *et al.*, 2019). But agriculture is also responsible for causing many of these trends, as the rampant intensification of livestock production systems is driven by increasing food demands of the rising population which ultimately comes at a high environmental cost at both local and global levels (Gerber *et al.*, 2013a). Livestock sector contributes to around 14.5 % of global anthropogenic greenhouse gas emissions (GHGs) in the form of methane (CH_4), carbon dioxide (CO_2), and nitrous oxide (N_2O), and the sources are mainly emissions from land use change, agricultural operations, manure deposition and application, mineral fertilizer manufacturing and application, and feed transportation (Figure 1) (Gerber *et al.*, 2013b; Grossi *et al.*, 2018).



Figure 1 Major emission sources in livestock sector based on Gerber *et al.* (2013b)

Enteric CH₄ emissions in ruminants are a significant source of emissions from the livestock sector, accounting for up to 39 %, followed by emissions from feed production and processing (30 %) (Gerber *et al.*, 2013b). The high global warming potential (GWP, over 100 years) of CH₄ (28 times) and N₂O (265 times) compared to CO₂, further exacerbates their impact on climate change (Allen *et al.*, 2018). Consequently, reduction in emissions from livestock sector will play a key role in the mitigation of GHG emissions. In order to transition towards sustainable livestock production systems, precise quantification of greenhouse gas (GHG) emissions and identification of their sources is a key to develop practical mitigation strategies and their successful implementation (Gerber *et al.*, 2013b). Simultaneously, a shift from biologically simple systems towards low input- high output- low carbon (C) footprint systems is indispensable to overcome the negative environmental effects the livestock sector (Ryschawy *et al.*, 2018).

1.2 Challenges for intensively managed grasslands in temperate regions

Grassland based ruminant production systems are considered to be a promising alternative to the conventional livestock production systems depending on the management of the systems, as they incur a positive impact on the environment as well as on the economy, (Lüscher *et al.*, 2014; Taube *et al.*, 2014; Hamacher *et al.*, 2021). Temperate grasslands cover around 70% of the global agricultural area, and are an important agroecosystem providing a wide range of ecosystem services ranging from feed supply to the ruminants, biofuels, soil C sequestration, and wildlife habitats (Taube *et al.*, 2014; Ghahramani *et al.*, 2019).

Furthermore, grasslands can sequester up to 0.6 gigatons CO₂ globally, and well managed pastures can potentially offset CH₄ emitted by the cattle by acting as a net C sink (Beauchemin *et al.*, 2009; Lagrange *et al.*, 2021). However, grassland systems are under threat as the management of these systems has been highly intensified over the last century, and characterized mainly by monocultures of high yielding forage species and high nitrogen (N) inputs. Grazing systems in north-western Europe, primarily comprise perennial ryegrass (*Lolium perenne* L.) swards or, with binary swards containing mixtures of perennial ryegrass with forage legumes, such as white clover (*Trifolium repens* L.) or red clover (*Trifolium pratense* L.) or lucerne (*Medicago Sativa* L.) (Lorenz *et al.*, 2020). Inclusion of legumes in the mixtures can substantially reduce the dependence on N fertiliser inputs, increase forage yields, and high animal intake, making these systems cheaper to operate (Hofer *et al.*, 2016; Garrett *et al.*, 2021). Although, these legumes are highly productive, they have been associated in providing excess N to grazing animals, thus leading to poor nutrient utilisation

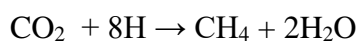
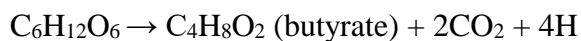
in ruminants, which affects both animal health and productivity (Mielmann, 2013). Additionally, the rapid protein degradation to ammonia (NH_3) during mastication by rumen microbes leads to bloating, i.e. the formation of stable foam in rumen, which if left untreated, could result in animal death (Aerts *et al.*, 1999; McMahon *et al.*, 2000). Concomitantly, the increase in urinary N excretion due to low N utilisation efficiency can lead to high metabolic energy costs for N excretion in the form of urine indicating decrease in metabolic protein supply in ruminants (Carulla *et al.*, 2005). Furthermore, increase in urinary N excretion contributes to environmental pollution via NO_3 leaching, NH_3 volatilisation and N_2O emissions (Chung *et al.*, 2013; Nyameasem *et al.*, 2021). The resistance of gastrointestinal nematodes to synthetic anthelmintic drugs is also a major concern for the livestock sector (Naumann *et al.*, 2017).

In addition to aforementioned disadvantages, the continuing loss of biodiversity resulting from the intensification of the grassland systems makes their productivity more susceptible to climate change. It is crucial, however, to maintain the productivity of these systems across increasing climatic variabilities as they are expected to provide high annual yields across several harvests in a year (Hofer *et al.*, 2016). Diverse swards comprising different forage herbs or legumes, have been found to prevent yield losses under drought events compared to simple grass-clover swards. Deep-rooted herbs, such as chicory (*Cichorium intybus* L.) and red clover, have been shown to tolerate low soil moisture conditions and maintain yields. Additionally, multi-species swards with six and nine species were found to more effective in weed suppression compared to swards with two or three species (Lorenz *et al.*, 2020; Loza *et al.*, 2021). Consequently, increasing species richness and species diversity with different functional groups in grasslands can help an efficient resource utilisation and positive interspecific interactions to provide long term yield stability (Lüscher *et al.*, 2014; Komainda *et al.*, 2020). Multispecies swards containing diverse grasses, legumes, and non-leguminous forbs have been found to be improve animal performance compared to simple swards which could be attributed to higher rates of herbage utilisation (Jerrentrup *et al.*, 2020). Studies have shown that higher biomass production associated with species diversity can reduce N_2O emissions from the field with increase in plant N uptake leading to decrease in soil mineral N availability as a substrate for microbial N_2O production (Cong *et al.*, 2017). Moreover, underutilised forbs, such as chicory and ribwort plantain (*Plantago lanceolata* L.), are found to be rich in plant secondary metabolites (PSMs), essential macrominerals and vitamins, and possess anthelmintic properties, which are beneficial for animal health. Simultaneously, dairy cows fed on sainfoin (*Onobrychis*

viciifolia) and birdsfoot trefoil (*Lotus corniculatus*), have been found to decrease the milk and blood urea concentration (Mueller-Harvey *et al.*, 2017). Therefore, it is essential to promote research on the underutilised forage species which have high agronomic potential, as they can not only curtail negative externalities of livestock sector on the environment, but also improve animal productivity to meet the growing demands of milk and production from ruminant systems.

1.3 Enteric methane emissions: An important determinant for high ecological footprint of livestock production systems.

Enteric fermentation enables the ruminants to digest the human inedible biomass to nutrient rich products, such as milk and meat, for human consumption (van Gastelen *et al.*, 2019). The complex organic material in the feed, such as structural carbohydrates, proteins, and other organic polymers, are digested in the rumen to produce their monomeric components in the presence of anaerobic microorganisms. These components are converted into volatile fatty acids (VFAs), CO₂, and hydrogen (H₂). Primarily, CO₂, H₂, and formate are used as a substrate for rumen methanogenesis as the end step of fermentation in the presence of methanogenic archaeal populations (methanogens) (Boadi *et al.*, 2004; Morgavi *et al.*, 2010)



Methanogenesis is an integral part of ruminal fermentation as it prevents H₂ accumulation in the rumen, which in exceeding levels can hinder the fermentation. The symbiotic relationship of methanogens with rumen protozoa allows them to promote their growth by utilising the H₂ produced from protozoa via hydrogenosomes. This association between methanogens and protozoal population can produce up to 37% of total CH₄ production in the rumen. Some methanogens can also potentially utilise methanol, methylamines, dimethyl sulfide, acetate, and alcohols as substrates for CH₄ production (Boadi *et al.*, 2004; Greening *et al.*, 2019). Although, CH₄ production is an important H₂ sink in the rumen, it is an energetically intensive process and can represent energy losses of around 2 to 12% of the gross energy intake (Johnson and Johnson, 1995; Beauchemin *et al.*, 2009). Consequently, curtailing CH₄

emissions is not only beneficial from the environmental perspective but it can also improve the production efficiency of livestock sector at both animal and farm level.

1.4 Strategies to mitigate enteric methane emissions

The emissions patterns from ruminant production systems vary a lot across different regions globally. In the Global South, environmental footprint of livestock sector arises from low production levels due to poor forage quality and digestibility, resulting in inefficient digestion and hence, high emissions intensities for animal products. Contrary to that, in developed countries, the technological advancements have substantially decreased the CH₄ intensity. Still, the intensification and resulting increase in animal numbers have resulted in net increments of CH₄ emissions worldwide (Gerber *et al.*, 2013b). Several factors have been found to influence CH₄ production in ruminants and these are either linked to the differences in rumen physiology or to forage quality parameters. With regards to daily management, factors such as diet intake level, passage rate, forage quality and their influence on rumen microbiota and fermentation parameters, as well as the interactions among them, can play an important role in reducing CH₄ intensity of animal products (van Gastelen *et al.*, 2019). On longer time scales, improving animal management, including selecting animal, animal health, and reproductive management, can drastically decrease emission intensity for each product (Beauchemin *et al.*, 2009; Haque, 2018). The effectiveness of a mitigation strategy is highly dependent on their mode of action to reduce CH₄ in ruminants. The strategies directly targeting methanogenesis-related fermentation pathways are more likely to be effective across different ruminant types than the strategies related to ruminant-specific factors such as rumen physiology (van Gastelen *et al.*, 2019). Additionally, it is important to promote accessibility and affordability of the existing mitigation measures, and research on new mitigation strategies with long-term applicability. The following sections will discuss some of these promising measures to mitigate CH₄ emissions in ruminants.

1.4.1 Improved forage quality and management

Forage quality and management are one of the most important determinants of enteric CH₄ emissions. Enhancing the quality of the diet increases the digestibility of the feed and concomitantly lowers the CH₄ intensity of an animal product. A meta-analysis by Archimède *et al.* (2011) indicated that grasses using C4 metabolic pathways (warm and tropical grasses) produce higher CH₄ compared to C3 grasses, and cool and warm legumes. This could be due to the higher fiber content in C4 grasses compared to C3 grasses, and there is a positive correlation between forage fiber concentration and CH₄ production. Forages containing lower

concentration of structural carbohydrates are associated with higher organic matter (OM) digestibility hence, lower CH₄ yield (g/kg of DMI) due to a shorter mean retention time in the rumen (Archimède *et al.*, 2011). Consequently, altering the diet composition of the animal which increases forage quality is one of the most promising strategies. This could be done in four ways: a) including additives which change VFA production patterns, b) increase passage rate of feed in rumen and shift part of digestion to small intestine, c) alter microbial population in rumen, and d) feed high quality diet to reduce CH₄ cost associated with maintenance requirements (Ellis *et al.*, 2008). For instance, increasing corn silage in animal diet has been found to increase DMI by 11 % and lower CH₄ yield (g/kg of DMI) by 5%. which promotes the propionate production at the expense of acetate and reduces H₂ availability for methanogenesis. However, this strategy has been found to be more effective on dairy cattle compared to beef cattle (van Gastelen *et al.*, 2019).

High grain content in the diets can reduce gross energy intake (GEI) losses in the form of CH₄ ranging from 4% to 6.5% or more, which is generally observed for forage-based diets (Beauchemin *et al.*, 2009). Replacing roughage by concentrate in the diet can enhance animal productivity and decrease the energy associated to produce CH₄. However, its inclusion of more than 50% of the diet has negative consequences on the milk quality. Furthermore, concentrate feeding has been associated with high costs, which makes it inaccessible to many parts of the world and has also been associated with high on- and off-farm emissions associated with their production, processing, and transportation (Beauchemin *et al.*, 2008). With the growing population, use of grains as animal feed will be limited as it might increase the competition with food source for humans or it will eventually become less profitable, if the costs related to grain production increases due to its close relationship with oil energy sources, which makes them unsustainable in the long term (Lagrange *et al.*, 2021).

1.4.2 Animal selection and manipulation

Increasing the productivity of cattle and breeding animals that produce lower CH₄ emissions can lead to an increase in production, but would decrease the CH₄ emission intensity i.e. lower CH₄ per unit animal product. Simultaneously, replacement of cattle with low productivity with more efficient breeds can decrease overall emissions at a herd level (Haque, 2018). In a case study, it was found that an increase in production efficiency of the dairy cattle to produce double the milk yields (from 5000 to 10000L annually) in EU, by using high grain diets or by improving the genetic merit of the cow, would lead to 23% higher CH₄ emissions (per animal) per year, but would reduce the CH₄ per unit of milk (kg of CH₄ kg

milk⁻¹) produced by 40% (Haque, 2018). Consequently, selection of animals which reduced residual feed intake are found to lower CH₄ production (g/day) and intensity (g/ kg gain) by approximately 25% due to their lower dry matter intake (DMI). It has been observed that CH₄ production (% of GEI) from Dutch/US cross Holstein cows produced approx. 8 to 11% less than New Zealand Friesian cows, and was not dependent on whether they were grazing or receiving a total mixed ration (TMR) (Knapp *et al.*, 2014).

1.4.3 Ruminal defaunation

A promising alternative to the reduced CH₄ emissions by increasing concentrate share in the diet via reduction of methanogenesis by targeting microbial population in the rumen due to their association with methanogens. Several treatments such as acids, surface-active chemicals, triazine, tannins, ionophores, and saponins are used for defaunation (Haque, 2018). However, their effect has been found to be highly variable and diet dependent. Supplementation of monensin at the concentration of 24 to 35 g/day to beef and dairy cattle was able to reduce CH₄ (g/day) by 4 to 13%. Although, its effect was not persistent in the long term and it was hypothesized that ciliate protozoal population get adapted to monensin with time (Beauchemin *et al.*, 2009). Sheep and lambs in which the protozoal populations have been completely eliminated can exhibit up to 26 % CH₄ reduction without affecting digestibility. It was also found that in a high concentrate diet, CH₄ reduction was higher with defaunation, as protozoal population is the main source of H₂ production in starch-based diet (Haque, 2018). Bromochloromethane and bromoethanesulfonate were found to inhibit methanogenesis by targeting methanogens leading to a strong CH₄ reduction (Morgavi *et al.*, 2010). Another strategy which directly targets methanogenesis, is the development of vaccines which produces antibodies against methanogens. A study by Williams *et al.* (2009), showed that antimethanogen vaccines targeting 20% of different methanogen species in the sheep was able to reduce around 7.7% CH₄ per unit DMI, but the vaccine targeting 52% of the population led to an 18% increase in CH₄ output, indicating that the later vaccine did not target the methanogens producing most of the CH₄. Furthermore, their implementation becomes strenuous due to their dependence of the host location and diet, and their long-term effect still remains uncertain (Williams *et al.*, 2009; Hook *et al.*, 2010).

1.4.4 Organic acids

Addition of organic acids as feed additive to animal diet is another emerging strategy for CH₄ mitigation. Organic acids provide an alternative H₂ sink, thereby reduces methanogenesis. Both fumarate and acrylate have been found to consistently reduce CH₄ in batch cultures

(Haque, 2018). For *in vivo* studies, organic acid supplementation of more than 2% is required to produce mitigation effect on CH₄ production. However, these concentrations must be carefully assessed for different ruminant types and diet. When included at higher concentration, they can reduce DMI and lower ruminal pH resulting in negative effect on fiber degradation. Furthermore, their effect is more pronounced on high-concentrate diets (Beauchemin *et al.*, 2009; Hook *et al.*, 2010).

1.4.5 Lipid supplementation

Inclusion of lipids, such as fatty acids and oils, in ruminant diet has been found to decrease methanogenesis by inhibiting protozoal population and promote propionic acid production. Sunflower oil as a feed additive reduced methanogenesis by 11 to 22%, and supplementation of linseed oil at 5% DM can result up to 56% reduction in CH₄ (g/day). Lipids are also known to affect palatability, diet intake level, animal performance, and milk quality, however, ruminant genotype and diet should also be accounted for before its supplementation (Hook *et al.*, 2010). Furthermore, the majority of *in vivo* studies on lipid mediated CH₄ reduction have been short-termed, and the persistence of their effect in long term still needs to be assessed. Inclusion of essential oils rich in bioactive compounds, such as terpenoids and phenolic compounds, can also decrease methanogenesis due to their antimicrobial effect. However, more research is needed for low cost lipid sources, such as distillers' dried grains (DDG) and microalgae, as the commercial vegetable oils and oilseeds are comparatively cost intensive supplements (Beauchemin *et al.*, 2009; Knapp *et al.*, 2014).

1.4.6 Inclusion of bioactive forages

Incorporation of forages rich in plant secondary metabolites (PSMs) have been found to benefit animal health and nutrition as well as have high potential to mitigate CH₄ in ruminants. Several PSMs have been identified which can suppress CH₄ in ruminants, and among these, tannins and saponins have garnered much attention (Haque, 2018). Saponins are known to exhibit antimicrobial properties by directly inhibiting rumen protozoal population, and altering their cell membrane permeability. Although, there was no effect of the supplementation of tea saponins on methanogens as observed in an *in vitro* study. Supplementation of saponin rich source *Yucca schidigera* at the concentration of up to 45 g/kg DM was observed to linearly decrease CH₄ production up to 26% (Beauchemin *et al.*, 2009).

Additionally, tannins have been found to reduce enteric CH₄ production by forming complexes with feed proteins in the rumen and shift the digestion to small intestine in ruminants. Simultaneously, they have also been found to reduce rumen bacterial, protozoal and methanogen population. Several studies have indicated that when provided in low to moderate concentration in the diet, tannin rich forages (TRFs) reduces CH₄ production in ruminants and also improve animal health and productivity. One of the major benefits of including tannins in ruminant diet is that in addition to their antimethanogenic property they exert wide range of benefits on animal health. This includes enhanced nutrient utilisation, reduction in bloating incidences and reducing intestinal parasites in ruminants (Naumann *et al.*, 2017). Additionally, inclusion of tanniferous forages reduces the GHG emissions at the farm level due to decrease in emissions related to the feed production and transport, and decrease in N-based fertilizers. The subsequent sections will discuss in detail the mitigation potential of TRFs in reducing CH₄ emissions from the livestock sector.

1.5 Can tannin rich forages be the answer to reducing methane emissions?

Recent emphasis on sustainable livestock production has encouraged the research on inclusion of alternative feed options that can improve the nutrient utilization in the ruminants as well as curtail the negative effects of production to the environment. Due to their harsh and astringent taste, tannins act as a chemical defense mechanism for the plants against the predators. They have been known to form reversible, and pH dependent complexes with protein, enzymes, cellulose, and nucleic acids (O'Donovan and Brooker 2001). And thereby, preventing the substrate from degradation and precipitating them in aqueous solutions. This trait describes the principle mechanism of tannins' anti herbivory activity and leads to the decrease in the dietary value of some important forage as well as fodder species (Haslam 1988; Zucker 1983; Salminen and Karonen 2011). Tannins are known to incur toxic effects in insects, however, their ability to bind with proteins can potentially benefit ruminants, depending on the concentration of tannin in the diet and nutrient levels (Barbehenn and Peter Constabel, 2011). Tannin rich forages (TRFs) have been found to slow down the degradation of the proteins in the rumen when provided as a substantial feed component or feed additive (Athanasiadou *et al.*, 2000). This helps in synchronization of the release of protein with other nutrients which in turn increases microbial efficiency. The excess protein is then diverted to the small intestine for digestion which increases the absorption of more amino acids from the tannin-containing feed compared to non-tannin containing feeds (Makkar, 2003; Mueller-Harvey *et al.*, 2017). This prevents feed energy losses in the form of CH₄, and shifts the N

losses from urine to feces (Mueller-Harvey, 2006; Jayanegara *et al.*, 2011; Hassanat and Benchaar, 2013; Gunun *et al.*, 2016). Less urinal N could lead to decrease in indirect N losses to the environment from the urine patches which are more susceptible to volatilization, nitrification, and denitrification (Sordi *et al.*, 2014). In addition to binding with dietary protein, tannins have been proven to modify rumen microbiota leading to increased microbial protein synthesis, reduced methanogenesis, modulate fatty acid biohydrogenation, and prevent bloating incidences in cattle (Morales and Ungerfeld, 2015; Diaz Carrasco *et al.*, 2017). Hence, inclusion of TRFs in animal diet is a promising mitigation strategy, which can not only reduce CH₄ emissions from ruminant production but also benefit overall animal health and productivity due to their anthelmintic properties, and proven ability to increase the N utilization (Acevedo-Ramirez *et al.* 2019).

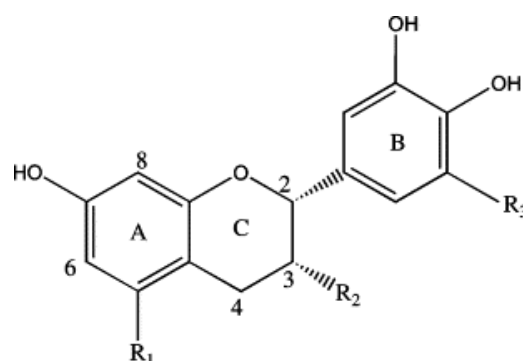
Tannin concentration in plants have been found to vary across different species, plant's phenological stage, and their environmental conditions. Several studies have tried to explore the bioactive effects of TRFs, however, tannin's structural attributes are rarely included in the studies which have also been found to affect their bioactivity. Concomitantly, inconsistent biological effects and lack of reproducible results, makes it difficult to assess their nutritional and antimethanogenic potential (Verma *et al.*, 2021).

1.5.1 Tannin synthesis and their structural diversity

Tannins normally occur in plant vacuoles and are formed through acetate/malonate and shikimate pathways. Tannins can be broadly divided into proanthocyanidins (PAs), hydrolysable tannins (HTs), and phlorotannins. Of these, phlorotannins are mainly found in marine plants, whereas PAs and HTs are predominantly found in terrestrial plants.

Proanthocyanidin synthesis follows the same pathway as anthocyanin synthesis till the formation of leucocyanidin, and then the pathways diverge. Leucoanthocyanidins are reduced to form catechins which is then polymerized to form PAs (Aerts, Barry, and McNabb 1999; James *et al.* 2017; Waghorn 2008). Proanthocyanidins are the second most abundant polyphenols after lignins, and exists in the polymerised form of flavan-3-ol subunits.

Primarily, PAs comprise procyanidin (PC; catechin and epicatechin) and prodelphinidin (PD; gallo catechin and epigallo catechin) subunits which differ from each other in terms of their hydroxylation pattern as shown in Figure 2. Tannin reactivity can be influenced by the hydroxylation pattern of the flavanol group, higher prodelphinidin/procyanidin ratio in a PA, and has been found to increase its ability of forming complexes with proteins.



R ₁	R ₃	Class
OH	H	Proanthocyanidin
OH	OH	Prodelphinidin
H	H	Profisetidin
H	OH	Prorobinetidin

Figure 2. The basic building blocks of proanthocyanidins (Schofield *et al.*, 2001)

Flavan-3-ol subunits are linked with each other via different interflavan linkages to form oligomers and polymers of PAs (Figure 3).

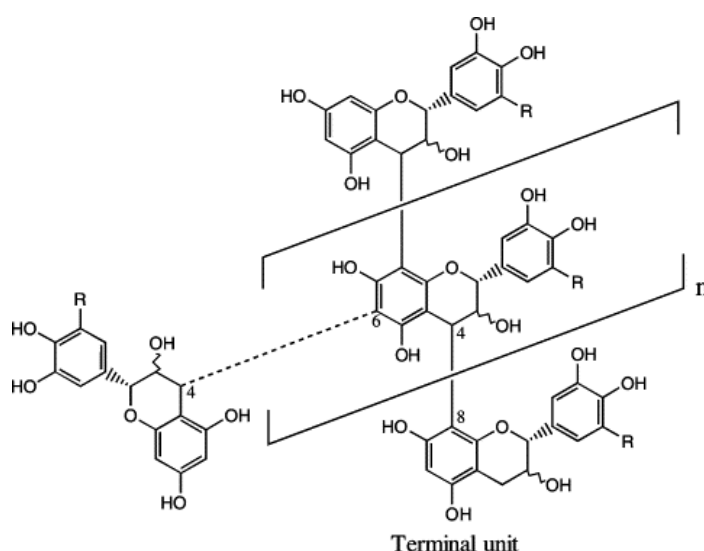


Figure 3. 4-8-B Type interflavan linkage (solid line) and 4-6 B type interflavan linkage (dotted line) between different flavan-3-ol subunits (Schofield *et al.*, 2001).

The interflavan linkage could be between C-4 and C-8 or between C-4 and C-6 which determines the structure of PA (Barry and McNabb 1999; Zeller, Sullivan, et al. 2015). The variability of PA structure originates on the basis of the nature of flavonoid skeleton, stereochemistry at the C-2, C-3, and C-4 (chiral centres) of the pyran ring, substitution pattern on aromatic ring, location, and type of interflavan linkage. These variations combined with the varying degrees of polymerization can lead to perpetual structures and hence, extensive biological properties of PAs (Naumann *et al.*, 2017; Zeller, 2019).

Hydrolysable tannins can be majorly subdivided into three groups, gallic acid derivatives, gallotannins (GTs) and ellagitannins (ETs), and the classification is based on whether the central core (glucose/polyol) is esterified with gallic acid or hexahydroxydiphenic acids respectively (Koleckar *et al.*, 2008; Ekambaram *et al.*, 2016). They are formed through shikimate pathway which leads to the formation of gallic acid and it is the primary building unit for all the HTs (Jourdes *et al.*, 2013). Through a series of steps, pentagalloyl glucose is formed from gallic acid which is a branching point of HT pathway (Mueller-Harvey, 2006; Jourdes *et al.*, 2013). Ellagitannins are formed from GTs through intra- and intermolecular oxidative coupling between at least two galloyl groups (Buzzini *et al.*, 2008; Smeriglio *et al.*, 2017). Ellagitannins are mostly found in high concentrations in young plant tissues and PA concentration is found to peak in mature leaves (Salminen and Karonen, 2011). Hydrolysable tannins are hydrolyzed in the presence of weak acids. Gallotannins yield gallic acid and sugar upon hydrolysis and ETs release sugar, gallic acids and ellagic acid upon hydrolysis (Smeriglio *et al.*, 2017). Intermolecular C–C coupling between at least two galloyl units of GTs result in the formation of ETs, such as hexahydroxyphenoyl (HHDP), dehydrohexahydroxydiphenoyl (DHHDP), and transformed dehydroellagitannin derivatives (Buzzini *et al.*, 2008; Jourdes *et al.*, 2013; Smeriglio *et al.*, 2017).

1.5.2 Influence of tannins in N utilization in ruminants

Tannins can reduce protein degradation in ruminants which influences the digestion and utilisation of nitrogenous compounds in rumen. This could be attributed to the tannins' ability to form complexes with protein and thus, rendering them unavailable for degradation by ruminal microbes (Hess *et al.*, 2006). These tannin-protein complexes inhibit the fermentation of protein to NH_3 in the rumen, shifts the protein to small intestine. The improved NH_3 utilization by ruminal microbes with TRFs, would the reduce the absorption of NH_3 across the gut wall of the ruminants, and decrease urea production in the liver, leading to reduction in urinary N excretion. Inclusion of birdsfoot trefoil hay in total mixed ration (0.51% PAs) was found to increase microbial protein and total VFA production, compared to alfalfa hay based TMR. The cows fed on TMR including birdsfoot trefoil hay were also found to produce higher milk N compared TMR including alfalfa hay, indicating higher N utilisation efficiency of milk (Christensen *et al.*, 2015).

Similarly, it was observed that PAs from birdsfoot trefoil has a strong effect on proteolytic bacteria in the rumen of the sheep when compared with the diet of ryegrass-white

clover. This was accompanied by decrease in plant protein degradation in rumen and enhanced non- NH_3 N flow to the small intestine (Min et al. 2002; Naumann et al. 2018a). An *in vivo* study by Ebert *et al.* (2017) found that the inclusion of PA extracts in the diet of beef cattle at the different concentration levels (0, 0.5, and 1.0% of diet, DM basis) did not affect the urinary N excretion when compared to a basal diet of steam-flaked corn-based finishing diet. However, the proportion of urinary N in total N excretion was found to decrease linearly with increasing the concentration of PAs. Although, various studies have shown a positive influence of PAs on N utilisation, there have also been studies which have failed to show positive effect on N balance in ruminants and found that it decreases apparent total tract digestibility of crude protein (Hess *et al.*, 2006). The differences in the results from these experiments could result from the differences in the ruminant genotype employed in the experiment, and the type of tannins, structure, and source.

1.5.3 Antimethanogenic property of tannins

As discussed before, one of the most promising strategies to reduce CH_4 in livestock production systems are TRFs. In addition to benefitting the environment by reducing CH_4 and N emissions, they offer a range of benefits, ranging from their contribution to the ecological benefits of biodiversity to their beneficial effects on animal health and production. Tannins can reduce CH_4 emissions in three ways: a) by binding to substrate for methanogenesis, such as fiber and protein, b) by impairing the growth of methanogens, and c) by inhibiting protozoal and methanogen symbiosis by negatively affecting rumen protozoal population (Mueller-Harvey, 2006; van Gastelen *et al.*, 2019; Verma *et al.*, 2021). Inclusion of TRFs in the diet of dairy cattle has been found to increase DMI by 20%, and reduce the CH_4 yield (g/kg of DMI) and CH_4 intensity (g/ kg of milk) by 16 and 18%, respectively (van Gastelen *et al.*, 2019). Both PAs and HTs, have been shown to reduce CH_4 in ruminants either linearly or quadratically, although, reduction in CH_4 was higher from HTs (Jayanegara *et al.*, 2015). Supplementation of mao (*Antidesma thwaitesianum*) rich in PAs at 1.6% and 2.4% of total DMI, increased volatile fatty acids (VFAs) production, propionate levels and reduced CH_4 emissions in goats (Gunun *et al.*, 2016). A meta-analysis study considering a range of plant sources, showed that tannins in general were able to limit CH_4 production in the ruminants. However, the correlation between *in vitro* and *in vivo* studies for CH_4 production is very low. It was suggested that major part of their antimethanogenic property is due to their direct inhibitory effect on the methanogens and their effect on protein digestibility was comparatively low (Jayanegara, Leiber, and Kreuzer 2012). The antimethanogenic activity of

TRFs has been found to vary widely across studies and have been rarely observed in *in vivo* studies as seen for sainfoin where there was no effect on CH₄ production (g/kg of DMI) in a study by Chung *et al.* (2013), and Huyen *et al.* (2016) observed a reduction of 6% in CH₄ (g/kg of DMI) when compared to control.

In addition to tannin concentration, structural properties of tannins such as mean degree of polymerisation (mDP), prodelphinidin percentage (PD%), and molecular weight (MW) have also been found to influence CH₄ production in ruminants. High MW PA fractions from leucaena were found to reduce methanogenic population in rumen at a greater extent compared to lower MW fractions in an *in vitro* study by Saminathan *et al.* (2016). The effect of small and large oligomeric ETs isolated from rosebay willowherb (*Epilobium angustifolium*) flowers was analysed on CH₄ production *in vitro*. Methane production per unit of OM was found to decrease with the increase in polymer size, however, the decrease was not linear. The strongest inhibition of methanogenesis was observed from dimeric, trimeric, and tetrameric ET, with the lowest influence on OM fermentation (Baert *et al.*, 2016). Similarly, mDP and PD % of PAs from sainfoin were found to be negatively correlated to *in vitro* CH₄ production (Hatew *et al.*, 2015). The results from these studies highlight the importance of incorporating tannin structural features of TRFs in assessing their impact on CH₄ production.

1.6 Research Justification

Although, the beneficial effects of tannins have been widely recognised, these results have not been translated from research to practice. There are several factors which impede the incorporation of TRFs in the livestock production systems. Primarily, there is a high variability in the results from both, *in vivo* and *in vitro* studies. Majority of animal nutrition studies assess the effect of tannins based on their concentration, and the structural features of tannins are rarely considered which can contribute to these inconsistent animal responses across different studies. The reliance on colorimetric assays for tannin quantification and the presence of several variations of the same method, can also result in variabilities (Grabber *et al.*, 2013). The analytical methods which are available to quantify tannins are mostly based on the evaluation of the determination of specific functional groups or their affinity for proteins (Crestini *et al.*, 2016). Consequently, the absence of a standard method of analysis makes it come difficult to consolidate the findings of other researchers.

Moreover, as tannins are highly plastic in nature, their concentration in plants is known to vary with the season as well as environmental factors (Barbehenn and Peter Constabel, 2011).

Abiotic stresses such as high temperatures, drought, and low nutrient availability can elevate their concentration in the plants (Makkar, 2003; Frutos *et al.*, 2004). Proanthocyanidin concentration in sulla was observed to be influenced by harvest time and the concentration was found to be higher in summer compared to when harvested in spring (Tibe *et al.*, 2011). Additionally, PA concentration in young leaves was found to be higher compared to leaves approaching senescence, as they are more prone to herbivory than mature plants (Waghorn, 2008; Theodoridou *et al.*, 2011). In sainfoin, PA structural features were found vary not only with growth stage but also across different cultivars (Malisch *et al.*, 2015). Nowadays, it has been clear that tannin structural features are important determinants for tannin's bioactivity, however, studies assessing the extent of their impact are still limited. The absence of the methodology which overcomes all these variances makes it difficult to understand their mode of action and physiological functions in ruminants. Fortunately, there have been rapid advancements of analytical techniques which quantify and characterise tannin composition with much higher accuracy. Assessing the inter- and intraspecies variability of tannin traits across different TRFs and determining the influence of environmental factors on their variability is an important niche for research. Understanding the structural diversity of tannins present in TRFs can enable us to comprehend the factors affecting plant-animal interactions and realize their potential of reducing the environmental impact of livestock agriculture.

1.7 Research Objectives

As discussed in previous sections, the high variability in the results from different studies has been one of the main hindrances in the inclusion of TRFs in animal diets. Furthermore, structural complexity of tannins is often not considered in animal nutrition studies, and assessing their bioactivity based only on their concentration leads to inconsistent results. To close the knowledge gaps as described in the previous section, the studies were designed to fulfil the following objectives:

- To analyse the polyphenolic composition and PA structural features in leaves of 17 cultivars from eight different species.
- To establish the variability of PA composition in different plant organs and how it was affected at different phenological stage.
- To analyse the inter- and intraspecies variation in CH₄ mitigation potential of the selected forage species and assess the contribution of tannins on the CH₄ reduction in the forages.

- To identify which PA structural features and forage quality parameters play an important role in determining the antimethanogenic activity of TRFs .

1.8 Outline of the thesis

The utilisation of TRFs in ruminant production systems is often limited by their varying effects on ruminant nutrition comprising both, beneficial and anti-nutritional effects on ruminants. Several studies have shown that in addition to tannin concentration, tannin composition as well as PA structural traits play an important role in determining their influence on biological systems. With the growing interest in the tannins' bioactivity and emerging novel analytical techniques, it is possible to characterise polyphenol subgroups as well as PA structural features. Integration of these techniques with animal nutrition studies enables the researchers to understand the functional attributes of individual metabolite subgroups and can help assess their bioactive effects on animal health. The aim of the present doctoral thesis is to improve the understanding of the functional attributes of the selected polyphenol containing forages with high agronomic importance. The current thesis explores the structure activity relationship of tannins in terms of their CH₄ mitigation potential.

The thesis has been submitted as a cumulative thesis comprising three scientific articles with three published papers (Chapter 2, Chapter 3 and Appendix) and one paper has been submitted to the journal (Chapter 4). The format and the styles of the Chapters 2, 3, and 4 follow the guidelines of the journal to which they have been submitted. The first part of the thesis (Chapter 2) provides an overview of livestock production systems from an agroecological point of view, and the share of enteric methane emissions in increasing environmental footprint of livestock production systems. Secondly, it illustrates the promising mitigation strategies to reduce enteric methane emissions and what role can tannin rich forages play in curtailing these emissions. Chapter 2 establishes the current obstacles which deter the inclusion of TRFs as a substantial feed component in ruminant nutrition and provides a roadmap for future research while highlighting the experimental and analytical incongruities which impede the inclusion of TRFs in livestock sector. Following the roadmap described in Chapter 2, Chapter 3 assesses the variation of tannins and PA structural features across the species as well as within the species. The distribution of different PA types (extractable and non-extractable PAs) in the aboveground plant organs (leaves, flowers, stems) across different phenological stage was also examined. The influence of these variations on *in vitro* CH₄ production from these species was discussed in Chapter 4. We examined the CH₄ reduction potential of seven species (16 cultivars) with the CH₄ production

from alfalfa, a conventional forage. The specific tannin-effect on *in vitro* fermentation products was also evaluated with the treatment with polyethylene glycol. Furthermore, links between forage chemical composition and PA traits were determined. Chapter 5 concludes the dissertation with general discussion elucidating the implications of these results and provides a future outlook on the inclusion of tannin rich forages in animal diet. The appendix includes a published paper about the antimethanogenic effect of inclusion of PSMs rich species in binary mixtures with perennial ryegrass.

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Chapter 2



Review

Examining the Variables Leading to Apparent Incongruity between Antimethanogenic Potential of Tannins and Their Observed Effects in Ruminants—A Review

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Examining the Variables Leading to Apparent Incongruity between Antimethanogenic Potential of Tannins and Their Observed Effects in Ruminants—A Review

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2.1 Abstract

In recent years, several secondary plant metabolites have been identified that possess antimethanogenic properties. Tannin-rich forages have the potential to reduce methane emissions in ruminants while also increasing their nutrient use efficiency and promoting overall animal health. However, results have been highly inconclusive to date, with their antimethanogenic potential and effects on both animal performance and nutrition being highly variable even within a plant species. This variability is attributed to the structural characteristics of the tannins, many of which have been linked to an increased antimethanogenic potential. However, these characteristics are seldom considered in ruminant nutrition studies—often because the analytical techniques are inadequate to identify tannin structure and the focus is mostly on total tannin concentrations. Hence, in this article, (i) we review previous research that illustrate the variability of the antimethanogenic potential of forages; (ii) identify the source of inconsistencies behind these results; and (iii) discuss how these could be optimized to generate comparable and repeatable results. By adhering to this roadmap, we propose that there are clear links between plant metabolome and physiology and their antimethanogenic potential that can be established with the ultimate goal of improving the sustainable intensification of livestock.

Keywords: proanthocyanidins; condensed tannins; secondary plant metabolites; methane; ruminants; climate change

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2.2 Introduction

Intensification and global expansion of livestock production systems have led to significant increased emissions of agricultural carbon dioxide (CO₂), nitrous oxide (N₂O), and methane

(CH₄), with agriculture contributing to almost 15 % of the total anthropogenic greenhouse gas (GHG) emissions [1, 2]. A major part of these emissions is in the form of CH₄ (44%), while the rest is divided between N₂O (29%) and CO₂ (27%) (proportions expressed in terms of CO₂-equivalent (CO₂-e)). From 1990 to 2012, global CH₄ emissions have increased by 11% from 1869 million tonnes to 2080 million tonnes CO₂-e. Methane has a shorter atmospheric lifespan (12 years) compared to N₂O (114 years) and CO₂ (up to thousands of years), and developing mitigation strategies for CH₄ abatement will help reach the global GHG-reduction targets and temperature stabilization goals [2, 3]. In addition to this potential for temperature stabilization, a reduction in CH₄ emissions could further allow a reduction in existing atmospheric CH₄, as the remaining CH₄ emissions are naturally removed from the atmosphere within a short timespan [4-6]. Methane is released as a product of microbial degradation of feed macromolecules in the digestive tract of ruminants [7]. Ruminant methane emissions are the result of an inefficient pathway in ruminant digestion of feed and reducing these emissions would also be efficacious in preventing metabolizable energy losses; these comprise between 2 and 15% of the digestible energy intake depending on the forage quality [8-10]. Hence, the development and adoption of strategies and approaches to reduce CH₄ emissions from livestock systems would have both environmental benefits and lead to improved feed utilization and animal productivity. Since CH₄ production cannot be eliminated entirely without the ruminant losing its ability to digest fiber, the focus should be on increasing nutrient use efficiency in ruminant livestock [11].

One strategy with promising potential is the use of tannin-rich forages (TRFs). Tannins are polyphenolic plant secondary metabolites, which can precipitate or crosslink the proteins, thus making them less prone to proteolysis [12, 13]. While several TRFs have been investigated for their antimethanogenic potential in numerous in vivo and in vitro trials, the results have so far been highly inconsistent. One such TRF is sainfoin (*Onobrychis viciifolia*). A study by Chung [14] indicated no difference between methane emissions from sainfoin and alfalfa hay in terms of dry matter intake (DMI), but identified 25% emission reductions from sainfoin based on the organic matter digested. In contrast, Huyen [15] showed that sainfoin silage diets decreased CH₄ emissions (per unit DMI) by 5.8% compared to grass and maize silage. On the other hand, other studies reported increments in CH₄ emissions when diets of sainfoin hay [16] and sainfoin silage [17] were fed. Similar discrepancies can be found in their effect on reducing bloat [18, 19] and shifting nitrogen (N) excretion from urine to faeces [14, 15, 20, 21]. These inconsistencies are still difficult to explain, although they may be partly explained by the lack of precise structural characterizations of tannins. Some studies have shown the

intraspecies variation of tannin concentration and structures in sainfoin, indicating the complexity of tannin composition in forages [22, 23], as well as the impact of the structures on antimethanogenic properties [24, 25]. The co-presence of other secondary plant metabolites such as flavanols and saponins can also exert potential mutualistic or antagonistic effects [10, 26]. Variations can also arise as a result of the growth conditions of the tested plants, which differed greatly across the reported experiments, and these can affect their secondary metabolite synthesis [27-30].

In this review, we (a) identify the potential of TRFs, specifically those containing condensed tannins (CTs, *syn.* proanthocyanidins), to affect rumen productivity and methanogenesis; (b) illustrate how the structural diversity within CTs is likely to contribute to explaining the inconsistencies observed; and (c) provide a roadmap to assess the bioactive potential of CT in livestock production systems. We aim to integrate the research on TRFs' potential to reduce methane emissions by understanding tannin synthesis, their mode of action in the animal and thereby, to indicate suitable analyses to improve their interpretability. If applied in practice, following this roadmap will increase the potential to extrapolate findings of antimethanogenic potential of forages.

2.3. Understanding Tannins and Their Functional Attributes

Previously, the sole function of tannins was regarded to be a part of a plant's defence mechanism against herbivory [13, 31-33]. This trait conferred antiherbivory effects through (a) the ability of CT to precipitate proteins, thus rendering them unavailable for animal nutrition, and (b) they can have oxidative activities, which create oxidative stress in the herbivore gut [34].

In terms of their role in herbivore diets, plant tannins have surpassed their reputation of being purely antinutritional compounds and several of their beneficial functions have been identified. Tannins have been shown to possess the potential to reduce the impact of drought by acting as antioxidants and detoxifying reactive oxygen species produced as a result of drought stress [28, 35]. Additionally, tannins and other polyphenols have been found to reduce the carbon and N mineralization rates in soil, by inhibiting the activity of soil microorganisms and enzymes [29, 36]. At an individual plant level, this can result in long-term nutrient availability due to slower litter decomposition [29], while at the plant community level, this will enable better adaption of microorganisms to adapt to TRFs, thus generating a general "home field advantage" for one species [36], as well as increasing soil carbon stocks [37-40]. With the discovery of the additional functions of tannins, TRFs have

emerged as a promising solution to help reduce CH₄ emissions in ruminants, while concomitantly providing a series of additional environmental or animal health benefits. A selection of relevant properties will subsequently be discussed in more detail.

2.3.1 What Are Tannins?

Tannins are the end products of energy demanding and extensive biosynthetic pathways, indicating that they play an important role in plant metabolism. They can be broadly divided into two groups—hydrolysable tannins (HTs) and CTs—depending on their structure [31, 41]. Hydrolysable tannins contain central polyol esterified with gallic acid molecules [12, 42]. They can be further divided into three groups: simple gallic acid derivatives, gallotannins (GTs), and ellagitannins (ETs). The two first classes contain only galloyl groups attached to the central core (glucose/polyol): simple gallic acid derivatives having only monogalloyls groups, but GTs having digalloyl or even trigalloyl groups in series attached to the polyol. In ETs, two of the galloyls are C-C linked to make the characteristic hexahydroxydiphenoyl (HHDP) group that can be modified even further [12, 42, 43]. Condensed tannins are the second most abundant polyphenols after lignins, and consist of two or more flavan-3-ol monomeric units. The most common flavan-3-ol subunits of CTs are characterized based on the number of hydroxyl groups on the A and B rings, and the relative stereochemistry between the B and C rings (Figure 1).

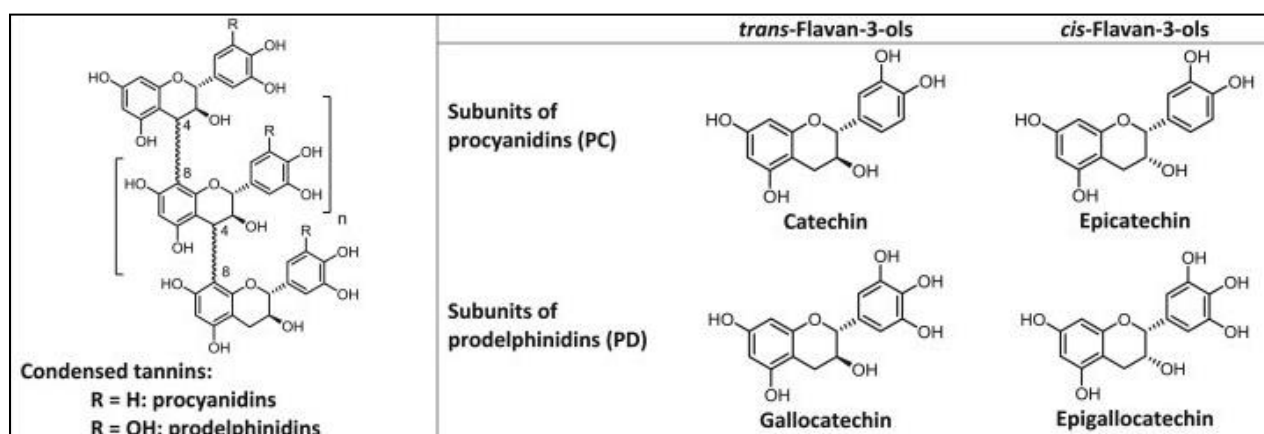


Figure 1. Structure of condensed tannin subunits [44].

Catechin and epicatechin have two hydroxyl groups present adjacent to each other on the B ring of flavon-3-ol subunits, and are categorized as procyanidin (PCs) units when found in CT structures. Gallocatechin and epigallocatechin have three hydroxyl groups adjacent to each other on the B ring and are categorized as prodelphinidin (PDs) units in CTs [33, 45-47]. Additionally, both PCs and PDs can differ in their relative orientation of the C-2/C-3 carbon substituents of the C-ring, where catechin and gallocatechin have a *trans*-configuration,

whereas epicatechin and epigallocatechin have a *cis*-configuration [48, 49]. These subunits are connected through interflavan linkages, the most common of which are B type linkages. In B type linkages, the bonds between the subunits are formed either between the C-4 carbon of the C ring and the C-8 carbon of the subsequent flavan-3-ol subunit ($4 \rightarrow 8$) or between the C-4 carbon and the C-6 carbon ($4 \rightarrow 6$) [50, 51]. When the covalent bond is formed between two flavon-3-ol subunits via a C-2 oxygen atom and a C-7 carbon in addition to the $4 \rightarrow 8$ B linkage, the linkage is known as A type (Figure 2) [49].

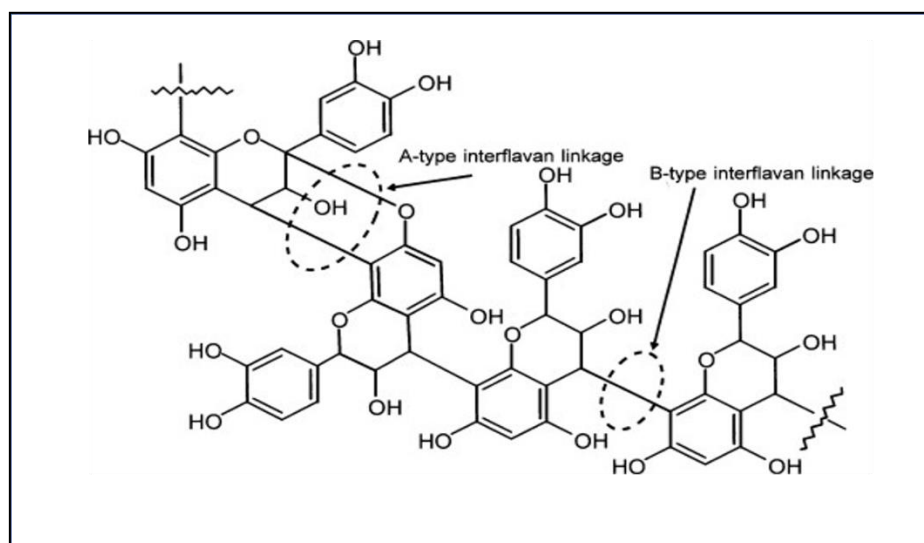


Figure 2. A- and B-type interflavan linkages in condensed tannin oligomers and polymers [52].

The proportions of PC and PD subunits, and also the type of linkages within CTs, vary substantially both across and within plant species [53, 54]. These variations combined with the varying degrees of polymerization can lead to a multitude of combinations in structures and hence, a wide range of bioactive properties of CTs [55]

2.2. Functional Attributes of Tannins

The bioactive properties of tannins are either a result of their protein precipitation capacity (PPC), or their anti- or pro-oxidant behaviour. The effect of tannins on biological systems is found to be dependent on pH, with protein precipitation capacity being generally efficient in slightly to moderately acidic environments, whereas the oxidative activity is expressed in alkaline environments or by oxidative plant enzymes, such as polyphenol oxidases [56]. The fate of ingested tannins in herbivores is dependent on the physiological conditions of their gut. Tannins when consumed by herbivores with high gut pH, such as in caterpillars, undergo auto oxidation to produce semiquinone radicals and quinones. These oxidation products can bind to the nutrients in the gut lumen of the caterpillar and cause damage to the surrounding gut tissues [57]. In contrast, the effect of tannins on mammalian herbivores is dependent mainly

on its PPC, as the mammalian gut has acidic to neutral gut conditions which provide an ideal environment for tannin–protein interactions [34]. When supplied in moderate quantities, the protein binding ability of tannins can improve nutrient utilisation in ruminants; however, in insects, both CTs and HTs had no impact on protein utilization [58, 59]. Additionally, the efficacy of these effects is dependent on the structure of tannins. Ellagitannin-rich plants were found to be more potent in terms of their oxidative behaviour compared to plants rich in galloyl glucoses or CTs [57], and CTs are found to precipitate proteins more actively than ETs [33]. The anthelmintic and antimethanogenic bioactivity of CTs in ruminants is linked to their precipitation capacity [60, 61] and their antioxidative behaviour [34, 49, 62]. Condensed tannins are known to form insoluble complexes with proteins by binding to the protein's surface and forming a coat and this leads to its precipitation [63, 64]. These complexes are generally based on non-covalent interactions such as hydrophobic interactions and hydrogen bonding [65]. However, there have been reports on ionic interactions and covalent bonds with amino acids or sulphur on proteins [66]. Additionally, under low pH and oxidative conditions, tannins can form covalent bonds with proteins [38, 65, 67, 68].

Independent of the bond type, within CTs, a higher PD percentage has been associated with a higher PPC, which is likely a result of the additional hydroxyl groups at carbon 5 of the B ring [49, 69, 70]. In addition to the PD/PC ratio, the *cis/trans* ratio, polymer size, and co-presence of galloyl groups have been identified as having effects on the PPC [71]. However, these results have been inconsistent, which is likely a result of multiple structural features being responsible for the tannins' astringency concomitantly and potentially imparting contrasting effects [59, 72, 73]. The polyphenolic polarity, as defined by the octanol-water partition coefficient (K_{OW}), can also influence the PPC of tannins [68]. Tannins with high K_{OW} values (e.g., acacia (*Acacia mearnsii*) leaves, $K_{OW} = 13.92$) are fat soluble and bind non-specifically to the proteins. They have the tendency to be adsorbed by animal tissues and exert toxic effects. Tannins with low K_{OW} values such as chestnut (*Castanea sativa*) extracts ($K_{OW} = 1$) bind more efficiently with proteins and lead to better nutrient utilization in animals [59]. However, the nature of these interactions is also dependent on the proteins. For example, the PCs were found to have a stronger affinity for larger proteins with open structures such as BSA (66 kD) compared to lysozyme (14.4 kD), which has a compact structure and is smaller in size [50, 59, 66, 68]. Additionally, the isoelectric point (pI) of proteins has generally been identified to affect the tannins' protein precipitation behaviour [38], and proteins aggregate faster when the pH is close to their pI [68]. The reaction conditions also play a significant role in the strength of tannin–protein complexes. The variability in dietary composition with

differences in protein chemistry (for example: proline content), amino acids, and CT composition, makes it exceptionally difficult to predict the response of CT–protein interactions. Finally, it should be mentioned that there appears to be at least a partial specificity, with plant tannins showing a higher precipitation of plant proteins, compared to animal protein. Accordingly, in a study by Zeller [51], tannins from birdsfoot trefoil (*Lotus corniculatus*) were better at precipitating proteins from lucerne (*Medicago sativa*), compared to BSA. Hence, the protein source should also be accounted for in the estimation of the PPC [74].

The link between PPC, oxidative properties, and the observed bioactivity of tannins is, however, still not clear because of the inadequate tests in many reported studies. Therefore, complementing their protein precipitation assays with the analysis of their anti-/pro-oxidative behaviour can provide a better overview and improve understanding of CT–animal interactions.

2.3. Potential of Incorporating Tannin Rich Forages in Ruminant Nutrition

As explained previously, tannins have long been considered to be non-specific anti-nutritive factors and potentially toxic, as they protect dietary protein from degradation, and because of their pro-oxidant properties [65]. These characteristics are undoubtedly true, as tannins have, indeed, been found to form strong, yet pH-dependent and reversible bonds to proline rich proteins and affect protein digestibility [75]. Some browsing herbivores have developed the ability to produce proline-rich salivary mucoproteins as an evolutionary adaptation to overcome the deleterious effects of tannins [66]. Herbivore palatability of TRFs is determined on the basis of astringency resulting from the interactions between CTs and the herbivore's salivary proteins. Tanniferous forages are often considered to be less palatable and therefore, less acceptable. At a CT concentration above 5% of the herbage dry matter (DM), intake and palatability of TRFs may be depressed and feed intake is reduced. However, reported results shows this is highly variable [49]. Despite its high CT concentration, sulla (*Hedysarum coronarium*) has been found to be highly acceptable by sheep [76]. Similarly, the acceptability (and assumed palatability) of sainfoin was found to be comparable to conventional temperate forages such as alfalfa and ryegrass/clover mixtures [77]. Sainfoin has also been reported to be more palatable than birdsfoot trefoil despite its higher tannin concentration [77, 78].

2.3.1. Impact of Tannins on Enteric Fermentation

Feed constituents such as carbohydrates, proteins, and other organic polymers are degraded to their monomer components in the presence of rumen microbes under anaerobic conditions [7, 79, 80]. Tannin-rich forages have been reported to cause alterations in rumen microflora, increase nutrient utilization efficiency, improve animal health, and consequently, influence their environmental effect [15, 46, 81, 82]. The presence of tannins in the feed has been found to slow down the degradation of the dietary proteins by forming tannin–protein complexes in the rumen [83]. These complexes are then transported from the rumen (pH = 6–7) to the small intestine (pH > 7), where they are partially dissociated under alkaline conditions. Through this process, the excess protein is initially protected from inefficient degradation in the rumen, so it reaches the small intestine as rumen bypass protein. As a result, there is an increased amino acids absorption throughout the entire digestive tract for tannin-containing feeds compared with non-tannin-containing feeds [32, 59, 84]. The decrease in excess protein degradation in the rumen also results in a decrease in methanogenesis and consequently, lower CH₄ emissions. Concomitantly, the non-ammonia N transported to the small intestine leads to a higher production of milk, meat, and wool. This deviation further decreases the urinary N and slightly increases faecal N [10, 59, 78, 85, 86]. The decrease in urinary N can lead to lower indirect N losses to the environment from the urine patches, as these spatially concentrated excretions have a high risk of volatilization, nitrification, and denitrification (Figure 3) [15, 87].

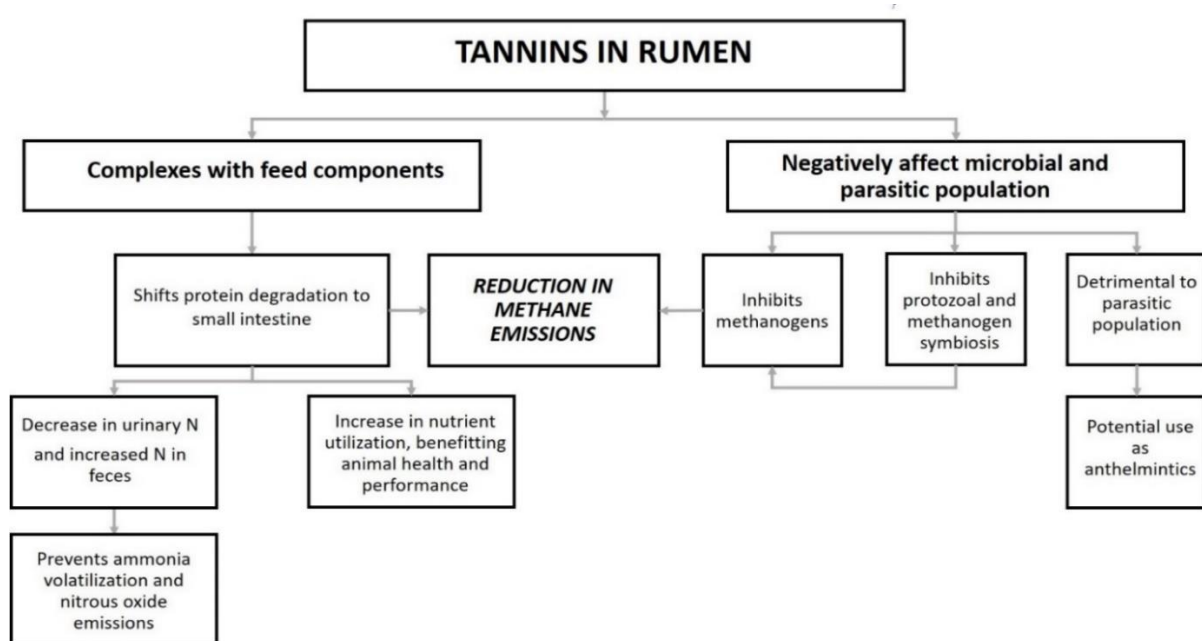


Figure 3. Beneficial effects of tannins on ruminant nutrition.

In temperate forage systems, the forage-protein concentrations in are generally higher than in tropical forages. Hence, the N use efficiency in temperate forages is often low, and in some

instances, as low as around 10–20% [78, 88]. Accordingly, reductions in available protein can be achieved without adversely affecting milk yields by increasing the N use efficiency, thereby concomitantly reducing the nitrogen emissions to the environment. However, the effects of tannins in the gastrointestinal tract of ruminants are complex. For example, CTs in birdsfoot trefoil have a strong effect on the proteolytic bacteria in the rumen of sheep. As a result, plant protein degradation in the rumen is decreased and non-ammonia N flow to the small intestine is increased, resulting in higher utilizable crude protein (uCP) in the small intestine [89, 90]. However, even within the *Lotus* genus, big trefoil (*Lotus pedunculatus*) and birdsfoot trefoil have different modes of action in their effect on nitrogen flows. A direct comparison of these species shows that CTs from big trefoil were more effective in the degradation of Rubisco compared to those of birdsfoot trefoil. Similarly, CTs from big trefoil were able to inhibit the degradation of protein in the rumen by forming strong tannin–protein complexes, whereas birdsfoot trefoil tannins reduced degradation of proteins by directly inhibiting the proteases [91]. Additionally, big trefoil was found to have a stronger potential to reduce CH₄ emissions than birdsfoot trefoil [59].

As a result of this complexity, in vivo experimentation has not yet been able to successfully show both a reduction in CH₄ emissions and incremental improvement in N use efficiency simultaneously from tannin-containing forages. To illustrate the existing research gaps, it is important to understand how tannins influence rumen microbiota, as well as the interactions between hydrogen producers (bacteria, protozoa, fungi) and consumers (methanogens) [92].

2.3.2. Mode of Action to Lower Methane Emissions

Several mechanisms have been hypothesized by which tannins might decrease CH₄ emissions in ruminants. Efficient nutrient utilization is considered to be one of the most likely explanations, and this might increase animal productivity and reduce CH₄ production per unit of animal product. The inclusion of tannins in feed has been found to improve nutrient utilization in the ruminants, thereby reducing metabolic energy losses that would otherwise occur through CH₄ emissions [79, 93, 94].

Another factor which could be linked to CT's potential in reducing CH₄ emissions is its affinity to form complexes with lignocellulose and preventing fiber degradation, thereby leading to lower microbial fermentation [95]. Microbial fermentation leads to the formation of volatile fatty acids (VFA) such as acetate, propionate, and butyrate, with CO₂ and H₂. These metabolic byproducts are either absorbed by the rumen wall and used as a source of energy for animals or used as substrates by microorganisms [7, 79, 94, 96]. Tannins have been known

to reduce the CH₄ emissions of ruminants either by directly inhibiting the ruminal methanogenic population [92, 97], or by hindering the methanogen-protozoa symbiosis [49]. Approximately 37% of the CH₄ from the ruminants is produced by protozoa-associated methanogens. In the methanogen-protozoa symbiosis, hydrogen (H₂) required by methanogens to produce CH₄ is provided by rumen protozoal population via transfer of H₂ produced in their hydrogenosomes. The subsequent utilization of H₂ by methanogens benefits the protozoal population as H₂ hinders their metabolism [10, 98]. As the accumulation of H₂ in the rumen can impede fermentation, methanogens play an important role in feed digestibility by utilizing the rumen borne H₂. Hence, before adapting feeding strategies to achieve defaunation of the rumen, it is important to provide alternative H₂ sinks to maintain the animal's productivity and improve the utilization of metabolizable energy from the feed [99, 100].

Here, tannins might be part of the solution as well, as some studies have hypothesized that tannins influence the VFA profile in rumen. They promote the shift towards the production of more propionate compared to acetate, which acts as a hydrogen sink. The reduced availability of H₂, which is the main substrate for CH₄ production, results in a reduction in methanogenesis [86, 100, 101]. The shift in acetate and propionate production could be attributed to changes in the composition of microbial communities and their activity [95]. However, the mechanism by which tannins influence methanogenesis and shift the VFA profile is still not well understood.

2.4 Current Findings on the Antimethanogenic Potential of TRFs

Recent studies have shown that the effect of tannins on ruminant nutrition is highly dependent on the tannin type, structural characteristics, dosage supplied, rumen morphology, and rumen physiology [102, 103]. Numerous plant species containing tannins have been studied to determine their efficacy in ruminant nutrition, either as forages or feed additives. These species include acacia, quebracho (*Schinopsis balansae*), chestnut, valonea (*Quercus Aegilops*), leucaena (*Leucaena leucocephala*), desmodium (*Desmodium ovalifolium*), sainfoin, birdsfoot trefoil, big trefoil, Chinese bushclover (*Lespedeza cuneata*), Japanese clover (*Lespedeza striata*), white clover (*Trifolium repens*), and sulla [59, 86, 101].

Antimethanogenic potential was found to vary across the species. Promising temperate forage species include sainfoin, birdsfoot trefoil, big trefoil, and sulla, and among tropical forages are leucaena, desmodium, and Chinese bushclover [24, 62, 104, 105]. A study of Friesian dairy cows found that cows that grazed on birdsfoot trefoil produced not only 17.5% less CH₄

emissions (per unit DMI) but also 32% less CH₄ emissions/kg milk solids when compared with cows grazing on perennial ryegrass (*Lolium perenne*) [106]. Similarly, leucaena, a tropical leguminous shrub, has been found to reduce CH₄ emissions in sheep and heifers without affecting DMI or organic matter intake in the animals [107, 108]. In another study, when supplied with 80% leucaena in the diet compared with a basal diet of *Pennisetum purpureum*, CH₄ emissions were reduced by 61% in heifers without negatively affecting DMI and VFA production [109]. The overall performance of the lambs (approx. 6 months age) was improved when CTs were included in their basal diet (wheat straw, oat hay, and concentrate mixture). Condensed tannins in the diet were supplied as leaf meal mixture of *Ficus infectoria* and *Psidium guajava* (70:30). The diet with 2% CTs was able to suppress CH₄ emissions by approx. 26%. Additionally, improved N metabolism, wool yield, and growth performance of lambs was reported. Inclusion of CTs in the feed did not affect the intake or apparent palatability of the feed [110].

Similarly, in a study conducted on adult sheep, hazel (*Corylus avellana*) leaves when supplemented at 50% of the total diet were able to reduce CH₄ emissions by 35% (per unit OM intake) compared to the control (ryegrass hay and lucerne pellets). Concomitantly, a substantial decrease in urinary N proportion of total N intake was observed without any negative effects on forage intake, apparent palatability, or body weight of the sheep [111]. However, despite the promising findings indicated by these studies, the antimethanogenic potential of the forages is not clearly linked to the tannin concentration, as evidenced by the high variability in results from different studies (Table 1). As summarized in Table 1, the variation in CH₄ abatement by forages also depends on the phenological stage at which they are harvested and by the method of forage preservation. In addition to the changes in forage chemical composition, phenological stage also affects the CT composition and structural features. The bioactivity of sainfoin CTs was found to decrease with maturity, as shown by the increase in phenological stage. This could be attributed to the lower proportion of extractable CTs (ECTs) resulting from increase in CT polymerization with maturity [112]. Similarly, when TRFs are ensiled, the process can rupture plant cells, allowing the CTs to release and bind to other molecules. This decreases the proportion of free CTs (ECTs) and hence, there is reduced bioactivity of CTs in conserved forages compared to fresh forages in terms of their ability to reduce CH₄ emissions [21].

Furthermore, the mode of action by which these forages reduce CH₄ emissions remains largely unclear. Tannins from chestnut, quebracho [113], and leucaena [114, 115] have been found to reduce CH₄ by reducing different methanogenic populations in the rumen. There was

a significant effect of high molecular weight (M_w) CT fractions from *Leucaena* on richness and species diversity of rumen methanogenic and bacterial population in rumen. The study showed that CTs with high M_w had a pronounced inhibitory effect on proteolytic bacteria, *Prevotella* spp., and *Methanobrevibacter* population [97, 116].

Similarly, birdsfoot trefoil and sainfoin were found to inhibit the proteolytic bacterial population [49]. A study analysing the effect of different tannin sources on CH_4 emissions found that CT-rich (acacia and quebracho tannins) and HT-rich (chestnut and valonea tannins) affect rumen fermentation differently. At concentrations above 5% DM, in addition to a significant decrease in CH_4 emissions, there was also a negative effect on total VFA production. CT-rich extracts reduced the acetate/propionate ratio significantly at a concentration higher than 5%. However, the ratio was not affected by HT extracts, indicating that they had a stronger impact on methanogen population in comparison with substrate fermentation. Only valonea extracts (5% w/w) were able to reduce CH_4 emissions without any negative impact on fermentation and VFA profile. This indicates that classification based solely on tannin concentration or the type of tannins (HTs and CTs) present in the feed is not sufficient to determine their potential to reduce CH_4 emissions [86]. Similarly, a study was performed on CT-rich forages from Texas to determine the effect of different functional features (PPC and antioxidative activity) on CH_4 emissions. No effect of PPC was found on CH_4 abatement, whereas the correlation between antioxidative property of tannins and decrease in CH_4 emissions was significant. In contrast to previous studies, the decrease in acetate/propionate in this study was not correlated with a decrease in CH_4 emissions [90]. The results from these studies further reinforce the need for CT structural characterization in addition to concentration, in order to make an accurate assessment of their impact on ruminant nutrition.

Table 2.1. A short overview of methane production potential of tropical and temperate forages.

Plant Species	Age	Fraction	Preservation	ECT * (%)	Animal (Rumen Fluid)	Methane (g/kg DM)	Study (Duration)	Reference
<i>Acacia. angustissima</i> var hirta (STX)	Mature	Leaves	Fresh	4.9	Steers	0.6	In vitro (48 h)	[90, 117]
<i>Acacia angustissima</i> var. hirta (STP5)	Mature	Leaves	Fresh	4.4	Steers	0.8	In vitro (48 h)	[90, 117]
<i>Desmanthus illinoensis</i> (Michx.) MacMill	Mature	Leaves	Fresh	5.1	Steers	24.9	In vitro (48 h)	[90, 117]
<i>Desmodium paniculatum</i> var. paniculatum	Mature	Leaves	Fresh	10.3	Steers	7.9	In vitro (48 h)	[90, 117]
<i>Lespedeza cuneata</i>	Mature	Leaves	Fresh	4.7	Steers	15.1	In vitro (48 h)	[90, 117]
<i>Lespedeza stuevei</i>	Mature	Leaves	Fresh	9.9	Steers	4.9	In vitro (48 h)	[90, 117]
<i>Leucaena retusa</i>	Mature	Leaves	Fresh	2.4	Steers	40.7	In vitro (48 h)	[90, 117]
<i>Mimosa strigillosa</i>	Mature	Leaves	Fresh	9.9	Steers	7.6	In vitro (48 h)	[90, 117]
<i>Neptunia lutea</i>	Mature	Leaves	Fresh	7.0	Steers	19.7	In vitro (48 h)	[90, 117]
<i>Onobrychis viciifolia</i> acc LRC 3519	Early stage	Herbage	Fresh	2.5	Cross bred heifers	28.2	In vivo (24 h)	[14]
<i>Onobrychis viciifolia</i> acc LRC 3519	Late stage	Herbage	Fresh	0.7	Cross bred heifers	24	In vivo (24 h)	[14]
<i>Onobrychis viciifolia</i> acc LRC 3519	Mature	Herbage	Hay	0.6	Cross bred heifers	22.5	In vivo (24 h)	[14]
<i>Medicago sativa</i>	Early stage	Herbage	Fresh	0	Cross bred heifers	26.6	In vivo (24 h)	[14]
<i>Onobrychis viciifolia</i> cv. Perly	Mature	Herbage	Silage	3.7	Brown Swiss cows	18.75	In vitro (24 h)	[17]
<i>Onobrychis viciifolia</i> cv. Shoshone ¹	Early Flowering	Herbage	Hay	3.9	Holstein dairy cows	12.9	In vitro (24 h)	[118]
<i>Lotus corniculatus</i> cv. Norcen ¹	Early Flowering	Herbage	Hay	0.4	Holstein dairy cows	11.7	In vitro (24 h)	[118]
<i>Lotus corniculatus</i> cv. Oberhaunstadter ¹	Early Flowering	Herbage	Hay	0.7	Holstein dairy cows	11.8	In vitro (24 h)	[118]
<i>Lotus corniculatus</i> cv. Bull	Mature	Herbage	Silage	2.2	Brown Swiss cows	17.64	In vivo (24 h)	[17]
<i>Lotus corniculatus</i> cv. Polom	Mature	Herbage	Silage	0.8	Brown Swiss cows	18.75	In vivo (24 h)	[17]
<i>Lotus corniculatus</i>	Vegetative	Herbage	Silage	2.5	Friesian dairy cows	26.9	In vivo (24 h)	[119]
<i>Lolium perenne</i>	Mature	Herbage	Fresh	0	Friesian dairy cows	24.15	In vivo (24 h)	[106]
<i>Lotus corniculatus</i>	Mature	Herbage	Fresh	0.2	Friesian dairy cows	19.9	In vivo (24 h)	[106]
<i>Lotus pedunculatus</i>	Mature	Herbage	Fresh	8	Sheep	14.5	In vivo (24 h)	[119]
<i>Hedysarum coronarium</i>	Mature	Herbage	Fresh	2.8	Friesian and Jersey dairy cows	19.5	In vivo (24 h)	[120]

¹ Refers to feed supplied in total mixed ration, * Extractable condensed tannins.

2.5 Existing Research Gaps and Future Directions

As discussed in the previous sections, several studies have tried to explore the properties that affect tannin astringency. However, the variations in the results obtained, and their lack of reproducibility, hinder their field-scale applicability. Furthermore, their structural complexity and the varied forage chemical composition among different species present difficulties for understanding the implications that CTs have for ruminant nutrition and particularly their antimethanogenic potential. Although several studies have identified a large variability in both the concentration and structure of CTs across species and their cultivars [22, 32, 74], few studies have analysed the implications of this variability on the observed bioactivity. In the following sections, we present a brief overview about the factors responsible for current situation, with an apparent incongruity regarding the influence of tannins on ruminant nutrition. We also discuss the frequently used analytical techniques for qualitative and quantitative analysis of tannins and the underlying problems associated with them. Our aim here is to illustrate the importance of optimized tannin analyses and inclusion of tannin structural features in animal studies to overcome inconsistent animal responses. By avoiding these factors, which cause substantial variation in the reported studies, we can focus more precisely on CT–animal interactions.

2.5.1. *Experimental and Analytical Incongruities*

Tannin concentration and composition in plant has been reported to be substantially influenced by changes in environmental conditions, as well as by plant species and its phenological stage [40]. The preparation and handling of tannin extracts can also cause alterations in quantification of tannins [121]. In order to ensure accurate determination of tannin concentration and composition in a plant tissue, handling and storage protocols should be followed, as CT concentration is highly influenced by the environmental factors.

Quantitative analyses are essential for determining CT bioavailability in the samples and spectrophotometric assays are routinely utilized due to their rapid and low-cost analysis. Due to their structural complexity, the number of derivatisation and analytical techniques are few and they have certain limitations. Substantial information about the activity of CTs can be obtained by analysing specific structural traits of tannins as it is difficult to isolate individual large polymeric CT units compared to dimers or trimers [122]. The complexity of CT structures means that they are frequently analysed by a method where multiple techniques with different functions are integrated together.

2.5.1.1 Growth Conditions of Experimental Plants

Tannin concentrations in plants can be up to 20% of their total dry weight [38]. Although, CTs are found in different parts of the plant and they are predominantly concentrated in young leaves and flowers [105, 123]. The concentration of tannins in tropical plants is, on average, higher than in temperate plants, yet there is substantial variation across seasons and environmental factors [62]. Drought, nutrient availability, and other conditions during plant growth have also been shown to affect CT concentration and composition. Although the effect of these abiotic stresses on the CT composition has not been well researched [27, 28, 124], they have been shown to produce incremental effects in CT concentrations [84, 125]. Accordingly, it was observed that the concentration of CTs in *sulla* was higher in the summer than in spring [54]. Similarly, *Quercus rubica* had higher tannin concentration and less polymerized tannins when grown in dry conditions compared with wet conditions [62]. Thus, it is important to account for and report the precise experimental conditions because of their potential to affect the observed tannin concentration, composition, and bioactivity [126].

2.5.1.2. Sample Preservation and Storage

After harvest, sample preservation plays an important role in the quantification of CTs, as their extraction and quantification are heavily influenced by biotic factors. For precise tannin concentration and composition analysis, samples should be freeze-dried, rather than air or oven dried. In a direct comparison, hay-drying of samples from purple prairie clover (*Dalea purpurea* Vent.) resulted in a slight decrease in ECTs from 70.2 to 64.1 g kg DM⁻¹, while the protein-bound CTs increased from 9.0 to 12.4 g kg DM⁻¹. With ensiled samples, the differences were even more pronounced, and in these samples, ECTs decreased to 27.4 g kg DM⁻¹, while protein-bound CTs increased their concentration to 44.3 g kg DM⁻¹ [127]. Thus, while the total CT concentration did not differ, without freeze drying at least a part of the CTs, it can change from the available form to the protein-bound form, which is often not accounted for in the studies analysing bioactivity of tannins. Similarly, when comparing different drying methods for the concentration of HTs in white birch (*Betula pubescens*), oven drying reduced the ET concentration significantly from 10.9 to 8.4 g kg DM⁻¹, while simultaneously increasing the concentration of insoluble ET from 0.8 to 2.4 g kg DM⁻¹. In this study, neither storing the samples at -20 °C for 3 months prior to drying nor vacuum or air drying resulted in a decrease in ellagitannins or total HTs despite a minor but non-significant decrease in air dried samples [121]. This indicates that the adverse effect from air and oven drying increases with the air temperature, yet the short-term effect at room temperature appears to be negligible. The effect of temperature during post drying storage is less clear, and storage at 25 °C for three weeks reduced tannins in walnuts by 20–40%

(dependent on the subsequent extraction technique), with large parts of the reduction having occurred in the first week [128]. However, with regard to the HTs from birch, a one-year storage period at room temperature (22 °C) yielded lower tannin concentrations compared to samples that were stored in a freezer, with 17.4 and 19.1 g kg DM⁻¹, respectively. This value was still higher than the HT concentration in the samples stored in a refrigerator at 4 °C, which yielded 15.7 g kg DM⁻¹ [121]. Contrasting results were obtained by Kardel [129], where samples stored in a refrigerator for one year had on average 3.5% higher CT concentrations than samples stored at room temperature. In this study, also storing the samples in an oven at 60 °C for 5 days had no measurable impact on CT concentrations.

2.5.1.3. Tannin Extraction

While the vast majority of studies use aqueous acetone to extract tannins of any kind, with either 70 or 80% acetone, there is no clear indication about the superiority of any extraction method yet. Some studies have also used methanol or hot water extraction as well. Accordingly, the extraction yields with hot water were 6% higher on average, compared to a water/methanol (1:1) extraction solvent, and 13% higher compared to an acetone/water/formic acid (70:29.5:0.5) solvent [129]. This study only determined the tannin concentration and did not evaluate potential changes in the structure due to the high temperatures. Contrary to these findings, Salminen [121] found that aqueous acetone extraction yielded on average 41% more extractable HT, compared to aqueous methanol extraction, although the study did not test hot water extraction. Pure acetone, however, yielded the lowest HT concentrations, with a reduction of almost 75% compared to acetone/water (70:30). The extraction yield of the 70% acetone was increased even further by 29%, if ascorbic acid was added to the acetone water mixture, presumably because it prevented oxidation of the HTs [121]. This is in accordance with the findings of Chavan [130] and Hagerman [131] for CTs, where 70% acetone also provided the highest extraction yields compared to all methanol mixtures and acetone mixtures with higher acetone concentrations. Acidification of aqueous acetone extraction solvent with 1mL of concentrated HCl further increased the extraction yields by around 10%.

Some recent studies have, however, indicated in general much lower performance from maceration-based techniques compared to techniques such as ultrasonic baths, microwave-assisted extraction (MAE), and Soxhlet extraction. Aspé [132] identified much larger cell wall destruction from these last three techniques, which resulted in generally much higher extraction yields compared to maceration techniques, where only minor cell wall damage has occurred. According to Chupin, et al. [133], MAE effectiveness depends on the particle size

and increases with small particles. However, they generally did not identify an effect of MAE on the structure of the tannins.

In addition to the solvent, the extraction conditions can also affect the efficiency of extraction. Extraction at 4 °C in darkness led to around 14–17% higher recovery of CTs compared to extraction at room temperature, also in dark conditions [134].

2.5.1.4. Major Analytical Techniques I: Spectrophotometric Assays

Spectrophotometric assays are routinely used in studies due to their rapid and low-cost analysis. Vanillin and HCl-butanol assays are conventionally employed to quantify CTs. Both these assays have been widely used in the majority of studies due to their accessibility and low cost for quantifying CTs. In principle, results from the HCl-butanol assay are more reproducible than the vanillin assay. Vanillin assay with methanol has been found to lack specificity as in addition to CTs, it complexes with flavan-3-ols and dihydrochalcones [84, 135]. In the HCl-butanol assay, CTs are depolymerized oxidatively to form bright coloured anthocyanidins in the presence of mineral acids [135]. The HCl- butanol assay in some instances lacks specificity and leads to over- or underestimation of results as the colour is dependent on the interflavonoid linkages and 5-OH groups [126]. For accurate quantification by the HCl-butanol assay, freeze-dried samples should be preferred as under heat treatment, tannins can bind to other macromolecules which could undergo oxidation. Macromolecules containing phenolic groups can be oxidised to form quinones which can lead to condensation reactions between tannins and other macromolecules, thereby preventing the release of anthocyanidins from the modified tannins [84]. Furthermore, the addition of iron increases the specificity of this assay [135]. The addition of acetone to the assay also leads to the complete dissociation of CTs from the plant material and this inclusion has further refined this method [136]. A wide range of studies use tannic acid, catechin, or leucocyanidin as standards for these assays. This is also one of the sources for which there is a huge variation in the results, as seen in Table 2 [84, 129].

Table 2.2. Variability of condensed tannins concentration in frequently studied species across different studies.

Species	Condensed Tannins (% DM)	Coefficient of Variation (%)	References
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<i>Acacia angustissima</i>	7.4–8.9	9.3	[117, 137]
<i>Acacia nilotica</i>	0.46–8	67.4	[137-140]
<i>Acacia senegal</i>	0.07–7.8	138.9	[137, 139]
<i>Acacia tortilis</i>	4.7–5.4	9.8	[137, 139]
<i>Lespedeza cuneata</i>	0.83–5.1	36.2	[117]
<i>Leucaena leucocephala</i>	0.52–18	112.7	[109]
<i>Mimosa caesalpinifolia</i>	1.8–12.4	105.5	[117, 141]
<i>Hedysarium coronarium</i>	0.4–3.8	68.1	[76, 142, 143]
<i>Onobrychis viciifolia</i>	2.4–14.1	113.1	[14, 18, 78, 144]
<i>Lotus corniculatus</i>	1.4–7.6	45.3	[78, 106, 145-148]
<i>Lotus pedunculatus</i>	0.25–0.8	50.9	[119, 147, 149]
<i>Onobrychis viciifolia</i> (Silage)	2.6–3.7	17.4	[17, 21]
<i>Lotus corniculatus</i> (Silage)	2.2–3.4	22.3	[17, 119]

2.5.1.5 Major Analytical Techniques II: Liquid Chromatography Coupled with Mass Spectrometry

High-Performance Liquid Chromatography (HPLC) has been proven to be a competent and rapid method for the analysis of polyphenols apart from the highly polymerized oligomers [48, 150]. Reverse phase LC (RPLC) is a commonly used chromatographical technique to analyse CTs, ranging from monomers to tetramers and in some cases, their isomers distinctly [151]. With increased CT polymerization, the intelligibility of the chromatogram in RPLC decreases due to the presence of unresolved peaks. The combination of fluorescence detection with RPLC leads to increases in selectivity and sensitivity of the method [152]. A UV-DAD detector is most frequently complemented with LC for the determination of CTs. It also helps in the direct classification of polyphenols into different subgroups such as flavonoids, ellagitannins, gallic acid derivatives, and caffeic acid derivatives, etc. [153]. Recently, to increase the specificity and resolution of the LC analysis, separation techniques have been coupled with ESI-MS or matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS [154]. These are used extensively for the analysis of CTs in plant and food material such as cocoa, grapes, wine, and birch species, etc. [155, 156]. Soft ionization methods such as ESI or MALDI are used to ionize non-volatile analytes such as biopolymers and detect highly polymerized CTs. MALDI-MS has identified procyanidins of degree of polymerization (DP) of 15 in unripe apples, for seed coats of soyabeans until DP of 30 [157]. Similarly, the combination of Hydrophilic Interaction Chromatography (HILIC) x RPLC with fluorescence detection and electrospray full scan mass spectrometry (ESI-MS) resulted in high resolution analysis. This method was able to detect procyanidins with DP value of 16 and gallolylolation degree of 8 in the grape seed extracts [158]. These methods are constantly evolving and are now able to provide rapid quantitative and qualitative results. One such method is the

Engstrom method which utilizes ultra-high performance liquid chromatography (UPLC) separation coupled with DAD and negative ion ESI-MS to generate a polyphenolic profile directly from plant extracts. In addition to the quantification of different polyphenolic groups, it provides an insight into the composition of flavonols, CTs, HTs, and structural features of condensed tannins [159, 160]. These methods provide a great deal of information on tannin structural diversity, but due to high operational costs, their use is not yet widespread.

2.5.2 Influence of CT Structural Features on Ruminant Nutrition Is Still Ambiguous

The additive influence of CT structural features on the antimethanogenic potential of CT forages remains largely unexplored. Although research on CT structure and functional features has progressed immensely [64, 158, 160], only a small number of studies have assessed their impact on CH₄ abatement. These studies have shown that CT composition, and the concentration of CTs present in forages, are both significant determinants of their antimethanogenic potential [24, 90, 95, 161]. One of the major reasons is that CTs exist as highly polymerized structures, so large polymers cannot be easily purified as individual compounds and are studied in terms of certain structural features such as molecular weight, polymer size, and prodelphinidin proportion [33]. High structural variability across and within the species adds to the difficulty for assessing their structure–activity relationship. This has been shown in studies on sainfoin cultivars where antimethanogenic potential was found to be highly variable. Hatew [24] studied the intraspecies variability by analysing 46 different accessions of sainfoin with CT concentrations ranging from 0.6 to 2.8% of DM for their antimethanogenic potential. Emissions were analysed based on CT structural properties, i.e., mDP (12 to 84), percentage of trans isomers (12 to 34%), and PD (52.7 to 94.8%) in CT. These properties have been associated with the astringency of CTs. It was observed that PD percentage was a primary CT structural characteristic responsible for reducing CH₄ emissions in this in vitro study [24]. Weight-average M_w of CTs was shown to have little impact on reduction in CH₄ production ($R^2 = 0.0009$) from North American native forage plants [117]. Nevertheless, it is important to note that the variation in CH₄ reduction potential of different species also arises from plant morphology, CT interaction with feed components, and the presence of other plant secondary metabolites [162, 163]. The impact of CT structural features was found to be more pronounced in the studies conducted on CT extracts from plants. The additive effect of other secondary metabolites and forage quality parameters could be voided by the addition of purified CT extracts in the feed. Studies have shown that inclusion of CT extracts (40 mg/g DM) from leucaena (hybrid-Bahru) and mangosteen (*Garcinia mangostana* L.) peel could reduce CH₄ emissions by 45 and 35 percent, respectively. In both studies,

Panicum maximum substrate was used as control. The inhibitory effect of mangosteen peel extracts ($M_w = 2081$) was milder than leucaena ($M_w = 2737$) owing to its lower M_w but it was associated with fewer negative effects on in vitro DM degradability and lower protein binding affinity [164, 165]. When the antimethanogenic potential of leucaena extracts with differing average molecular weights was tested, extracts with the highest M_w were able engender CH_4 to the maximum [161, 166]. Table 3 summarizes the data from two different in vitro studies where at the same concentration, the effect of the molecular weight was more pronounced and it had a strong negative correlation with CH_4 emissions.

Table 2. 3. Influence of molecular weight on methane emissions from two in vitro studies using Kedah-Kelantan cattle rumen fluid.

Extracts	CT ¹ (%)	M _w ² (Da)	Total Gas (mL g ⁻¹ DM)	Methane (mL g ⁻¹ DM)	Reference
<i>Leucaena leucocephala</i> hybrid-Rendang	3	1265.8	57	8.07	[161]
<i>Leucaena leucocephala</i> hybrid-Rendang	3	1028.6	61.6	9.2	[161]
<i>Leucaena leucocephala</i> hybrid-Rendang	3	652.2	67	9.35	[161]
<i>Leucaena leucocephala</i> hybrid-Rendang	3	562.2	67.3	10.27	[161]
<i>Leucaena leucocephala</i> hybrid-Rendang	3	469.6	69.7	11.06	[161]
<i>Leucaena leucocephala</i> hybrid-Bahru	3	1348	49.8	4.6	[166]
<i>Leucaena leucocephala</i> hybrid-Bahru	3	857	51.8	5.6	[166]
<i>Leucaena leucocephala</i> hybrid-Bahru	3	730	56	7.8	[166]
<i>Leucaena leucocephala</i> hybrid-Bahru	3	726	55.5	9.7	[166]
<i>Leucaena leucocephala</i> hybrid-Bahru	3	494	57.5	9.5	[166]
Correlation between molecular weight and methane production					-0.72

¹ CT: Condensed tannins, ² M_w: Molecular weight of condensed tannins.

Some studies have also analysed the impact of multiple structural features simultaneously from CT extracts. Extracts from multiple sainfoin cultivars and diverse CT sources were analysed for CT structural features such as PD percentage, *cis* flavan-3-ols percentage, and average polymer size (mean degree of polymerization). PD percentage and average polymer size were found play an important role in determining antimethanogenic potential of CTs, in addition to the actual CT concentration [25, 95]. This shows that high reproducibility of the results can be attained by incorporating the structural features of CTs in ruminant nutrition studies.

2.5.3 A Roadmap to Close the Missing Links and Possible Future Directions

As discussed above, there is an apparent incongruity between measured tannin concentrations and their bioactive effects. This may be explained by a combination of four factors: (a) the variability of tannins and their composition is large both within and amongst species, and it is affected, at least partially, by the environment; (b) most studies have used too few plants and have been conducted under non-controlled environmental conditions or not comparable

conditions, to capture the variability of the tannins; (c) many studies have used inadequate or unsuitable analytical techniques (often due to lack of alternatives or resources), which do not capture the structural characteristics; and (d) the studies that investigated the antimethanogenic potential of CTs while accounting for structural attributes are still limited.

To overcome these inconsistencies, in future studies, every aspect that might affect the results, from the growing conditions to growth stage of the plant at harvest, and from sampling to extraction should be carefully considered in future studies. In the absence of laboratory infrastructure for the structural characterisation of CTs, assays to determine their astringency could be employed. Protein precipitation and radial diffusion assays are frequently used to measure the protein binding ability of tannins [167-169]. Furthermore, assays that determine their antioxidative and oxidative (at high pH) behaviour could also be utilized. They have been associated with the negative impact of tannins on the rumen microbial population [90] and their antiherbivore effect [33], respectively. Additional treatments with polyethylene and polyvinylpyrrolidone in vitro studies could be employed to elucidate the tannin effect on CH₄ emissions, as they bind specifically to tannins. These studies could be instrumental in distinguishing between the effect of tannins and forage chemical composition on CH₄ emissions. In vitro fermentation/CH₄ production techniques could be useful for screening these forages and determining their adequate supplementation. Using CT extracts of tanniferous forages in in vitro studies can illustrate the structure-activity relationship of CTs with methane emissions more distinctly. Condensed tannin supplementation has been found to impact the diversity and composition of the rumen microbial community [170].

Understanding the dynamics of microbial populations in rumen, and how CT-containing forages influence their abundance and diversity, can provide significant insights into their mode of action. Employing novel techniques such as metagenome and metatranscriptome analysis of the rumen microbiome under CT treatment can help in identifying the microbial population and the functional shifts in rumen microbiome which lead to CH₄ abatement [171]. As we gather more information about the relationship between the structure of CTs and their bioactivity, there are prospects for breeding plants with desired concentrations and composition of CTs [105, 172]. Molecular approaches have already made it possible for white clover to reach moderate levels of CT in its leaves [173] and efforts are also being made in directions to improve the persistence of TRFs, as may be seen for birdsfoot trefoil [174]. Several questions still remain unanswered, and these are critical for ensuring a comprehensive understanding of the fate of CTs in biological systems.

- How do different environmental conditions influence the structural features of CTs?

- To what extent are the structural features responsible for the functional attributes of tannins (PPC and oxidative property) and whether these assays could be utilized as an indicator of their antimethanogenic activity?
- How does the presence of other secondary plant metabolites affect the influence of tannins on CH₄ emissions?
- How does tannin supplementation affect mineral and vitamin bioavailability in ruminants? Which properties are primarily responsible for these interactions?
- How do forage conservation methods (ensiling vs. hay drying vs. fresh material) influence the palatability/acceptability and DMI by livestock, and anthelmintic and antimethanogenic potential of TRFs?
- To what extent are the anthelmintic effects of tannins sustained during long-term trials? Is it possible for gastrointestinal parasites to develop resistance to tannins?
- How do the PPC and oxidative capacity of tannins influence their antimethanogenic potential? What is the magnitude of their effect on antimethanogenic potential?
- How do different tannin sources influence rumen microbiome diversity and abundance and whether these effects are short or long term?
- How do CTs interact with feed constituents and how do structural characteristics play a role in this?

2.6 Conclusions

In recent years, there have been remarkable new insights into CT structural diversity and functions with more sensitive analytical methods. However, CT bioactivity is a complex process which results from a multitude of variations occurring simultaneously in plants as well as in their effects in animals. The variability in the results from different studies focuses our attention on the need for developing and adapting a course of action for the investigation of potential of CTs to reduce CH₄ emissions. The comparison of CT fingerprints of different species could help us understand not only the factors which define their antimethanogenic potential but also provide a vital framework to assess their interactions with plant constituents and rumen microflora, benefitting overall ruminant health.

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Chapter 3

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Article

Large Inter- and Intraspecies Variability of Polyphenols and Proanthocyanidins in Eight Temperate Forage Species Indicates Potential for Their Exploitation as Nutraceuticals

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A large inter- and intraspecies variability of polyphenols and proanthocyanidins in eight temperate forage species indicates potential for their exploitation as nutraceuticals

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3.1 Abstract

Substantial efforts have been made in incorporating tannin-rich forages into grassland-based livestock production systems. However, structural and functional diversity of tannins in different species limit their potential use at field scale. We conducted a greenhouse experiment with 17 cultivars from eight forage species and their cultivars. UPLC-MS/MS was used to analyse their polyphenolic profile and proanthocyanidin (PA) structural features in leaves. Our results highlight large inter- and intraspecies variability of plants, in terms of polyphenol and tannin concentrations in the leaves. A concomitant and significant variation was also registered in the structural features of PA-rich forages such as mean degree of polymerisation and prodelphinidin percentage. Concentration of PA also varied within plant organs, highest concentration was in flowers but leaves had the highest contribution to harvestable PA biomass. Our research highlights that identifying these variations can help identifying the representativeness of bioactivity and provides the basis for targeted breeding programmes.

KEYWORDS: temperate grasslands, UPLC-MS/MS, polyphenols, proanthocyanidins, breeding, tannin containing forages

3.2 Introduction

Grasslands occupy around 70% of the global agricultural area and play an important role in - global food security. In addition, they provide ecosystem services such as biodiversity and carbon sequestration.¹ Climate change, associated with elevated atmospheric CO₂ and emissions of other greenhouse gases is considered to be responsible for increased temperatures and more extreme weather events such as drought stress, floods, and wildfires.² These altered climatic patterns present threats to grasslands worldwide, with long-term impact on habitats and abundance of grassland forage species. Incorporating diverse forage species

with complementary functional traits can potentially make the grasslands more resource-use efficient and more resilient to the effects of climate change.³ Furthermore, bioactive forages such as sainfoin (*Onobrychis viciifolia*) and birdsfoot trefoil (*Lotus corniculatus*) have the potential to enhance productivity and sustainability of grassland-based livestock systems.⁴

The role of polyphenols, such as flavonoids and tannins, was previously considered to be limited to the plant's anti-herbivory defense mechanism. However, with progress in the understanding of polyphenolic structural diversity at molecular level, several of their beneficial effects on animal production and diverse ecological functions have been identified.^{5, 6} There is an increasing focus on environmentally friendly systems of food production, and in this context research on the ecological role of polyphenols and their impact on biological systems is rapidly gaining momentum. Flavonoids and tannins are a diverse group of plant secondary metabolites (PSMs) and they are widely distributed among the forage species as well as trees and shrubs.⁶⁻⁸ Flavonoids are known to possess a wide range of pharmacological functions, including anti-oxidative, anti-microbial, and anti-inflammatory functions.⁷⁻⁹ Similarly, tannin rich forages are found to have beneficial effects on ruminant health, by promoting an efficient nutrient utilization of dietary protein, decreasing populations of intestinal parasites, as well as improving growth rate and milk yields.^{10, 11} In addition to these effects, they also render a positive effect on the environment by decreasing methane emissions and shifting the N flow from urinary N to fecal N, leading to less ammonia volatilization and nitrous oxide emissions.^{4, 12} Finally, tannins in plant litter have been found to play an important role in nutrient cycling by reducing carbon (C) and nitrogen (N) mineralization rates by regulating the microbial activity in the soil.^{13, 14}

Tannins can be broadly categorized as proanthocyanidins (PAs, syn. condensed tannins) and hydrolysable tannins (HTs). Hydrolysable tannins can be further classified as gallic acid derivatives, gallotannins (GTs) and ellagitannins (ETs). Proanthocyanidins are commonly formed as a result of polymerization of procyanidin (PC) and prodelphinidin (PD) subunits linked with interflavan linkage.¹⁵ Although the benefits of tannins have been widely recognized, there have been conflicting number of studies showing beneficial, or almost no or even adverse effects on the animal health.¹⁶ The plasticity of tannin concentration in plants is one of the main factors responsible for this variation. It is known that tannin concentrations are not only affected by the plant species and their cultivars but also vary with the plant's phenological stage, and growing conditions.^{17, 18} In addition, the lack of characterization of the structural properties of PAs in these forages may lead to further inconsistencies in the results.¹⁹ For example, the PC and PD subunits in PAs have been shown to result in

substantial differences in mode of bioactivity, as well as the potency. Similar effects were shown for the polymer size.^{4, 16} This is important because the majority of studies on PA containing forages are based on the total concentration of tannins present in the forage, and have not considered the heterogeneity of PA structures in the plants, which makes it difficult to predict their impact on biological systems.^{20, 21} As it is difficult to isolate individual large PA polymers, PA activity is predicted on the basis of their general structural characteristics such as PC to PD ratio, mean degree of polymerization (mDP), and the distribution of polymers in terms of their size.^{22, 23} In order to predict the impact of polyphenol and tannin containing forages on grassland based ruminant production systems, it is crucial to understand whether the differences in the distribution of polyphenols and tannins in these forages are representative for any given species and whether they are affected by the development stage or environmental conditions. Furthermore, understanding the influence of environmental factors on tannin structural characteristics is an important niche for future research as they have been found to play an important role in determining bioactivity of PA-containing forages.¹⁶ Hence, this study investigated two main hypotheses: a) The variability among species (interspecies variability) is larger compared to the variability among cultivars of the same species (intraspecies variability), and b) these concentrations, and more importantly the structural features of any cultivar will be comparable across different harvests and phenological stages at harvest. Both of these factors would result in theoretically reproducible bioactive effects from any forage species, thus providing immediate relevance for their potential use as bioactive forages (nutraceuticals). To test these hypotheses, we conducted a greenhouse experiment using eight different species, with 2-3 cultivars of each being tested with the exception of lucerne (*Medicago sativa*), which was used as a negative control. The plants were grown under homogeneous and optimized environmental conditions to prevent shifts in polyphenolic profiles due to environmental stresses. Using this setup, we analyzed (i) the variability of the polyphenolic and tannin profiles in leaves across different species and their cultivars (ii) the effect of maturity stage at the harvest on PA concentration and structural features in leaves of different PA containing forages, and (iii) the effect of plant maturity stage on PA composition in different plant organs.

3.3 Materials And Methods

3.3.1 Experimental specifications

The study took place in a greenhouse located at Kiel University, northern Germany (54°20' N, 10°6' E). It included 17 cultivars of eight forage species which were sown on March 19, 2019

(Table 1). Lucerne (*Medicago sativa*) was used as a tannin free negative control. The pots ($25 \times 25 \times 40 \text{ cm}^3$ (L \times W \times H)) were arranged in a complete randomized design with 4 replicates for each cultivar leading to a total of 68 pots. The nutrients in the soil were present in the following concentration; P_2O_5 , 24 (mg/100g); K_2O , 14 (mg/100g); Mg, 60 (mg/100g); Mn, 12 (mg/kg); Cu, 1 (mg/kg); B, 0.53 (mg/kg); Na, 110 (mg/kg); Zn, 2.7 (mg/kg); N, 8 (mg/kg); Org.S, 14 (mg/kg) and C/N, 11. After 3 weeks, 0.5 M $\text{Ca}(\text{NO}_3)_2$ and 0.5 M K_2SO_4 solution was applied in the pots to increase the concentration of N to 100 mg/kg DM soil and of K to 150 mg/kg DM soil respectively. Five seeds were planted in each pot and plants from each pot were pooled to enhance the representativeness within cultivars. Optimal growth conditions for each species were envisaged and implemented by adjusting the pH to meet the species requirements. Irrigation was done to maintain the soil water filled pore space at a minimum of 60% and the volumetric soil moisture content was monitored continuously using 5TM soil moisture sensors (Meter AG, Munich, Germany). The illumination to the plants in the greenhouse was provided using the Sanlight Q6W LED (215W, SANlight, Bludenz, Austria) grow light system. The system was optimized to provide a homogeneous illumination with a photosynthetic photon flux of $624 \mu\text{mol/s}$. The light duration was controlled and simulated to provide 14 h of day and 10 h of night. To enhance the homogeneous distribution of illumination, and in response to plant's growth and thus potential shading effect on smaller plants, highly reflective foils were installed to ensure that also smaller plants were not limited by light. The effectiveness of this method was controlled using a LAI-2200C plant canopy analyzer (LI-COR Environmental, Lincoln, NE, USA).

Table 3.1. List and origins of forages species and cultivars used in the study. The pH of the soil was adjusted according to the species requirement using powdered limestone

Species	Cultivar	Common name	Origin	pH
<i>Onobrychis viciifolia</i>	Visnovsky	Sainfoin	Czech Republic	7.6
<i>Onobrychis viciifolia</i>	CPI 63750	Sainfoin	Turkey	7.6
<i>Onobrychis viciifolia</i>	Premier	Sainfoin	Switzerland	7.6
<i>Lotus corniculatus</i>	Bull	Birdsfoot trefoil	Canada	6.3
<i>Lotus corniculatus</i>	Lotar	Birdsfoot trefoil	Czech Republic	6.3
<i>Lotus corniculatus</i>	Rocco	Birdsfoot trefoil	Germany	6.3
<i>Lotus pedunculatus</i>	Lot 29	Big trefoil	Portugal	6.2
<i>Lotus pedunculatus</i>	Wild type	Big trefoil	NA ¹	6.2
<i>Sanguisorba minor</i>	Sang 10	Salad burnet	Germany	7.3
<i>Sanguisorba minor</i>	PI 308861	Salad burnet	Israel	7.3
<i>Cichorium intybus</i>	Plumato	Chicory	Netherlands	7.1
<i>Cichorium intybus</i>	Diente de Leon	Chicory	Spain	7.1
<i>Cichorium intybus</i>	Spadona	Chicory	Italy	7.1
<i>Plantago lanceolata</i>	PLA60	Ribwort plantain	Germany	7.3
<i>Plantago lanceolata</i>	Svatojansky	Ribwort plantain	Czech R.	7.3
<i>Hedysarum coronarium</i>	Sudda	Sulla	Italy	7.2
<i>Hedysarum coronarium</i>	Grimaldi	Sulla	Tunisia	7.2
<i>Medicago sativa</i>	Galaxy	Lucerne	NA ¹	7.5

¹Origin could not be determined

The temperature of the greenhouse was continuously monitored with a VP-4 air temperature and humidity sensor coupled with EM50 data logger (Meter AG, Munich, Germany). The average monthly air temperature of the greenhouse ranged from 24 to 27 °C (March (24.9 °C); April (24.9 °C); May (26.5 °C); June (27.3 °C); July (25.5 °C); August (26.0 °C)).

3.3.2 Sample Collection

The forages were harvested three times during 2019 and cut to a residual height of 5 cm (1st harvest: flowering stage, 2nd harvest: vegetative stage, and 3rd harvest: vegetative stage). The harvested plants were immediately separated into the plant organs (leaves, stems, inflorescences) before being frozen at -80 °C. At the first harvest, plants were harvested at the stage when the majority of pots for each cultivar were at full flowering. As sulla and salad burnet did not reach the flowering stage in the given time frame, these were harvested at the vegetative stage. The plants harvested at the vegetative stage were divided into leaves and stems, only. The samples were freeze dried before being milled into fine powder using a ZM 200 centrifugal mill with a sieve size of 0.25 mm (Retsch, Haan, Germany). Subsequently, they were stored at -80 °C until further analysis.

3.3.3 Extractable PAs

All the samples were extracted for quantitative analysis by weighing 20 mg of plant material in a 2 mL Eppendorf tube and adding 1400 μ L of 80% acetone/water (80:20, v/v). The samples were vortexed and then macerated overnight on a planar shaker at 4 °C in the dark. After the maceration, samples were centrifuged at $13,500 \times g$ for 10 mins and the supernatant was transferred to a new Eppendorf tube and the acetone in the supernatant was evaporated under vacuum using a concentrator plus (Eppendorf AG, Hamburg, Germany) for 90 mins. The extraction residues were simultaneously extracted a second time with 1400 μ L of acetone/water (80:20, v/v) for a duration of 3 h, before being centrifuged again and the supernatant from the second extraction was combined with the extract from the first extraction. Subsequently the acetone was evaporated again for 90 mins and the aqueous extracts were freeze dried and stored at -80 °C for the determination of extractable PAs (E-PAs). The extracts for the leaf samples were analyzed through UPLC-MS/MS to achieve their in-depth polyphenolic profile, PA concentration and structural characteristics of the PAs.

3.3.4 UPLC-MS/MS Analysis

The leaf samples were analyzed for their polyphenolic profile, E-PA concentration and composition according to Engström et al.²⁴ Group specific multiple reaction monitoring (MRM) methods for PC and PDs, PA polymer size, PA structural features, flavonoids, and phenolic acids were utilised for characterisation and quantification of these compounds. The freeze-dried E-PA extracts were dissolved in 1 mL ultrapure water and vortexed for 5 mins. The samples were filtered with 0.20 μ m polytetrafluoroethylene (PTFE) filter and 50 μ L of it was transferred into the UPLC vial. The analysis was performed using an Acquity UPLC system coupled with a Xevo triple-quadrupole tandem mass spectrometer. The system was equipped with an Acquity UPLC BEH Phenyl 1.7 μ m, 2.1 mm \times 100 mm column (Waters Corp., Wexford, Ireland) and diode array detector. The eluent comprised of two solvents, acetonitrile (A) and 0.1% aqueous formic acid (B), and the flow rate of the eluent was set to 0.5 mL/min. The gradient profile was applied as follows: 0–0.5 min, 0.1% A (isocratic); 0.5–5.0 min, 0.1–30% A (linear gradient); 5.0–8.0 min, 30–45% A (linear gradient); 8.0–11.5 min, column wash and stabilization. The data was recorded from 0 to 6 min for UV-vis (190–500 nm) and MS (m/z 100 to 2000). Negative electrospray ionization was used with following specifications; capillary voltage: 2.4 kV, desolvation temperature: 650 °C, source temperature: 150 °C, flow rate of desolvation and cone gas (N₂): 1000 and 100 L/h, respectively, and collision gas: argon (0.15 mL/min).

The system's stability was monitored with the performance of the standards which were injected at the beginning and end of the run. The standards comprised a flavonoid mix containing $4 \mu\text{g mL}^{-1}$ each of kaempferol-7-*O*-glucoside, kaempferol-7-*O*-neohesperoside, kaempferol-3-*O*-glucoside, quercetin-3-*O*-galactoside, and quercetin-3-*O*-glucoside, in an acetonitrile/0.1% aqueous formic acid (1:4 v/v) mixture, which was injected twice prior to each run. In addition to the flavonoid mix, PC ($1.0 \mu\text{g mL}^{-1}$, *Salix caprea* leaves: 95% pure), PD (*Trifolium repens* flowers: 98% pure) standards were also used to evaluate the system's stability. Before and after every 10 samples, catechin solution ($1 \mu\text{g mL}^{-1}$ in acetonitrile/0.1% aqueous formic acid, v/v) was injected five times to monitor the stability of ionization.

3.3.5 Tannin purification by column chromatography

For the preparation of species-specific tannin standards to use in the colorimetric assays for PA quantification, a 10 g pooled sample from the leaves of each plant was used. The tannins were extracted from the different plant species as described for extractable tannins with few modifications. The extraction step was repeated three times with 700 mL of 80% acetone/water (80:20, v/v) (for 10 g plant sample) to maximize recovery. The acetone was evaporated at 30 °C with the rotary evaporator and aqueous extracts were freeze dried. Freeze dried extracts were dissolved in water and filtered with 0.2 μm PTFE filters. The extracts were then loaded on a Sephadex LH-20 column (40×4.8 cm, Cole-Parmer, Vernon Hills, IL, USA) with a solvent gradient as described by Salminen and Karonen.²² Briefly, the column was first eluted with water (500 mL) followed by 500 mL of following solvent mixtures in the following order, F1: methanol/water (0.5:0.5, (v/v)), F2: acetone/water (3:7, (v/v)), F3: acetone/water (1:1, (v/v)), F4: acetone/water (4:1, (v/v)). The flow rate was set to 5 mL/min. The organic solvents from each fraction were simultaneously evaporated with the rotary evaporator. The remaining aqueous extracts were freeze dried. The fraction F3 and F4 were pooled and subsequently analyzed with UPLC-MS/MS as described in the previous section, to determine the purity of the tannin extracts.

3.3.6 Non-extractable PAs

The species-specific purified standards were used to prepare the standard curve for HCl butanol assay and the values were adjusted according to the purified concentration. Non-extractable PAs (NE-PAs) were obtained as sum of protein-bound PAs (P-PAs) and fiber-bound PAs (F-PAs) as described by Terrill et al.²⁵ Briefly, 600 μL of extraction solvent, containing 10 g L^{-1} sodium dodecyl sulfate and 50 g L^{-1} of 2-mercaptoethanol solution in 10 mM of Tris/chloride (pH= 8) was added to the plant residue. The samples were vortexed for 5

mins and then added to a Thermomixer C (Eppendorf AG, Hamburg, Germany) and heated at 95 °C for 60 mins. After the incubation, the samples were placed on ice for 5 mins and then centrifuged at $10\,000 \times g$ for 10 mins. The supernatant was decanted and stored in the fridge. The extraction step was repeated again and supernatants were combined to quantify P-PAs. The remaining residue obtained after the P-PA extraction was used to quantify F-PAs by performing the HCl-butanol assay directly on the residues.

3.3.7 HCl- Butanol Assay

The composition of PAs in terms of E-PAs, P-PAs and F-PAs in the plant organs, was determined by HCl- butanol assay according to Terrill et al.²⁵ The reagent for the assay was prepared with concentrated hydrochloric acid (37%) and n-butanol (5:95 v/v). The reagent (960 µL) was added to 160 µL P-PA extract to quantify P-PA concentration respectively. The F-PA concentration was determined by adding 1200 µL of HCl-butanol reagent and 120 µL of a pure SDS solution (10 g/L) to the remaining plant residue. The samples were vortexed for 5 mins and then transferred to Thermomixer C (Eppendorf AG, Hamburg, Germany) at the temperature of 90 °C for 90 mins. After incubation, the samples were cooled down and analyzed with Libra S22 photometer (Biochrom, Cambridge, UK) at 550 nm. The purified tannin extracts from the Sephadex LH-20 column with known purity, were used as standards for this assay.

3.3.8 Statistical Analysis

The data was analyzed using linear mixed models with the statistical programming language R²⁶, with harvest, cultivar and their interaction effects as the fixed factors. Harvest nested within pot was used as a random factor. The effect of the factors on the distribution of polyphenols and PA structural characteristics in leaves was analyzed using ANOVA and the variability between the species and their cultivars were determined using multiple contrast tests. The response variables were total flavanol, total polyphenol, total tannin, total PA concentration and the structural characteristics of PA. An additional linear mixed model was used to analyse PA composition of the plant organs of PA rich forages. Here, the fixed factors were PA containing cultivars, harvests, and their interaction effects, with harvest nested within the pot as a random factor. The response variables were E-PA, NE-PA, and total PA (sum of E-PA and NE-PA, T-PA) concentration in leaves and stems. The data for both the models was assumed to be normally distributed and heteroscedastic based on graphical residual analysis.

3.4 Results

3.4.1 Distribution and composition of extractable polyphenols in the leaves varied widely among the species but remained uniform across the harvests

The polyphenol concentration in the leaves varied widely across the cultivars ($P < 0.0001$, $F_{16,51}$: 1976.8) and harvests ($P < 0.001$, $F_{2,98}$: 8.6). The significant interaction effect between harvest and cultivar ($P < 0.0001$, $F_{32,98}$: 4.2) indicates that the effect of harvest was dependent on the type of cultivar. As seen in Figure 1, the significant effect of harvest was seen only in sainfoin, birdsfoot trefoil, ribwort plantain and lucerne whereas in other species concentration was not affected by the harvest. The flavonoid concentration was also affected by cultivars ($P < 0.0001$, $F_{16,51}$: 184.5), harvests ($P < 0.0001$, $F_{2,98}$: 23.4) and the interaction between harvest and cultivar ($P < 0.0001$, $F_{32,98}$: 4.8).

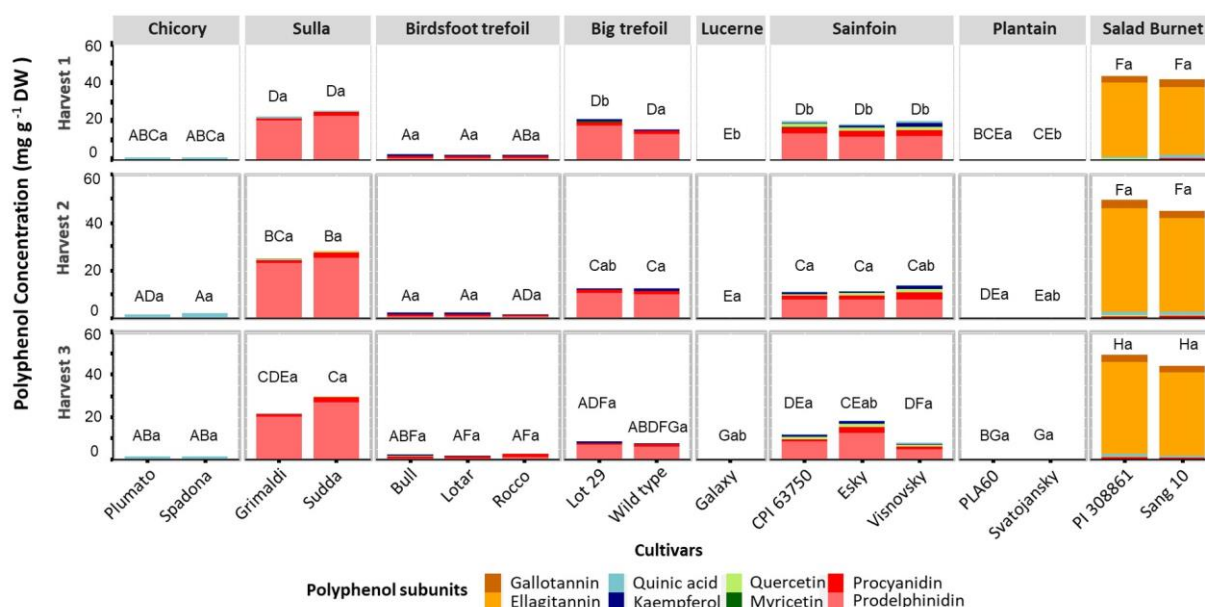


Figure 4 Polyphenolic profile of leaves from different cultivars across the three harvests. Means followed by different uppercase letters represent significant differences ($P < 0.05$) between the cultivars for each harvest and different lower-case letters represent the significant differences within the harvests for each cultivar.

Salad burnet consistently had highest polyphenol concentrations in all the three harvests and consisted primarily of HTs with concentration in the range 39-48 mg g⁻¹ DM (Table S1). As expected, lucerne which was used as a negative control, was found to be devoid of polyphenols. The polyphenols in ribwort plantain (0.2-0.6 mg g⁻¹ DM) and chicory (1-2.5 mg g⁻¹ DM) cultivars comprised mainly quinic acid derivatives. Proanthocyanidin containing species were sainfoin, birdsfoot trefoil, big trefoil and sulla. Among these species, birdsfoot trefoil had the lowest E-PA concentration in leaves (1.5-2.5 mg g⁻¹ DM) and the highest concentration was found in sulla (21-29 mg g⁻¹ DM). In big trefoil (Lot 29) and sainfoin

cultivars, the concentration of total polyphenols was found to decrease significantly with the harvest ($P < 0.05$). However, polyphenol concentration for the cultivars were not significantly different within the species with the exception of sainfoin cultivars in the third harvest.

3.4.2 Effect of harvests on PA structural features in the leaves was dependent on the plant species

Proanthocyanidin structural features were obtained by analysing the leaves of PA-containing forages with UPLC-MS/MS. These structural features are known to be associated with PA's bioactivity. They were evaluated in the terms of the readily bioavailable PA fraction (E-PA) present in the leaves, mean degree of polymerization (mDP) of PA polymers and proportion of prodelphinidins (PD percentage) in PAs (Figure 2). The interaction between harvest and cultivar was significant for all of these characteristics (mDP: $P < 0.0001$, $F_{18,55}$: 3.2; PD percentage: $P < 0.0001$, $F_{18,55}$: 6.0; E-PA: $P < 0.0001$, $F_{18,55}$: 3.3). Extractable-PA concentration in the leaves ranged from 10 to 40 mg g⁻¹ DM. The cultivars from birdsfoot trefoil had the lowest E-PA concentration (10 to 20 mg g⁻¹ DM) and concentration remained stable with the harvests and there were no significant differences between the cultivars. A significant variation between the birdsfoot trefoil cultivars, Bull, Lotar, and Rocco, could be observed in terms of mDP and PD percentage. Among the birdsfoot trefoil cultivars, Rocco has the highest mDP values (12.3- 13.5) and proportion of PD (48.6-52.3%) compared to Bull (mDP: 7.9-9.7, PD: 28.4-33.3%) and Lotar (mDP: 8.9-10.6, PD: 34.3-39%).

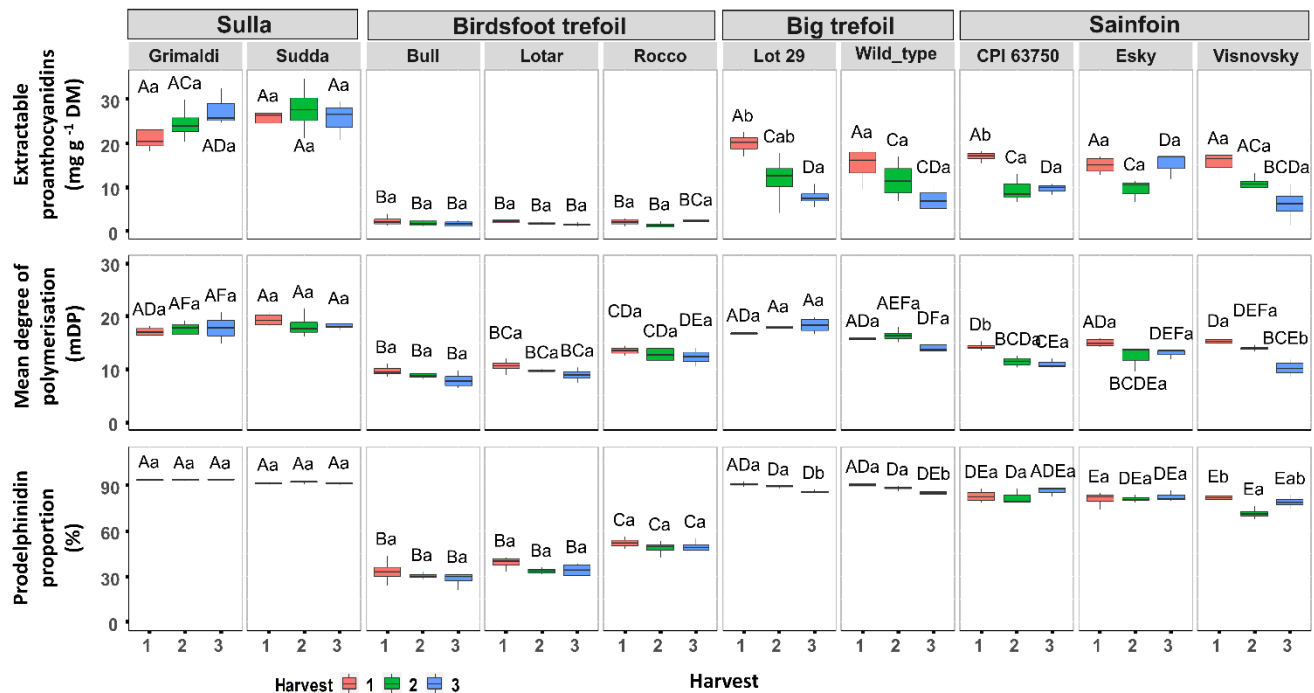


Figure 5 Features responsible for proanthocyanidin bioactivity across different harvests and species. Means followed by different uppercase letters represent significant differences ($P < 0.05$) between the cultivars for each harvest and different lower-case letters represent the significant differences within the harvests for each cultivar.

In addition to having the highest E-PA concentrations ($42 \text{ mg g}^{-1} \text{ DM}$), sulla had the highest mDP values (19.3) and PD percentage (93.6 %) and neither the PD percentage of sulla (91-93%) nor its mDP (16-19) were affected by the harvest and remained comparable between the cultivars. A significant effect of harvest was, however, observed in sainfoin cultivars, where E-PA concentration for Visnovsky declined from 15 to $6 \text{ mg g}^{-1} \text{ DM}$ and for CPI 63750, it declined from 17 to $10 \text{ mg g}^{-1} \text{ DM}$ respectively ($P < 0.05$). Similarly, mDP and PD percentage values were found to decrease with the progression of harvests in Visnovsky and CPI 63750. In big trefoil, harvest had no influence on mDP but, PD percentage decreased by 5% from the 1st to 3rd harvest ($P < 0.05$).

3.4.3 Aboveground dry matter yields decreased with the harvests

Dry matter (DM) yield was found to differ significantly with cultivar ($P < 0.0001$; $F_{16,51} = 6.39$ and harvest ($P < 0.0001$; $F_{16,51} = 42.1$). There was also a significant interaction effect of cultivar and harvest ($P < 0.0001$; $F_{32,98} = 27.8$). For the majority of species, the first harvest produced the highest yields and this resulted in high variation in yields across the harvests. The duration required for plants to reach flowering stage also varied considerably, and reflected the inclusion in the experiment of the diverse range of species and their cultivars (Table 2). Plants

of sulla and salad burnet never reached the flowering stage. In the first harvest, chicory cultivars had the highest DM yields while ribwort plantain cultivars produced the lowest yields. The average cumulative DM yield ranged from 12.2 g DM per pot in ribwort plantain cv. PLA60 to 38.8 g DM per pot in chicory cv. Spadona. Within the cultivars of these species there were no significant differences in DM yields. A prominent harvest-effect was seen on the yield of sainfoin cultivars ($P < 0.05$). Similar to DM yield, there was a significant effect of cultivar ($P < 0.0001$; $F_{16,51}$: 47.7), harvest ($P < 0.0001$; $F_{2,98}$: 485.8) and interaction between cultivar and harvest ($P < 0.0001$; $F_{32,98}$: 27.8). The highest leaf proportion was found in sainfoin cultivars and this was in the range of 30-100 % and the lowest proportion was found in lucerne (28-36 %). The high variability of leaf proportion in sainfoin cultivars could be attributed to the heterogeneous growth of the plants in different pots and only a few plants showed stem development. Similar trends were observed in chicory cv. Spadona and ribwort plantain cv. PLA60.

Table 3.2. Mean average aboveground biomass yield per pot and the proportion of leaves for each cultivar (grouped by species) across the three harvests.

Cultivar	Flowering time (days after sowing)	Aboveground yield (g DM per pot)			Leaf proportion (%)		
		Harvest 1	Harvest 2	Harvest 3	Harvest 1	Harvest 2	Harvest 3
Chichory							
Plumato	98	26.3 ± 3 ^{AB}	17.9 ± 4 ^{ABCD}	15.8 ± 2 ^{AB}	68 ± 11 ^{ABC}	68 ± 17 ^{AB}	85 ± 11 ^{AD}
Spadona	118	38.8 ± 3 ^{Ab}	13.3 ± 3 ^{ABCDEa}	9.2 ± 0 ^{ABa}	77 ± 14 ^{AB}	80 ± 11 ^{ABC}	100 ± 0 ^{ABCD}
Sulla							
Grimaldi	-	11.2 ± 1 ^{AB}	7.6 ± 2 ^{ABCDEF}	4.6 ± 1 ^{AC}	60 ± 3 ^A	63 ± 1 ^A	62 ± 12 ^A
Sudda	-	20.1 ± 2 ^{AB}	8.7 ± 3 ^{ABCDEF}	8.6 ± 2 ^{ABC}	55 ± 3 ^{ABC}	64 ± 2 ^{AB}	66 ± 3 ^{ABCD}
Birdsfoot trefoil							
Bull	66	19.3 ± 2 ^{Bab}	20.6 ± 1 ^{Ba}	16.3 ± 2 ^{Bb}	48 ± 2 ^{DEF}	58 ± 1 ^{DEF}	55 ± 3 ^{EF}
Lotar	66	16 ± 1 ^B	17.2 ± 1 ^{BCD}	13.1 ± 2 ^{AB}	50 ± 3 ^{CDE}	64 ± 3 ^{DEFG}	52 ± 2 ^{B^{EF}G}
Rocco	66	12.5 ± 2 ^B	11.8 ± 2 ^{ABCDE}	15.3 ± 2 ^B	44 ± 4 ^{ABCD}	60 ± 2 ^{BCDG}	52 ± 2 ^{BCEG}
Big Trefoil							
Lot 29	66	15.4 ± 2 ^B	17.8 ± 1 ^{ABCD}	14.3 ± 2 ^{AB}	54 ± 3 ^{Fb}	49 ± 1 ^{Ea}	45 ± 3 ^{Fa}
Wild Type	66	13.2 ± 2 ^{Bab}	17 ± 2 ^{ABCDa}	11.2 ± 1 ^{ABb}	54 ± 5 ^{EFb}	44 ± 3 ^{EFab}	62 ± 7 ^{EFa}
Sainfoin							
CPI 63750	122	23.5 ± 2 ^{ABb}	5.5 ± 1 ^{EFa}	8.1 ± 2 ^{ABCa}	85 ± 3 ^{Gb}	100 ± 0 ^{Ha}	67 ± 8 ^{Ha}
Esky	102	21.4 ± 4 ^{ABb}	3.9 ± 1 ^{Fa}	11.8 ± 1 ^{ABb}	81 ± 6 ^{Gb}	100 ± 0 ^{Ha}	30 ± 4 ^{Ib}
Visnovsky	78	19.1 ± 0 ^{ABb}	7.1 ± 2 ^{AEFa}	3.1 ± 1 ^{Ca}	70 ± 1 ^{Gc}	86 ± 3 ^{Ia}	100 ± 0 ^{Hb}
Ribwort plantain							
PLA60	98	12.2 ± 4 ^B	6.8 ± 3 ^{ACEF}	5.9 ± 2 ^{ABC}	64 ± 5 ^{AB}	78 ± 6 ^A	85 ± 5 ^A
Svatojansky	78	11.3 ± 1 ^B	12 ± 3 ^{ABCDE}	7.4 ± 1 ^{ABC}	36 ± 2 ^A	100 ± 0 ^A	100 ± 0 ^A
Salad burnet							
PI 308861	-	17.9 ± 2 ^{AB}	9.7 ± 2 ^{ACDEF}	13.4 ± 1 ^{AB}	64 ± 1 ^{BCDE}	76 ± 2 ^{CDFG}	100 ± 0 ^{EFG}
Sang 10	-	21 ± 5 ^{AB}	8.8 ± 2 ^{ABCDEF}	17 ± 1 ^B	68 ± 4 ^{ABCD}	74 ± 3 ^{BCG}	68 ± 3 ^{BCDG}
Lucerne							
Galaxy	66	17.3 ± 1 ^B	22 ± 3 ^{BD}	13.4 ± 2 ^{AB}	36 ± 4 ^{ABC}	28 ± 2 ^A	28 ± 3 ^{ACD}

Note: The species, sulla and salad burnet, did not reach flowering stage. Values identify means and standard error of dry matter yield of the cultivar per pot. Different uppercase letters represent significant differences ($P < 0.05$) between the yield of cultivar for each harvest and different lower-case letters represent the significant differences within the harvests for each cultivar.

3.4.4 Variation in PA content in different plant organs and their proportions varied significantly among plant species

The comparison of different PA fractions categorized according to their extractability (bioavailability) is considered here only for the PA-containing species. The concentration of E-PA and NE-PA fractions in different plant organs was determined by HCl-butanol assay. The contribution from each plant organ to total DM yield was determined to attain the PA yield in each plant (Table S2, Eq-S2). It was found that the total PA (T-PA, sum of E-PA and NE-PA) content in the plant varied significantly among cultivars ($P < 0.0001$, $F_{9,30}$: 78.5) ranging from 6-13 mg per plant in Bull to 72-239 mg per plant in Sudda (Figure 3).

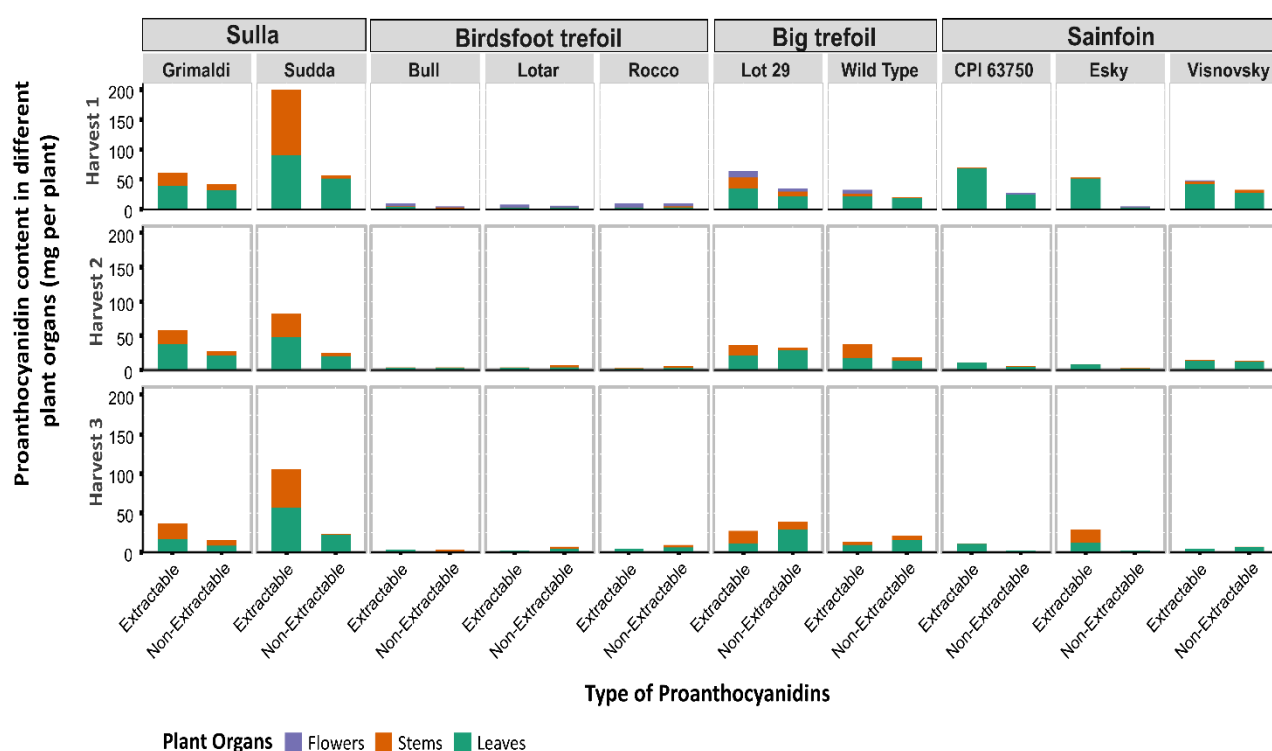


Figure 6. Distribution of extractable and non- extractable proanthocyanidins in different plant organs. Means followed by different uppercase letters represent significant differences ($P < 0.05$) between the cultivars for each harvest and different lower-case letters represent the significant differences within the harvests for each cultivar.

Across the cultivars, the effect of harvest ($P < 0.0001$, $F_{2,55}$: 96.3) as well as the cultivar x harvest interaction ($P < 0.0001$, $F_{18,55}$: 7.7) were significant for the T-PA content of the plants (Table 3). The PA concentration in flowers was measured for the 1st harvest and the highest T-PA content was found in big trefoil flowers (Lot 29: 19.4 ± 7 mg plant⁻¹, Wild type: 19.4 ± 3 mg plant⁻¹) followed by birdsfoot trefoil (Bull: 7.1 ± 0.5 mg plant⁻¹, Lotar: 7 ± 1 mg plant⁻¹, Rocco: 9.1 ± 3.5 mg plant⁻¹) and sainfoin (Esky: 0.4 ± 0.2 mg plant⁻¹, Visnovsky: 3 ± 0.7 mg plant⁻¹, CPI 63750: 2.5 ± 1.2 mg plant⁻¹).

Table 3.3 Distribution of proanthocyanidin (PA) type for each species and their cultivars in different plant organs

Species	Cultivar	Harvest	E-PA (mg per plant)	NE-PA (mg per plant)	Proportion of E-PA (% T-PA)	T-PA (mg per plant)	Leaves T-PA (mg per plant)	Stems T-PA (mg per plant)
Sulla	Grimaldi	1	70 ± 8 ^{ADb}	61.2 ± 4 ^{Aa}	52.8 ± 4 ^{ABC}	131.2 ± 9 ^{ADa}	88 ± 3 ^{ACb}	43.2 ± 9 ^{ADb}
Sulla	Sudda	1	141.7 ± 12 ^{Ab}	38.8 ± 4 ^{Ab}	78.6 ± 1 ^A	180.5 ± 15 ^{Ab}	104.1 ± 4 ^{Ab}	57.4 ± 3 ^{Aa}
Birdsfoot trefoil	Bull	1	8.2 ± 1 ^{BCb}	4.5 ± 1 ^B	64.8 ± 3 ^{ABC}	12.7 ± 2 ^B	6 ± 2 ^B	0.3 ± 0 ^B
Birdsfoot trefoil	Lotar	1	7.3 ± 1 ^{Bb}	5 ± 0 ^B	57.6 ± 6 ^{ABCb}	12.3 ± 1 ^B	5.5 ± 1 ^B	1 ± 1 ^B
Birdsfoot trefoil	Rocco	1	9.3 ± 2 ^{BCb}	8.9 ± 2 ^{BC}	49.8 ± 2 ^{Bb}	18.2 ± 4 ^{BCb}	4.7 ± 0 ^B	2.4 ± 1 ^{BC}
Big trefoil	Lot 29	1	63.8 ± 12 ^{AD}	33.5 ± 7 ^A	65.7 ± 3 ^{ABC}	97.3 ± 17 ^{AD}	54.9 ± 9 ^{AC}	27.8 ± 2 ^{AD}
Big trefoil	Wild type	1	32.1 ± 8 ^{BCD}	20.2 ± 4 ^{AC}	58.8 ± 8 ^{AB}	52.4 ± 8 ^{CD}	39.7 ± 6 ^C	5.8 ± 0 ^{CEb}
Sainfoin	CPI 63750	1	75.7 ± 11 ^{ADb}	28.4 ± 6 ^{ACb}	73.5 ± 2 ^{ABC}	104 ± 17 ^{ADb}	100.5 ± 17 ^{ACb}	1.6 ± 0 ^{BCb}
Sainfoin	Esky	1	52.6 ± 9 ^{Dc}	2.8 ± 1 ^B	95.2 ± 1 ^{Cb}	55.4 ± 10 ^{Db}	52.8 ± 9 ^{ACb}	2.4 ± 1 ^{Bc}
Sainfoin	Visnovsky	1	47.2 ± 3 ^{ACDb}	33 ± 2 ^{Ab}	58.8 ± 3 ^{ABC}	80.2 ± 1 ^{ADb}	67.9 ± 2 ^{ACb}	10 ± 2 ^{DEc}
Sulla	Grimaldi	2	39.7 ± 8 ^{ADab}	33.5 ± 8 ^{Aa}	54.2 ± 11 ^{ABC}	73.1 ± 3 ^{ACa}	49.8 ± 2 ^{AEab}	23.4 ± 2 ^{Aab}
Sulla	Sudda	2	72.4 ± 2 ^{Aa}	21.7 ± 3 ^{ABab}	77.3 ± 3 ^A	94 ± 4 ^{Aa}	59.1 ± 3 ^{Aa}	35 ± 2 ^{Aa}
Birdsfoot trefoil	Bull	2	4.2 ± 1 ^{BCab}	3.6 ± 2 ^C	56.5 ± 11 ^{AB}	7.8 ± 2 ^B	6.8 ± 2 ^{BC}	1 ± 0 ^{BC}
Birdsfoot trefoil	Lotar	2	3.9 ± 0 ^{BCab}	6.7 ± 1 ^C	37.7 ± 5 ^{BCab}	10.6 ± 1 ^B	8 ± 0 ^{BC}	2.6 ± 1 ^B
Birdsfoot trefoil	Rocco	2	2 ± 1 ^{Ba}	5 ± 1 ^C	27.2 ± 2 ^{Ca}	7.1 ± 2 ^{Ba}	5.1 ± 1 ^B	2 ± 0 ^B
Big trefoil	Lot 29	2	36.4 ± 8 ^{AD}	33.1 ± 9 ^A	53.6 ± 8 ^{AB}	69.6 ± 11 ^{AC}	49.2 ± 11 ^A	20.4 ± 5 ^A
Big trefoil	Wild type	2	38.1 ± 10 ^{AD}	19.3 ± 4 ^{AB}	64.4 ± 7 ^{AB}	57.4 ± 13 ^{AC}	31 ± 7 ^{ADE}	26.4 ± 10 ^{Aa}
Sainfoin	CPI 63750	2	11 ± 3 ^{CDa}	5.5 ± 2 ^{BCab}	66.2 ± 14 ^{AB}	16.5 ± 2 ^{Ba}	16.5 ± 2 ^{CDEa}	0 ± 0 ^{Ca}
Sainfoin	Esky	2	7.2 ± 2 ^{Ca}	2.3 ± 1 ^C	79.4 ± 6 ^{Aa}	9.5 ± 3 ^{Ba}	9.5 ± 3 ^{BCDa}	0 ± 0 ^{BCa}
Sainfoin	Visnovsky	2	13.8 ± 4 ^{CDab}	12.2 ± 2 ^{ABCab}	51.6 ± 7 ^{AB}	26 ± 4 ^{BCab}	24.3 ± 4 ^{ACDEab}	1.6 ± 1 ^{BCa}
Sulla	Grimaldi	3	19.7 ± 5 ^{DEa}	11.8 ± 4 ^{ACb}	63.9 ± 3 ^{ACE}	31.5 ± 9 ^{CDb}	24.5 ± 9 ^{CDEa}	7 ± 3 ^{ABa}
Sulla	Sudda	3	53.7 ± 7 ^{Aa}	18.2 ± 3 ^{ABa}	74.9 ± 1 ^A	71.8 ± 10 ^{Aa}	62.4 ± 6 ^{Aa}	9.4 ± 4 ^{ABb}
Birdsfoot trefoil	Bull	3	3.1 ± 1 ^{Ba}	2.9 ± 1 ^{CD}	52.4 ± 9 ^{ABC}	6 ± 1 ^B	4.1 ± 1 ^B	1.9 ± 0 ^A
Birdsfoot trefoil	Lotar	3	2.2 ± 1 ^{Ba}	7 ± 1 ^C	22.8 ± 4 ^{Da}	9.2 ± 1 ^B	7 ± 1 ^{BC}	2.2 ± 0 ^A
Birdsfoot trefoil	Rocco	3	3.9 ± 0 ^{BCab}	8.8 ± 1 ^{AC}	31 ± 3 ^{BDa}	12.7 ± 1 ^{BCab}	9.9 ± 1 ^{BCD}	2.7 ± 1 ^A
Big trefoil	Lot 29	3	26.5 ± 8 ^{AD}	39.6 ± 6 ^B	38.2 ± 8 ^{BCD}	66.1 ± 10 ^{AD}	38.2 ± 4 ^{AE}	27.9 ± 8 ^B
Big trefoil	Wild type	3	12.4 ± 3 ^{CDE}	21.2 ± 5 ^{AB}	36.7 ± 4 ^{BCD}	33.6 ± 7 ^{ACD}	24.8 ± 7 ^{DE}	8.8 ± 3 ^{ABab}
Sainfoin	CPI 63750	3	10.6 ± 1 ^{CDEa}	2 ± 1 ^{CDa}	85.5 ± 8 ^{AE}	12.7 ± 2 ^{BCa}	12.7 ± 2 ^{BCDa}	0 ± 0 ^{Ca}
Sainfoin	Esky	3	28.7 ± 3 ^{Db}	1.5 ± 1 ^D	95.9 ± 3 ^{Eb}	30.2 ± 4 ^{CDb}	12.5 ± 4 ^{BCDa}	17.7 ± 1 ^{Bb}
Sainfoin	Visnovsky	3	4.7 ± 2 ^{BCEa}	7.2 ± 2 ^{ACDa}	34 ± 5 ^{BCD}	11.8 ± 4 ^{BCa}	11.8 ± 4 ^{BCDa}	0 ± 0 ^{Cb}

E-PA, extractable proanthocyanidins; NE-PA, non-extractable proanthocyanidins; T-PA, total proanthocyanidins; E-PA, proportion of extractable proanthocyanidins in total proanthocyanidins; Leaves T-PA, total proanthocyanidins in leaves; Stems T-PA, total proanthocyanidins in stems; Values identify means and standard error of the concentration. Within a column, different uppercase letters represent significant differences ($P < 0.05$) between the different cultivars for each harvest and different lower-case letters represent the significant differences within the harvests for each cultivar ($P < 0.05$).

Similar to T-PA, the amounts of E-PA and NE-PA amount in plants were affected significantly by the cultivar (E-PA: $P < 0.0001$, $F_{9,30}$: 54.3; NE-PA: $P < 0.0001$, $F_{9,30}$: 40.4), harvest (E-PA: $P < 0.0001$, $F_{2,55}$: 244.6; NE-PA: $P < 0.05$, $F_{2,55}$: 4.27), and the interaction between harvest and cultivar was also significant (E-PA: $P < 0.0001$, $F_{18,55}$: 13.7; NE-PA: $P < 0.0001$, $F_{18,55}$: 5.4). A significant effect of harvest was observed in T-PA content in sulla ($P < 0.05$) and sainfoin ($P < 0.05$) whereas in birdsfoot trefoil and big trefoil the variation was not significant. The highest amount of T-PA in leaves were found in Sudda; however, the amount of T-PA decreased significantly from 104.1 ± 4 mg in the 1st harvest to 62.4 ± 6 mg in the 3rd harvest ($P < 0.0001$). A high variability T-PA content in stems was also observed within the sainfoin species.

3.5 Discussion

3.5.1 Polyphenolic profiling of the forages can help understand the interspecies and intraspecies variation in their bioactivity

For decades, researchers have been trying to assess the role of polyphenols in grassland ecosystem health. The vast diversity of polyphenols present in different forages makes it difficult to comprehend their entire functional implications for biological systems.^{6, 17} Additionally, limited knowledge of structure-activity relationship of tannins hinders the incorporation of polyphenol rich forages in conventional grassland systems.²¹ Consequently, this study, which included 17 different cultivars from the most prominent temperate forage species that contain tannins, and which were grown under identical growing conditions, it was of utmost importance to identify the polyphenolic variability due to biotic factors. Furthermore, it has provided unique insights into the dynamics of flavonoids and tannin distribution in the forages and on their inter-and intraspecies diversity. As expected, there was a significant variation in the distribution of polyphenols across the species, and this was generally larger than the variability within species.

The sulla cultivars comprised predominantly PAs, accounting for up to 99% of the total polyphenols. The species differed extensively in terms of their flavonoid composition and concentration. Sainfoin leaves were found to contain the most diverse profile of flavonoids such as kaempferol, quercetin, and quinic acid derivatives. Sainfoin has emerged as a promising tanniferous forage for livestock feed due to its high palatability and its antiparasitic properties.²⁷ The presence of diverse flavonoids concomitantly with elevated tannin concentrations could also be an important factor in the beneficial effects of sainfoin on

livestock. It has been hypothesized that PSMs such as flavonoids when combined with tannins can exert either additive, synergistic, or deleterious matrix effects.^{14, 21} Although, the profile of flavonoids in birdsfoot trefoil and big trefoil followed a similar pattern, they differed in their tannin profile. The leaves of big trefoil were found to have approx. 5 to 10-fold more E-PA compared to birdsfoot trefoil. While the individual flavonoids were not quantified in detail as part of this study, according to previous reports published on these species, flavonoids in sainfoin¹⁸, birdsfoot trefoil²⁸, big trefoil²⁹ and sulla¹¹ were dominated by diglycosides of kaempferol and quercetin. Among the tested species, salad burnet was the only HT-rich species, it contained approximately 96% HTs of total polyphenols. Salad burnet also contained minute quantities of kaempferol (0.2%), quercetin (0.9%) and quinic acid (2%) derivatives, and PAs (0.9%). Hydrolysable tannins in salad burnet were mainly present in the form of ellagitannins ranging from 87-93%. These findings were in accordance with a study by Finimundy et al.³⁰ where ellagitannins were the major tannin constituents in salad burnet tannin extracts (leaves and stems).

While the focus of the current study was on tannins, and in particular on PAs, the polyphenols in chicory cultivars comprised mainly phenolic acids and quercetin derivatives. Studies have also shown that the major phenolic acids found in chicory leaves are caffeic, chlorogenic, *p*-hydroxybenzoic and *p*-coumaric acid.^{31, 32} In the present study, chicory and ribwort plantain were void of PAs, which was in accordance with the study conducted by Peña-Espinoza et al.³³ Different previous findings from colorimetric assays can be linked to methodological issues. For instance, a study by Hoskin et.al.³⁴ found that the PA concentration in chicory was 10 times higher when measured with vanillin assay compared to when quantified by HCl-butanol assay. Despite their low polyphenol concentration, both ribwort plantain and chicory have been associated with antimicrobial and anthelmintic properties.³⁵ Chicory is more commonly used as a sown forage species compared to ribwort plantain, due to its higher DM yield, crude protein and *in vitro* true digestibility,³⁶ although ribwort plantain is able to persist in a wide variety of soil types and occur at high plant density in swards.³⁷ Chicory and ribwort plantain are promising companion forage plants in swards with cool season grass species. Their high tolerance to elevated temperatures and drought enables them to overcome the yield deficits during summer and thereby increase overall annual herbage production.^{31, 36, 37}

Incorporation of these flavonoids even in small quantities is reported to improve ruminant health by increasing rumen efficiency, improving antioxidative status and

immunity. Owing to the antimicrobial and anthelmintic properties of flavonoids, they have been proposed as one of the alternatives to synthetic anthelmintic drugs.^{9, 38}

3.5.2 Intraspecies variation of PA structural features was less compared to PA concentration

Although there have been many studies on PA concentration in plants, the effect of phenological stage on the dynamics of PA structural features in plants have seldom been analyzed. These features can play an important role in assessing the biological and functional implications of these forages on biological systems. The average polymer size (mDP) of PAs in plants has been found to range from two to over 40 and to vary even within the same species.^{4, 39} In addition to mDP, the composition of PA containing various mixtures of PC and PD subunits can lead to the formation of highly diverse PA compounds. In the present study, sainfoin, sulla and big trefoil cultivars, were found to be PD dominant, whereas birdsfoot trefoil comprised mainly PC. Forages rich in PD were found to be more bioactive compared to PC-rich forages. This is due to the presence of a third hydroxyl group in PD subunits, compared to two hydroxyl groups in PC subunits which provides an extra binding site for interactions with macromolecules, and thereby, rendering PD subunits more biologically active.¹⁹ It is promising that, while there was a significant variation in PA features observed within species, in the majority of the species considered here, both mDP and PD percentage in the leaves were not affected by harvest and plant phenological stage. Contrary to that, the PA composition in the leaves of sainfoin cultivars was found to vary highly with the harvests. Additionally, the sainfoin cultivars CPI 63750 and Visnovsky were significantly different in terms of PD percentage across harvests. Similarly, birdsfoot trefoil cultivars also differed from each other in terms of mDP and PD percentage but not in their concentration.

The finding that cultivars of species differed in their PA concentration and composition was expected and in line with the findings by Theodoridou et al.⁴⁰ and Malisch et al.¹⁸ A study by Scioneaux et al.⁴¹ on *Populus* species found that the genetics of the plant strongly controls PA subunit composition and polymer length. This is in accordance with the findings of the present study, as the observed significant cultivar x harvest interaction indicated that the effect of harvest was highly dependent on the cultivar ($P < 0.0001$). This emphasizes that the variation in PA concentration and composition in a plant is highly dependent on its genetic composition and it is difficult to extrapolate the results from one cultivar to the whole species. Consistent PA profiles of forages can improve the predictability

of PA traits and can accelerate their incorporation at field-scale applications. Nevertheless, the large differences across harvests and cultivars in PA concentration compared to PA structural features, as well as the larger differences across different species compared to cultivars of the same species, indicate potential for the estimation of bioactivity from tannin-rich forages in the field. This is particularly relevant for *sulla* and big trefoil, as these forages are known to be highly astringent (and hence more bioactive), which could be attributed to their high mDP and PD percentages in addition to their high PA concentration. In the present study, the PA features of *sulla* were not affected by harvest which is in accordance with a study by Tibe et al.¹¹ in which seasonal changes were found to have had a limited effect on the PA composition in *sulla*.

In addition to their forage nutrition role, the stability of PA features across the harvests increases the value of these tannin-rich forages for their incorporation into animal diets. Our results on the profiling of the important features responsible for PA bioactivity; have shown that there is a variation among species and their cultivars at a finer level, and this information provides a basis to identify the consequences of these variations with regard to their biological reactivity. We found that the interspecies variability was much higher than intraspecies variation in these forages in terms of PA composition. *In vivo* studies can further help to evaluate the impact of these variations in different species and their cultivars, in terms of their biological consequences.

3.5.3 Proanthocyanidins comprise mainly extractable proanthocyanidins in the majority of species.

Proanthocyanidin concentration in growing plants are highly variable in nature. The allocation of PAs in different plant organs depends highly on the plant phenological stage and their growing conditions.⁴² Our analyses showed that flowers had the highest PA concentration based on per unit DM (Table S2). However, due to the negligible contribution of flowers to the harvested biomass, the harvestable PA concentration per plant was derived primarily from the leaves in all the harvests with the exception of birdsfoot trefoil. Although the high tannin concentration in the flowers did not contribute much to the tannin yield due to their low proportion in the biomass, the flowering first cut had higher total PA concentrations (T-PA) compared to the 2nd and 3rd harvests. This is consistent with Theodoridou et al.⁴⁰, who found that the highest PA concentrations in sainfoin plants were found at an early flowering stage. A possible explanation is that upon reaching the flowering stage, growth is reduced as

the plant matures. Thus, the excess fixed carbon from photosynthesis is remobilized towards the synthesis of carbon-based secondary metabolites such as tannins. This leads to higher tannin content in the plants at the flowering stage compared to vegetative stage when biomass formation is the primary sink for fixed carbon.⁴²

The E-PA concentration in sainfoin had previously been reported to range from 23 to 48 mg g⁻¹ DM in leaves¹⁸ and 30 to 46 mg g⁻¹ DM in the whole plant.⁴³ It should be noted that in the majority of studies, PA are quantified for the whole plant and quantification of PAs is limited to determining E-PA. The E-PA concentration in sulla leaves (12 to 40 mg g⁻¹ DM) found in the present study is comparable to previously reported concentrations ranging from 22 to 40 mg g⁻¹ DM however, the PA concentration in stems was higher in the present study (16-26 mg g⁻¹ DM) than reported previously (4-14 mg g⁻¹ DM) (Table S2).^{44, 45} In the present study, PAs in sulla and sainfoin were dominated by E-PAs. A slight shift from E-PA to NE-PA was observed in big trefoil with the succession of harvests. Additionally, the degree of polymerisation (DP) of PA compounds also increased with plant maturity. As the increase of DP, strengthens the PA's binding capacity to plant protein or fiber,⁴⁰ this can further reduce the extractability of PAs. Hence, determining the PA content and extractability in the plant at different phenological stages can also help in the determining the optimal stage of harvest based on the nutritional demands of the livestock.

3.5.4 Profiling of PA concentration and composition establishes a basis for future breeding and selection opportunities for PA-rich forages

The majority of the selection and breeding programmes are focused mainly on increasing PA concentrations in conventional forages such as lucerne and clovers to exploit the beneficial effects of PAs in these forages. In lucerne, the PAs are found the seed coats only. In white clover, they accumulate mainly in the flowers, with leaves containing only trace amounts of PAs. Breeding trials on white clover were able to achieve flowers with elevated PA concentration and increased flowering prolificacy and this substantially increased the PA concentrations in the whole plant. However, increase in floral PA concentration and higher flower density led to a decrease in white clover persistence and agronomic performance in the fields. The results from that study suggested future research should focus on modulating PA expression in white clover leaves which can concomitantly maintain their agronomic performance in the field.^{46, 47}

An improved understanding of the source of variability of PA expression in PA-rich forages may substantially further the progress of knowledge-driven breeding programmes. In recent years, the genetic improvement of PA-rich forages has also started to gain momentum and efforts have been made to improve the persistence of birdsfoot trefoil and sainfoin in the field.^{48, 49} The extensive variation of PA distribution within the plant presents an opportunity to optimise the PA concentration to achieve desired bioactivity by altering the proportion of biomass yield from different plant organs. However, as seen in the case of white clover, altering the foliar PA expression has higher potential compared to floral PA levels, and PA levels in white clover leaves were able to reach up to 0.6% of DM in a genetic transformation experiment.⁵⁰ Similarly, screening of PA structure characteristics in forages is necessary for plant breeders to select and engineer forages with novel PA composition as these are known to play an important role in determining their bioactivity.⁴ Both the generally observed variability and particularly the stability of the structural PA characteristics, are important considerations for these breeding schemes.

In conclusion, our study showed that the variability of PA structural features was lower compared to PA concentration in PA-rich forages across the harvests. Similarly, the variability across cultivars of the same species was larger than compared to the variability across species. This indicates that the observed bioactivity in field experiments is likely to be representative for a range of cultivars of the same species and across several harvests, which ultimately is a main condition for the implementation of nutraceuticals in the field. In the long run, sufficient variability is available to attempt the alteration of PA structural characteristics in forages by breeding. This provides another important opportunity for plant breeders to enhance or tailor bioactivity of PA-rich species to meet particular needs. These features have been found to influence their antimethanogenic potential and their anthelmintic properties, which have important consequences to provide nutritional and environmental benefits for livestock production systems. However, further research is required in elucidating the structure-activity relationships of PA in biological systems and extrapolating these results from *in vitro* to *in vivo* studies. Concomitantly, research on possible interactions between PA and other PSMs such as saponins, flavonols, and terpenes could provide an insight into their fate in the biological systems.

ASSOCIATED CONTENT

Supporting information

Table S1: Concentration of different polyphenolic subunits across the different species and their cultivars; Table S2: Extractable (E-PA) and Non.extractable proanthocyanidin (NE-PA) concentration in different plant organs of the analyzed species and their cultivars; and Eq S1 and S2: Determination of proanthocyanidin amount in the plant organs.

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ABBREVIATIONS USED

PAs, proanthocyanidins; UPLC-MS/MS, ultraperformance liquid chromatography tandem mass spectrometry; PSMs, plant secondary metabolites; HTs, hydrolysable tannins; GTs, gallotannins; ETs, ellagitannins; PCs, procyanidin; PD, prodelphinidins; mDP, mean degree of polymerization; DM, dry matter; MRM, multiple reaction monitoring

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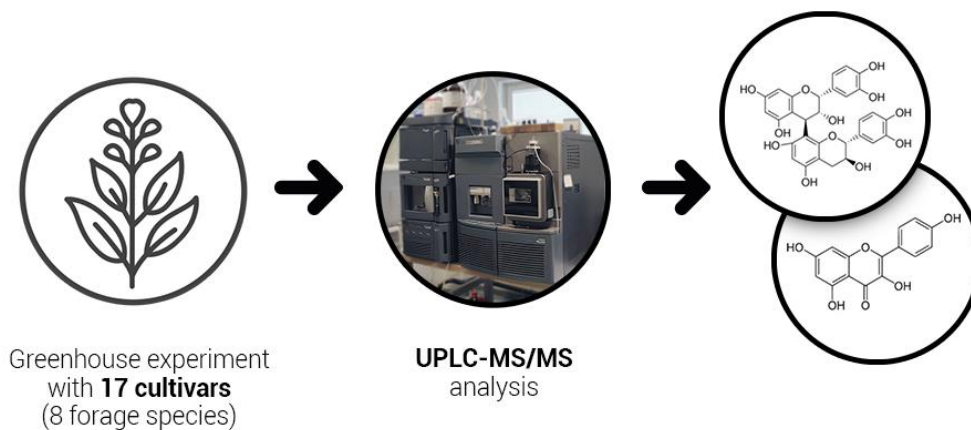
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CHARACTERISATION & QUANTIFICATION OF DIFFERENT POLYPHENOLIC GROUPS



3.7 Supporting Information

Table S1 Concentration of different polyphenolic subunits across the different species and their cultivars

Species	Cultivar	Harvest	Gallotannins (mg/g DM)	Ellagitannins (mg/g DM)	Quinic acid derivatives (mg/g DM)	Kaempferol derivatives (mg/g DM)	Quercetin derivatives (mg/g DM)	Myricetin derivatives (mg/g DM)	Procyanidin (mg/g DM)	Prodelfinidin (mg/g DM)
Chicory	Plumato	1	0 ± 0	0 ± 0	1.4 ± 0.8	0 ± 0	0.1 ± 0.1	0 ± 0	0 ± 0	0 ± 0
Chicory	Spadona	1	0 ± 0	0 ± 0	1.2 ± 0.8	0 ± 0.1	0.1 ± 0.1	0 ± 0	0 ± 0	0 ± 0
Sulla	Grimaldi	1	0 ± 0	0 ± 0	0.5 ± 0.2	0 ± 0	0 ± 0.1	0 ± 0	1.4 ± 0.2	20.7 ± 4.7
Sulla	Sudda	1	0 ± 0	0 ± 0	0.2 ± 0.1	0 ± 0	0.1 ± 0.1	0 ± 0	2.2 ± 0.2	23 ± 2.7
Birdsfoot trefoil	Bull	1	0 ± 0	0 ± 0	0 ± 0	0.7 ± 0	0 ± 0	0 ± 0	1.5 ± 0.5	0.8 ± 0.6
Birdsfoot trefoil	Lotar	1	0 ± 0	0 ± 0	0 ± 0	0.5 ± 0.1	0 ± 0	0 ± 0	1.3 ± 0.3	0.8 ± 0.3
Birdsfoot trefoil	Rocco	1	0 ± 0	0 ± 0	0 ± 0	0.4 ± 0	0 ± 0	0 ± 0	1 ± 0.4	1.1 ± 0.4
Big trefoil	Lot 29	1	0 ± 0	0 ± 0	0 ± 0	1.3 ± 0.8	0 ± 0.1	0.1 ± 0	1.8 ± 0.4	18.1 ± 2
Big trefoil	Wild type	1	0 ± 0	0 ± 0	0 ± 0	0.9 ± 0.1	0 ± 0	0.1 ± 0.1	1.4 ± 0.3	13.7 ± 3.8
Lucerne	Galaxy	1	0 ± 0	0 ± 0	0 ± 0	0.2 ± 0.1	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Sainfoin	CPI 63750	1	0 ± 0	0 ± 0	0.6 ± 0.1	0.7 ± 0.2	1.6 ± 0.3	0.2 ± 0.1	2.9 ± 0.6	14.1 ± 1.4
Sainfoin	Esky	1	0 ± 0	0 ± 0	0.5 ± 0.2	1 ± 0.2	1.7 ± 0.2	0.2 ± 0.1	2.8 ± 0.5	12.2 ± 2.1
Sainfoin	Visnovsky	1	0 ± 0	0 ± 0	0.9 ± 0	1.8 ± 0.4	1.6 ± 0.4	0.3 ± 0.1	2.7 ± 0.3	12.7 ± 2.6
Plantain	PLA60	1	0 ± 0	0 ± 0	0.5 ± 1.1	0 ± 0.1	0 ± 0.1	0 ± 0	0 ± 0	0 ± 0
Plantain	Svatojansky	1	0 ± 0	0 ± 0	0.3 ± 0.2	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Salad burnet	PI 308861	1	3.3 ± 0.5	38.7 ± 2.4	1 ± 0.1	0.1 ± 0.1	0.4 ± 0.1	0 ± 0	0.4 ± 0.5	0 ± 0
Salad burnet	Sang 10	1	4 ± 1.2	35.3 ± 3.5	1.5 ± 0.9	0 ± 0	0.3 ± 0.1	0 ± 0	0.6 ± 0.8	0.2 ± 0.1
Chicory	Plumato	2	0 ± 0	0 ± 0	1.6 ± 0.5	0 ± 0	0.1 ± 0.1	0 ± 0	0 ± 0	0 ± 0
Chicory	Spadona	2	0 ± 0	0 ± 0	1.8 ± 0.2	0 ± 0	0.1 ± 0.1	0 ± 0	0 ± 0	0 ± 0
Sulla	Grimaldi	2	0 ± 0	0.1 ± 0.1	0.3 ± 0.3	0 ± 0	0 ± 0.1	0 ± 0	1.5 ± 0.2	22.9 ± 3.8
Sulla	Sudda	2	0 ± 0	0.2 ± 0.4	0.1 ± 0.1	0 ± 0	0.1 ± 0	0 ± 0	2.1 ± 0.3	25.4 ± 5.6
Birdsfoot trefoil	Bull	2	0 ± 0	0 ± 0	0 ± 0	0.6 ± 0.1	0 ± 0	0 ± 0	1.2 ± 0.4	0.5 ± 0.2
Birdsfoot trefoil	Lotar	2	0 ± 0	0 ± 0	0 ± 0	0.5 ± 0.1	0 ± 0	0 ± 0	1.2 ± 0.2	0.6 ± 0.1
Birdsfoot trefoil	Rocco	2	0 ± 0	0 ± 0	0 ± 0	0.3 ± 0	0 ± 0	0 ± 0	0.7 ± 0.4	0.7 ± 0.3
Big trefoil	Lot 29	2	0 ± 0	0 ± 0	0 ± 0	0.8 ± 0.2	0 ± 0	0 ± 0	1.3 ± 0.7	10.4 ± 5
Big trefoil	Wild type	2	0 ± 0	0 ± 0	0 ± 0	0.8 ± 0.2	0 ± 0	0 ± 0	1.4 ± 0.7	10.2 ± 3.8
Lucerne	Galaxy	2	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Sainfoin	CPI 63750	2	0 ± 0	0 ± 0	0.5 ± 0.1	0.4 ± 0.1	0.8 ± 0.2	0 ± 0.1	1.6 ± 0.2	7.8 ± 3.2

Table S1 contd..

Sainfoin	Esky	2	0 ± 0	0 ± 0	0.3 ± 0.3	0.8 ± 0.1	0.9 ± 0.1	0 ± 0	1.8 ± 0.4	7.7 ± 2.2
Sainfoin	Visnovsky	2	0 ± 0	0 ± 0	0.5 ± 0.1	1.3 ± 0.3	1.3 ± 0.2	0.2 ± 0.1	3 ± 0.7	7.6 ± 1.7
Plantain	PLA60	2	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Plantain	Svatojansky	2	0 ± 0	0 ± 0	0.2 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Salad burnet	PI 308861	2	3.8 ± 1	43.3 ± 1.7	1.4 ± 0.7	0 ± 0	0.4 ± 0.2	0 ± 0	0.7 ± 1	0 ± 0
Salad burnet	Sang 10	2	3.2 ± 0.7	39.1 ± 3.3	1.5 ± 0.8	0 ± 0	0.3 ± 0.1	0 ± 0	0.8 ± 0.7	0.1 ± 0
Chicory	Plumato	3	0 ± 0	0 ± 0	1.5 ± 0.3	0 ± 0	0.1 ± 0.1	0 ± 0	0 ± 0	0 ± 0
Chicory	Spadona	3	0 ± 0	0 ± 0	1.3 ± 0.3	0 ± 0.1	0.1 ± 0.1	0 ± 0	0 ± 0	0 ± 0
Sulla	Grimaldi	3	0 ± 0	0 ± 0	0.2 ± 0.3	0 ± 0	0 ± 0	0 ± 0	1.4 ± 0.7	20.1 ± 12.1
Sulla	Sudda	3	0 ± 0	0 ± 0	0.2 ± 0.1	0 ± 0	0.2 ± 0	0 ± 0	2.4 ± 0.5	27.2 ± 8.4
Birdsfoot trefoil	Bull	3	0 ± 0	0 ± 0	0 ± 0	0.5 ± 0.1	0 ± 0	0 ± 0	1.2 ± 0.5	0.5 ± 0.3
Birdsfoot trefoil	Lotar	3	0 ± 0	0 ± 0.1	0 ± 0	0.3 ± 0.1	0 ± 0	0 ± 0	1 ± 0.2	0.5 ± 0.2
Birdsfoot trefoil	Rocco	3	0 ± 0	0 ± 0	0 ± 0	0.3 ± 0	0 ± 0	0 ± 0	1.3 ± 0.2	1.2 ± 0.4
Big trefoil	Lot 29	3	0 ± 0	0 ± 0	0 ± 0	0.6 ± 0.1	0 ± 0	0 ± 0	1.1 ± 0.3	6.7 ± 1.8
Big trefoil	Wild type	3	0 ± 0	0 ± 0	0 ± 0	0.5 ± 0	0 ± 0	0 ± 0	1 ± 0.4	6.1 ± 2.6
Lucerne	Galaxy	3	0 ± 0	0 ± 0	0 ± 0	0.1 ± 0.1	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Sainfoin	CPI 63750	3	0 ± 0	0 ± 0	0.4 ± 0.1	0.6 ± 0.2	1 ± 0.1	0 ± 0.1	1.3 ± 0.1	8.3 ± 1.3
Sainfoin	Esky	3	0 ± 0	0 ± 0	0.4 ± 0.1	1.1 ± 0.1	1.5 ± 0.1	0.2 ± 0	2.6 ± 0.7	12.6 ± 2.6
Sainfoin	Visnovsky	3	0 ± 0	0 ± 0	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.2	0 ± 0	1.2 ± 0.8	4.9 ± 3.2
Plantain	PLA60	3	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Plantain	Svatojansky	3	0 ± 0	0 ± 0	0 ± 0.1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Salad burnet	PI 308861	3	3.6 ± 0.8	43.8 ± 4.1	0.9 ± 0.1	0.1 ± 0.1	0.4 ± 0.1	0 ± 0	1.1 ± 0.8	0.1 ± 0.1
Salad burnet	Sang 10	3	3 ± 0.4	39.3 ± 1.6	0.9 ± 0.2	0 ± 0	0.2 ± 0	0 ± 0	0.6 ± 0.7	0.1 ± 0.1

Table S2 Extractable (E-PA) and Non.extractable (N-PA) proanthocyanidin concentration in different plant organs of the analysed species and their cultivars

Species	Cultivar	Harvest	E-PA _{leaves} (mg/g DM)	N-PA _{leaves} (mg/g DM)	E-PA _{stems} (mg/g DM)	N-PA _{stems} (mg/g DM)	E-PA _{flowers} (mg/g DM)	N-PA _{flowers} (mg/g DM)
Sulla	Sudda	1	25.17 ± 2.82	13.16 ± 4.73	16.61 ± 5.28	8.82 ± 1.41	0	0
Sulla	Grimaldi	1	22.04 ± 4.86	17.22 ± 1.81	31.86 ± 11.68	2.42 ± 3.41	0	0
Birdsfoot trefoil	Bull	1	2.34 ± 1.14	0.8 ± 0.57	0	0.18 ± 0.35	16.54 ± 4.43	11.48 ± 5.16
Birdsfoot trefoil	Lotar	1	2.09 ± 0.55	1.4 ± 0.55	0	0.68 ± 1.15	34.47 ± 9.38	15.39 ± 5.4
Birdsfoot trefoil	Rocco	1	2.06 ± 0.78	2.4 ± 0.47	0	1.84 ± 0.58	39.7 ± 12.82	24.6 ± 6.91
Big trefoil	wild type	1	15.08 ± 4.12	13.77 ± 5.93	17.52 ± 10.72	6.78 ± 3.95	65.26 ± 12.2	21.44 ± 4.23
Big trefoil	Lot 29	1	19.9 ± 2.33	12.2 ± 3.94	3.6 ± 0.57	1.78 ± 2.1	83.45 ± 9.26	2.33 ± 3.51
Sainfoin	Esky	1	15 ± 1.99	0.59 ± 0.14	5.51 ± 2.7	6.05 ± 1.4	8.92 ± 2.99	17.14 ± 1.97
Sainfoin	CPI 63750	1	17 ± 1.19	6.04 ± 1.61	2.31 ± 1.14	0	7.73 ± 1.76	1.67 ± 0.58
Sainfoin	Visnovsky	1	15.43 ± 2.78	10 ± 1.29	2.99 ± 2.46	0.98 ± 0.85	6.83 ± 2.85	4.37 ± 0.72
Sulla	Grimaldi	2	24.39 ± 4.03	12.88 ± 1.45	23.05 ± 4.99	7.19 ± 2.33	0	0
Sulla	Sudda	2	27.48 ± 5.84	10.99 ± 1.17	26.88 ± 7.69	4.67 ± 3.1	0	0
Birdsfoot trefoil	Bull	2	1.7 ± 0.68	1.06 ± 1.25	0	0.53 ± 0.38	0	0
Birdsfoot trefoil	Rocco	2	1.37 ± 0.68	2.14 ± 0.45	0	2.03 ± 0.55	0	0
Birdsfoot trefoil	Lotar	2	1.76 ± 0.3	1.91 ± 0.58	0	2.08 ± 0.86	0	0
Big trefoil	Wild type	2	11.62 ± 4.42	8.89 ± 4.27	8.32 ± 4.14	3.03 ± 2.71	0	0
Big trefoil	Lot 29	2	11.76 ± 5.72	16.08 ± 9.99	10.86 ± 9.68	3.48 ± 1.54	0	0
Sainfoin	Esky	2	9.5 ± 2.57	2.86 ± 1.96	0	0	0	0
Sainfoin	CPI 63750	2	9.4 ± 3.25	4.91 ± 3.72	0	0	0	0
Sainfoin	Visnovsky	2	10.61 ± 2.22	10.44 ± 4.27	8.4 ± 4.98	6.84 ± 1.14	0	0
Sulla	Grimaldi	3	21.49 ± 12.8	12.83 ± 4.7	4.31 ± 0.61	8.69 ± 1.58	0	0
Sulla	Sudda	3	29.58 ± 8.76	10.71 ± 3.65	8.04 ± 3.66	1.47 ± 0.32	0	0
Birdsfoot trefoil	Bull	3	1.68 ± 0.72	0.6 ± 0.69	0	1.44 ± 1.08	0	0
Birdsfoot trefoil	Lotar	3	1.51 ± 0.42	3.56 ± 0.92	0	1.81 ± 0.39	0	0
Birdsfoot trefoil	Rocco	3	2.48 ± 0.56	3.87 ± 0.81	0	1.88 ± 0.72	0	0
Big trefoil	Wild type	3	7.08 ± 2.96	10.14 ± 3.28	3.29 ± 3.87	7.51 ± 1.8	0	0
Big trefoil	Lot 29	3	7.77 ± 2.18	24.01 ± 10.65	9.71 ± 4.57	7.84 ± 5.26	0	0
Sainfoin	CPI 63750	3	9.61 ± 1.24	1.99 ± 1.98	0	0	0	0
Sainfoin	Esky	3	15.21 ± 2.93	1.68 ± 1.92	0	0	0	0
Sainfoin	Visnovsky	3	6.12 ± 3.88	10.95 ± 4.12	11.01 ± 1.94	0	0	0

E-PA_{leaves}: Extractable proanthocyanidins in leaves; N-PA_{leaves}: Non-extractable Proanthocyanidins in leaves; E-PA_{stems}: Extractable proanthocyanidins in stems; N-PA_{stems}: Non-extractable proanthocyanidins in stems; E-PA_{flowers}: Extractable proanthocyanidins in flowers, N-PA_{flowers}: Non-extractable proanthocyanidins in flowers

Determination of proanthocyanidin amount in the plant organs

The amount of Proanthocyanidins (Extractable (E-PA) and Non-extractable (N-PA) Proanthocyanidins in the plant organs were determined as below

$$(Eq-S1) \quad E-PA_{\text{leaves}} \text{ (mg per plant)} = E-PA_{\text{leaves}} \times Lr \times DW_{\text{plant}}$$

Where $E-PA_{\text{leaves}}$ was the E-PA concentration (mg/g DM) in the leaves, Lr was the leaf ratio per plant and DW_{plant} was the dry weight of the plant.

Similarly, $E-PA_{\text{stems}}$ and $E-PA_{\text{flowers}}$ were calculated and consequently, $E-PA_{\text{plant}}$ was calculated as follows,

$$(Eq-S2) \quad E-PA_{\text{plant}} = E-PA_{\text{leaves}} + E-PA_{\text{stems}} + E-PA_{\text{flowers}}$$

Chapter 4



Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Linking Metabolites in Eight Bioactive Forage Species to Their in Vitro Methane Reduction Potential Across Several Cultivars and Harvests

Accepted in Nature Scientific Reports

Linking metabolites in eight bioactive forage species to their *in vitro* methane reduction potential across several cultivars and harvests

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4.1 Abstract

An *in vitro* Hohenheim gas test was conducted to analyze the fermentation end-products from 17 cultivars of eight polyphenol containing forage species. The polyphenol composition and proanthocyanidin (PA) structural features of all the cultivars were analyzed with UPLC-MS/MS in leaves of vegetative or generative plants. The samples were incubated with and without polyethylene glycol (PEG, a tannin-binding agent) to separate the tannin-effect on methane (CH₄, ml/200mg DM) production from that of forage quality. Sulla and big trefoil, two particularly PA rich species, were found to have the highest CH₄ reduction potential of up to 47% when compared to the samples without PEG. However, concomitant reduction in gas production (GP, ml/200mg DM) of up to 44% was also observed. An increase in both GP and CH₄ production under PEG treatments, confirms the role of tannins in CH₄ reduction. Moreover, PA structural features and concentration were found to be an important source of variation for CH₄ production from PA containing species. Despite having low polyphenol concentrations, chicory and plantain were found to reduce CH₄ production without reducing GP. Additionally, interspecies variability was found to be higher than intraspecies variability, and these results were consistent across growth stages, indicating the findings' representativeness.

4.2 Introduction

One of the main environmental issues from ruminant livestock production are enteric methane (CH₄) emissions, which contribute to up to 40 % of the total livestock sector emissions worldwide¹. The radiative forcing capacity of CH₄ is 63 times higher than that of CO₂ over the first 20 years, and 27 times higher over the 100-year horizon. That makes CH₄ the second most important greenhouse gas after CO₂, regarding anthropogenic climate change². On the other hand, the very shorter atmospheric lifetime of CH₄ (around 12 years), results in a much higher potential to reduce the radiative forcing in the short term, compared to CO₂ and N₂O. Thus, it can provide a valuable opportunity to limit the extent of anthropogenic climate change until long term solutions are in place³. Although, CH₄ emissions from ruminants cannot be completely eliminated without losing the ability of ruminants to digest cellulose, their reduction is also desirable from an economic point of view, as energy losses in the form of CH₄ can amount up to approximately 2 to 12% of gross energy intake in ruminants⁴.

The inclusion of forages rich in plant specialized metabolites (PSMs) (e.g. tannins and saponins) in the animal diet can potentially reduce CH₄ emissions in the ruminants⁵. Particularly tannin-rich forages (TRFs) are considered a promising solution due to their multifunctionality and their beneficial effects on animal health and productivity. Tannin-rich forages can exert beneficial and anti-nutritional effects on ruminants, however their potential depends on tannin type and concentration supplied in the feed⁶. Generally, tannins are known to bind with feed constituents such as protein, fiber, and carbohydrates. They can potentially improve protein utilization of the forages by making protein-tannin complexes in the rumen (pH = 6-7), and transporting them to abomasum (pH < 3.5) and small intestine (pH < 7) where they will eventually dissociate due to post ruminal pH shifts and release proteins. This makes the protein available for gastric or pancreatic digestion, which can reduce feed energy losses in form of CH₄ emissions⁷. This is especially relevant for ruminant production systems in temperate regions where despite having high protein content, the conventional forages, such as lucerne (*Medicago sativa* L.) and white clover (*Trifolium repens* L.), have been associated with bloating and inefficient N utilisation^{8,9}. The high extent of protein degradation in the rumen increases high urinary N excretion, which could potentially lead to increased ammonia (NH₃) volatilization from the cattle manure and concomitantly negatively affect the environment⁶.

Tannins can be broadly classified as proanthocyanidins (PAs; *syn.* condensed tannins), hydrolysable tannins (HTs) and phlorotannins, and the latter are mainly found in marine organisms. Hydrolysable tannins can be further subdivided into gallic acid derivatives, ellagitannins and gallotannins¹⁰, while PAs exist in the form of polymers and oligomers of flavan-3-ol subunits, most often catechin or epicatechin procyanidins (PCs), and gallocatechin and epigallocatechin (prodelphinidins, PD)¹¹. Procyanidins and PDs differ from each other in terms of their hydroxylation pattern of their flavan-3-ol B-ring, with PD subunits containing three hydroxyl groups and PC subunits containing two hydroxyl groups in the B-ring⁶. The structural traits are important, particularly the percentage of PD subunits from total PAs (PD%) and the polymer size have been shown to affect the mode and extent of the desired bioactive properties from PAs¹². Both forages rich in PAs, such as sainfoin (*Onobrychis viciifolia* L.) and birdsfoot trefoil (*Lotus corniculatus* L.), as well as forages rich in HTs, such as salad burnet (*Sanguisorba minor*), have emerged as promising species that can improve N utilisation in the ruminants while simultaneously curtailing CH₄ emissions^{13,14}. When supplied in low to moderate concentrations, tannins (both PAs and HTs) in forages can potentially improve animal performance, reduce NH₃ volatilization from manure by redirecting N excretion from urine to feces, lower CH₄ emissions and decrease bloating incidences in cattle^{7,15}. Birdsfoot trefoil, one of the promising TRFs, has been found to increase milk yield and milk protein content compared to perennial ryegrass in Friesian cows, and improved wool growth in sheep¹⁶.

Several *in vivo* and *in vitro* studies have analyzed the antimethanogenic potential of TRFs; however, a high variability in the results from these studies makes it difficult to harness their benefit in the livestock systems¹⁷. This is likely due to the abovementioned high structural diversity of tannins, which makes it difficult to assess their bioactivity and understand their effect on animal physiology and nutrition. It has been widely recognized that PA structural characteristics are important determinants of bioactivity, however, in the majority of animal nutrition studies the structural characteristics or the composition of PAs and HTs are rarely considered. These structural characteristics are known to vary not only across different plant species but also within their cultivars and have been found to be affected by plant's phenological stage¹⁸. However, the variation within the cultivar for each species was found to be less than the variation across the species¹⁹. To date, no study has identified the antimethanogenic effect of several cultivars each from multiple forage species grown under identical environmental conditions and across multiple harvests to determine not

only the interspecies variability but also the reproducibility of these findings within species and across harvests, which is ultimately a main prerequisite for their use in practice.

Consequently, the study aimed to assess the *in vitro* CH₄ reduction potential while minimizing the aforementioned variations and accounting the often-neglected PA structural features. To our knowledge, this is the first study to analyze the CH₄ mitigation potential of eight agronomically important temperate forage plants grown under identical environmental conditions and harvested at the same phenological stage (Figure 1). Main objectives of this study were, (i) to analyze the inter- and intraspecies variation of the CH₄ mitigation potential of multiple forage species, (ii) to assess the tannin-effect on the CH₄ reduction from the TRFs, and (iii) determine the influence of PA structural features, such as PD% and PA polymer size, on the CH₄ production.

4.3 Results

Forage quality and tannin composition

The forage chemical composition of the leaves varied extensively across the species. While general trends existed, and herbs had lower CP and higher NDF concentrations compared to legumes, there was a large variation across different functional groups. In general, highest CP concentration was found in big trefoil and birdsfoot trefoil cultivars (224-272 g/kg DM) when plants were harvested at the flowering stage (first harvest) (Table 1), while sainfoin cultivars had the lowest CP concentrations (157-180 g/kg DM) of all legumes and generally the highest NDF concentrations (518-548 g/kg DM). In the vegetative stage at the second harvest, however, two sainfoin cultivars (CPI 63750 and Esky) had the highest CP concentrations of all species. However, generally the differences in CP concentration decreased with the vegetative stage and no clear differences were observed between legumes and herbs at this stage. Additionally, differences between cultivars of same species were mainly observed for CP concentration and the species ribwort plantain, salad burnet and chicory.

Significant differences among cultivars were observed for total polyphenol (TP), total flavonols (TF, sum of myricetin, quercetin and kaempferol concentration), and total tannin (TT) concentration ($P < 0.001$). The lowest TP was found in chicory, ribwort plantain, and lucerne. Lucerne was found to be void of polyphenols, and was used as tannin-free negative control in the experiment. Highest TF concentrations were found in sainfoin cultivars with a range from 1.3 to 3.8 g/kg DM, while ribwort plantain cultivars generally contained no

flavonols¹⁹. Salad burnet was the only HT containing forage in our study and had the highest TP concentrations ranging from 42 to 50 g/kg DM. Proanthocyanidin containing species were sainfoin, sulla, big trefoil and birdsfoot trefoil with concentrations ranging from 1 to 25 g/kg DM across both harvests. Proanthocyanidin structural features, PD% and mean degree of polymerisation (mDP), generally differed significantly ($P < 0.001$) across species, while differences across the cultivars of a single species were restricted to birdsfoot trefoil (Table 2)¹⁹. In birdsfoot trefoil, Rocco was consistently found to have significantly higher mDP values (12-14) and PD% (48-52%) than Bull (mDP: 9-10, PD%: 30-33.3%) and Lotar (mDP: 9-11, PD%: 33-39%)¹⁹.

Differences in gas and methane production were observed across species and their cultivars

The *in vitro* gas production (GP) from the forages differed significantly across the cultivars ($P < 0.001$) with the GP ranging from 25 to 54 ml/200mg DM across both harvests (Figure 2, -PEG). At the species level, chicory had the highest GP (44 to 54 ml/200 mg DM), while sulla had the lowest GP (25 to 32 ml/200 mg DM). The variability within the cultivars of any species for GP was only observed among the cultivars of sainfoin and ribwort plantain, with the plantain cultivars showing the largest differences, as Svatojansky had produced 28% less gas compared to PLA60 in the first harvest. The effect of harvest ($P = 0.63$) on GP was not significant; however, a significant interaction effect of cultivar and harvest ($P < 0.001$) on GP was observed. This was apparent for ribwort plantain, where the cultivar Svatojansky produced 40% more gas in the second harvest compared to the first harvest and as a result, its GP then exceeded that of the cultivar PLA60. For salad burnet on the other hand, the GP was reduced in the second harvest compared to the first harvest by approx. 26%, yet this difference was homogeneous across both the cultivars.

The CH₄ production was also influenced by the plant species and their cultivars ($P < 0.001$) and the effect of harvest ($P = 0.06$) was not significant (Figure 3, -PEG treatment). The lowest CH₄ production was observed for the ribwort plantain cultivar Svatojansky (5.6 ml/200 mg DM) and the sulla cultivar Sudda (5.6 ml/200 mg DM), followed by the big trefoil cultivar Lot 29 (6.8 ml/200 mg DM). Despite containing PAs, CH₄ production from birdsfoot trefoil (9-11 ml/200 mg DM) was the highest and was similar to lucerne (10.8 ml/200 mg DM). In general, the CH₄ production from the cultivars in the first harvest was lower compared to the second harvest and there was a significant cultivar-harvest interaction ($P < 0.001$). Within

species variation in CH₄ production was observed in the cultivars from birdsfoot trefoil, sainfoin and ribwort plantain. As seen for GP, the variability in CH₄ production between ribwort plantain cultivars was larger in the first harvest, with Svatojansky producing 35% less CH₄ than PLA60. In the second harvest, the variation between the cultivars decreased and Svatojansky produced 5% more CH₄ than PLA60. In birdsfoot trefoil, Rocco generally produced lowest CH₄ and had on average 10% lower CH₄ formation compared to other birdsfoot trefoil cultivars (Bull and Lotar). Largely, GP and CH₄ production from the cultivars were stable across the harvests.

The cultivars were observed to differ significantly in terms of CH₄ percentage in total gas (MP, ($P < 0.001$)). The MP ranged from 17 to 25% in the first harvest and from 20 to 26% in the second harvest. The effect of harvest was not significant ($P = 0.06$) on MP, however the interaction between harvest and cultivar was found to be significant ($P < 0.001$). Within species variability in MP was again limited to cultivars from sainfoin, ribwort plantain, and birdsfoot trefoil (Figure 4; -PEG treatment). Generally, the MP from tannin containing forages (20-26%) was found to be higher compared to ribwort plantain and chicory (17-21%). The lowest CH₄ produced per unit total gas production was found in ribwort plantain followed by chicory.

In general, there was a concomitant reduction of GP and CH₄ production from all the tannin containing species when compared to lucerne. It was observed that the decline in CH₄ production (7-47%) with TRFs (sulla, big trefoil, salad burnet and sainfoin), was higher than the GP reduction (5-43%) when compared to lucerne (Supplementary Table S2). Contrary to that, chicory cultivars were found to increase GP (7-21%) and reduce CH₄ production (5-25%) with respect to lucerne in both the harvests.

Addition of PEG, significantly increased gas and methane production from tannin rich forages

The addition of PEG resulted in increments of GP ($P < 0.001$) and decrements in CH₄ production ($P < 0.001$) during the *in vitro* fermentation (Figure 2 and 3, +PEG). These changes were, however, limited to the TRFs. Ribwort plantain and chicory cultivars did not change their fermentation patterns with the addition of PEG. As a result, significant interaction effect of cultivar and PEG treatment existed for GP ($P < 0.001$) and CH₄ production ($P < 0.001$). The GP in the +PEG treatment was found to range from 33 to 53 ml/200mg DM, while the CH₄ ranged from 5 to 12 ml/200mg DM. In the first harvest,

ribwort plantain cultivar Svatojansky produced the lowest CH₄ (5 ml/200mg DM) under the +PEG treatment, the salad burnet cultivar, Sang 10 had the lowest CH₄ production (8.9 ml/200mg DM) under the +PEG treatment. The highest change in GP and CH₄ production caused by the PEG addition was found with sulla cultivar, Sudda, where the +PEG treatment increased GP and CH₄ by 46 and 45 percent respectively, compared to the control (-PEG). Conversely, the lowest effect from the PEG addition in tannin containing forages, was observed on birdsfoot trefoil cultivars. While the effect of PEG treatment on the MP was significant ($P < 0.001$, Figure 4), there was no significant interaction between treatment and harvest for the MP. There was, however, a significant effect of three-way interaction between cultivar, treatment, and harvest ($P < 0.001$) observed for MP. Still, the major variations in MP were observed for birdsfoot trefoil and sainfoin in the first harvest, while in the second harvest, a significant treatment effect was observed on the MP from birdsfoot trefoil, big trefoil, salad burnet and plantain.

Covariates generally explain the variability of fermentation parameters

A model comparison was done by substituting fixed factors, cultivar and harvest, sequentially with measured covariates, to determine whether the measured covariates explain the formation of fermentation end products (CH₄, GP and MP) equally well, compared to the fixed factors. Yet, replacing the fixed factors with the covariates was not able to improve the quality of the model in terms of the AIC value for neither CH₄ (AIC=312) nor GP (AIC=1749; Table 3). Similarly, for MP the AIC was -646. The marginal R² for CH₄ and GP of the final models were generally high with 0.81 and 0.68, respectively. This was despite them being lower than the respective marginal R² values of the reference model, with 0.89 and 0.88 for CH₄ and GP, respectively. With regards to covariates, the covariates which significantly affected the fermentation products differed among CH₄, GP and MP. Accordingly, the GP was significantly increased by the TF concentration, and decreased by HHDP, PD%, and mDP, while the CH₄ production was decreased by PA concentration, CP, and mDP. The MP was only significantly decreased by the PD%.

4.4 Discussion

The inclusion of forages with diverse functional groups (e.g. legumes) in mixtures generally improves the yield stability in the grassland-based livestock production systems^{20,21}. Forages rich in tannins and other polyphenols provide even more benefits as they have been shown to decrease CH₄ and N emissions from ruminant based production systems^{22,23}. Although,

several studies have analyzed TRFs for their effect on animal performance and CH₄ reduction potential, the results till date have been contrasting, making it difficult to adapt the forages on the large scale. The reason for these contrasting results is assumed to be at least partially on the environmental conditions during plant growth, as these can affect tannin synthesis, as well as on colorimetric tannin assays that do not identify tannin structural characteristics or other compounds with potential interactions^{17,24}. Simultaneously, CH₄ emissions can vary highly across different animal species as well as the measurement methods. Both *in vivo* and *in vitro* techniques have been found to have their own set of advantages and disadvantages, leading to inconsistent results across different studies. *In vivo* techniques can accurately identify the impact of TRFs in an animal, yet they require large amounts of feed and are highly cost intensive thus, limiting the number of replicates or treatments. Additionally, they are still affected by the pre-diet and breed of the experimental animals hence, variability still exists across several *in vivo* studies²⁵. On the other hand, *in vitro* studies are inexpensive and can be used to test several feedstuffs simultaneously with sufficient number of replicates, yet they fail to take into account the changes in rumen processes such as shifts in ruminal pH as well as ruminal buffering capacity during the fermentation of the feed^{26,27}. However, *in vitro* studies are crucial in screening the promising feed additives on ruminal fermentation and predicting their effects *in vivo*. In the present study, the comparative assessment of 17 cultivars from eight agronomically promising temperate forage species, across two harvests, provides a novel insight of how the bioactive properties of these forages vary across the different species, and how stable are their antimethanogenic activity across different species and their cultivars under these harvests.

PA structural characteristics were found to be more stable than forage chemical composition across harvests

The CP levels in legumes are known to generally be in the range of 180 to 300 g/kg of DM and this mostly exceeds animal requirements (130-170 g/kg of DM)²⁸. As expected, in the present study, CP concentrations in the forages were higher in the second harvest where the plants were harvested at the vegetative stage compared to the first harvest (flowering stage). This can be explained by the much higher leaf shares in second harvest, as leaves are richer in CP than stems²⁹. Higher CP content increases the digestibility of the forage, as it is easily degraded by microbes³⁰. Conversely, high NDF and ADF content in the forages is known to be an indicator of lower forage digestibility²⁸. A meta-analysis by Maccarana, et al.³¹ found that NDF content in the feed is negatively correlated to GP in the rumen. It can reduce the

availability of rapidly fermentable carbohydrates leading to lower microbial activity in the rumen and hence a decrease in GP³². The flavonoid content of the cultivars was also determined, due to potential matrix effects between tannins and flavonoids, as well as because of known anti-inflammatory and antimicrobial properties that are beneficial for ruminant immunological system (Olagaray and Bradford 2019). Assessing the effect of tannins on ruminants based only on their concentration has given rise to ambiguous results in the past as structural traits of tannins have been found to play an important role in determining their bioactivity¹¹. Consequently, PA structural features were also analyzed in addition to concentration as they have been hypothesised to play an important role in determining their bioactivity, yet are rarely analysed in the animal studies. In the present study, these features were found to be stable across the harvests, and within the cultivars of the same species, the differences were comparatively lower than the variability across the species.

Methane and gas production from TRFs were consistently lower than lucerne in both the harvests

Numerous studies have demonstrated that TRFs reduce enteric fermentation in ruminants, resulting in lower GP and CH₄ production³³⁻³⁵. In the first harvest, across all the species with varying forage quality and plant metabolite composition, the largest reductions in CH₄ and GP were observed from TRFs, which were sulla, big trefoil, sainfoin and salad burnet, in comparison with lucerne. This could be attributed to the high TT concentration in these forages compared to lucerne. This is in line with the previous studies, where TRFs reduced the fermentation in the ruminants resulting in lower GP³⁴. Tannins have the propensity to bind with protein and carbohydrates (both structural and non-structural carbohydrate), preventing fermentation and degradation of the molecules, thus lowering GP³⁵. A study by Jayanegara, et al.³⁶ found that HTs are more potent compared to PAs. In the present study, however, this was not confirmed, as both the GP and CH₄ production of PI 308861 (salad burnet) were found to be higher than those of Sudda (sulla) and Lot 29 (big trefoil). This was despite the higher TT and TP concentrations in salad burnet, which reaffirms the results from studies, indicating that the extent of CH₄ reduction from TRFs does not only depend on tannin concentration but also on the tannin source and structural features^{37,38}. At the animal scale, the differences between PAs and HTs bioactivity is also likely to be dependent on their fate in biological systems. Proanthocyanidins exist in the form of large polymers, enabling them to bind to the feed components strongly and they do not get easily degraded in the

rumen. On the other hand, HTs have been shown to undergo hydrolysis, hence degrading to form low molecular weight phenolics in the rumen, and can thereby, lose part of their bioactivity^{39,40}. However, the enzymatic or auto-oxidation of the initial HTs or their hydrolysis products could also produce large bio-oligomers or polymers with tannin-like functions in the rumen or small intestine³⁹. Consequently, this makes it difficult to assess the fate of tannins in biological systems from *in vitro* studies alone. A general trend of higher GP and CH₄ production, when plants were harvested at the vegetative stage (second harvest), was recorded compared to when they were harvested at the flowering stage (first harvest) which could be a result of lower tannin content in the plants in the second harvest. Furthermore, lower content of instantly fermentable fractions, such as water-soluble carbohydrates in the leaves at the flowering stage can result into lower GP⁴¹. Interestingly, the species with lower TP and TT content, such as ribwort plantain and chicory, were also found to reduce CH₄, but it was not accompanied by reduction in GP. This could result from PSMs other than tannins, found in these forages. A study by Navarrete, et al.⁴², found that chicory is rich in inulin and sesquiterpene lactones and, ribwort plantain leaves are known to contain acteoside, aucubin, and catapol.

Additionally, it is important to look at the CH₄ percentage (MP) in the total gas, as this can be used as an indicator of the potential CH₄ emissions per unit of organic matter (OM) degraded. Due to the concomitant reductions in GP and CH₄ from TRFs, their MP ranged between 21% and 26% and hence was comparable to that of lucerne (24%) (Supplementary, Table S3). Chicory and ribwort plantain (17-21%) had the lowest MP compared to the other tested species in this study. The reduction in the CH₄ from these species was not accompanied by the reduction in GP indicating their higher potential for CH₄ abatement in ruminants without negatively affecting ruminal fermentation

PEG treatment affirmed the antimethanogenic activity of TRFs, however no influence on methane percentage in total gas was observed

The inclusion of PEG as a tannin inactivating agent, enabled the quantification of the specific tannin-effect from different species on the ruminal fermentation *in vitro*. Polyethylene glycol can inactivate tannins by displacing proteins from tannin-protein complexes, and binding with tannins to form tannin-PEG complexes. Increased GP and CH₄ production from TRFs under +PEG treatment could be a result of high availability of protein after being dissociated from tannin-protein complexes. The increased availability of N compounds that can no longer

bind to tannins with the addition of PEG leads to more substrate for microbial degradation and higher microbial growth. This in turn results into increased ruminal fermentation and higher GP^{38,43}. Simultaneously, as the tannins are inactive they can no longer influence the methanogens in the rumen which could affect CH₄ production from these species. As expected, no differences in GP and CH₄ production were observed from the samples of ribwort plantain, lucerne and chicory, due to the absence of tannins in these species. Generally, the variation in the extent of GP and CH₄ reduction from across the different TRFs under -PEG and +PEG treatments was in line with the variation in tannin concentration and composition. Accordingly, stronger PEG-treatment effect was observed in cultivars with higher tannin concentrations, which is in line with the studies by Basha, et al.⁴³ and Jayanegara, et al.³⁵.

Although, it is evident that tannins reduce CH₄ emissions, the mode of action by which they reduce CH₄ in ruminants is still not well understood. A study by Hassanat and Benchaar³³ found that the PA (acacia and quebracho) and HT (chestnut and valonea) extracts had a more pronounced effect on rumen methanogenesis compared to the substrate degradation³³. Tannins are found to modify the rumen microflora by either directly inhibiting methanogenic population or indirectly affecting methanogen-protozoa symbiosis by inhibiting protozoal population in rumen^{7,17}. In a study by O'Donovan and Brooker⁴⁴, PAs from acacia were able to change the morphology of *Streptococcus bovis*, *Butyrivibrio fibrisolvens* and *Prevotella ruminicola*, and, hence, affected their activity. Additionally, HTs and PAs from different plant sources were also found to decrease CH₄ production by suppressing the methanogenic archaea and protozoal populations *in vitro*⁴⁵.

PA structural features were able to explain the variability in methane production from PA containing species but model was not improved

As mentioned above, not only the differences between HT and PA are relevant, but also their structural characteristics affect their potential bioactivity. Both mDP and PD% are among the most relevant features of PAs, determining their protein precipitation activity, which in turn is linked to anthelmintic and antimethanogenic bioactivity of PAs^{11,46}. Hence, in the present study, a stepwise regression was performed to analyze which forage parameters were best suited to explain the total gas and CH₄ production. By identifying covariates as potential predictors, fermentation patterns and CH₄ reduction potential could be estimated from plant traits. This would enable extrapolations to any other species in terms of their bioactivity and

would potentially help in predicting their influence on ruminants. However, the updated model (covariate-based model) with forage composition parameters as covariates did not improve the base model in terms of AIC value. Nevertheless, the conditional R^2 for CH_4 and GP remained the same and even increased for MP. This indicates that the overall updated model, including both fixed and random effects, was able to explain the variance of CH_4 and GP in the present study for PA containing species. However, in order to predict the fermentation parameters, cultivar remains the better estimate compared to the sum of all covariables, indicating that due to the large number of factors with interconnected effects, the tannin source remains more relevant compared to the traits alone. This could also arise from the fact that in addition to PAs, plants may produce other bioactive PSMs such as terpenes, organic acids, saponins and others which can also influence the CH_4 production from the substrate^{6,47} which were not quantified in this study. Furthermore, the influence of matrix effects between tannins and other PSMs on tannin bioactivity remains unexplored. Still, the model quality was sufficient to identify parameters which had a strong influence on the *in vitro* fermentation end-products. Particularly in case of CH_4 , the variables CP, PA concentration and mDP, were found to exhibit a negative relationship with CH_4 production. This is in accordance with the study by Hatew, et al.⁴⁸, which observed that the polymer size (mDP) was an important determinant of *in vitro* CH_4 and GP production from the substrate. With regards to MP, however, both PA and PD% were found to have limited influence as indicated by their near-zero coefficient and low marginal R^2 (0.05). One solution to improve the predictive capabilities of CH_4 reductions from tannin traits compared to forage quality traits might be the inclusion of tannin extracts in *in vitro* incubation instead of the whole plant sample.

The tested forage species are promising alternatives as sustainable feedstock in livestock production systems

The results in the present study underpin that TRFs have high potential for their use in livestock production as their CP content across both harvests was comparable to lucerne. This was further demonstrated by the GP production in the presence of PEG, which did not vary significantly from lucerne. The inclusion of PEG treatment further established the role of tannins in the antimethanogenic activity of TRFs. Hydrolysable tannins have been observed to affect methanogens in the rumen, however, the mode of action of PA containing forages has been found to be variable²⁴. A significant treatment effect was observed when sulla and big trefoil were employed as a substrate, indicating their higher antimethanogenic activity

which resulted from their high PA content and potent PA structural characteristics. Sulla is a highly palatable forage and its supplementation can reportedly reduce the dependency of the livestock production systems on proprietary anthelmintics⁴⁹. The supplementation of fresh fed big trefoil was found to reduce CH₄ emissions by 26% (g CH₄ kg⁻¹ DM intake) compared to lucerne in sheep⁵⁰. However, the nutritional value of sulla and big trefoil need to be evaluated before providing them as a substantial feed component. Reportedly, owing to the high tannin content, sulla and big trefoil can compromise both, N digestibility and microbial activity in rumen which leads to lower GP, in addition to CH₄ reduction⁵¹. Furthermore, forages containing PAs with high mDP and high PD% (big trefoil, sulla and sainfoin) are able to reduce protein degradation more effectively than PC-rich forages such as birdsfoot trefoil³⁸. Additionally, PA concentration in the leaves of birdsfoot trefoil in our study, was found to be too low (approx. 0.1%) to exhibit detectable antimethanogenic activity. The concomitant reduction in GP and CH₄ from TRFs in the present study, makes it essential to research on how these forages can be incorporated in animal feed and in which proportion, to exploit their beneficial effects. A study by Orlandi, et al.⁵² found that even though total-tract N digestibility by PA inclusion decreased linearly with the increased concentration, there was a linear increase in N retention and the efficiency of N utilization was improved with the inclusion of tannin extracts of *Acacia mearnsii* until the concentration of 18 g/kg DM, implying that the positive impact of duodenal flux of amino acid supply can outweigh the reduction in protein digestibility when tannins are supplied at optimum levels⁵². Although more research is required to determine the relationship between PAs and post ruminal amino acid availability, it is well established that low to moderate PA concentrations leads to increased rumen undegraded protein and post ruminal amino acid flux which improves the N utilization efficiency in ruminants⁶.

Furthermore, the mechanism by which tannins exert their antimethanogenic activity needs to be investigated. There is a possibility of rumen microbiome can get adapted to these bioactive compounds which could influence their effect in the long term and, thus, their CH₄ reduction potential^{6,53}. However, the research on this topic is still limited, and further research is warranted to understand long-term feeding effects of tannin-containing legumes rumen microbiome and their antimethanogenic activity. The stability of PA structural features across the different harvests is a promising ground to include PA structural features for future structure-activity relationship studies of tannins.

Additionally, this study also showed that underutilized forbs, such as chicory and ribwort plantain, have a high CH₄ reduction potential despite being void of tannins. The CH₄ reduction from these species did not result in a negative impact on GP when compared to the other tested species in this study. This is in line with an *in vivo* study by Niderkorn, et al.⁵⁴, which found that sheep produced 23% lower CH₄ per kg DM intake with pure chicory compared to when fed with pure perennial ryegrass (*Lolium perenne* L., cv. AberAvon) which no significant differences in the DM digestibility. The bioactive compounds, aucubin and acteoside, in these forages are found to reduce net NH₃ production during ruminal fermentation, and increase potential total GP⁴². Both chicory and ribwort plantain are promising alternatives to produce high quality feed to overcome feed-deficits during dry seasons due to their drought tolerance, and their inclusion in grass-clover mixtures can enhance above- and below-ground primary production in temporary grasslands^{55,56}. Furthermore, they are known to benefit animal health due to their antimicrobial and anthelmintic properties which could be attributed to the presence of the PSMs other than polyphenols which were not analyzed in this study^{23,57}.

4.5 Conclusions

In summary, low intraspecies variation compared to interspecies variation in antimethanogenic potential of these species is a promising basis for breeding and selection opportunities. Our study found that the intraspecies variability was much lower compared to the interspecies variability in terms of polyphenolic composition as well as PA structural features, and these features were generally stable across the harvests. Furthermore, these variations were translated to the antimethanogenic activity of the species. The reproducibility of the bioactive effects of TRFs across both the harvests provides a promising outlook of their potential to reduce CH₄ in the livestock production systems. However, as the measurements were conducted under controlled conditions, it is crucial to assess their potential under field conditions. Additionally, inclusion level of these species in ruminant diet should also be assessed to prevent their negative effects on digestibility and animal performance. Inclusion of PEG treatment was able to establish the role of tannins in the antimethanogenic activity of TRFs. Furthermore, it was illustrated that the antimethanogenic property of the PA containing forages is at least in part a consequence of PA structural characteristics, as the highest CH₄ reduction was observed from sulla containing highest PD% and mDP value. Moreover, the stability of PA structural characteristics across the harvests and less variation within the cultivars of the same species than across the species,

provides an opportunity for breeding and selection programmes to modulate PA structural characteristics in the forages. Hence, follow up studies to screen the species with high antimethanogenic property for their variation in PA structural features and bioactivity are suggested, which can in turn improve the predictability of structure-activity relationship of tannins. Assessing the effect of PA structural features in addition to PA concentration on the bioactivity of these forages could be a useful criterion to understand the diverse effects of different plant sources containing similar PA concentration, as these differences have been shown to produce quantifiable effects.

4.6 Methods

Experimental design

A greenhouse experiment was conducted with 17 different forage cultivars from 8 species (Table 4). The seeds for the plants were obtained from the seedbank of IPK Leibniz Plant Genetics and Crop Plant Research, Gatersleben. The plants were harvested twice at different developmental stages. In the first harvest, plants were harvested at the flowering stage and in the second harvest, which was done after a 6-week regrowth interval, plants were harvested at the vegetative stage. The plants were grown in flower pots of 24x24x40 cm³ (LxWxH) each and 5 plants were sown in each pot. Each cultivar had 4 pots as replicates. The pots were arranged in a complete randomized design and reflective foils were installed to ensure even lighting. For details see Verma, et al. ¹⁹. The plants were consequently separated into leaves, stems and flowers. The samples were freeze dried and stored at -80 °C for further analysis. The leaf samples were subsequently ball milled, and stored at -80 °C to preserve the structural integrity of the polyphenols in the sample. The average monthly temperature in the greenhouse during the plant growth ranged from 24-27 °C (March to August). The experimental research on plants complies with the relevant institutional, national, and international guidelines and legislation.

Chemical composition of forages

Nitrogen concentration of the forages was determined by the DUMAS rapid combustion method using a CN elemental analyser (Vario Max Elementar Analysensysteme, Hanau, Germany). Crude protein (CP) was calculated by multiplying the N concentration with 6.25. The concentrations of neutral detergent fiber (NDF) was analysed according to Van Soest detergent procedure ⁵⁸ and AOAC method 973.18. The methods were adapted to be used with fiber analyser Ankom A220 (Ankom Technology, Macedon, NY, USA).

Polyphenol and tannin analysis with UPLC-DAD-MS/MS

The leaf tannin extracts were prepared for the UPLC-DAD-MS/MS as described by Verma, et al.¹⁹. The samples were analyzed with an Acquity UPLC (Waters Corp., Milford, MA, USA) coupled with a XEVO triple-quadrupole mass spectrometer (Waters Corp., Milford, MA, USA) as described by Engström, et al.⁵⁹. The system consisted of a sample manager, a binary solvent manager and a column, coupled with diode-array detector. The UV spectra was recorded. For the elution, acetonitrile (A) and 0.1% aqueous formic acid (B) were used with a constant flow rate of 0.5 mL min⁻¹ with the following elution profile: 0–0.5 min, 0.1% A (isocratic); 0.5–5.0 min, 0.1–30% A (linear gradient); 5.0–8.0 min, 30–45% A (linear gradient); 8.0–11.5 min, column wash and stabilization. The data was recorded from 0 to 6 min for UV-vis (190–500 nm) and MS data (m/z 100 to 2000). The specifications for the negative electrospray ionization are as follows; capillary voltage: 2.4 kV, desolvation temperature: 650 °C, source temperature: 150 °C, flow rate of desolvation and cone gas (N₂): 1000 and 100 L/h, respectively, and collision gas: argon.

Hohenheim gas test

From the 4 pots per cultivar, due to analytical constraints, 3 pots were selected for analysis with the Hohenheim gas test (HGT) in the first harvest. For the same reason, in the second harvest, the biomass from all pots for each cultivar was pooled. The GP and CH₄ production from the samples were analyzed using *in vitro* HGT⁶⁰. The plant samples (200 ± 1 mg) were added to 100 mL calibrated glass syringes (Haeberle Labortechnik, Lonsee-Ettlenschieß, Germany) and rotated inside the incubator (39°C) for 24h. The samples were run in triplicates and each run was performed on two different days leading to 6 replicates per sample. Additionally, to standardize each run, four blanks (no plant material) as well as hay and concentrate standards from the Institute of Animal Science, University of Hohenheim, were used as a reference, and were included in triplicates. The animals were fed a ration consisting of grass hay (3 kg) and concentrate (3 kg) divided into 2 meals (7 a.m. and 4 p.m.). Ruminal fluid was collected prior to morning feeding from two ruminally-cannulated, non-lactating crossbred heifers (Jersey × German Black Pied). It was filtered with cheese cloth into a prewarmed insulated flask, immediately transferred to the lab, and mixed with a freshly prepared buffer solution at 1:2 ratio (v/v). The mixture was continuously stirred and flushed with CO₂ while maintained in a water bath 39°C. The buffered ruminal fluid (30ml) was added to the syringes containing plant samples and syringes were placed back in the

incubator with the rotor set to about one rotation per minute during the period of incubation (24 hours). The GP and CH₄ production from the samples were measured at the time interval of 8 and 24 hours. The GP was recorded from the scale printed onto the syringes and methane concentration in the fermentation gas was measured with an infrared spectrometer (Methan AGM 10, Firma Sensors Europe, Ratingen, Germany). The infrared spectrometer was calibrated with pure nitrogen gas (zero point) and a gas mixture of methane and CO₂ (60:40) as standard. The measured GP volume from the samples after 24 h was corrected with an average of the factors obtained from hay and concentrate standards. These constant values were calculated based on the measured and the targeted GP values of 45.9 ml/200 mg DM and 65.13 ml/200 mg DM after 24 h incubation of hay and concentrate standards, respectively.

Additionally, to evaluate the specificity of tannin effect on the gas production parameters, PEG treatment was included to attenuate the effect of tannin on fermentation. The samples were incubated with 200 mg PEG (MW: 6,000 kDa). The PEG treatments were referred as +PEG (samples with PEG) and -PEG (samples without PEG).

Statistical Analysis

The statistical analysis was done with using the software R⁶¹. A linear mixed model was used with cultivars, harvests, treatment, and their interactions (two- and threefold) as fixed factors. Analytical replicates from the HGT per experimental pots and per date of experiment were used as random factor. Graphical residual analysis was performed and based on that, the data was assumed to be normally distributed and heteroscedastic. An ANOVA and multiple contrast tests were conducted to compare the significance of the influence factors on different levels. All tests of significance were made at $P < 0.05$.

Second, to assess whether the measured forage quality parameters of PA containing species can explain the fermentation end products, a (forward) model selection was done based on the AIC value (Akaike information criterion) as described by Venables and Ripley⁶². Therefore, the same model as above was used here as a reference model containing a subset of only PA containing species and the “-PEG” values (without the corresponding PEG treatment effects). In the reference model, the fixed factors were cultivar (only PA containing species), harvest and their interaction effect, and the random effect were the analytical replicates from the HGT per experimental pots and per date of experiment. Subsequently, the previous fixed factors were removed and replaced consecutively with forage trait derived

covariates (forage quality and tannin traits). The decision for or against a certain covariate was also based on its significance and its variance inflation factor (VIF; <5) until the optimal model with the lowest complexity was achieved. The final, covariate-based model was then compared with the reference model.

Data availability statement. Data sharing not applicable. The data generated during the study is included within this article and supplementary information.

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Author contributions

Conceptualization, C.S.M.; data curation, C.K. and S.V.; formal analysis, S.V., M.H., and C.S.M.; funding acquisition, C.S.M.; investigation, S.V.; methodology, S.V., M.H. and R.B.; project administration, C.S.M.; supervision, C.S.M. and F.T.; visualization, S.V.; writing—original draft, S.V.; and writing—review and editing, S.V., S.W., J-P. S., M.H., A.S., R.B., C.K., F.T., and C.S.M. All authors have read and agreed to the published version of the manuscript

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information

4.8 Figures

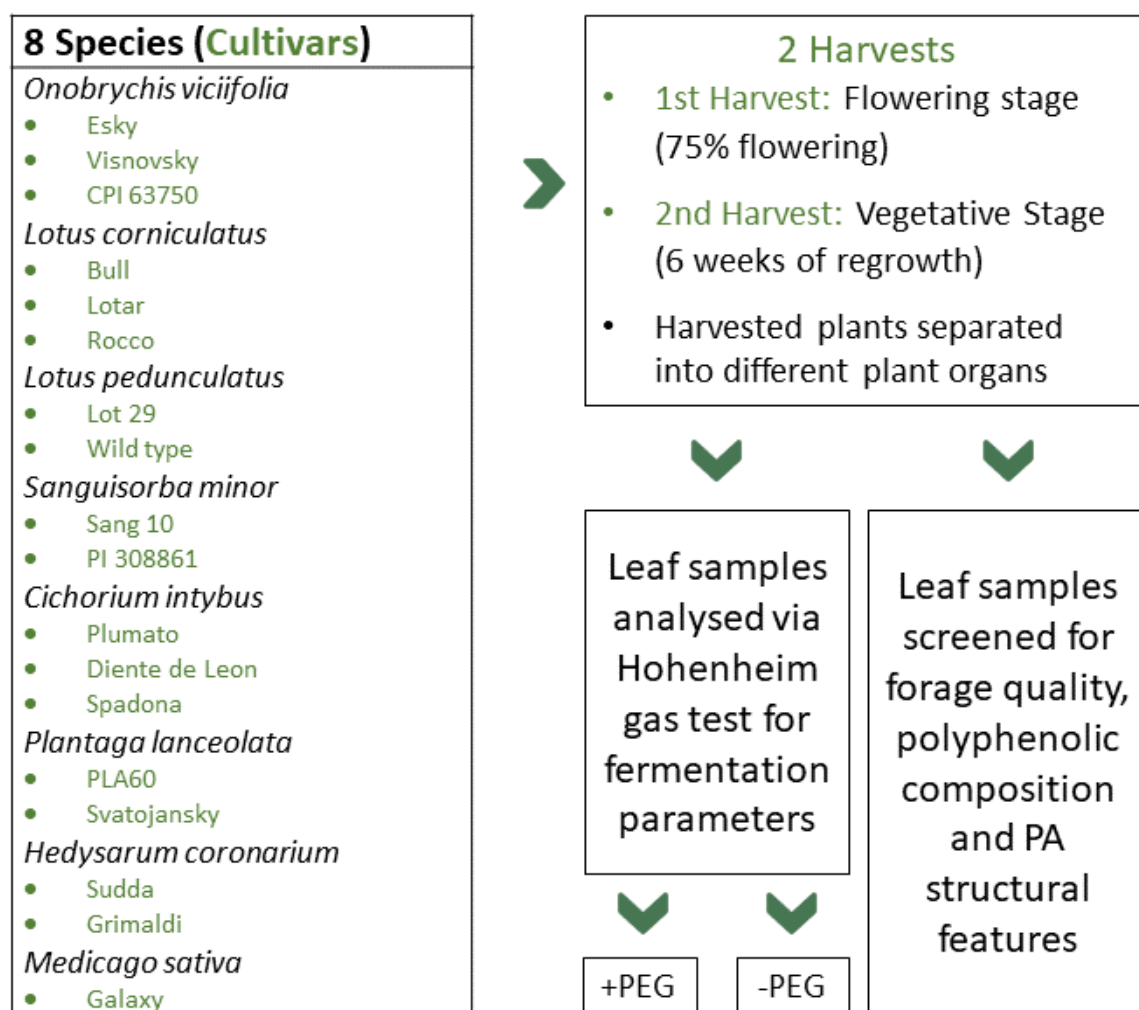


Figure 7 A greenhouse experiment was conducted with 17 cultivars from eight forage species. The cultivars were harvested twice at different phenological stages (Flowering and vegetative stage). Harvested plants were analysed for their polyphenolic composition, PA structural features, and antimethanogenic potential.

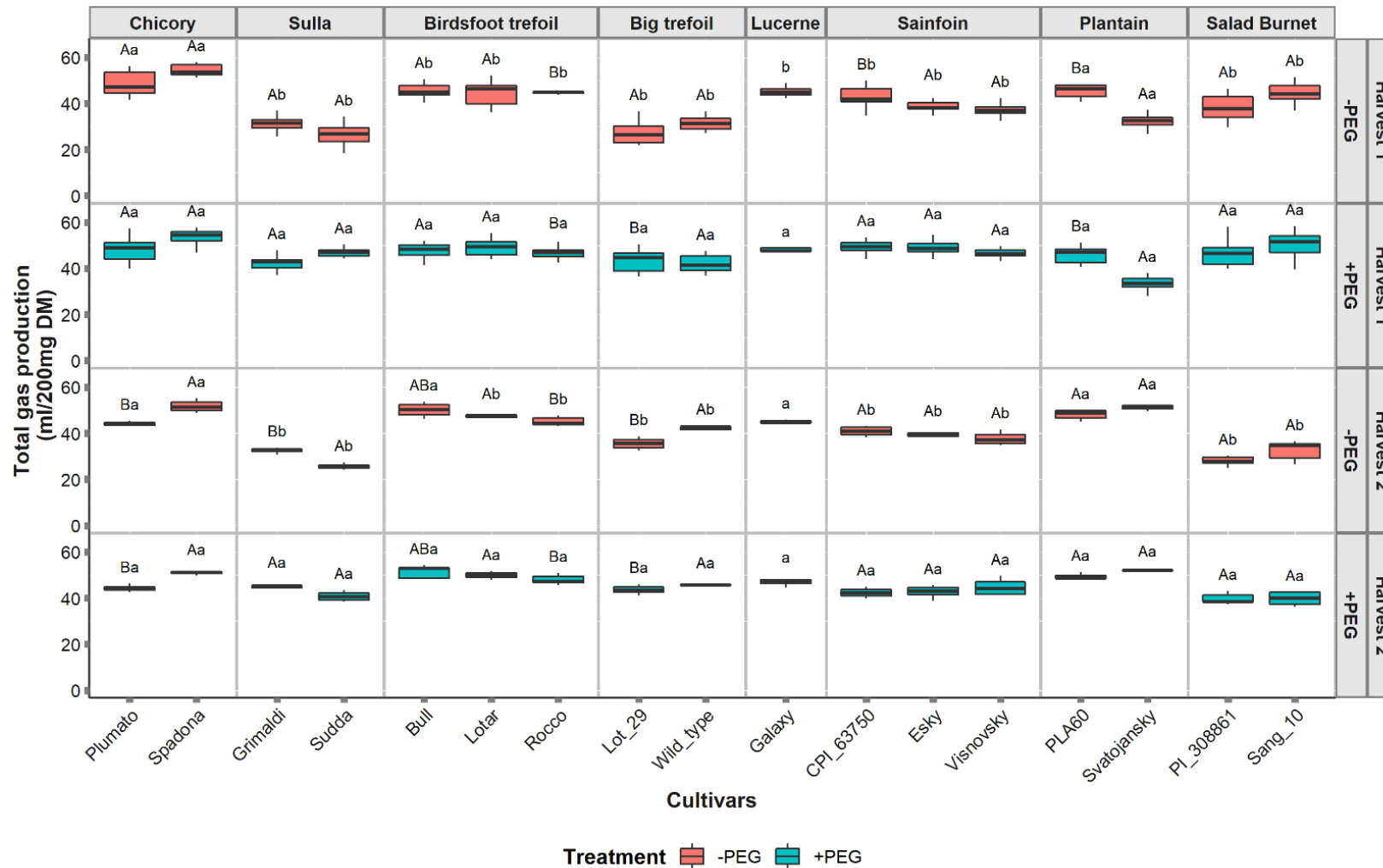


Figure 2 Gas production from 17 cultivars of 8 species under different treatments for two harvests. -PEG indicates the samples incubated without polyethylene glycol (PEG) and +PEG refers to the samples incubated with PEG. Different uppercase letters represent significant differences ($P < 0.05$) between the cultivars for each species, and different lowercase letters represent the significant differences within the treatments for each cultivar.

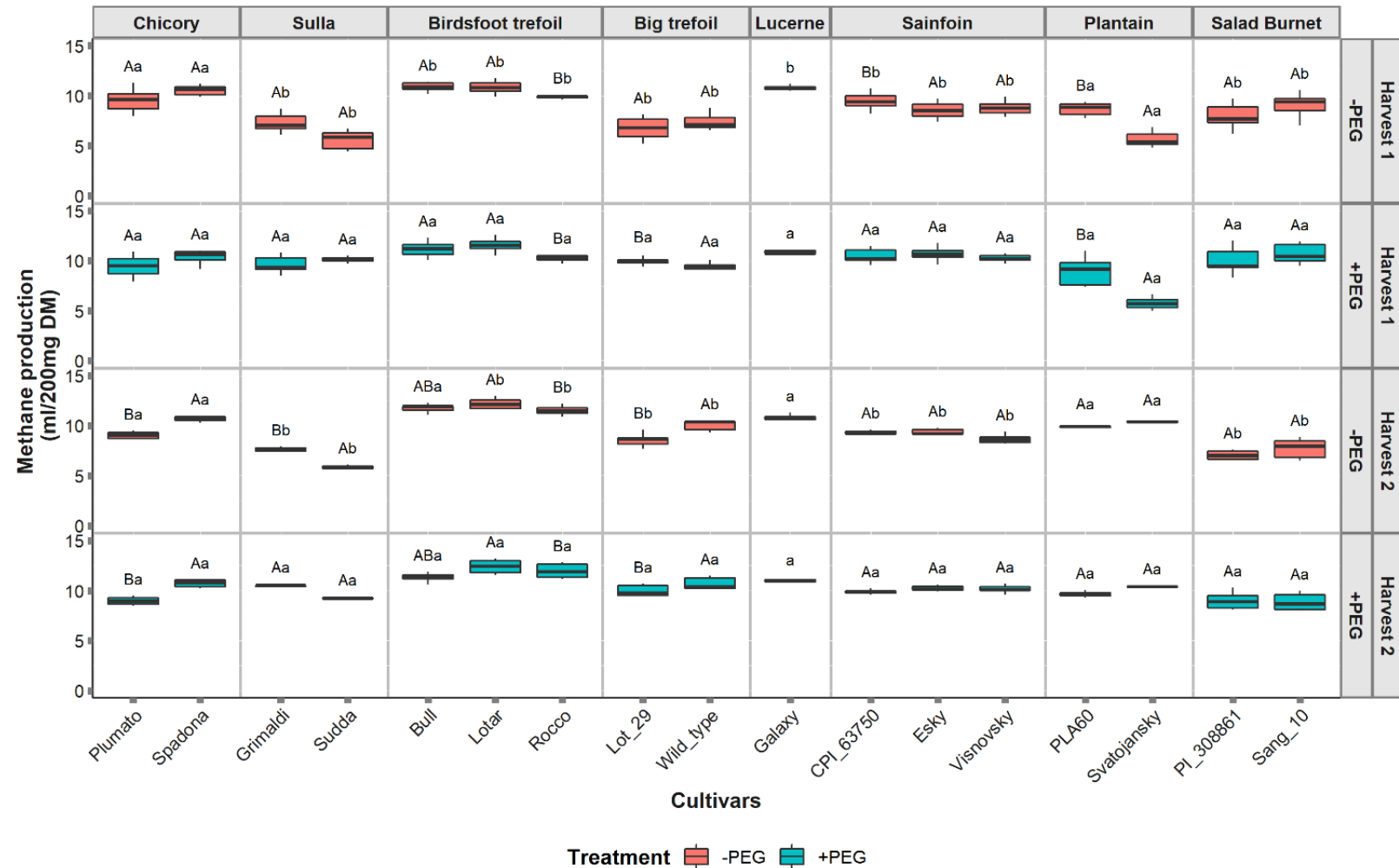


Figure 3 Methane production from 17 cultivars of 8 species under different treatments for two harvests. -PEG indicates the samples incubated without polyethylene glycol (PEG) and +PEG refers to the samples incubated with PEG. Different uppercase letters represent significant differences ($P < 0.05$) between the cultivars for each species, and different lowercase letters represent the significant differences within the treatments for each cultivar.

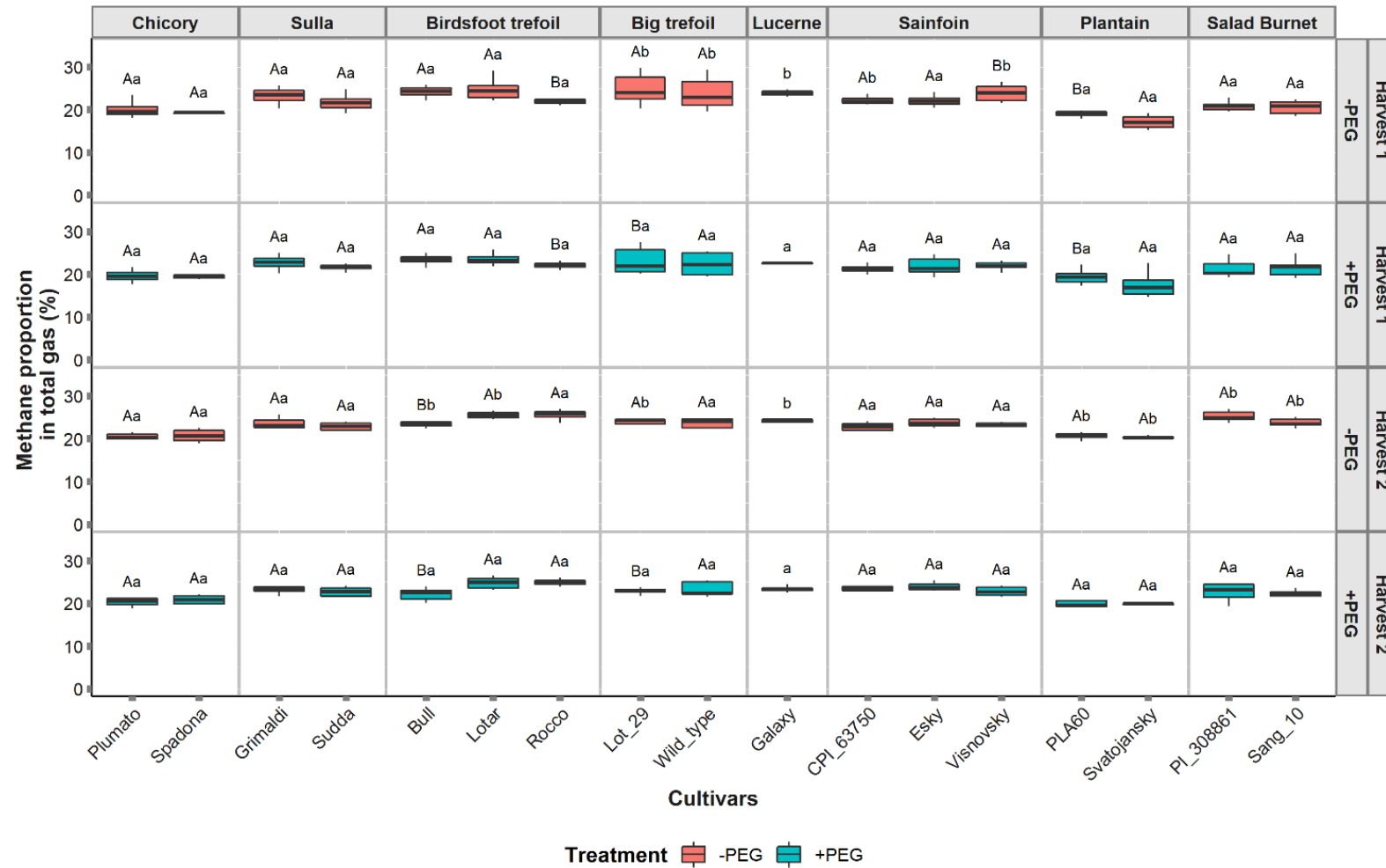


Figure 4 Methane percentage in total gas from 17 cultivars of 8 species under different treatments for two harvests. -PEG indicates the samples incubated without polyethylene glycol (PEG) and +PEG refers to the samples incubated with PEG. Different uppercase letters represent significant differences ($P < 0.05$) between the cultivars for each species, and different lowercase letters represent the significant differences within the treatments for each cultivar

4.9 Tables

Table 1. Chemical and polyphenol composition of the plants evaluated in the trial

Species	Cultivar	Harvest	CP	NDF	ADF	TP	TF	TT
Chicory	Plumato	1	181	346	212	1.5 ^{AB}	0.1 ^{ABC}	0 ^A
Chicory	Spadona	1	122	464	281	1.3 ^{ABC}	0.1 ^{ABC}	0 ^A
Sulla	Grimaldi	1	232	496	363	22.5 ^D	0 ^A	22 ^{BC}
Sulla	Sudda	1	186	495	366	25.5 ^D	0.1 ^{ABC}	25.2 ^B
Birdsfoot trefoil	Bull	1	224	469	202	3.1 ^B	0.7 ^{DEF}	2.3 ^D
Birdsfoot trefoil	Lotar	1	239	389	236	2.6 ^B	0.5 ^{BDE}	2.1 ^D
Birdsfoot trefoil	Rocco	1	272	349	239	2.4 ^{AB}	0.4 ^{ABCD}	2 ^D
Big trefoil	Lot 29	1	250	482	340	21.4 ^{DE}	1.5 ^{Fb}	19.9 ^{BCb}
Big trefoil	Wild type	1	266	518	376	16 ^D	0.9 ^{EF}	15.1 ^C
Lucerne	Galaxy	1	259	412	219	0.2 ^{Cb}	0.2 ^{ABCa}	0 ^{Aa}
Sainfoin	CPI 63750	1	157	519	351	20.2 ^{Db}	2.6 ^{Gb}	17 ^{BCb}
Sainfoin	Esky	1	206	530	407	18.4 ^{Db}	2.9 ^{Gb}	15 ^C
Sainfoin	Visnovsky	1	181	549	411	20.1 ^D	3.8 ^{Gb}	15.4 ^C
Ribwort plantain	PLA60	1	211	443	237	0.6 ^{AC}	0.1 ^{AC}	0 ^A
Ribwort plantain	Svatojansky	1	129	512	319	0.3 ^C	0 ^A	0 ^A
Salad burnet	PI 308861	1	163	523	95	43.8 ^E	0.4 ^{BCDE}	42.4 ^E
Salad burnet	Sang 10	1	110	426	112	42 ^E	0.3 ^{ABCD}	40.1 ^E
Chicory	Plumato	2	220	393	232	1.7 ^{AB}	0.1 ^{AB}	0 ^A
Chicory	Spadona	2	137	449	301	1.9 ^{AB}	0.2 ^{AB}	0 ^A
Sulla	Grimaldi	2	240	549	455	24.9 ^{CD}	0 ^A	24.5 ^B
Sulla	Sudda	2	224	467	393	27.9 ^C	0.1 ^{AB}	27.7 ^{BC}
Birdsfoot trefoil	Bull	2	229	418	247	2.3 ^A	0.6 ^{CD}	1.7 ^D
Birdsfoot trefoil	Lotar	2	232	408	234	2.2 ^A	0.5 ^{CDE}	1.8 ^D
Birdsfoot trefoil	Rocco	2	242	393	211	1.7 ^A	0.3 ^{BE}	1.4 ^D
Big trefoil	Lot 29	2	250	554	375	12.6 ^{DE}	0.8 ^{Ca}	11.8 ^{Ea}
Big trefoil	Wild type	2	229	493	344	12.4 ^E	0.8 ^{CD}	11.6 ^E
Lucerne	Galaxy	2	235	433	245	0 ^{Fa}	0 ^{Aa}	0 ^{Aa}
Sainfoin	CPI 63750	2	263	540	372	11.2 ^{Ea}	1.3 ^{Fa}	9.4 ^{Ea}
Sainfoin	Esky	2	273	520	385	11.5 ^{Ea}	1.7 ^{Fa}	9.5 ^E
Sainfoin	Visnovsky	2	216	554	426	13.8 ^E	2.8 ^{Ga}	10.6 ^E
Ribwort plantain	PLA60	2	157	419	239	0 ^{BFG}	0 ^A	0 ^A
Ribwort plantain	Svatojansky	2	122	511	342	0.2 ^G	0 ^A	0 ^A
Salad burnet	PI 308861	2	221	552	156	49.7 ^H	0.4 ^{DE}	47.9 ^C
Salad burnet	Sang 10	2	221	553	187	45.1 ^H	0.3 ^{BE}	43.3 ^C

CP, crude protein (g/kg DM); NDF, neutral detergent fiber (g/kg DM); ADF, acid detergent fiber (g/kg DM); TP, total polyphenols (g kg⁻¹ DM); TF, total flavonols (sum of myricetin, quercetin and kaempferol concentration, g kg⁻¹ DM); TT, total tannins (g kg⁻¹ DM). Values identify mean concentration of the measured parameters. Within a column significant differences ($P < 0.05$) between the different cultivars for each harvest is represented by different uppercase letters and the significant differences within the harvests for each cultivar ($P < 0.05$) is represented by different lower-case letters. For CP, NDF and ADF, a pooled sample for each cultivar was used for analysis due to biomass limitation.

Table 2. Proanthocyanidin (PA) concentration and the structural traits of the PA containing species evaluated in the trial

Species	Cultivar	Harvest	PA	mDP	PD%
Sulla	Grimaldi	1	22 ± 4.9 ^{ADE}	17.2 ± 0.9 ^{ADE}	93.6 ± 0.5 ^{AD}
Sulla	Sudda	1	25.2 ± 2.8 ^A	19.3 ± 1.3 ^A	91.4 ± 0.9 ^A
Birdsfoot trefoil	Bull	1	2.3 ± 1.1 ^B	9.7 ± 1 ^B	33.3 ± 7.9 ^B
Birdsfoot trefoil	Lotar	1	2.1 ± 0.5 ^{BC}	10.6 ± 1.3 ^{BC}	39 ± 4.1 ^B
Birdsfoot trefoil	Rocco	1	2.1 ± 0.8 ^{CD}	13.5 ± 0.8 ^{CD}	52.3 ± 3.3 ^C
Big trefoil	Lot 29	1	19.9 ± 2.4 ^{AE}	16.7 ± 0.2 ^{AE}	90.8 ± 1.7 ^{AD}
Big trefoil	Wild type	1	15.1 ± 4.1 ^{ADE}	15.8 ± 0.2 ^{ADE}	90.5 ± 0.8 ^{AD}
Sainfoin	CPI 63750	1	17 ± 1.2 ^{DEb}	14.3 ± 0.7 ^{DEb}	82.9 ± 4 ^D
Sainfoin	Esky	1	15 ± 2 ^{DE}	15 ± 0.8 ^{DE}	81 ± 4.7 ^D
Sainfoin	Visnovsky	1	15.4 ± 2.8 ^{DE}	15.3 ± 0.5 ^{DE}	82 ± 2.3 ^{Db}
Sulla	Grimaldi	2	24.5 ± 4 ^{ADE}	17.2 ± 2.3 ^{ADE}	93.7 ± 0.4 ^{ADE}
Sulla	Sudda	2	27.7 ± 5.5 ^A	18.2 ± 2.3 ^A	92.3 ± 1 ^A
Birdsfoot trefoil	Bull	2	1.7 ± 0.7 ^B	9 ± 0.9 ^B	30.3 ± 1.9 ^B
Birdsfoot trefoil	Lotar	2	1.8 ± 0.3 ^B	9.7 ± 0.2 ^B	33.8 ± 1.9 ^B
Birdsfoot trefoil	Rocco	2	1.4 ± 0.7 ^{CD}	12.8 ± 1.3 ^{CD}	48.7 ± 4.3 ^C
Big trefoil	Lot 29	2	11.8 ± 5.7 ^{Aa}	18 ± 0.5 ^{Aa}	89.1 ± 0.9 ^A
Big trefoil	Wild type	2	11.6 ± 4.4 ^{AE}	16.4 ± 1.2 ^{AE}	88.3 ± 1.6 ^{AD}
Sainfoin	CPI 63750	2	9.4 ± 3.2 ^{BCa}	11.5 ± 1.1 ^{BCa}	81.9 ± 4.9 ^{CE}
Sainfoin	Esky	2	9.5 ± 2.6 ^{BCD}	12.3 ± 2.4 ^{BCD}	81.1 ± 2.2 ^{CDE}
Sainfoin	Visnovsky	2	10.6 ± 2.2 ^{CDE}	13.9 ± 0.5 ^{CDE}	71.7 ± 3.8 ^{Ea}

PA, proanthocyanidin concentration (g/kg DM); mDP, mean degree of polymerisation; PD%, prodelphinidin percentage in PA. Values identify mean concentration of the measured parameters. Within a column significant differences ($P < 0.05$) between the different cultivars for each harvest is represented by different uppercase letters and the significant differences within the harvests for each cultivar ($P < 0.05$) is represented by different lower-case letters.

Table 3. The relationship between forage quality traits and the *in vitro* fermentation end-products (methane production (ml/200mg DM), gas production (ml/200mg DM), and methane percentage in total gas (%)) was estimated by stepwise regression using forward selection. The table shows the model performance of the covariate- based model using measured forage quality parameters (PA, proanthocyanidins (g/kg DM); CP, crude protein (g/kg DM), mDP, mean degree of polymerisation; PD%, prodelphinidin percentage in PA; TF, total flavonoids (g/kg DM); ET, ellagitannins (g/kg DM)) as covariates and its comparison with the reference model. For the R² values of the model, marginal R² values (variance explained by fixed effects only) and conditional R² values (variance explained by the entire model including both fixed and random terms) are presented.

Response parameters	Covariance parameter estimate	Covariates						Model parameters	Model Comparison	
		PA	CP	mDP	PD%	TF	ET		Reference Model	Covariate Based Model
Methane production	F-values	105.36	49.16	7.57				AIC	312.56	325.37
	P values	<0.001	<0.001	<0.01				Marginal R ²	0.89	0.81
	Coefficient	-0.17	-0.15	-0.12				Conditional R ²	0.96	0.96
Total gas production	F-values		0.05	13.32	7.26	4.82	6.61	AIC	1749.47	1781.65
	P values		0.83	<0.001	<0.01	<0.05	<0.05	Marginal R ²	0.88	0.68
	Coefficient		0.18	-5.72	-0.55	5.17	-177.75	Conditional R ²	0.98	0.98
Methane percentage in total gas	F-values	0.93			5.41			AIC	-650.08	-646.86
	P values	0.34			<0.05			Marginal R ²	0.26	0.05
	Coefficient	0.001			-0.001			Conditional R ²	0.75	0.81

Table 4 The plant species and their cultivars used in the greenhouse experiment

Species	Cultivar	Common name	Plant family
<i>Onobrychis viciifolia</i>	Visnovsky	Sainfoin	Fabaceae
<i>Onobrychis viciifolia</i>	CPI 63750	Sainfoin	Fabaceae
<i>Onobrychis viciifolia</i>	Esky	Sainfoin	Fabaceae
<i>Lotus corniculatus</i>	Bull	Birdsfoot trefoil	Fabaceae
<i>Lotus corniculatus</i>	Lotar	Birdsfoot trefoil	Fabaceae
<i>Lotus corniculatus</i>	Rocco	Birdsfoot trefoil	Fabaceae
<i>Lotus pedunculatus</i>	Lot 29	Big trefoil	Fabaceae
<i>Lotus pedunculatus</i>	Wild type	Big trefoil	Fabaceae
<i>Sanguisorba minor</i>	Sang 10	Salad burnet	Rosaceae
<i>Sanguisorba minor</i>	PI 308861	Salad burnet	Rosaceae
<i>Cichorium intybus</i>	Plumato	Chicory	Asteraceae
<i>Cichorium intybus</i>	Spadona	Chicory	Asteraceae
<i>Plantago lanceolata</i>	PLA60	Ribwort plantain	Plantaginaceae
<i>Plantago lanceolata</i>	Svatojansky	Ribwort plantain	Plantaginaceae
<i>Hedysarum coronarium</i>	Sudda	Sulla	Fabaceae
<i>Hedysarum coronarium</i>	Grimaldi	Sulla	Fabaceae
<i>Medicago sativa</i>	Galaxy	Lucerne	Fabaceae

4.10 Supplementary Information

Supplementary Table S1. Gas production from different species and cultivars across the two harvests with and without polyethylene glycol (PEG, a tannin binding agent) treatments and the reduction potential of the tested cultivars with respect to control. Red_MS refers to the reduction in gas production from the cultivar when compared with lucerne, Red_PEG refers to reduction in gas production from the cultivar in the absence of PEG (-PEG) compared to the treatment with the addition of PEG (+PEG). Numbers between brackets correspond to the standard error of the mean (SEM).

Species	Cultivar	Harvest 1				Harvest 2			
		-PEG (ml/200mg DM)	+PEG (ml/200mg DM)	Red_MS (%)	Red_PEG (%)	-PEG (ml/200mg DM)	+PEG (ml/200mg DM)	Red_MS (%)	Red_PEG (%)
Chicory	Plumato	47.93 (1.23)	48.12 (1.16)	-6	0	44.07 (0.35)	44.30 (0.77)	2	1
Chicory	Spadona	54.42 (0.56)	53.40 (0.78)	-20	-2	51.65 (1.06)	51.35 (0.55)	-15	-1
Sulla	Grimaldi	31.27 (0.77)	42.11 (0.80)	31	26	32.48 (0.52)	45.20 (0.81)	28	28
Sulla	Sudda	26.26 (1.06)	47.02 (0.46)	42	44	25.62 (0.46)	40.78 (0.87)	43	37
Birdsfoot trefoil	Bull	45.25 (0.72)	47.67 (0.68)	0	5	50.10 (1.21)	51.54 (1.20)	-11	3
Birdsfoot trefoil	Lotar	44.58 (1.32)	49.26 (0.76)	-1	8	47.73 (0.49)	49.97 (0.55)	-6	4
Birdsfoot trefoil	Rocco	44.75 (0.24)	47.04 (0.78)	4	5	45.05 (0.84)	47.93 (0.84)	0	6
Big trefoil	Lot 29	27.54 (1.36)	43.23 (1.05)	39	36	35.60 (1.01)	43.60 (0.76)	21	18
Big trefoil	Wild type	31.27 (0.83)	42.21 (1.13)	31	25	42.38 (0.36)	46.08 (0.56)	6	8
Lucerne	Galaxy	45.16 (1.36)	48.1 (0.3)	0	6	44.78 (0.41)	47.35 (1.23)	1	5
Sainfoin	CPI 63750	42.81 (0.95)	49.29 (0.72)	5	13	40.83 (0.91)	42.33 (0.83)	9	4
Sainfoin	Esky	38.74 (0.77)	49.29 (0.90)	14	20	39.56 (1.01)	42.77 (1.03)	12	7
Sainfoin	Visnovsky	36.94 (0.76)	46.47 (0.44)	18	21	37.63 (1.11)	44.75 (1.43)	16	16
Plantain	PLA60	45.44 (0.63)	46.12 (0.82)	-1	1	48.07 (0.86)	48.70 (1.03)	-7	-4
Plantain	Svatojansky	32.46 (0.59)	33.33 (0.77)	28	3	51.55 (0.66)	51.98 (0.40)	-14	1
Salad burnet	PI 308861	38.48 (1.24)	46.90 (1.30)	15	18	27.97 (0.86)	39.65 (1.00)	38	29
Salad burnet	Sang 10	44.28 (1.10)	49.98 (1.13)	2	11	32.60 (1.75)	39.75 (1.27)	28	18
Factors		F-value	p-value	Factors		F-value	p-value		
Cultivar		334.35	<0.001	Cultivar x Harvest		87.12	<0.001		
Treatment		611.19	<0.001	Treatment x Harvest		22.39	<0.001		
Harvest		0.02	0.89	Cultivar x Treatment x Harvest		5.78	<0.001		
Cultivar x Treatment		97.52	<0.001						

Supplementary Table S2. Methane production from different species and cultivars across the two harvests with and without polyethylene glycol (PEG, a tannin binding agent) treatments and the reduction potential of the tested cultivars with respect to control. Red_MS refers to the reduction in methane production from the cultivar when compared with lucerne, Red_PEG refers to reduction in methane production from the cultivar in the absence of PEG (-PEG) compared to the treatment with the addition of PEG (+PEG). Numbers between brackets correspond to the standard error of the mean (SEM).

Species	Cultivar	Harvest 1				Harvest 2			
		-PEG (ml/200mg DM)	+PEG (ml/200mg DM)	Red_MS (%)	Red_PEG (%)	-PEG (ml/200mg DM)	+PEG (ml/200mg DM)	Red_MS (%)	Red_PEG (%)
Chicory	Plumato	19.88 (0.37)	19.64 (0.30)	17	-1	20.52 (0.28)	20.32 (0.55)	15	-1
Chicory	Spadona	19.33 (0.08)	19.49 (0.09)	19	1	20.72 (0.62)	20.87 (0.45)	14	1
Sulla	Grimaldi	23.29 (0.38)	22.76 (0.31)	3	-2	23.64 (0.60)	23.17 (0.35)	2	-2
Sulla	Sudda	21.55 (0.35)	21.57 (0.16)	10	0	22.90 (0.39)	22.73 (0.46)	5	-1
Birdsfoot trefoil	Bull	24.16 (0.24)	23.47 (0.30)	-1	-3	23.47 (0.28)	22.08 (0.68)	3	-6
Birdsfoot trefoil	Lotar	24.58 (0.57)	23.44 (0.24)	-2	-4	25.57 (0.33)	24.78 (0.59)	-6	-3
Birdsfoot trefoil	Rocco	22.02 (0.16)	21.92 (0.32)	5	2	25.62 (0.47)	24.95 (0.30)	-6	-3
Big trefoil	Lot 29	24.98 (0.81)	23.26 (0.66)	-4	-8	24.03 (0.27)	22.88 (0.27)	0	-5
Big trefoil	Wild type	23.71 (0.86)	22.44 (0.77)	1	-8	23.66 (0.51)	23.28 (0.77)	2	-2
Lucerne	Galaxy	23.90 (0.36)	22.59 (0.03)	0	-6	24.2 (0.14)	23.35 (0.39)	0	-3
Sainfoin	CPI 63750	22.13 (0.18)	21.32 (0.22)	7	-4	22.82 (0.42)	23.40 (0.25)	6	2
Sainfoin	Esky	22.09 (0.25)	21.82 (0.43)	8	-1	23.73 (0.45)	23.93 (0.38)	2	1
Sainfoin	Visnovsky	23.90 (0.44)	22.03 (0.19)	1	-8	23.10 (0.39)	22.80 (0.45)	4	-1
Plantain	PLA60	19.07 (0.17)	19.36 (0.31)	20	1	20.67 (0.29)	19.90 (0.33)	14	-2
Plantain	Svatojansky	17.19 (0.31)	17.20 (0.52)	28	0	20.35 (0.13)	19.98 (0.15)	16	-2
Salad burnet	PI 308861	20.70 (0.21)	21.20 (0.38)	13	2	25.29 (0.53)	22.66 (0.88)	-5	-12
Salad burnet	Sang 10	20.61 (0.32)	21.45 (0.33)	14	4	23.75 (0.42)	22.30 (0.30)	2	-7
Factors		F-value	p-value	Factors		F-value	p-value		
Cultivar		505.22	<0.0001	Cultivar x Harvest		14.92	<0.0001		
Treatment		13.78	<0.05	Treatment x Harvest		0.42	0.52		
Harvest		6.50	<0.1	Cultivar x Treatment x Harvest		3.15	<0.0001		
Cultivar x Treatment		3.73	<0.0001						

Supplementary Table S3. Methane percentage in total gas (MP) from different species and cultivars across the two harvests with and without polyethylene glycol (PEG, a tannin binding agent) treatments and the reduction potential of the tested cultivars with respect to control. Red_MS refers to the reduction in MP from the cultivar when compared with lucerne, Red_PEG refers to reduction in MP from the cultivar in the absence of polyethylene glycol (-PEG) compared to the treatment with the addition of PEG (+PEG). Numbers between brackets correspond to the standard error of the mean (SEM).

Species	Cultivar	Harvest 1				Harvest 2			
		-PEG (ml/200mg DM)	+PEG (ml/200mg DM)	Red_MS (%)	Red_PEG (%)	-PEG (ml/200mg DM)	+PEG (ml/200mg DM)	Red_MS (%)	Red_PEG (%)
Chicory	Plumato	19.88 (0.37)	19.64 (0.30)	17	-1	20.52 (0.28)	20.32 (0.55)	15	-1
Chicory	Spadona	19.33 (0.08)	19.49 (0.09)	19	1	20.72 (0.62)	20.87 (0.45)	14	1
Sulla	Grimaldi	23.29 (0.38)	22.76 (0.31)	3	-2	23.64 (0.60)	23.17 (0.35)	2	-2
Sulla	Sudda	21.55 (0.35)	21.57 (0.16)	10	0	22.90 (0.39)	22.73 (0.46)	5	-1
Birdsfoot trefoil	Bull	24.16 (0.24)	23.47 (0.30)	-1	-3	23.47 (0.28)	22.08 (0.68)	3	-6
Birdsfoot trefoil	Lotar	24.58 (0.57)	23.44 (0.24)	-2	-4	25.57 (0.33)	24.78 (0.59)	-6	-3
Birdsfoot trefoil	Rocco	22.02 (0.16)	21.92 (0.32)	5	2	25.62 (0.47)	24.95 (0.30)	-6	-3
Big trefoil	Lot 29	24.98 (0.81)	23.26 (0.66)	-4	-8	24.03 (0.27)	22.88 (0.27)	0	-5
Big trefoil	Wild type	23.71 (0.86)	22.44 (0.77)	1	-8	23.66 (0.51)	23.28 (0.77)	2	-2
Lucerne	Galaxy	23.90 (0.36)	22.59 (0.03)	0	-6	24.2 (0.14)	23.35 (0.39)	0	-3
Sainfoin	CPI 63750	22.13 (0.18)	21.32 (0.22)	7	-4	22.82 (0.42)	23.40 (0.25)	6	2
Sainfoin	Esky	22.09 (0.25)	21.82 (0.43)	8	-1	23.73 (0.45)	23.93 (0.38)	2	1
Sainfoin	Visnovsky	23.90 (0.44)	22.03 (0.19)	1	-8	23.10 (0.39)	22.80 (0.45)	4	-1
Plantain	PLA60	19.07 (0.17)	19.36 (0.31)	20	1	20.67 (0.29)	19.90 (0.33)	14	-2
Plantain	Svatojansky	17.19 (0.31)	17.20 (0.52)	28	0	20.35 (0.13)	19.98 (0.15)	16	-2
Salad burnet	PI 308861	20.70 (0.21)	21.20 (0.38)	13	2	25.29 (0.53)	22.66 (0.88)	-5	-12
Salad burnet	Sang 10	20.61 (0.32)	21.45 (0.33)	14	4	23.75 (0.42)	22.30 (0.30)	2	-7
Factors		F-value	p-value	Factors		F-value	p-value		
Cultivar		505.22	<0.0001	Cultivar x Harvest		14.92	<0.0001		
Treatment		13.78	<0.05	Treatment x Harvest		0.42	0.52		
Harvest		6.5	<0.1	Cultivar x Treatment x Harvest		3.15	<0.0001		
Cultivar x Treatment		3.73	<0.0001						

Chapter 5

General Discussion

Rampant agricultural intensification has led to highly specialized production systems with increments in herd size, high fertilizer inputs and specialized feed products. Due to an increase in energy and fertilizer costs combined with the high cost of concentrates, these systems are not only cost intensive but are also associated with high environmental costs (Beauchemin, McAllister, and McGinn 2009). Thus, European livestock production systems face the challenge of producing more meat and milk products to meet the increasing consumer demands, while also adhering to strict regulations to reduce emissions (Hamacher et al. 2021). Grassland based ruminant production systems are more environmentally sustainable as they do not rely on energetically intensive feed products, and ruminants can derive their energy from human inedible biomass such as forages rich in cellulose (van Gastelen, Dijkstra, and Bannink 2019). Moreover, climate change is already beginning to alter weather patterns, resulting into extreme climatic events, such as frequent droughts and floods worldwide thus, one important task is to design grasslands to be resilient to a range of environmental conditions (Lüscher et al. 2014). One way to do so is increasing sward phytodiversity, thereby increasing the range of ecological niches that are available to the swards, and including for example deep rooted species that can access deeper water layers when the top soil is already dry (Suter, Huguenin-Elie, and Lüscher 2021; Hofer et al. 2016). These diverse pastures can also provide additional benefits, such as, ruminants feeding on diverse pastures comprising various forbs and legumes, allow them to have more balanced diet of water-soluble carbohydrates from the grasses, as well as protein from the legumes (Lagrange et al. 2020). Additionally, if herbs containing PSMs are included in ruminant diet, the range of benefits can be further exacerbated. Bioactive forages comprising polyphenols, such as tannins and flavonoids, when supplemented in moderate quantities have been found to incur anthelmintic and antimethanogenic effects on ruminants (Verma, Taube, and Malisch 2021; Gerber et al. 2013). Reduction in CH₄ emissions from livestock sector is particularly crucial as it is one of the biggest environmental burdens from this sector (Beauchemin, McAllister, and McGinn 2009; Aboagye et al. 2018). Additionally, inclusion of these herbs has been found to increase not only net primary productivity of the grasslands by improved niche utilization and resource use efficiency, but also enhances animal health and productivity (Hamacher et al. 2021; Lorenz et al. 2020). A field experiment conducted in Germany for a period of five years, found that inclusion of forage species with complementary functional traits improved animal productivity. Diverse swards, containing grasses, legumes, and forbs, were found contain higher CP and lower ADF concentrations compared to grass-dominated swards with no differences in DM yields under both the

treatments. Additionally, there was 17% increase in lamb live weight gain in diverse swards when averaged over 5 years (Jerrentrup et al. 2020).

As discussed earlier in Chapter 1, the utilisation of legumes which are prevalent in temperate-zone temporary grasslands is almost entirely limited to lucerne, white clover and red clover (*Trifolium pratense*), with alternative forages being much less exploited despite being potentially beneficial for ruminant production. The first chapters of the doctoral dissertation (**Chapter 1 and 2**) highlights the potential of TRFs in ruminant production and the factors which have led to inconsistent animal responses across different studies. The experiments entailed in the doctoral dissertation were focused on identifying the variabilities in different polyphenol containing forages with high agronomic potential which is crucial to understand the bioactive effects on ruminants. The second part of the dissertation characterises and quantifies the polyphenolic subunits present in different species and their cultivars (**Chapter 3**). Using these variabilities as a starting point, the third part focuses on analysing the effect of tannins and flavonoids on CH₄ production from these forages *in vitro* and identify the parameters which influence their bioactivity (**Chapter 4**). This interdisciplinary research was conducted with the aim to unravel the links between polyphenolic compounds present in the forages and their CH₄ mitigation potential. The elucidation of structure-activity relationship of tannins as well as establishing the inter- and intraspecies variability across the forages in terms of their polyphenolic profile, will enable their future exploitation at the farm level.

5.1 Establishing the inter- and intraspecies variation in polyphenolic and tannin composition in temperate forage

Polyphenolic subgroups, such as tannins and flavonoids, are present in varied mixtures in the forages and characterising them into specific subgroups could provide more detailed insight into their functional implications on ruminants. It has also been found that species containing similar polyphenol concentration can differ widely in terms of their polyphenolic profile, and different polyphenolic subgroups vary in their biological responses (Mueller-Harvey 2006; Malisch et al. 2015).

Tannins are a chemically diverse group of polyphenolic compounds and are recognised mainly for their protein binding ability. Contrary to the previous notion of tannins' function being limited to their antiherbivore effects, they have been identified to possess diverse functional attributes (Marsh et al. 2020; Naumann et al. 2017). Forages rich in tannins have gained a lot of attention, among others due to their antimethanogenic

properties, as CH₄ is one of major environmental burdens from the livestock sector. There have been a number of studies which have analysed the potential of including TRFs in ruminant diet, however, the results on their effects on ruminant health and performance, so far have been variable (Naumann et al. 2018; Verma, Taube, and Malisch 2021). There are several factors which can lead to contrasting results from different studies as described in Chapter 2. Tannin concentration in plants is known to be plastic in nature (Barbehenn and Peter Constabel 2011) and their concentration has been observed to differ substantially not only across the species but also within the cultivars of the same species (Malisch et al. 2015; Hatew et al. 2015). Additionally, growing conditions and plant's phenological stage have been found to influence tannin content in the plants. These sources of variabilities as well as variability resulting from the use of different analytical techniques for tannin quantification across different studies are often neglected in the animal trials (Mueller-Harvey et al. 2017; Salminen and Karonen 2011). Simultaneously, the influence of tannins' structural traits on their bioactivity cannot be underestimated and could contribute to their contrasting effects on ruminant nutrition. Due to the diversity and complexity of tannins' structure which makes it difficult to analyse individual PAs, structural features, such as mDP and PD%, are often determined (Verma et al, 2021; Zeller 2019; Aboagye and Beauchemin 2019). To circumvent the aforementioned sources of variabilities, a greenhouse experiment was conducted where 17 cultivars from eight species were grown under controlled conditions and progressively harvested three times at the same phenological stage. As hypothesised in Chapter 3, the differences in the concentration and distribution of flavonoids and tannins across different plant species and their cultivars were evident.

Based on the results, the species used in the studies can be divided into three categories, (i) PA containing species comprising sainfoin, birdsfoot trefoil, big trefoil, and sulla, (ii) salad burnet which rich in HTs, and (iii) low polyphenol containing species such as lucerne, chicory and plantain. A large variability was observed across the species, however, the variability across the cultivars was comparatively less. Similar trend was observed when PA containing species were analysed in detail for PA composition and structural features. The distribution of PAs in plant organs varies with the plant's phenological stage and usually they are not uniformly distributed across the entire plant. This makes it essential to understand their distribution in different plant organs, and how growth stage affects these dynamics. With the changes in the phenological stage of the plant, PA content per plant was shifted and varied depending on the species. This study revealed that flowers had the highest PA concentration followed by leaves and stems. According to growth rate hypothesis, during

the plant's growth period, there are limited amount of resources available, and these resources could be invested either for its growth or its defense mechanisms such as production of PSMs (e.g. PAs). At the flowering stage, as the growth slows down, excess carbon is may be available for PA synthesis, this could potentially be the reason for high PA concentration in flowers (Theodoridou *et al.*, 2011; Malisch *et al.*, 2015). However, as the leaf biomass was higher compared to flowers for the plants, PA content for an individual plant was found to be dependent on PA concentration in the leaves. This is of particular importance for plant breeders as this means that the bioactivity of the forage could be increased by increasing the proportion of leaves in the plants. Nevertheless, the distribution of PAs also varied across the species and harvests. This was evident for birdsfoot trefoil where the tannin concentration (E-PA) of the entire plant (leaves, flowers, and stems) at the flowering stage was 7 to 9 mg per plant compared to the concentration in the vegetative stage (2-4 mg per plant). This could be attributed to the higher PA concentration in flowers (16-40 mg/g DM) compared to leaves (2-2.5 mg/g DM), and biomass for flowers (4-10% DM) was higher compared to other species. For Sudda, PAs in stems contributed more to harvestable PAs per plant compared to other aerial plant parts. A similar trend was observed for sainfoin in a study by Häring *et al.* (2007) where the PA concentration per plant decreased with maturation and was dependent on the changing proportions of PA-rich plant organs during plant development (Theodoridou *et al.*, 2011).

Furthermore, not only the concentration, but also the bioavailability of PAs present in the plant tissues determines their potency in ruminants. PAs in plants are known to exist in two forms, extractable PAs (E-PAs) and non-extractable PAs (N-PAs). Proanthocyanidins have the tendency to bind with protein and fiber in plant tissues which are difficult to extract, and together they are known as N-PAs (Terrill *et al.* 1992; Kagiya *et al.* 2019). In the present study, the proportion of E-PAs was higher compared to N-PAs in the majority of PA containing species, ranging from 30 to 90 % of total PAs (E-PAs and N-PAs). It is well known that the E-PA concentration influences the bioactivity of the forages, however, research on the effect of N-PAs on their bioactivity is still limited. There are few studies which have been conducted on determining the role N-PAs in PA forages, and it has been hypothesised that N-PAs can potentially function as anthelmintic in small intestine after being released from the complexes under acidic conditions ($\text{pH} < 3.5$) (Desrues *et al.* 2017). In addition to the forage quality, understanding these dynamics is essential in selecting the harvesting stage of a forage to meet the animal requirements.

Tannin's astringency is commonly measured in terms of its efficacy to aggregate or precipitate protein or other feed components (McAllister et al. 2005). Our analyses found that the selected cultivars comprised a wide range of mDP (8-21) and PD% (24-94%) values. Both sulla and big trefoil had similar PA composition and concentration. Studies have reported them to be highly astringent (Douglas and Foote 2000), which is at least in part a consequence of their high PD% and mDP (Mueller-Harvey 2006). Large variability in sainfoin's structural characteristics is in accordance with the previous studies where an extensive range of sainfoin cultivars from different were analysed (Hatew et al. 2015; Malisch et al. 2015). Interestingly, the variability of PA structural characteristics within the cultivars of the same species was much less compared to the intraspecies variability of PA concentration. This is particularly useful for plant breeders to develop forages with novel PA traits as these traits have been linked to PA bioactivity (Mueller-Harvey et al. 2017).

5.2 Role of PA structural features on antimethanogenic potential of PA containing forages and their potential implications on ruminant health

Several *in vivo* and *in vitro* studies have been conducted on the effect of TRFs on CH₄ production in ruminants but the animal responses varied across different studies, making their on-farm exploitation difficult. Recent research has highlighted that tannin structural complexity plays an important role in determining their bioactivity (Marsh et al. 2020; Leppä, Laitila, and Salminen 2020). Particularly PA structural features, such as PD% and mDP, have been identified to be important determinants of antimethanogenic activity of TRFs. Compared to other TRFs, sainfoin and birdsfoot trefoil have been the focus of previous studies due their low to medium range of tannins, however, the beneficial effects of these forages have not been consistent across the studies (Zeller, 2019; Verma *et al.*, 2021). This provides an incentive to do an extensive screening of TRFs for their antimethanogenic potential and determine the factors influencing it. As hypothesised, all the TRFs were found to produce lower CH₄ relative to lucerne which was used as a negative tannin control. Simultaneously, to determine the isolated-effect of tannins on CH₄ production from these forages, a treatment with the tannin-binding (and thus deactivating) agent polyethyleneglycol (+PEG) was included in the study. In the absence of PEG (-PEG), the CH₄ production from sulla, salad burnet, sainfoin, big trefoil, and birdsfoot trefoil was lower compared to the +PEG treatment with the reduction ranging from 2 to 44%. Furthermore, a general trend of lower CH₄ production was recorded when plants were harvested at the vegetative stage (second harvest) compared to when plants were harvested at the flowering stage (first

harvest). This could be a result of lower tannin content in the plants during the second harvest. Moreover, a tannin-effect was also observed on GP, as the GP in the -PEG treatment was found to be lower compared to the +PEG treatment.

Methane proportion in total GP is an important indicator for estimating whether or not the reduction in CH₄ is resulting from a decrease in digestibility of the forage. In this study, the comparison between the +PEG and -PEG treatment indicated that tannins in the forages were able to reduce CH₄ production, but the reduction was at the cost of decrease in forage digestibility. The objective of utilising TRFs as a feed component should be to reduce CH₄ emissions without affecting animal productivity. Forages such as sulla and big trefoil with high tannin concentrations, and have been shown to negatively affect the digestibility of the forage which can be potentially detrimental to animal health (Mueller-Harvey 2006). The negative effect of tannins can be a result of reduced protein digestibility combined with reduced fiber degradation due to partial inactivation of microbial activity (Molle et al. 2009). However, PAs in sulla have been found to increase the absorption of essential amino acids, live weight gain, milk protein concentration and yield. Simultaneously, they have shown to improve lamb carcass characteristics, meat quality, and reduced parasitic burdens in lambs (Tibe et al. 2011).

The adverse effects of high PA containing forages can be countered by supplementing them with non-PA forages to dilute the PA concentration in the diet. This has been observed in a study by where when 45 % of corn-lucerne diet was replaced by for *Leucaena cuneata* and *Desmodium paniculatum*, there was a decrease in CH₄ without any negative effect on GP in an in vitro study (Naumann et al. 2014). The antimethanogenic potential of birdsfoot trefoil and sainfoin have been proven also in *in vivo* studies. Birdsfoot trefoil was found to reduce CH₄ (g CH₄/kg DM) by 17% compared to ryegrass in dairy cows. Simultaneously, sainfoin hay reduced CH₄ per unit OM digested compared to lucerne hay by 24% in beef heifers (Chung et al. 2013). An *in vivo* study by Lagrange et al. (2020) found that even though the CH₄ emitted daily (g/d), per unit of intake from lucerne, birdsfoot trefoil, and sainfoin did not differ significantly, the CH₄/kg BW gain from sainfoin and birdsfoot trefoil was 25 and 17% lower in heifers compared to lucerne. Interestingly, heifers grazing on mixed pastures containing lucerne, sainfoin and birdsfoot trefoil reduced CH₄ yield by 14% and CH₄ intensity by 27% compared to the average from their respective monocultures with an increase in DMI and body weight gain (Lagrange et al. 2020). Inclusion of tanniferous forages (sainfoin, and birdsfoot trefoil) with lucerne in ternary mixtures was also found to

improve voluntary intake by lambs and enhance the digestibility of the diet relative to their monocultures. Moreover, sainfoin was found to shift N excretion from urine to feces which may reduce N pollution, as urinary N is prone to rapidly degrade to NH_3 and then oxidized to N_2O compared to fecal N (Lagrange and Villalba 2019). The results from these studies demonstrate the high potential of TRFs in reducing CH_4 in ruminants, however there is need for extensive research and long-term feeding trials in order to optimise their on-farm utilisation.

The present study found that there was a significant variation of CH_4 production across these species, however, the variation across the cultivars from the same species was considerably less and their reduction potential was consistent in both the harvests. This is in agreement with the patterns of variability established in the previous section where there was high interspecies variability compared to intraspecies variability in terms of PA structural features and polyphenolic composition. Simultaneously, the relationship between the antimethanogenic potential of PA containing forages and measured forage composition parameters was assessed with a stepwise regression. Here, the aim was to determine whether including the covariates (tannin concentration and composition, forage quality parameters) would be able to predict the antimethanogenic activity of a forage equally well or better than using species and cultivar as a predictor (base model). Subsequently, it was analysed which parameters play an important role in determining their CH_4 reduction potential. Although, the model generated from this procedure did not improve the base model, we found that mDP, CP and PA concentration in the forages played an important role in determining their antimethanogenic potential, and the type of forage species remains the crucial factor. This is in line with the previous studies where in addition to PA concentration, the structural characteristics of PA were found to be responsible for their antimethanogenic property (Hatew et al. 2015; Hatew et al. 2016; Naumann et al. 2013). In addition to tannins, there are several factors which comes into play to determine CH_4 production in ruminants such as carbohydrates, fat, protein, mineral composition, and other PSMs present in the forages (Gaviria-Urbe et al. 2020). The potential interaction effects on their bioactivity resulting from the coexistence of these dietary components are also important to assess in order to separate the effect of forage quality and other PSMs from PA traits.

5.3 Antimethanogenic potential of non-leguminuous forbs

Salad burnet was the only HT rich forage in this study and had the highest polyphenolic concentration (Chapter 2). Its cultivars were found to decrease CH_4 production

by 13 to 22% in the -PEG treatment (tannin effect). Interestingly, the CH₄ proportion of the GP for salad burnet was comparatively lower than PA containing forages indicating that HTs reduced GP less compared to PAs which in line with the study by Loza et al. (2021). In the present study, HTs in salad burnet were found to mainly comprised ETs, but the mean polymer size for ETs was not determined. However, as seen for PAs, a study by Baert et al. (2016) reported that polymer size of ETs plays a significant role in decreasing CH₄ production in an *in vitro* study. It was found that the antimethanogenic activity of purified ETs isolated from rosebay willowherb (*Epilobium angustifolium*) was found to increase with the increase in polymer size of ETs, but it was accompanied with reduction in GP. However, dimeric, trimeric, and tetrameric ETs were found to reduce CH₄ by lowest reduction in GP.

Generally, the inclusion of non-leguminous forbs, such as ribwort plantain, salad burnet, and chicory in temperate grasslands is rare with the exception of some organic farms or extensively used (semi-) natural grasslands (Hamacher et al. 2021; Elgersma, Søgaard, and Jensen 2014). They have been identified to possess either anthelmintic or antimethanogenic effects, however research on their bioactivity and chemical composition has been limited (Williams et al. 2016). Both, chicory and ribwort plantain were found to reduce CH₄ production compared to lucerne in the present trial. Even though, their CH₄ reduction potential was lower compared to TRFs, it was not accompanied with decrease in GP. This resulted in the lowest MP compared to other forages, making them ideal candidates for feed supplements as they did not affect the digestibility of the forage. Both ribwort plantain and chicory are deep-rooted perennial forbs which have shown potential to enhance the productivity of intensively managed grasslands and lower GHG emissions. Simultaneously, ribwort plantain has been found to provide high and stable yields in grass-clover mixtures compared to chicory (Cong et al. 2017). Still, for most of the important forage species in temperate grassland systems, the characterization of polyphenols has not been thorough enough to comprehend their differences in bioactivity, particularly not within cultivars of these species.

5.4 Future Outlook

Till date, the legume-utilisation in temperate regions has primarily been limited to lucerne, white clover, and red clover, thereby, breeding strategies have been limited to mainly these species (Lagrange et al. 2020; Lagrange and Villalba 2019). The other forage legumes remain less exploited within forage plant breeding programmes on selection of varieties for increased production (Abberton et al. 2008). The results from the experiments presented in the

dissertation establishes the high potential of polyphenolic rich forages for sustainable intensification of the livestock sector and it doesn't stem only from their antimethanogenic potential, but also due to their diverse functional implications on grassland-based livestock systems (Naumann et al. 2017; Lagrange et al. 2020). The present study reveals the major patterns of the selected species and their cultivars in terms of their polyphenolic composition and antimethanogenic activity, and further assessed their links under controlled conditions. The trials in the present study provided preliminary evidence of the influence of PA structural features on their antimethanogenic activity. However, to establish a clear relationship between tannin structures and their bioactivity, examining pure tannin extracts for their antimethanogenic activity is suggested as this would void their potential matrix effects with other PSMs. The focus on elucidating the factors influencing tannin bioactivity would enable the progress in the application of TRFs from *in vitro* to *in vivo* systems. Recent research as well as the results from the trials present in the dissertation have shown that tannin structural features were more stable compared to tannin concentration which provides an opportunity for plant breeders to engineer stable PA composition in forages to achieve desirable bioactivity. Further *in vivo* and long-term studies are warranted to comprehend the impact of these bioactive forages on ruminal health and productivity. Simultaneously, the aim should to be enhance efficacy of their antimethanogenic potential by modulating the tannin concentration in the offered forage without negatively affecting the palatability or nutritional quality of the feed. On a field scale, inclusion of tanniferous forages with non-tanniferous forages in ruminant diet could be a viable strategy to counter the detrimental effects of high tannin containing forages. Finally, it is crucial to improve the agronomic characteristics of tanniferous forages, such as birdsfoot trefoil and sainfoin, and underutilised forbs, in terms of their yield, regrowth capacity after grazing, persistence and adaptability to different environments, which will provide the producers a broader range of options under different environments to achieve sustainable grazing systems.

5.5 References

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Appendix



Article

Assessing the Potential of Diverse Forage Mixtures to Reduce Enteric Methane Emissions In Vitro

Cecilia Loza ^{1,*} , Supriya Verma ¹ , Siegfried Wolffram ² , Andreas Susenbeth ², Ralf Blank ² ,
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Assessing the Potential of Diverse Forage Mixtures to Reduce Enteric Methane Emissions In Vitro

Cecilia Loza ^{1,*}, Supriya Verma ¹, Siegfried Wolffram ², Andreas Susenbeth ², Ralf Blank ², Friedhelm Taube ^{1,3}, Ralf Loges ¹, Mario Hasler ⁴, Christof Kluß ¹, and Carsten Stefan Malisch ¹

A.1 Simple Summary

Changes in agriculture towards simpler and more intensive systems have contributed to increased environmental problems. In temperate sown grasslands this has resulted in ryegrass dominance and forage legume use is limited mainly to three species: red clover, white clover and lucerne. Other dicot forages such as *Lotus pedunculatus* and *Sanguisorba minor* are of interest as they contain plant specialized metabolites (PSM), especially tannins, which can potentially reduce methane from ruminants, an important source of agricultural greenhouse gas emissions. In an *in vitro* study, we compared binary mixtures of perennial ryegrass with one of eight dicot species including PSM-rich species in different proportions, to assess their potential to reduce methane production. An additional aim was to determine whether moderate additions of these forage species can be sufficient to reduce methane formation, or whether the relationship is linearly dose-dependent. Results show that all dicot species studied, including the non-tannin containing species, reduced methane production. While all plant species rich in PSM reduced methane production, they also decreased digestibility. Additionally, they did not persist in the pasture into the second year. The lowest methane emissions per digestible forage unit were obtained with chicory (*Cichorium intybus*), a promising forage herb with both agronomic and bioactive potential.

A.2 Abstract

Methane emissions from ruminants are a major contributor to agricultural greenhouse gas emissions. Thus, eight different forage species were combined in binary mixtures with *Lolium perenne* in increasing proportions, *in vitro*, to determine their methane reduction potential in ruminants. Species were sampled in two consecutive years where possible. The aims were: a) to determine if mixtures with specific forages, particularly those rich in plant specialized metabolites (PSM), can reduce methane emissions compared to ryegrass monocultures, b) to identify whether there is a linear-dose effect relationship in methane emissions from the legume or herb addition, and c) whether these effects are maintained across sampling years. Results showed that all dicot species studied, including the non-tannin containing species, reduced methane production. The tannin-rich species, *Sanguisorba minor* and *Lotus pedunculatus*

showed the greatest methane reduction potential of up to 33%. Due to concomitant reductions in digestibility of the forage, *Cichorium intybus* yielded the lowest methane emissions per digestible forage unit. Contrary to total gas production, methane production was less predictable, with a tendency for the lowest methane production being obtained with 67.5% share of the legume or herb partner species. Thus, linear increments in the partner species share did not result in linear changes in methane concentration. The methane reduction potential differed across sampling years, but the species ranking in methane concentration was stable.

A.3 Introduction

Simplification of agricultural systems towards crop or grass-ley monocultures and uniform, intensive production systems has contributed to a number of negative environmental externalities from agriculture, including soil organic carbon losses, excessive nutrient inputs into water bodies and increased greenhouse gas emissions (Lemaire et al., 2015). For temperate grasslands, previous experiments and research networks have repeatedly shown potential advantages of swards based on mixtures of grasses and dicot (forb) species, particularly forage legumes and also some forage herbs, compared with grass monoculture swards. These advantages occur notably under conditions with substantially reduced nutrient inputs, resulting in lower emissions to the environment, attributed mainly to improved niche utilization and facilitation effects (Nyfeler et al., 2009). In one example, in a 31-site plot experiment, four-species mixtures including legumes, and receiving 150 kg N/ha/a showed greater herbage yield than grass monocultures receiving 450 kg N/ha/a across a wide range of environments (Finn et al., 2013). In these mixtures, legume shares of approximately 1/3 were sufficient to obtain 95% of the highest total N yield (N_{tot}) and exceeded the N_{tot} of grass monocultures by 57% (Suter et al., 2015). The improved niche utilization also offered potential for weed suppression, as 58% of all sites had significantly lower weed abundance than even the best performing monoculture sward (Conolly et al., 2018).

Changes in grassland management regimes, with higher use intensities and shorter regrowth periods under both grazing and cutting systems, have restricted the role of legume utilization in temperate-zone temporary grasslands to three main species: red clover (*Trifolium pratense*), white clover (*Trifolium repens*), and lucerne (*Medicago sativa*). All have been subject to varietal improvement by plant breeders and are widely used globally, while other forage legumes remain less exploited (Lüscher et al., 2014). This is despite the potential role for many alternative forages, legumes and also non-legume forage herbs, to increase resource use efficiency further, to reduce environmental impacts particularly from dairy production, and

to provide additional benefits for animal health due to their content of plant specialized metabolites (PSM) (Mueller-Harvey et al., 2019, 2006; Min et al., 2003; Niderkorn, 2011). Among these beneficial effects are anthelmintic properties, a shift in nitrogen excretion pathways from the volatile urine to the more stable form in dung, potentially higher animal productivity, and reductions in methane emissions (Mueller-Harvey et al., 2019). The latter is particularly important since methane emissions from enteric fermentation are currently one of the biggest environmental burdens arising from ruminant livestock production (Thorpe et al., 2009), and there is considerable interest in adapting ruminant nutrition to reduce this impact. Moreover, methane emissions from enteric fermentation represent a massive loss of feed energy, and global emissions have been calculated to represent an energy loss equivalent of 144 million tons of oil (Gerber et al., 2013). Furthermore, unlike losses by ruminants via their urine and faeces, these gaseous losses cannot be recycled within the agricultural system, but they contribute to the global pool of greenhouse gas emissions and thereby contribute to climate change.

Among the approaches that have potential to reduce enteric methane emissions, the use of forages that contain PSM is particularly promising, as these might reduce methane emissions while at the same time provide high quality forage. Of the PSM, polyphenols, and particularly tannins (both hydrolysable tannins (HTs) and proanthocyanidins (PAs, syn. condensed tannins)) have potential for use in ruminant nutrition, and will be the focus of our research. PAs bind predominantly via hydrogen bonds with the dietary protein in the rumen, preventing it from enzymatic hydrolysis. Additionally, they inhibit methanogens, protozoa, and generally can affect the rumen microbiota (Verma et al., 2021). Due to these factors, they can reduce enteric methane production. The PA-protein complexes dissociate in the intestine as a result of the increased pH, thereby enabling increased amounts of utilizable protein at the duodenum (uCP), which is subsequently absorbed as amino acids. This can increase the forage nutritive value, without depressing rumen fiber digestion or daily intake (Min et al., 2003; Aerts et al., 1999). Among the PA-containing forages, the legumes birds'foot trefoil (*Lotus corniculatus*), big trefoil (*Lotus pedunculatus*) and sainfoin (*Onobrychis viciifolia*) have all shown good potential as sources of feed for livestock (Waghorn, 2008). However, their anti-methanogenic effects have been variable in previous findings (e.g. Chung et al., 2013; Huyen et al., 2016). The variability in the results could be attributed, at least partially, to the often-insufficient characterization of the structural characteristics of the tannins (Verma et al., 2021). Additionally, the co-presence of other PSM such as flavanols and saponins has been shown to interact with tannins, with potentially mutualistic or antagonistic effects (Jayanegara et al.,

2013, 2011). Furthermore, the growth conditions of the tested plants varied across experiments, affecting not only the synthesis of PSM (Top et al., 2017; Malisch et al., 2016; Selmar et al., 2013), but also the forage chemical composition (Moore et al., 2020), hence constituting an additional source of variability in methane emissions. Finally, many previous studies have been performed with plant extracts and purified compounds, with little applicability for grazing animals or even current indoor systems.

Several other non-legume forage species with high feeding value, such as chicory (*Cichorium intybus*) with lower CT contents but rich in other phenolic compounds and rapidly fermentable carbohydrates, and ribwort plantain (*Plantago lanceolata*), which lacks CT but has a high nitrogen use efficiency in dairy cows, potentially due to a favourable water soluble carbohydrates (WSC):N ratio (Minnée et al., 2020), also appear to be beneficial, but there have been few studies on their inclusion in diets related to methane emissions. Furthermore, and in contrast to the nitrogen-derived benefits of diverse swards, there has not yet been any study that has identified the required shares of potentially bioactive components in forage mixtures needed to obtain a desired anti-methanogenic effect. Hence, the study reported in this paper aimed to provide the first comparison of specifically designed binary forage mixtures of perennial ryegrass (*Lolium perenne*) with one of eight different forage species in increasing shares and across two different years to test the following hypotheses: a) tannin-containing forages reduce methane emissions compared to non-tannin forage species, b) the anti-methanogenic effect is not linear, and moderate additions of partner species can be sufficient to reduce methane emissions with lower reductions of digestibility, and c) these findings will be affected by the growth conditions, but the general species-specific reduction potential is replicable across harvest years.

A.4 Materials and methods

The in-vitro analysis was performed between August and December 2019, at the laboratory of the Institute of Animal Nutrition and Physiology of Kiel University, Germany.

Eight species including legumes and non-legume forbs (hereafter called “partner species”) were combined in binary mixtures with perennial ryegrass (*Lolium perenne*) used as the common species, in increasing proportions (25%; 37.5%; 50%; 62.5%; 75% and 100%). The *Lolium perenne* monoculture represents the treatment with 0% partner species. Table 3.1 lists all partner species used in the binary mixtures. Species listed only in one year (2018) were outcompeted and did not persist in the second year in the field.

Table A.1. Plant species combined for the *in vitro* Hohenheim gas test (HGT), cultivar, common name, abbreviation, plant family and year of sampling.

	Species/Cultivar [†]	Common name	Abb [*]	Plant family	Year
Common	<i>Lolium perenne</i>	Perennial ryegrass	<i>L. perenne</i>	<i>Poaceae</i>	2018, 2019
	<i>Carum carvi</i>	Caraway	<i>C. carvi</i>	<i>Apiaceae</i>	2018, 2019
	<i>Cichorium intybus</i>	Chicory	<i>C. intybus</i>	<i>Asteraceae</i>	2018, 2019
Partner	<i>Lotus corniculatus</i> "Leo"	Birds'foot trefoil	<i>L. corniculatus</i> Leo	<i>Fabaceae</i>	2018, 2019
	<i>Lotus corniculatus</i> "Lotanava"	Birds'foot trefoil	<i>L. corniculatus</i> Lotanava	<i>Fabaceae</i>	2018
	<i>Lotus pedunculatus</i>	Big trefoil	<i>L. pedunculatus</i>	<i>Fabaceae</i>	2018
	<i>Trifolium pratense</i>	Red clover	<i>T. pratense</i>	<i>Fabaceae</i>	2018, 2019
	<i>Trifolium repens</i>	White clover	<i>T. repens</i>	<i>Fabaceae</i>	2018, 2019
	<i>Plantago lanceolata</i>	Ribwort plantain	<i>P. lanceolata</i>	<i>Plantaginaceae</i>	2018, 2019
	<i>Sanguisorba minor</i>	Salad burnet	<i>S. minor</i>	<i>Rosaceae</i>	2018

^{*}Hereafter, species are referred to as abbreviations that are presented above.

[†]Except for *Lotus corniculatus*, the samples of each species harvested from the forage mixture is a combination of up to 4 cultivars in *L. perenne*, with most species consisting of 2 cultivars to manage risk of unsuccessful establishment and ensure representativeness at the species level.

A.4.1 Sample collection

All forage species were sown in autumn 2016, in plots of approximately 2.2 ha in a diverse mixture, integrating a two-year grass-clover ley system in an organic 4-year crop rotation (2 years of grass-clover, followed by 2 years of crops: oats/winter triticale, faba beans/winter spelt, consecutively), at "Lindhof", the experimental farm for organic production of Kiel University (54°27'56.0"N 9°57'56.0"E). Samples of each species for the *in vitro* analysis were collected from these multispecies swards before grazing in spring, in two consecutive years (2018 and 2019) when the sward was at a late vegetative growth stage. Some species (*L. corniculatus* Lotanava, *L. pedunculatus* and *S. minor*) were harvested only in 2018, due to their low persistence. A sample of approximately 300 g of fresh plant material was cut to a residual height of 4 cm. Samples were immediately frozen and stored at -28°C. Before analysis, samples were freeze-dried to a constant weight and milled to a particle size of 1 mm (Ultra centrifugal mill, ZM200, Retsch GmbH, Haan, Germany).

A.4.2 In vitro incubation

Total gas and methane production of these forage mixtures were determined using the Hohenheim gas test (HGT) according to the procedure described by Menke and Steingass (1988). Each forage mixture was incubated in triplicate, and these incubations were repeated two times within an interval of one week. In addition to the samples, four blank syringes (rumen

fluid without added plant material) and three replicates of hay and concentrate standards (200 ± 1 mg) were included to correct the gas production each time.

Incubations were run for 24 h with recording of total gas production and methane production at 8 and 24 h. Subsequently, these values were adjusted to the dry matter and digestible organic matter (DOM) content of the incubated substrates. Methane concentration in the total gas was measured by an infrared methane analyzer (Pronova Analysentechnik GmbH & Co. KG, Berlin, Germany). Total gas production was obtained according to the following equation: $((V_{24}-V_0+V_8-Z-Gb_0) * F * 200)/W$, where Gb is the total gas produced (ml/200 mg DM); V_0 , V_8 and V_{24} is the gas volume (ml) at 0, 8 and 24 h from the beginning of the incubation time, respectively; Z is the volume of sample without gas; Gb_0 is the gas volume before the incubation; F is the average of the correction factor for hay and concentrate and W is the weight of the sample (mg).

The rumen fluid for this analysis was obtained from four rumen-cannulated, non-lactating Jersey \times German Black Pied heifers, with average body weight of 565 ± 29 kg, belonging to the Institute of Animal Nutrition and Physiology of Kiel University. Animals were fed a ration consisting of 3 kg of grass hay and 3 kg of concentrate divided into 2 meals (7 a.m. and 4 p.m.). Approximately 750 ml of rumen fluid was collected before the morning feeding, for each incubation run. The fluid was subsequently filtered through cheese-cloth and transferred into a pre-warmed thermos flask and immediately transported to the laboratory. The preparation of incubation media, filling of syringes and all additional procedures were done according to the protocol of Menke and Steingass (1988), and 200 mg (± 1 mg) of each replicate were weighed individually and filled into 100 ml fermentation syringes with 30 mL of rumen fluid, for incubation.

A.4.3 Sample analysis

A.4.3.1 Analysis of the chemical composition

All forage samples used in the *in vitro* incubation were analysed using near-infrared reflectance spectroscopy (NIRS) with a NIRSystems 5000 monochromator (FOSS, Silver Spring, USA). Mathematical evaluation of the spectra was performed using the Modified Partial Least Squares method (WinISI software version 3, Infrasoft International, USA). Calibration and validation were based on sample subsets of perennial ryegrass, legumes and forage herb species, which represented the whole spectral and chemical variability.

The following analyses of the subset samples were performed as follows: N concentration was directly determined with an elemental analyzer (Vario Max CN, Elementar

Analysensysteme, Hanau, Germany); crude protein content (CP) was calculated from the N content ($CP = N \times 6.25$). The concentrations of neutral detergent fibre (assayed with heat-stable amylase (aNDF)) and acid detergent fibre (ADF) were analysed using a Fiber Analyzer Ankom A2000 (Ankom Technology, Macedon, NY, USA). ADF values are expressed exclusive of residual ash (ADF_{om}). Ash (A) was determined by combustion in a muffle furnace (24 h at 550 °C). Digestibility and metabolizable energy (ME) content of herbage samples were determined using the *in vitro* cellulase technique developed by De Boever, et al. (1988). The percentage of digestible organic matter (DOM) was calculated using the enzymatic soluble organic matter (ELOS) and the enzymatic insoluble organic matter (EULOS).

The following equations were used:

$$DOM (\%) = 100 (940 - A - 0.62 \text{ EULOS} - 0.000221 \text{ EULOS}^2) / (1000 - A) \quad (1)$$

$$\text{EULOS (g/kg DM)} = 1000 - A - \text{ELOS} \quad (2)$$

$$\text{ME (MJ/kg DM)} = 5.51 + 0.00828 \text{ ELOS} - 0.00511 \text{ A} + 0.02507 \text{ CL} - 0.00392 \text{ ADF}_{om} \quad (3)$$

Where: A, CL (crude fat), ELOS, EULOS are expressed in g/kg DM

The statistical key figures of the NIRS calibration and validation are given in Table A1.

A.4.3.2 Analysis of tannin composition

Tannin extraction and sample preparation were done as described by Malisch, et al. (2015). Briefly, forage samples were freeze-dried and subsequently ball-milled using a MM 200 mill (Retsch Technology GmbH, Haan, Germany). Plant samples (20 mg) were weighed into 2 ml Eppendorf tubes and stored at -80 °C until further analysis. For extraction, 1.4 mL of acetone/H₂O (80:20, v/v) was added into the Eppendorf tubes, which were then shaken for 15 min in a vortex shaker. The plant and solvent mixture was macerated in a refrigerator overnight to enhance the extraction efficiency of the especially large PAs. While macerating in the refrigerator, the tubes were shaken in a planar shaker before centrifuging at 9000g and decanting of the solvent. Solvent samples were concentrated for approximately 2 h in an Eppendorf concentrator plus (Eppendorf AG, Hamburg, Germany) to remove the acetone, and the plant residues were extracted with 1.4 mL of a new acetone and H₂O solution (80:20, v/v) for an additional 3 h. The two extracts were then combined and concentrated into the water phase. The extracts were then frozen and freeze-dried (Beta 1-8 LD plus, Martin Christ GmbH, Osterode, Germany) overnight and stored at -20 °C. Prior to injection in the ultra performance liquid chromatography - tandem mass spectrometer (UPLC-MS/MS), samples were dissolved

in 1 mL of ultrapure water, shaken for 10 min, filtered with 0.2 µm PTFE syringe filters (VWR International, Radnor, PA, USA), and diluted 2-fold with ultrapure water.

After milling, partner species were homogenized and a subsample was taken for analysis via UPLC-MS. The UPLC-MS/MS analysis of all forage samples was conducted according to Engström, *et al.* (2014). Therefore, the setup was identical to the Acquity UPLC system (Waters Corp., Milford, MA, USA), connected to a Xevo TQ triple-quadrupole mass spectrometer (Waters Corp., Milford, MA, USA) with electrospray ionization. For the UPLC system a 100 mm × 2.1 mm i.d., 1.7 µm, Acquity UPLC BEH Phenyl column was utilized (Waters Corp., Wexford, Ireland). Quantification was made via a diode array detector and the Xevo TQ mass spectrometer, using multiple reaction monitoring (MRM) methods. Before each run, to determine the system's performance, a flavonoid mix stock solution was injected twice. Additionally, prior to and after each 10 samples, 5 samples of a catechin stock solution (1.0 µg mL⁻¹) were run to determine the stability of the system's ionization efficiency for polyphenols over the course of the analysis, and to correct the measured concentrations accordingly.

A.4.4 Statistical analysis

The effect of the differing partner species on methane and total gas emissions was analysed with the statistical software R Core Team (2020) in three different approaches:

- i. a linear mixed model was used (Pinheiro et al., 2016), with the partner species, the sampling year and the partner proportion, as well as their interactions (two- and threefold) as fixed factors, while partner species nested in the date of analysis was the random factor. Residuals were assumed to be normally distributed and heteroscedastic, based on a graphical residual analysis, and the heteroscedasticity was accounted for. Subsequently, multiple contrast tests (Hothorn et al., 2008) were conducted in order to compare the different levels of the influence factors. With Y being the response variable, the model was:

$$Y_{ijlm} \sim a_i + (ab)_{ij} + c_k + (cd)_{kl} + e_{ijklm} \quad (4)$$

Where: Y_{ijlm} is the response variable of the m th piston of each partner share i in the combination of partner species and sampling year j , at each individual measurement date k for each species l respectively. The levels of the respective fixed factors were:

$i = 1, \dots, 6$ (partner share)

$j = 1, \dots, 17$ (combination of partner species and sampling year)

while the random terms had the levels

$k = 1, \dots, 14$ (date of in vitro analysis)

$l = 1, \dots, 10$ (partner species)

and the error term included additionally:

$m = 1, 2, 3$ (piston replicate).

- ii. To analyse the change in methane and total gas formation as a slope for each individual species, the model from I) was modified to now include the proportion of the partner species as a quantitative factor, together with a pseudo factor representing the combination of the actual factors partner species and sampling year. The pseudo factor was necessary, as the actual factors were not fully orthogonal due to the lack of three species in the 2019 sampling. The partner species nested in the analysis date was again the random factor and residuals were again assumed to be normally distributed and heteroscedastic based on a graphical residual analysis, and the heteroscedasticity was accounted for. Based on this model, Pseudo R^2 (coefficient of determination) values for each species were calculated according to Nakagawa and Schielzeth (2013). Slopes were tested to be significantly different from zero to identify which species would result in significant increases or decreases in methane or total gas emissions with increasing partner shares. Additionally, due to the large number of species, the grand mean was used to determine which species would perform better (or worse) than the average.
- iii. Additionally, covariates with prediction potential for changes in CH_4 or total gas emissions were identified, using parameters from forage quality or PSM. For this, it was tested whether a model based on these covariates would explain the emissions similarly and well, compared to the linear mixed model, with the aim of generating a formula that could predict CH_4 emissions and digestibility, independently of the plant species. In total 18 covariates were assessed in this approach, including the main forage quality parameters listed in Table 3.2, as well as the plant secondary metabolite concentrations listed in Table 3.3. Subsequently, an analysis of covariance was performed based on the model described in eq 1, adding covariates stepwise using a forward selection, based on Akaike information criterion (Akaike, 1974). Finally, covariates with a variance inflation factor (VIF) of 5 or greater were removed.

A.5 Results

The results are presented below as follows: i) the measured values of the *in vitro* analysis and for methane and total gas are presented for the individual mixtures, including the pairwise comparisons between the increasing legume/herb shares. With this, the observed variability across the mixtures can be evaluated and the potential linearity of dose-response effects assessed. Additionally, the analysis of covariance (ANCOVA) is presented to identify whether forage characteristics alone suffice to explain methane and total gas production. This is followed by ii) the modelled slopes for each individual species, which are tested against the grand mean to identify their general methane reduction potential and the relative change in digestibility. Finally, the model performance against the measured values is presented.

A.5.1 Measured parameters

A.5.1.1 Forage quality was high for all species and across both years

The energy concentration calculated from NIRS-based forage quality parameters was validated against the energy concentration as derived from total gas formation in the HGT (Table 3.2). The general coefficient of determination was $R^2 = 0.44$. When excluding the forage species with the highest tannin concentrations, *L. pedunculatus* and *S. minor*, this increased to $R^2 = 0.70$. The best coefficient of determination was achieved in the group of non-tannin containing forage legumes and herbs with $R^2 = 0.88$. Independent of the estimation method, the energy concentration was still high for all species throughout both years, despite general decrements for legumes and herbs in the second year. The species with the greatest energy content as ME in 2018, according to the total gas formation, was *L. perenne*, with 11.6 MJ ME/kg DM, followed by *L. corniculatus* Leo, *C. carvi* and *C. intybus*, all of which had energy concentrations greater than 11.0 MJ ME/kg DM. The OM digestibility (DOM) was greatest in *C. carvi*, *C. intybus* and *P. lanceolata* with 920 g/kg DM on average. *Sanguisorba minor* and *L. corniculatus* Leo had lower mean values of DOM (on average 870 g/kg DM), while *L. pedunculatus* was the species with the least DOM (800 g/kg DM, -9% when compared to the average of all partner species). *Carum carvi* showed exceptionally low CP contents (67 g/kg DM, ~55% less than the average of all partner species) while *T. repens* and *T. pratense*, the most common forage legumes, showed the greatest CP contents (211 g/kg DM, on average).

In 2019, when compared to 2018, the energy concentration of *L. perenne* increased even further and reached 12.1 MJ/NEL kg DM and a DOM of 910 g/kg DM. Almost all partner species showed a decrease in energy content (-4% NEL, on average) and *T. repens* was the only species that exceeded 11.0 MJ NEL/kg DM. Also, the DOM of partner species generally decreased (-5%, on average) as a consequence of a greater NDF concentration (+21%; Table 3.2).

Table 3.2. Chemical composition and energy content of the species evaluated, harvested in 2018 and 2019.

Year	Species	ME _{gas}	ME _{NIRS}	NEL	NDF	ADF	DOM	CP
		(MJ/kg DM)		(g/kg DM)				
2018	<i>L. perenne</i>	11.6	10.5	6.5	508	271	850	108
	<i>C. carvi</i>	11.0	11.0	6.7	303	233	930	67
	<i>C. intybus</i>	11.1	11.3	6.7	346	236	920	100
	<i>L. corniculatus</i> Leo	11.2	11.2	6.8	351	217	840	178
	<i>L. corniculatus</i> Lotanava	10.7	10.9	6.6	375	240	830	182
	<i>L. pedunculatus</i>	9.6	10.3	6.1	449	290	800	173
	<i>T. pratense</i>	10.9	10.8	6.6	412	221	860	214
	<i>T. repens</i>	11.0	10.6	6.6	418	257	860	209
	<i>P. lanceolata</i>	11.1	11.3	6.9	341	232	910	133
	<i>S. minor</i>	10.6	12.2	7.0	417	253	890	149
2019	<i>L. perenne</i>	12.1	11.7	7.2	394	200	910	100
	<i>C. carvi</i>	10.2	10.3	6.3	414	291	870	145
	<i>C. intybus</i>	10.4	10.4	6.4	513	276	870	162
	<i>L. corniculatus</i> Leo	9.3	9.9	5.9	425	310	810	186
	<i>T. pratense</i>	NA	11.3	6.9	359	204	850	172
	<i>T. repens</i>	11.4	11.4	7.1	338	189	890	214
	<i>P. lanceolata</i>	9.9	9.8	6.1	582	310	790	169

A.5.1.2 Plant secondary metabolites varied across species with *L. pedunculatus* and *S. minor* showing highest tannin concentrations

In 2018, the greatest concentration of polyphenols was observed in *S. minor* (47.6 mg/g DM; Table 3.3), of which 86% were hydrolysable tannins, and the rest were flavonols. The second highest polyphenolic concentrations were measured in *L. pedunculatus*, with 20.8 mg/g DM, of which 88% were proanthocyanidins (PA), with a prodelfphinidin (PD) share of 79%. The *L. corniculatus* Lotanava showed greater shares of total polyphenols, as well as total tannins, compared to *L. corniculatus* Leo, with CT concentrations of 4.1 and 1.7 mg/g DM, respectively. Both *Trifolium* spp. showed greater shares of flavonols (89% and 66% of total polyphenols for *T. pratense* and *T. repens*, respectively), but almost no proanthocyanidins (0.3 and 1.8 mg/g DM for *T. pratense* and *T. repens*, respectively). The lowest polyphenol concentrations were generally observed in *P. lanceolata*.

As the species with greatest tannins content in 2018 (i.e. *L. pedunculatus* and *S. minor*) could not be sampled for analysis in 2019, *L. corniculatus* Leo showed the greatest tannins contents compared to the other species in 2019. However, these concentrations were substantially lower at a total tannin concentration of 2 mg/g DM. Highest polyphenolic concentrations were measured in *C. carvi* with 5.9 mg/g DM. While the metabolic profile in *L. corniculatus* Leo and *P. lanceolata* were stable between 2018 and 2019, the polyphenolic concentrations in *C. carvi*, *C. intybus* and *T. repens* decreased from the 2018 to the 2019

sampling, yet not uniformly, as larger declines were observed in *T. repens* and *C. intybus* with 55% less total polyphenols, each, and no measurable PA concentration in *T. repens*.

Table 3.3. Tannin composition (mg/g DM) of the species evaluated, harvested in 2018 and 2019. (Polyphenols (PP), Flavonols (F), Tannins (T), hydrolysable tannins (HT), Proanthocyanidins (PA), Prodephinidins (PD)).

Year	Species	PP	F	T	HT	PA	PD share
		(mg/g DM)					(%)
2018	<i>C. carvi</i>	8.8	4.1	0.0			
	<i>C. intybus</i>	9.3	2.4	0.0			
	<i>L. corniculatus</i> Leo	3.1	1.4	1.7	0.2	1.5	25
	<i>L. corniculatus</i> Lotanava	5.6	1.5	4.1	0.3	3.9	36
	<i>L. pedunculatus</i>	20.8	2.4	18.4	0.2	18.2	79
	<i>T. pratense</i>	4.5	4.0	0.3	0.0	0.3	44
	<i>T. repens</i>	6.1	4.0	1.8	0.0	1.8	100
	<i>P. lanceolata</i>	1.2	0.1	0.0			
	<i>S. minor</i>	47.6	5.6	41.0	41.0	0.0	0
2019	<i>C. carvi</i>	5.9	2.9	0.0			
	<i>C. intybus</i>	4.2	1.4	0.0			
	<i>L. corniculatus</i> Leo	3.6	1.3	2.0	0.1	1.9	30
	<i>T. pratense</i>	3.3	3.1	0.1	0.0	0.1	0
	<i>T. repens</i>	2.8	2.8	0.0			
	<i>P. lanceolata</i>	1.1	0.3	0.0			

A.5.1.3 Total gas production was generally linked to the mixture composition linearly, while changes in methane were less predictable

In 2018, total gas production was generally dose-dependent and decreased as the proportion of the partner species increased (Table 4a, b). Accordingly, the largest reduction when comparing with *L. perenne* was always obtained with the pure partner species. The largest reduction was 32%, which was obtained with *S. minor* (Table 4b). Of these species, particularly *S. minor* and *L. pedunculatus* showed high coefficients of determination with $R^2 = 0.74$ and $R^2 = 0.72$, respectively (Table 5). In contrast, methane formation was not dose dependent, and the largest methane reduction was not achieved with pure partner species but was obtained at the ratio of 67.5% partner species with 32.5% *L. perenne*, with emissions having the tendency to increase again with a proportion of partner species in the ratio. However, the differences across partner shares were generally not significant within any species, and only in *L. pedunculatus*, *S. minor* and *P. lanceolata* were these lowest emissions different from even the highest emissions in the pairwise comparison (Table 3.4a, b). Also, due to the non-linear relationships of methane formation per increasing partner share, coefficients of determination were generally low and only significant for *L. pedunculatus* and *S. minor* (Table 3.5).

In 2019, with the exception of *C. intybus*, there was again a dose-dependent effect and this obtained the lowest total gas formation at 100% partner share. However, in the absence of the species with the highest tannin concentrations, it was *L. corniculatus* Leo that now obtained the lowest total gas formation with reductions of 21% compared to *L. perenne* (Table 3.4a) and *L. corniculatus* Leo also showed the highest coefficient of determination ($R^2 = 0.74$) (Table 3.5). With regards to methane formation, while there was again no dose-dependent effect in 2019, there also was no trend of lowest methane formations occurring at 67.5% of partner species. In fact, there was no discernable trend at all (Table 3.4a). Accordingly, the highest coefficients of determination were obtained with *C. intybus* and *L. corniculatus* Leo, with $R^2=0.34$ and $R^2= 0.39$, respectively (Table 3.5).

Table 3.4. a. Mean values of measured *in vitro* fermentation parameters in a 24h incubation period, of species harvested in 2018 and 2019, consecutively. Numbers between brackets correspond to standard error of the mean (SEM).

Species	Partner prop.	Total gas (ml/200 mg DOM)				Methane (ml/200 mg DOM)			
		2018		2019		2018		2019	
<i>L. perenne</i>	1	93.4	(3.23)	80.8	(2.38)	19.2	(1.68)	15.6	(0.24)
<i>C. carvi</i>	0.25	94.2	(0.68)	83.9	(0.68)	16.1	(0.07)	16.4	(0.32)
	0.375	89.5	(1.94)	82.4	(0.64)	16.5	(0.45)	16.2	(0.33)
	0.5	90.0	(0.72)	74.9	(0.41)	15.3	(0.34)	14.5	(0.16)
	0.675	86.8	(1.14)	82.2	(0.72)	14.3	(0.09)	16.1	(0.08)
	0.75	87.7	(1.22)	77.8	(0.64)	16.0	(0.43)	15.4	(0.45)
	1	83.8	(0.95)	77.3	(5.31)	16.6	(0.46)	15.1	(1.29)
<i>C. intybus</i>	0.25	94.3	(0.28)	81.2 ^A	(1.10)	16.0	(0.06)	15.9 ^{AB}	(0.28)
	0.375	86.2	(1.40)	81.3 ^A	(0.55)	14.4	(0.17)	16.2 ^A	(0.13)
	0.5	88.1	(1.57)	75.3 ^{AB}	(0.74)	15.4	(0.22)	14.4 ^{AB}	(0.07)
	0.675	86.2	(1.66)	80.9 ^A	(0.69)	13.6	(0.22)	15.9 ^{AB}	(0.09)
	0.75	84.8	(1.10)	75.5 ^B	(1.49)	15.3	(0.38)	14.7 ^B	(0.55)
	1	79.5	(0.69)	72.7 ^{AB}	(0.25)	15.7	(0.62)	14.0 ^{AB}	(0.15)
<i>L. corniculatus</i> Leo	0.25	91.4 ^{AB}	(2.25)	78.8 ^{AB}	(1.16)	16.7 ^{AB}	(0.24)	15.3 ^A	(0.19)
	0.375	88.0 ^{AC}	(1.34)	79.6 ^A	(0.50)	16.9 ^A	(0.41)	16.3 ^{AB}	(0.08)
	0.5	83.6 ^{CD}	(1.28)	76.7 ^{AB}	(1.00)	16.2 ^{AB}	(0.18)	14.9 ^A	(0.18)
	0.675	81.9 ^{ABCD}	(2.03)	75.7 ^{ABC}	(1.46)	14.7 ^{AB}	(0.17)	15.8 ^{AB}	(0.22)
	0.75	83.6 ^{jBD}	(1.05)	70.9 ^{kBC}	(0.73)	16.2 ^B	(0.44)	14.8 ^{AB}	(0.72)
	1	75.7 ^{BD}	(0.69)	63.8 ^C	(2.23)	16.5 ^{AB}	(0.37)	12.8 ^B	(0.23)
<i>T. pratense</i>	0.25	87.1 ^A	(1.84)	86.5	(0.87)	16.0 ^A	(0.34)	17.1 ^A	(0.28)
	0.375	82.8 ^{ABC}	(2.33)	87.6	(0.40)	16.5 ^A	(0.53)	17.5 ^{AB}	(0.37)
	0.5	82.9 ^{AB}	(0.72)	86.3	(1.07)	16.6 ^{AB}	(0.27)	17.5 ^{AB}	(0.34)
	0.675	80.2 ^{ABC}	(1.82)	89.4	(2.02)	14.9 ^{AB}	(0.15)	18.3 ^B	(0.35)
	0.75	80.1 ^{BC}	(3.20)	87.2	(2.06)	16.8 ^B	(0.35)	18.5 ^B	(0.08)
	1	72.0 ^C	(0.78)	82.8	(1.06)	16.7 ^{AB}	(0.62)	17.3 ^B	(0.20)
<i>T. repens</i>	0.25	96.0 ^A	(0.80)	81.4 ^{AB}	(0.67)	17.1	(0.03)	16.4	(0.37)
	0.375	82.9 ^{AB}	(1.62)	80.1 ^{AB}	(0.23)	16.5	(0.44)	16.4	(0.41)
	0.5	89.1 ^{ABC}	(0.55)	75.6 ^{AC}	(0.44)	16.7	(0.15)	15.0	(0.43)
	0.675	81.5 ^{ABC}	(0.69)	79.3 ^{AB}	(1.04)	15.3	(0.08)	16.6	(0.22)
	0.75	77.9 ^C	(1.16)	73.5 ^{CD}	(1.19)	16.3	(0.51)	16.3	(0.41)
	1	75.4 ^{BC}	(0.90)	69.8 ^{BD}	(0.20)	17.5	(0.55)	15.1	(0.04)
<i>P. lanceolata</i>	0.25	95.3 ^A	(0.95)	82.4	(1.83)	16.4 ^{ABCD}	(0.11)	16.2	(0.57)
	0.375	87.0 ^{AB}	(0.84)	79.6	(0.82)	15.2 ^{AB}	(0.10)	15.8	(0.20)
	0.5	87.5 ^A	(0.82)	82.6	(0.53)	16.5 ^{AC}	(0.23)	16.4	(0.09)
	0.675	83.9 ^{AB}	(1.54)	79.5	(1.14)	14.3 ^{CD}	(0.24)	15.9	(0.36)
	0.75	81.2 ^{AB}	(1.40)	77.5	(2.36)	15.4 ^{ABCD}	(0.43)	15.7	(0.45)
	1	77.3 ^B	(1.50)	72.6	(4.55)	16.1 ^{BD}	(0.37)	13.7	(0.75)

¹ DOM: digestible OM.

j-k non-common lowercase letters indicates differences between years.

A-D non-common uppercase letters indicates differences between partner proportions.

Table 3.4. b. Mean values of measured *in vitro* fermentation parameters in a 24h incubation period of *Lotus corniculatus* cv. Lotanava, *Lotus pedunculatus*, and *Sanguisorba minor* samples, harvested in 2018.

Species	Partner prop.	Total gas			Methane		
		(ml/200 mg DOM)					
<i>L. corniculatus</i> Lotanava	0.25	90.7	AB	(2.70)	16.3	AB	(0.42)
	0.375	87.8	A	(1.59)	16.7	A	(0.42)
	0.5	81.7	BC	(1.18)	15.6	AB	(0.35)
	0.675	82.7	ABC	(1.51)	14.6	AB	(0.11)
	0.75	80.7	BC	(1.29)	15.7	B	(0.46)
	1	71.9	C	(2.46)	15.8	AB	(0.47)
<i>L. pedunculatus</i>	0.25	87.7	AB	(3.70)	15.5	AB	(0.56)
	0.375	82.1	AC	(0.96)	14.1	A	(0.06)
	0.5	79.7	ABCD	(0.29)	14.9	AB	(0.06)
	0.675	73.4	BD	(3.14)	12.5	B	(0.25)
	0.75	71.7	CD	(1.07)	13.7	AB	(0.38)
	1	63.6	D	(1.59)	13.3	AB	(0.52)
<i>S. minor</i>	0.25	89.3	A	(1.17)	15.4	ABCD	(0.21)
	0.375	79.8	A	(1.58)	14.5	AB	(0.38)
	0.5	79.5	AB	(1.96)	15.0	AC	(0.44)
	0.675	72.8	ABC	(2.32)	11.9	BD	(0.14)
	0.75	71.8	BC	(1.15)	13.0	CD	(0.34)
	1	63.3	C	(1.31)	13.4	BD	(0.68)

¹ DOM: digestible OM.

A-D non-common uppercase letters indicates differences between partner proportions.

The methane concentration (i.e. methane/total gas ratio) expresses how methane changes compared to total gas, which is a proxy of digestibility. Here, the coefficients of determination were again generally higher in 2018, compared to 2019 (Table 3.5). However, *T. pratense* and *T. repens* were the species with the highest coefficients of determination in both years.

Table 3.5. Mean adjusted R^2 and P values derived from basic linear regression for methane production (ml/200 mg DOM), total gas production (ml/200 mg DOM) and methane concentration after 24h-rumen fermentation of the studied forage species harvested in 2018 and 2019. (Significance levels are: *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$ and ns $P > 0.05$).

Year	Species	Total gas		Methane		Methane/ total gas	
				adjusted R^2			
2018	<i>C. carvi</i>	0.19	**	-0.02	ns	0.12	*
	<i>C. intybus</i>	0.49	***	0.00	ns	0.31	***
	<i>L. corniculatus</i>	0.52	***	0.00	ns	0.26	***
	<i>Leo</i>						
	<i>L. corniculatus</i>	0.48	***	0.03	ns	0.28	***
	<i>Lotanava</i>						
	<i>L. pedunculatus</i>	0.72	***	0.12	*	0.46	***
	<i>T. pratense</i>	0.33	***	-0.02	ns	0.48	***
	<i>T. repens</i>	0.46	***	-0.02	ns	0.47	***
	<i>P. lanceolata</i>	0.59	***	0.00	ns	0.39	***
2019	<i>S. minor</i>	0.74	***	0.22	**	0.20	**
	<i>C. carvi</i>	0.23	**	0.09	ns	-0.03	ns
	<i>C. intybus</i>	0.40	***	0.34	***	0.01	ns
	<i>L. corniculatus</i>	0.74	***	0.39	***	0.00	ns
	<i>Leo</i>						
	<i>T. pratense</i>	-0.01	ns	0.11	ns	0.23	**
	<i>T. repens</i>	0.69	***	0.02	ns	0.27	**
	<i>P. lanceolata</i>	0.25	**	0.17	**	-0.03	ns

A.5.1.4 Covariates did not improve the model's ability to predict rumen fermentation parameters

The ANCOVA did not show any covariates that improved the basic model with the fixed factors partner proportion and the interaction of partner proportion, and a pseudo factor derived from partner species and sampling year, for either the total gas or the methane production. For total gas production per g DOM, only the interaction of partner proportion with the pseudo factor of partner species and harvest year was significant ($F_{14,416} = 31.6$, $P \leq 0.001$), while the partner proportion alone was not significant ($F_{1,416} = 0.7$, $P = 0.39$; results not shown). The marginal R^2 for the final model was 0.70, and the conditional R^2 was 0.84, indicating that the fixed factors were able to explain most of the variability.

With regards to the methane production per g DOM, again only the interaction of the partner proportion with the pseudo factor derived from partner species and sampling year ($F_{14,413} = 43.6$, $P \leq 0.001$) was significant, with the partner proportion alone being less relevant ($F_{1,413} = 3.81$, $P \leq 0.05$; results not shown). The marginal R^2 for the final model was 0.42, while the conditional R^2 was 0.91, indicating that the fixed factors explained less than half of the observed variation. None of the covariates were able to improve the quality of the model.

For the methane concentration in the total gas, several covariates were significant and improved the quality of the model. Accordingly, a measured impact was determined for NDF content ($F_{1,423}=16.7$, $P\leq 0.001$), hydrolysable tannins ($F_{1,423}=63.6$, $P\leq 0.001$), total myricitins ($F_{1,423}=9.7$, $P\leq 0.001$), total quinic acid ($F_{1,423}=266.9$, $P\leq 0.001$), total kaempferols ($F_{1,423}=28.2$, $P\leq 0.001$) and total prodelphinidins ($F_{1,423}=63.6$, $P\leq 0.001$; results not shown). Despite the large number of significant covariates, the marginal R^2 for the final model was 0.13, while the conditional R^2 was 0.98, indicating a low predictive capacity of the fixed factors. Of these factors, NDF, total myricitins and total kaempferols had a positive effect on the methane concentration, while hydrolysable tannins, total quinic acid and total prodelphinidins had negative relationships (results not shown).

A.5.2 Modelled fermentation parameters

A.5.2.1 Tannin containing species reduce methane formation most, but do not provide the lowest methane concentrations

There was generally a decrease in methane production with increasing shares of partner species (Figure 3.1). In 2018, the greatest methane reductions ($P\leq 0.001$) were predicted by the model for the species with the highest tannin concentrations, *L. pedunculatus* and *S. minor*, with slopes of -4.9 and -5.7, respectively (Figure 3.1). The third highest reductions ($P\leq 0.001$) were obtained with *C. intybus*, with a slope of -3.0, which was the only other species which had emission reductions significantly lower than the grand mean of all partner species ($P\leq 0.001$). However, for *L. pedunculatus* and *S. minor* the model also predicted the highest reductions ($P\leq 0.001$) in total gas, with slopes of -28.0 and -27.7, respectively. Contrary to that, *C. carvi* and *C. intybus* reduced total gas formation significantly less compared to the grand mean, with slopes of -7.2 ($P\leq 0.001$) and -11.2 ($P\leq 0.01$). As a result, the methane concentration of *L. pedunculatus* exhibited a slope of 0.005 and thus constituted a slight increment compared to that of pure *L. perenne*. Contrary to that, *S. minor* was predicted to reduce ($P\leq 0.001$) the methane concentration, but only slightly, with a slope of -0.005. However, the strongest reduction was achieved with *C. intybus*, with a slope of -0.009, thus being almost twice the slope of the best tannin-containing species.

In 2019, in absence of the species with the highest tannin concentration, the strongest methane reduction potential was predicted for *L. corniculatus* Leo, with a slope of -1.9, which was not significantly less compared to the grand mean but was different from zero ($P\leq 0.001$; result not shown). The second greatest reduction was predicted for *C. intybus*, with a slope of -1.7. However, when looking at total gas formation, *L. corniculatus* Leo was also predicted by

the model to result in the greatest reduction of -17.9, being much higher than the slope of *C. intybus*, which was estimated to be -10.3. While both were not significantly less than the grand mean, both slopes were significantly different from 0 ($P \leq 0.001$, result not shown). As a result, the slope of *C. intybus* for methane concentration was the lowest with 0.003, which was not significantly different from 0 (result not shown), while the methane concentration of *L. corniculatus* Leo was predicted to increase with a slope of 0.02, which was not significantly different from the grand mean (Figure 3.1) but was different ($P \leq 0.001$) from 0 (result not shown). The strongest increments in methane concentration were predicted for the most widely used forage legumes, white and red clover, with slopes of 0.03, which were greater ($P \leq 0.001$) than the grand mean and different from 0 ($P \leq 0.001$, result not shown).

A.5.2.2 Despite differences in total gas and methane formation, trends in methane concentration are stable across both experimental years

Despite the fact that the most important of the tannin-containing species could not be sampled in 2019 again, six partner species were harvested in both of the two years and their responses can be compared. Due to changes in forage quality in perennial ryegrass, the intercept of the model changed between these two years and were 17.2 and 16.1 ml/200 mg DOM for the methane formation, 92.3 and 84.4 ml/200 mg DOM for the total gas, and 0.185 and 0.191 for the methane concentrations of samples from 2018, and 2019, respectively (Figure 3.1). Due to the non-orthogonal design, interactions between partner species and year cannot be evaluated, yet both slopes and the order of species slopes differed between years for both methane and total gas formation. Accordingly, *L. corniculatus* Leo, which was best of the tested treatments at reducing methane formation in 2019, was second lowest in 2018, while *T. pratense* reduced methane emissions with a slope of -1.8, yet increased methane formation in 2019, with a slope of 2.4, both of which were significantly different from 0 ($P \leq 0.001$, result not shown). Similarly, *T. pratense* was predicted to reduce total gas formation in 2018 with a slope of -20.0, compared to 0 ($P \leq 0.001$), yet was predicted to have a non-significant tendency to increase with a slope of 2.6 (result not shown). Despite that, the ranking of species for methane concentration was the same across both years regardless of the fact that *C. carvi* and *C. intybus* had not been predicted to result in negative slopes in 2019, compared to 2018. Furthermore, when testing all slopes from 2018 against those of 2019, the coefficient of determination equals $R^2 = 0.94$ (result not shown), which indicated that generally the change in methane concentration with increasing share of partner species remains stable across species and years.

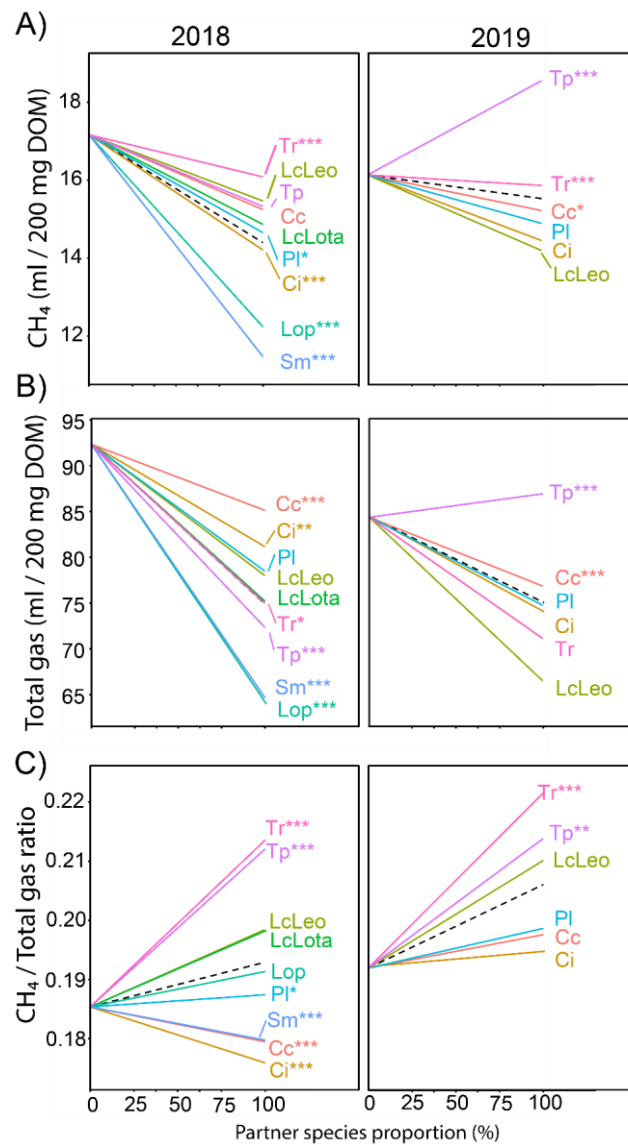


Figure 3.1. Methane production (ml/200 mg DOM), total gas production (ml/200 mg DOM) and methane/total gas ratio, after 24h-rumen fermentation of the selected forage species harvested in 2018 and 2019, mixed in increasing proportions with perennial ryegrass (Significance levels are: ***P ≤ 0.001, ** P ≤ 0.01, * P ≤ 0.05 and † P > 0.05). The dashed line corresponds to the Grand Mean. Abbreviations are as follows (in alphabetical order): Cc: *Carum carvi*, Ci: *Cichorium intybus*, LcLeo: *Lotus corniculatus* cv. Leo, LcLota: *Lotus corniculatus* cv. Lotanova, LoP: *Lotus pedunculatus*, Pl: *Plantago lanceolata*, Sm: *Sanguisorba minor*, Tp: *Trifolium pratense*, Tr: *Trifolium repens*.

A.5.3 Performance of model compared to measured data

When comparing the model performance against measured data, slopes were less than 1 for both the methane production (slope=0.41, Figure 3.2 A) and total gas production (slope = 0.63, Figure 3.2 B). Both intercepts were positive with 9.1 and 29.8, for methane production and total gas production, respectively. Thus, the model generally tended to overestimate lower emissions, while underestimating higher emissions. Across both years, the general model

performance was better for total gas ($R^2 = 0.67$, root-mean-square error (RMSE) = 3.19) than for methane ($R^2 = 0.36$, RMSE= 0.87).

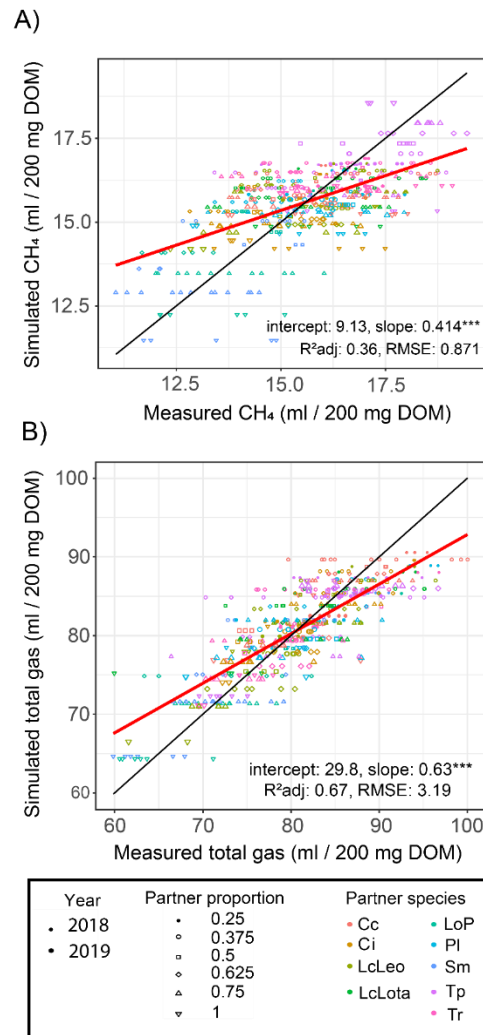


Figure 3.2. Comparison of simulated vs. measured data for methane production (A) and total gas production (B). 2(Significance levels a'e: *** $p \leq 0.001$). Abbreviations are as follows (in alphabetical order): Cc: *Carum carvi*, Ci: *Cichorium intybus*, LcLeo: *Lotus corniculatus* cv. Leo, LcLota: *Lotus corniculatus* cv. Lotanava, LoP: *Lotus pedunculatus*, PI: *Plantago lanceolata*, Sm: *Sanguisorba minor*, Tp: *Trifolium pratense*, Tr: *Trifolium repens*.

A.6 Discussion

A.6.1 Experimental setup was suitable to answer research question

The lack of a linear dose-dependent reduction in measured methane emissions due to the variability between increments in the share of partner species was surprising. This variability does not appear to be a methodological error, as a) standard errors were relatively small, indicating a reliable performance of the Hohenheim gas test, b) all samples were mixed

individually rather than as pool samples, which were then split for the replicates, making weighing mistakes unlikely, and c) total gas production showed trends that were much more linear and also showed a much better performance of our models. Consequently, the variation in methane production suggests that methane production is much harder to predict and either requires more inputs than forage quality and the main plant secondary metabolites, or several of these compounds interact with matrix effects, thereby leading to less predictable results. In the present study, it is probable that the composition of the diet fed to the donor cows (from which the rumen fluid for the *in vitro* test was extracted) may have had an effect on CH₄ production, as the donor cows were fed a diet with 50% concentrate, to evaluate the performance of the different forages, with greater contents of fiber. Considering that methanogenic archaea are very sensitive to changes in the rumen pH, with their optimum range between 6.5 and 7 (achieved with diets rich in fiber), a rumen environment adapted to diets rich in concentrate, with lower pH, would limit the methanogenic activity (Beauchemin et al, 2008). Therefore, it is possible that CH₄ production recorded in this experiment may have been underestimated due to this effect of the diet composition. However, it should be noted that the procedure for the performed *in vitro* test (HGT) is standardized, ensuring the comparability of the results. Additionally, in a review from Yáñez-Ruiz, *et al.* (2016), it is mentioned that when the rumen fluid is taken in the morning, immediately before feeding, as was the case in our experiment, the differences in the gas production profile from donors fed a grass silage:barley grain diet (80:20) or a barley straw diet were minimal. Furthermore, given the lower persistence of the PSM-rich species in study, which results in low shares in the forage mixtures offered to the cattle, as also found in our previous *in vivo* study (Loza et al., 2021), the addition of these forages in high energy diets (with greater shares of concentrate) for high yielding cows must be considered as a strategy. In this case, the use of rumen fluid from donors fed a diet rich in concentrates will make our results more comparable for these systems. Additionally, it is worth noting that the choice of *L. perenne* as the grass partner species was made according to its representativeness of the pasture-based systems. However, because of the high quality of this forage, the reduction in CH₄ production by the additions of bioactive herbs may have been underestimated. Conversely, if the reference species had been a grass with a lower content in WSC, or generally a lower digestibility, then the reduction in methane emissions had likely been larger. On the other hand, Jayanegara, et al. (2013), highlighted that the variation in methane production per digestible OM *in vivo* was particularly high when the dietary tannins were low (≤ 20 g/kg DM), as was the case for most of the species, and especially for almost all mixtures assessed in the present study. According to Jayanegara et al., (2013), this was

probably due to the influence of other dietary components masking the effects of tannin at low levels, and this effect was particularly pronounced when testing the effects of PSM forages in mixtures *in vitro*. This is consistent with previous studies, in which only one of these two factors was varied, by using purified tannin extracts which were added to a constant feed source. This generally provided clearer results; for example, Tiemann, *et al.* (2008) observed clear plant-specific effects ($P \leq 0.001$) of purified tannin extracts at different levels (0, 25, 50, 75 and 100 mg/g of forage dry matter (DM)) of purified PA from four seminiferous shrub legumes, added to a grass-legume mixture). Nevertheless, these two approaches represent different practical implications, with one being the supplementation of tannins in a total mixed ration (TMR) diet, whereas the mixing of plant species represents an approach that is suitable for pasture-based forage provision. Thus, despite the larger variability, both approaches are required to identify the most suitable improvements for both systems.

Additionally, several studies have identified the incubation time to be relevant when analysing mixtures. Accordingly, Niderkorn *et al.* (2011) and Robinson, *et al.* (2009) found that non-additive effects of feed mixtures on total gas production *in vitro* are clearer after short incubation periods of 3h to 8h, when compared to 24h. These findings are in accordance with Goel and Makkar (2012), who reported a lack of significant non-additive effects on methane emissions after 24h incubation. Therefore, the variation observed in measured methane production could also be attributable, at least in part, to the incubation time of 24 hours used in the present study. However, the variation during the initial hours is mainly due to the fermentation dynamics, as the fermentation of most soluble fractions and the protein synthesis occur within the first 3 to 5 hours of incubation, while the non-soluble fraction degrades in the later hours. Thus, the 24h are generally more representative of the overall feed performance and hence are also most frequently used when estimating the energy content of feeds (Menke and Steingass, 1988).

Regarding the overall model performance, the model generally performed better at estimating total gas production than for estimating methane production. For methane, when comparing the modelled values against measured data, the model tended to generally overestimate lower emissions (i.e. the high partner shares) while underestimating higher methane emissions (i.e. the *L. perenne* monoculture and low partner shares). Accordingly, the model can be considered conservative in its statements and generally underestimates the methane reduction potential of the partner species. As mentioned previously, this suggests that methane is much more complex to predict than total gas, as a result of a combination of factors that might include the characteristics of the rumen fluid utilized, and the NDF (and other

carbohydrates) content (as substrate for enteric fermentation). Nevertheless, independent of the reasons for the observed variability, the fact that the methane concentration was generally comparable across both years, and that also all pseudo-regressions exhibited slopes which showed a good fit and mostly were significantly different from 0 indicates that the reduction in methane formation and in part in methane concentration of the tested species is a valid statement.

A.6.2 Tannin rich forages reduced methane formation most, but chicory was the best species for reducing methane concentration

One of our hypotheses in this study was that tannin-containing forages were able to reduce methane emissions more than non-tannin containing forages. This was indeed the case for *S. minor* and *L. pedunculatus*, as both of these species showed the greatest methane reduction potential. However, both cultivars of *L. corniculatus* were less efficient at reducing methane than several of the tested non-tannin-containing forages. Nevertheless, within *L. corniculatus*, the ranking of the two cultivars was in accordance with the tannin concentrations, as Lotanava had greater tannin concentrations and also resulted in greater methane reductions than Leo.

Additionally, while the methane emissions were indeed reduced most by the species with the greatest tannin concentrations, this was different for the methane concentration. The methane concentration is an indicator for methane emissions per unit of digestible forage, and as such is important for estimating whether or not methane reductions also decrease per unit of digestible forage. In this regard, *C. intybus* and *C. carvi* outperformed *S. minor* and *L. pedunculatus*, indicating that for the latter two species there was a greater decrease in digestibility than of methane, whereas *C. intybus* and *C. carvi* maintained high digestibility while also reducing methane. This is important, as *C. intybus* particularly is recognized as a species of high agronomic value for its dry matter yield potential and drought resistance, and is frequently included in pasture-based systems (Loza et al., 2021; Lee et al., 2015), whereas *S. minor* and *L. pedunculatus* were outcompeted in the second sampling year of the present study and hence they could be sampled only once. The anti-methanogenic activity of *C. intybus* and *C. carvi* could be explained by the presence of PSM other than tannins. The results were generally in agreement with values reported by Waghorn, *et al.* (2008) in an *in vivo* experiment, who found that sheep fed diets including chicory had lower methane emissions than those fed ryegrass-white clover, and that these emissions were similar to those from sulla (*Hedysarum coronarium*), despite sulla containing PA concentrations of 3.5 to 6.8%. Similarly, Durmic, *et al.* (2016) reported that chicory and plantain reduced methane production by 25%, when compared with *Trifolium vesiculosum*, in an *in vitro* batch-fermentation assay. Among the

bioactive compounds present in chicory, sesquiterpene lactones and coumaric acid are of particular interest, as they have been shown to be beneficial for livestock production. The presence of sesquiterpene lactones in chicory has also been associated to anthelmintic properties. In an *in vitro* experiment carried out by Williams, et al. (2016), chicory extracts from two different cultivars were investigated for their anthelmintic effect against swine nematodes, and significant suppression of these parasites when exposed to sesquiterpene lactones from chicory was shown, although this effect was cultivar dependent. Anthelmintic effects in nematodes from lambs fed chicory have also been reported, for example by Peña-Espinoza, et al. (2020).

The clover species *T. repens* and *T. pratense* are the most widely used legume forage species in European pasture systems (Lüscher et al., 2014). Hence, the fact that they showed on average the lowest reductions in methane emissions, and even increased methane concentration substantially compared to *L. perenne*, is important. It is not very surprising, however, as neither of these species contains the important metabolites that have a role in reducing methane emissions. Regarding their metabolic profile, *T. repens* may contain PA, but only in the flowers (Foo et al., 2000). As the flowers comprise only a small proportion of the forage biomass of *T. repens*, to obtain sufficient amounts of flowers to meet the PA requirement would likely require an excessive proportion of *T. repens* in the sward, with an associated risk of bloat (Burgraaf et al., 2008). Nevertheless, the PA contents of the samples harvested in 2018 suggest the presence of flowers in the samples, whereas the absence of PA in the samples from 2019 suggests that the sample comprised only leaves. This between-years difference in tannin composition could explain the greater methane reduction potential observed 2018 (-9 %) when compared to 2019 (-3%), as the slope of the latter was not significantly different from zero. For *T. pratense*, the major component that has been researched for its bioactivity is polyphenol oxidase (PPO). However, while PPO has been found to increase the nitrogen-use efficiency in silage making [49] and live-weight gains in ruminants (Copani et al., 2016), no benefits have previously been identified regarding methane emissions (Copani et al., 2015; Gidlund et al., 2017). Consequently, due to the absence of bioactive substances and their concomitantly lower energy concentrations compared to *L. perenne* is a likely explanation for their increments in methane concentration.

A.6.3 Unlike digestibility, methane formation cannot be estimated accurately based on the share and type of partner species, or from forage quality parameters alone

While the total gas reduction showed comparably linear dose-effect responses with increasing share of forage legumes or herbs, and the general methane reduction potential and

improvements in methane concentration of all species was illustrated satisfactorily, estimating the methane formation of any particular plant species mixture remains difficult. Similarly, it was difficult to estimate the methane formation based on covariates that described forage quality or plant secondary metabolites. Regarding the forage quality covariates, this might be because the forage quality parameters were estimated based on NIRS. While these estimates were satisfactory for most partner species, the energy concentration and digestibility were overestimated compared to the estimates based on total gas for tannin-containing species. Consequently, this is likely to have affected their performance as covariables. The plant secondary metabolites on the other hand were very different, with almost no two species having comparable profiles, and this also makes their use as covariates difficult. Based on these factors, the estimation of methane formation from a distinct mixture of forage plant species remains difficult. Nevertheless, it remains unlikely that any minor addition of bioactive forage species is likely to achieve large benefits when applied at the field scale.

A.7 Conclusion

The present study has shown the potential of several forage species to reduce methane emissions from ruminants compared to diets based on highly digestible perennial ryegrass monocultures. The forbs salad burnet and big trefoil have shown substantial methane reduction potential. These were, however, accompanied by concomitant reductions in digestibility, as was evident by reduced total gas production. Accordingly, in terms of methane concentration, it was chicory, rather than the species that were richest in tannins, which resulted in the lowest methane emissions per unit of digestible forage. This finding is of particular interest, as chicory also has a greater agronomic potential as a component of sown forage mixtures for grazing. However, in contrast to the total gas production, methane production was less predictable and linear increments in the legume or herb share did not result in linear changes in methane concentration, but instead exhibited a comparably large variability. As a result, the extrapolation of findings to defined species mixture compositions for pasture swards remains difficult. Additionally, *in vivo* experiments with defined shares of the most promising species are required to identify whether these results can be extrapolated to the vastly more complex and diverse conditions in ruminants.

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visualization, C.S.M.; writing—original draft, C.L.; and writing—review and editing, S.W., A.S., R.B., C.K., S.V. and C.S.M. All authors have read and agreed to the published version of the manuscript.

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Appendix A

Table A1. Statistical data of the NIRS calibration and validation (SEC, standard error of calibration; SEP, standard error of prediction) for the relevant quality parameters by plant group.

Parameter	Plant group	N	Mean	Range	SEC	R ²	SEP
ME (MJ/kg DM)	whole sward	251	10.810	8.38 - 12.62	0.179	0.959	0.195
ME (MJ/kg DM)	Grasses	248	10.806	8.38 - 12.62	0.173	0.956	0.194
	Legumes	168	10.737	8.55 - 12.41	0.15	0.961	0.196
	Herbs	117	10.691	8.38 - 12.54	0.154	0.963	0.211
NEL (MJ/kg DM)	whole sward	249	6.567	4.79 – 7.81	0.138	0.955	0.173
NEL (MJ/kg DM)	Grasses	250	6.569	4.79 - 7.87	0.136	0.949	0.193
	Legumes	167	6.512	4.92 - 7.77	0.106	0.965	0.152
	Herbs	115	6.514	4.80 - 7.784	0.112	0.964	0.163
DOM (g/kg DM)	whole sward	249	809.45	642.6 - 919.0	8.85	0.967	9.14
DOM (g/kg DM)	Grasses	248	821.04	632.1 - 918.8	10.83	0.956	10.97
	Legumes	173	803.63	657.6 - 902.6	10.36	0.944	12.96
	Herbs	118	817.42	642.6 - 904.3	13.51	0.943	12.45
N (g/kg DM)	whole sward	268	26.69	8.7 - 54.2	0.951	0.991	1.071
N (g/kg DM)	Grasses	277	22.59	9 - 54.9	0.802	0.991	0.914
	legumes	178	35.07	14.7 - 57.1	1.116	0.981	1.131
	herbs	86	26.39	10.3 - 39.9	0.749	0.995	1.317

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