Norwegian University of Life Sciences
Faculty of Biosciences
Department of Animal and Aquacultural Sciencesw

Philosophiae Doctor (PhD)
Thesis 2024:30

Strategies in silage production to mitigate enteric methane emissions from ruminants

Strategier i surfôrproduksjonen for å redusere enterisk metangassutslipp fra drøvtyggere

Kim Viggo Paulsen Weiby

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Table of contents

List of abbreviations

AAT	Amino acids absorbed in the small intestine
AIA	Acid insoluble ash
AIC	Akaike information criteria
aNDFom	ash corrected neutral detergent fiber
CH_4	Methane
CO_2	Carbon dioxide
CP	Crude protein
DM/ DMI	Dry matter/dry matter intake
dOM	digestible organic matter
ECM	Energy corrected milk
FFA	Free fatty acids
GF	Greenfeed
GHG	Greenhouse gas
GWP	Global warming potential
H_2	Dihydrogen
iNDF	indigestible neutral detergent fiber
IPCC	International panel on climate change
K_d	Fractional rate of degradation
NAD^+/NADH	Nicotinamide adenine dinucleotide (hydrogen)
NH_3- N	Ammonia nitrogen
NIRS	Near infrared spectroscopy
NEl	Net energy lactation
O_2	Oxygen
OM	Organic matter
OMD	Organic matter digestibility
PBV	Protein balance in the rumen
SCFA	Short chained fatty acids
TMR	Total mixed ration
WSC	Water soluble carbohydrates

List of papers

1. **Weiby, K. V**., Kriszan, S. J., Eknæs, M., Schwarm, A., Whist, A. C., Schei, I., Steinshamn, H., Lund, P., Beauchemin, K. A. & Dønnem, I. Associations among nutrient concentration, silage fermentation products, in vivo organic matter digestibility, rumen fermentation and in vitro methane yield in 78 grass silages. Anim. Feed Sci. Tech. **2022**, 285, 115249. https://doi.org/10.1016/j.anifeedsci.2022.115249

2 **Weiby, K. V**., Kriszan, S. J., Dønnem, I., Østrem, L., Eknæs, M. & Steinshamn, H. Effect of grassland cutting frequency, species mixture, wilting and fermentation pattern of grass silages on in vitro methane yield. Scientific Reports. **2023**, 13:4806. https://doi.org/10.1038/s41598-023-31964-3

3 **Weiby, K. V**. Årvik, L., Eknæs, M., Schwarm, A., Steinshamn, H., Beauchemin, K. A., Lund, P., Schei, I. & Dønnem, I. Effects of grassland species and harvest frequency on milk production and methane emissions in dairy cows (manuscript).

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Abstract

Emission of enteric methane (CH_4) from ruminants have become a growing concern for policymakers globally as CH_4 now account for 6% of global greenhouse gas (GHG) emissions and the warming effect in the atmosphere is 28 times that of carbon dioxide (CO_2). The Norwegian agricultural sector has made an agreement with the government to reduce GHG emissions by 5 million tonnes of CO_2 equivalents from 2021 to 2030, and improved forage quality is one of the main strategies to achieve this reduction.

Grass-clover silage constitutes a large part of ruminant diets in Northern and Western Europe, as well as in North America. Timothy (*Phleum pratense L*) has been the dominating perennial grass species in Norway, Sweden, Finland and Iceland for centuries, but as climate is getting warmer perennial ryegrass (*Lolium perenne L*) species has become more commonly used, especially in the coastal areas with mild winters. Due to increased temperatures and extended growing season, it is now possible to increase the number of cuts per season, and increased harvest frequency is already used as a strategy to harvest high quality forage for ruminants.

However, the impact of silage chemical composition, ley species, harvest frequency, wilting, fermentation pattern, and use of mixed silages from different cuts on *in vitro* and *in vivo* CH_4 production is largely unknown. Therefore, the overall objective of this doctoral thesis was to develop strategies in silage production to mitigate enteric methane emissions from ruminants. We aimed to identify the quality attributes of grass and clover silage associated with variation in *in vitro* CH_4 production, and to test the effects of grassland species, cutting frequency, wilting and fermentation pattern on *in vitro* CH_4 production. Further, we aimed to investigate the effect of ley species (timothy, perennial ryegrass and red clover) and cutting frequency (two vs. three cuts per season) on dry matter intake (DMI), milk production and CH_4 production in lactating dairy cows.

In **Paper I** we found that among all investigated silage composition variables, neutral detergent fiber (NDF) and indigestible neutral detergent fiber (iNDF) were the most important correlating negatively ($r = -0.63$ and $r = -0.48$, respectively, $P < 0.001$) with *in vitro* CH_4 production, while water soluble carbohydrates (WSC) and organic matter digestibility (OMD) were the most important correlating positively ($r = 0.57$ and $r = 0.44$, respectively, $P < 0.001$) with *in vitro* CH_4 production. In **Paper II** we found that *in vitro* CH_4 production was, on average, 8.2% lower (31.3 vs. 34.1 mL/g OM, respectively, $P < 0.001$) for

the two-cut system than for the three-cut system, and 5.6% lower (32.2 vs. 34.1 mL/g OM, $P < 0.001$) in timothy than in perennial ryegrass. Silage DM concentration did not affect CH_4 production but using formic acid additive increased CH_4 production 3.7% (32.4 vs. 33.6 mL/g OM, $P = 0.003$) compared to untreated silage. In **Paper III** we found that *in vivo* CH_4 production (g/day) and yield (g/kg DMI) did not differ between three-cut system and two-cut system in timothy, but CH_4 intensity was 6.8% lower (16.5 vs. 17.7 g/kg energy corrected milk (ECM), $P = 0.003$) for the three-cut system compared to the two-cut system. Further we found that timothy obtained 5.6% (22.1 vs. 23.4 g/kg DMI, $P = 0.05$) and 5.2% (16.5 vs. 17.4 g/kg ECM, $P = 0.02$) lower CH_4 yield and intensity, respectively, compared to perennial ryegrass. Increasing the red clover proportion in the diet from 0 to 100% linearly increased CH_4 production by 3.8% (476 vs. 495 g/d, $P = 0.05$), linearly increased CH_4 yield by 10.9% (22.1 vs 24.8 g/kg DMI, $P < 0.001$) and linearly increased CH_4 intensity by 9.8% (16.5 vs. 18.3 g/kg ECM, $P < 0.001$).

In conclusion, the present results show that it is a viable strategy for farmers in Northern and Western Europe, as well as in North America, to mitigate enteric CH_4 emissions in dairy cows by increasing harvest frequency and to use timothy rather than perennial ryegrass or pure red clover silage in the diet.

Norsk sammendrag

Utslipp av enterisk metan (CH_4) fra drøvtyggere har blitt en økende bekymring for politiske beslutningstakere globalt ettersom CH_4 nå står for 6% av globale utslipp av klimagasser og oppvarmingseffekten av CH_4 i atmosfæren er 28 ganger kraftigere enn CO_2. Jordbrukssektoren i Norge har inngått en avtale med myndighetene der de har forpliktet seg til å kutte utslipp tilsvarende 5 millioner tonn CO_2 ekvivalenter i perioden fra 2021 til 2030, og økt fôrkvalitet er en av hovedstrategiene for å nå dette målet.

Surfôr av gras og kløver utgjør en stor andel av fôret til drøvtyggere i nord, - og vest Europa, i tillegg til i Nord Amerika. Timotei (*Phleum pratense L*) har vært den dominerende flerårige grasarten i Norge, Sverige, Finland og Island i århundrer, men ettersom klimaet blir varmere har også flerårig raigras blitt vanligere, spesielt i kystnære områder med milde vintre. På grunn av økte temperaturer og en lengre vekstsesong er det nå mulig å øke antallet slåtter per sesong. Økt slåttefrekvens er allerede en velkjent strategi for å øke kvaliteten i drøvtyggerfôret.

Det er imidlertid ukjent hvilken påvirkning surfôrets kjemiske innhold, bruk av ulike engarter, høstefrekvens, fortørking, gjæringskvalitet eller bruk av ulike slåtter har på *in vitro* og *in vivo* CH_4 produksjon. Derfor var det overordnede målet i denne doktorgrads-avhandlingen å utvikle strategier i surfôrproduksjonen for å redusere enterisk metangassutslipp fra drøvtyggere. Vi hadde som mål å identifisere kvalitetsparametre i surfôr av gras og kløver som har sammenheng med variasjon i *in vitro* CH_4 produksjon, samt å teste effekten av ulike engarter, høstefrekvenser, fortørkingsnivåer og gjæringsmønstre på *in vitro* CH_4 produksjon. Videre ønsket vi å undersøke effekten av engarter (timotei, flerårig raigras, rødkløver) og høstefrekvens (to vs. tre slåtter per sesong) på tørrstoffopptak, melkeproduksjon og CH_4 produksjon hos mjølkekyr.

I **artikkel 1** fant vi at blant alle surfôrvariabler som ble undersøkt var NDF og iNDF de aller viktigste med negativ korrelasjon ($r = -0.63$ og $r = -0.48$ respektiv, $P < 0.001$), mens WSC og OMD var de viktigste med positiv korrelasjon ($r = 0.57$ og $r = 0.44$ respektiv, $P < 0.001$) til *in vitro* CH_4 produksjon. I **artikkel 2** fant vi at *in vitro* CH_4 produksjon var gjennomsnittlig 8.2% lavere (31.3 vs. 34.1 mL/g OM respektivt, $P < 0.001$) i toslåttsystemet sammenlignet med treslåttsystemet, og 5.6% lavere (32.2 vs. 34.1 mL/g OM, $P < 0.001$) i timotei sammenlignet med flerårig raigras. Surfôrets tørrstoffkonsentrasjon hadde ingen effekt på CH_4 produksjonen, mens bruk av maursyretilsetning økte CH_4 produksjonen med

3.7% (32.4 vs. 33.6 mL/g OM, P = 0.003) sammenlignet med ubehandlet surfôr. I **artikkel 3** fant vi ingen forskjell i *in vivo* CH_4 produksjon i gram CH_4 per dag eller i gram CH_4 per kg tørrstoffopptak hos mjølkekyr som fikk enten timotei fra toslåttsystem eller fra treslåttsystem. Metanintensiteten (g/kg EKM) var 6.8% lavere (16.5 vs. 17.7 g/kg EKM, P = 0.003) hos kyr som hadde fått timotei fra treslåttsystem sammenlignet med kyr som hadde fått timotei fra toslåttsystem. Videre fant vi at kyr som hadde fått surfôr av timotei i reinbestand hadde 5.6% lavere CH_4 ytelse (22.1 vs. 23.4 g/kg TS opptak, P = 0.05) og 5.2% lavere CH_4 intensitet (16.5 vs. 17.4 g/kg EKM, P = 0.02) sammenlignet med kyr som hadde fått flerårig raigras i reinbestand. En økning i andelen rødkløver fra 0 til 100% i rasjonen til mjølkekyr ga en lineær økning i CH_4 produksjonen på 3.8% (476 vs 495 g/d, P = 0.05), en lineær økning i CH_4 ytelsen på 10.9% (22.1 vs 24.8 g/kg TS opptak, P < 0.001) samt en lineær økning i CH_4 intensiteten på 9.8% (16.5 vs. 18.3 g/kg EKM, P < 0.001).

Resultatene i denne avhandlingen viser at en god strategi for bønder med drøvtyggerproduksjoner i Nord, - og Vest-Europa og Nord Amerika som ønsker å redusere enterisk CH_4 gassutslipp er å øke høstefrekvensen i surfôrproduksjonen. I tillegg kan det anbefales å øke bruken av timotei heller enn flerårig raigras og rødkløver i reinbestand i rasjonen til drøvtyggere.

1. Introduction

Emissions of greenhouse gases (GHG) from livestock production systems have become a growing concern for policymakers globally over the last decade as the global demands for meat and milk are expected to rise by 73% and 58%, respectively, within 2050 compared to 2010 levels. Emissions of GHG from livestock (animals, manure, feed production and expansion of land into forested areas) already account for 14.5% of global anthropogenic GHG emissions, and enteric methane (CH_4) from ruminants account for 6% of global GHG emissions and 40% of all livestock emissions (Gerber et al., 2013). The global warming potential (GWP) of CH_4 in the atmosphere is estimated to be 28 times greater than that of carbon dioxide (CO_2) when compared over a 100-year period (IPCC, 2019), and concentration of CH_4 in the atmosphere is rapidly increasing (Saunois et al., 2016). In Norway the contribution from the agricultural sector is 4.6 million tonnes of CO_2 equivalents annually, or 9.4% of the annual Norwegian GHG emissions. Enteric CH_4 accounted for 52% of these emissions in 2022 (SSB, 2022).

In 2019 the Ministry of Climate and Environment and the Ministry of Agriculture and Food signed an agreement with the agricultural sector in Norway (Norges Bondelag/Norges Bonde og Småbrukarlag) where they agreed to reduce the total emissions from the agricultural sector by 5 million tonnes CO_2 equivalents in the period from 2021 to 2030. In the "Agricultural Climate Plan", which was prepared based on this agreement, it is estimated that approximately half of the reductions in the agricultural GHG emissions can be achieved by: "a targeted effort for increased roughage quality, animal breeding, better animal health and use of feed ingredients for CH_4 reductions". However, the impact of different silage management strategies is unclear (Beauchemin et al., 2020).

Silage (primarily made of perennial grasses and clovers) is the dominating preserved forage in ruminant diets in Norway, constituting approximately 45% of total feed intake on energy basis in milk production, and up to 70-80% of the total energy intake in meat production on cattle and sheep. As farmers are encouraged to reduce CH_4 emissions, changes in feeding regimes are a promising mitigation option (Beauchemin et al., 2020). Substituting forage with concentrate is often suggested as a feeding strategy to mitigate enteric CH_4 emissions from ruminants, but this is not a sustainable strategy in Norway due to the low percentage of arable land (only about 3%) and that about 2/3 of the arable land is best suited for cultivation of grassland (NIBIO 2024). The importance of using national feed resources such as grass silage is also emphasized by the government (St.meld. nr 11, 2016-2017).

Increasing the quality and digestibility of grass silages would also reduce the need for concentrates and imported protein sources (Álvarez et al., 2020), and thereby reduce the rivalry between human and animal food and feed resources (de Vries, 2023). To achieve this, it is necessary to find ways to produce silage with lower enteric CH_4 emission potential.

The main topic of this thesis is to present strategies in silage production to mitigate enteric CH_4 emissions in ruminants. I aimed to explore which silage compositional factors that were associated with increasing and reducing *in vitro* CH_4 yield. In addition, I wanted to investigate the effect of harvest regimes, grassland species, wilting and fermentation pattern on *in vitro* CH_4 production. One important research question in this thesis has been to use *in vivo* techniques to elucidate the effect of harvesting regimes and different grassland species on dry matter intake (DMI), energy corrected milk (ECM) yield, digestibility and enteric CH_4 production, yield, and intensity.

The dairy and meat industry need to reduce their environmental footprint in the production systems, while at the same time increase food production to a growing population. Methane is the most important GHG in animal agriculture today, but it is also maybe the one that is most challenging to reduce due to complex relationships between animal, microbial biology and feed related factors.

This PhD thesis will contribute to solving one of the biggest challenges in today's ruminant agricultural systems: How to reduce CH_4 emissions in a way that sustain consumer acceptance, while at the same time increase animal productivity and economy for farmers.

1.1 Grass and legume silage quality

The nutritive value of grass and legume silages is highly variable, which is challenging when trying to increase the proportion of locally produced feeds and reduce the concentrate share in feed rations. One of the objectives in silage production is to "close the gap" between the nutritional quality and feeding value of the original crop and the resulting silage. For several decades, research in silage production has investigated the effect of manipulating the silage quality by timing of harvest (Rinne, 2000). Silage quality can be defined as the feeding value and the ruminant's ability to utilize the silage for production of milk, meat, fiber (e.g. wool) or fetus production (Chavez et al., 2006). Parameters involving silage quality can be divided into crop related factors and fermentation related factors (Charmley, 2001). In addition, the net energy content of the silage, expressed as net energy lactation (NEL), the protein value, expressed as amino acids absorbed from the intestine

(AAT) and the protein balance in the rumen (PBV), are calculated values indicating what the feed provides for maintenance and production. Crop related factors are related to maturity stage at harvest, hence the chemical and physiological changes in the plant. Forages that are harvested at an early vegetative growth stage have lower aNDFom concentration, greater crude protein (CP) concentration and a greater concentration of digestible organic matter (DOM) per kg dry matter (DM) compared to more mature forages harvested at the later flowering stage.

The chemical composition of the feed involves, among other, the concentration of energy and protein in the feed, and in the Northern countries the feed evaluation system NorFor (Volden, 2011) is commonly used to establish the nutritional value of the feed for milk and/or meat production (**Figure 1**). The DM content of the feed consists of organic matter (OM) and ash. The OM of silage is further divided into CP, aNDFom, starch, crude fat, water soluble carbohydrates (WSC) and a calculated rest carbohydrate fraction. In addition, the OM contains fermentation products which includes e.g. lactic acid, volatile fatty acids, and alcohol. The nutrients are further divided into subgroups according to **Figure 1**.

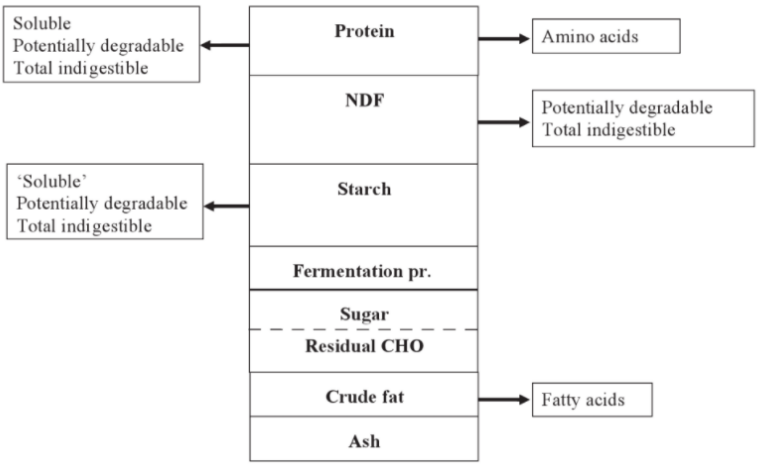

Figure 1. Feed fractions in the Norfôr system (Volden, 2011).

The naturally occurring sugars in the herbage are rapidly fermented to various organic acids, and Mo et al. (2001) reported as many as 51 different fermentation products in collected field samples of grass silage. The most common way to evaluate fermentation intensity includes pH, concentration of organic acids, alcohols and ammonia nitrogen (NH_3-

N) (Kung et al., 2018), in addition to the size of various microbial populations like *Enterobacteriaceae*, *Bacillus*, *Clostridium Tyrobutyricum,* yeast, mould and *E. Coli* (McDonald et al., 1991). Defining silage quality also depends on animal requirements. The nutrient requirement of a dairy cow in early lactation is very different from dry dairy cow or beef cattle. In general terms, high yielding animals (milk or meat) and youngstock have greater energy and protein requirements than animals with lower production or animals that don't produce milk or meat.

1.1.1 Phenological development and harvest frequency

Changes in the plants phenological developmental stages largely affects the forage nutritional quality and concentration of nutrients. In early growth phases the cell content constitutes as much as 65% of DM, while the cell walls only constitute 35%. However, in later phases when the plant is heading the relationship is opposite with cell wall fractions constituting 60% and cell content only 40% (Mo, 2005). The general changes in the chemical composition of plants with advancing phenological development are decreased CP concentration and increased aNDFom concentration. Lignification of the cell wall fraction accelerates with increased plant maturity, as lignin interacts with cell wall components to provide structural integrity. Increased maturity and lignification of the cell walls in plants lowers overall digestibility, as lignin is resistant to hydrolysis by rumen microorganisms (McDonald et al., 1991). Cherney et al. (2003) showed that it is possible to measure significant decrease in feed quality every second and third day. Changes occur both in the relationship between leaves vs. stems, but also in the chemical concentration within the leaves and stem (Chavez et al., 2006, **Figure 2**).

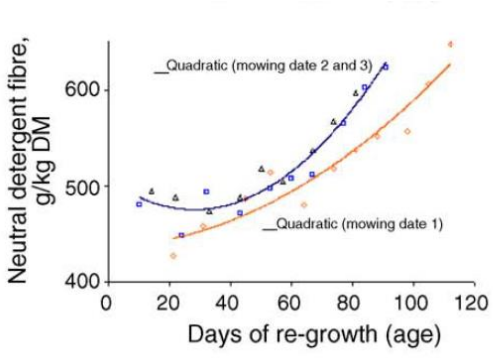

Figure 2. Changes in NDF concentration (g/kg DM) with increased maturity in perennial ryegrass from 0-120 days of re-growth after harvest. The one-year-old sward was harvested either 21st August (mowing date 1), 11th September (mowing date 2) or 21st September (mowing date 3) (Chavez et al., 2006).

The phenological development stage of perennial grass can be calculated as a numerical value based on the number of tillers (Moore et al., 1991), and this method has been developed further to fit Norwegian conditions by Bakken et al., (2005). According to Van Soest (1978) temperature is a factor involved in conversion of photosynthetic products into structural matter like cell walls and lignin, while light intensity affects soluble carbohydrates and digestibility of grasses. Nitrogen fertilization and water availability also have measurable effects on forage composition.

In Norway, climatic conditions limit the number of harvests per season due to the low number of growing degree days. In the Southern and South-Western parts of the country it is common with three or four harvests per season, while in the mountainous regions of the South and in the North, it is common with two harvests per season. In the Northernmost regions of Norway, they usually only harvest once per season. Increasing the harvest frequency due to extended growing season may include going from two to three harvests per season, while for some farmers it includes going from three to four harvests per season.

Harvest frequency (e.g., two cuts vs. three cuts per season) will affect nutrient composition and digestibility as the crop (especially the first cut) in a two-cut system is harvested at a more mature phenological developmental stage. In addition, when harvesting three times compared to two times, and feeding a mix of these different cuts, the proportion of regrowth in the total ration increases for the three-cut system compared to the two-cut system. Regrowth grass typically contains more leaves (Rinne and Nykänen, 2000; Gustavsson and Martinsson, 2004), and less cell wall carbohydrates (Kuoppala et al., 2008), but at the same time the regrowth grass is less digestible (Huhtanen et al., 2006) compared to the corresponding spring growth grass. The lower digestibility of regrowth grass is related to the increased iNDF concentration in the cell walls compared to the spring growth grass (Huhtanen, 2006). High temperatures in the summer increase the accumulation of lignin in the cell walls and decrease digestibility (Van Soest, 1994). Regrowth grass has a slower phenological development with a greater proportion of leaves before the elongation phase begin (Fagerberg, 1988). It has also been reported that regrowth herbage contains more weeds

and dead tissue which lower digestibility of the forage (Kuoppala, 2010). Kuoppala (2010) reported that the regrowth of red clover had a greater concentration of ash, CP, iNDF and lignin, but less NDF compared to the spring growth, but the regrowth of red clover was less homogenous as the red clover plants were in different developmental stages when the growth started after the first harvest. Buds emerged from remaining stems and started flowering at the same time as new stems developed (Fagerberg, 1988). Pang et al. (2021) reported that second regrowth silage had a greater energy value and was more digestible compared to the first regrowth, and that it was similar to the early cut spring growth in NDF concentration.

Most research on silage quality, feed intake and milk production are performed using silage from spring growth only (Kuoppala et al., 2008, Pang et al., 2021), although regrowth silages constitute a large part of ruminant feed consumption. There is a lack of knowledge in the effect of using a mixture spring growth and regrowth silages on dairy cow performance and enteric CH_4 production, yield and intensity. Furthermore, using a mixture of spring growth and regrowth silages in ruminant feeding regimes is becoming the industry standard as bunker silos and mixer wagons are becoming more common. By mixing silages from different cuts, the difference in feed quality between cuts is evened out. Therefore, study III was designed using a mixture of spring growth and regrowth proportional to the DM yield of each cut.

1.1.2 Botanical composition

Timothy (*Phleum pratense* L., **Figure 3 a**) is the most used forage grass species in Norway, Sweden, Finland, and Iceland (Höglind et al., 2005), and it is also an important herbage species in other temperate regions of the world. Timothy is more resistant to winter damage compared to e.g. perennial ryegrass which is more common in other parts of Europe (Höglind et al., 2001). Timothy is a perennial grass that grows in small patches and can be 0.5-1.0 m tall when fully grown. The plant is easily recognized with its internodes between the nodes. The lower node of the stem is called the proaxis, and above is the haplokorm where the plants energy reserves are stored. The leaves of the timothy are 4-12 mm wide and are comprised inside the leaf sheath before shooting. Timothy is a palatable herbage for ruminants, and it normally yields good the first year, but normally yields less after 3-4 years (Collins and Nelson, 2018), which is the reason for using timothy together with other herbage species. Timothy is not so tolerant to frequent defoliation which is seen in the slow regrowth after cutting (Collins and Nelson, 2018).

Perennial ryegrass (*Lolium perenne* L., **Figure 3 b**) is one of the most used grass species for sowing grasslands in temperate areas. It is particularly popular in Europe where it represented 50% of the marketed grass seeds in 2010 (Humphreys et al., 2010). Perennial ryegrass has a lot of leaves and tillers, making it a high yielding grass with good feed value for ruminants in suitable conditions (Sampoux et al., 2011).

In Norway, perennial ryegrass has become increasingly important, especially in the southern, - and western coastal parts of Norway where temperatures are greater than the inland and mountainous areas. Westerwold ryegrass (*Lolium westerwoldicum*) and Italian ryegrass (*Lolium multiflorum*) are used as annual crops, as these species normally don't survive Norwegian winters. Perennial ryegrass has thick, dark green and shiny leaves. The leaves are folded before they exit the leaf sheath. The stem is approximately 40 cm tall and without many leaves, but ryegrass has separate leaves coming from the root giving it a characteristic appearance with patches and roots spreading out from the sides filling up the area from other plants not growing anymore. Perennial ryegrass is suitable in mild and humid areas, and it quickly starts growing after harvest (Jetne, 1973).

Genotypes of perennial ryegrass have been bred for high concentrations of water-soluble carbohydrates (WSC) as a measure to improve animal performance (Parssons et al., 2011). One of the features of such high sugar grasses is that they are more prone to display extensive lactic acid fermentation during the ensiling process (Ellis et al., 2012). The readily available WSC in grass silage is subjected to fermentation where lactic acid is the dominating end-product in well-fermented silage. It has been speculated that this might lower the amount of enteric CH_4 produced as lactic acid is transformed to propionate in the rumen. Propionate acts as a H^+ sink and thereby reducing the CH_4 producing potential of silages. However, our *in-vitro* study (Weiby et al., 2022) showed no such relationship. In fact, CH_4 production increased as concentration of WSC increased, possibly due to increased butyrate and acetate concentrations in the rumen fluid.

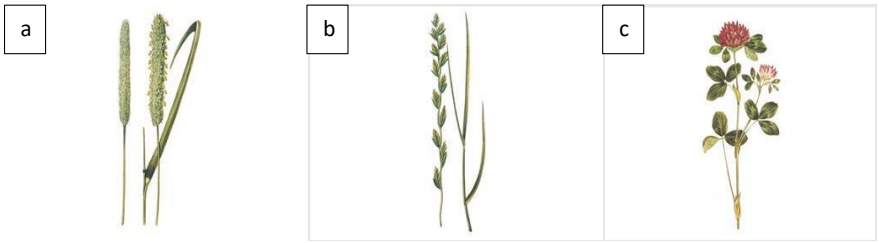

Figure 3 a, b, c. Timothy (*Phleum pratense* L.) (a), perennial ryegrass (*Lolium perenne* L.) (b) and red clover (*Trifolium pratense* L.) (c).

Red clover (*Trifolium pratense* L., **Figure 3 c**) originated from Southeast Eurasia and has been cultivated by farmers in Europe since the third century (Taylor and Quesenberry, 1996). Red clover is used as a forage legume in the temperate parts of the world and is commonly known as a good source of proteins, minerals and for its nitrogen fixating properties which benefits the soil and other plants (McKenna et al., 2018). Red clover has been an essential and commonly used forage crop in Norwegian agriculture since the 17th century, often used in combination with grasses (Jetne et al., 1973). Red clover has an important role in many organic farming systems as it utilizes atmospheric nitrogen for the crop and the farm. Other crops can then utilize this nitrogen in a crop rotation system (Nykänen, et al., 2000). Red clover gives good yields the first years, but it often diminishes after 2-3 years in conventional systems because of mineral fertilizing and poor over-wintering (Jetne et al., 1973). Red clover contains less aNDFom compared to perennial ryegrass (Johansen et al., 2017), and less aNDFom compared to timothy (Hetta et al., 2004). Reduced aNDFom concentrations might increase the propionate production and reduce the acetate production in the rumen fluid. Propionate gives rise to less H_2 thereby reducing the overall CH_4 production (Janssen, 2010; Boadi et al., 2004).

Most research investigating the effect of silage quality on enteric CH_4 yield and intensity is done using a mixture of different grass and legume species making it impossible to test for possible effects of separate grass and legume species on e.g. dry matter intake, daily milk production and CH_4 emission. As this is a gap of knowledge, we decided to design the experiment using pure stands of timothy, perennial ryegrass and red clover in study III.

1.1.3 Silage fermentation pattern

The ensiling process can be divided into four phases; (1) the aerobic phase, (2) the fermentation phase, (3) the stable phase and (4) the feed out phase (Bolsen et al., 1996). In the early phases of ensiling, plant sugars are broken down to carbon dioxide (CO_2) and water in the respiration process. In this process, oxygen (O_2) is consumed, and heat is produced. At the same time plant enzymes such as proteases break down proteins primarily to single amino acids and ammonia (McDonald et al., 1991). Changes in carbohydrate content due to respiration are more likely to be reflected in the WSC concentration rather than in the structural carbohydrates. However, acid hydrolysis or microbial breakdown can degrade

structural carbohydrates such as hemicellulose during the fermentation phase (McDonald et al., 1991).

Fermentation intensity can be controlled through wilting or by using silage additives (Bolsen et al., 1996; Charmley, 2001). Wilting of grass before ensiling has become a widely used strategy for improved silage fermentation quality and reduced effluent production (Dawson et al., 1999). In addition, wilting reduces proteolysis in the silage (Slottner and Bertilsson, 2006) resulting in increased amounts of rumen utilizable protein due to less soluble non protein nitrogen in the silage (Van Vuuren et al., 1990; Tamminga et al., 1991). Wilting grass before ensiling is a way of restricting fermentation as wilting reduces water activity with immediate reduction in microbial activity and fermentation intensity during preservation (Charmley, 2001). Elevated DM concentrations and reduced fermentation intensity in silage retain more WSC in the silage (Müller and Udén, 2007; Rupp et al., 2021).

As wilting has been shown to favour lactic acid fermentation, it also reduces the risk of fermentation taking unfavourable pathways (McDonald et al., 1991). Fresh grass has a high-water content, about 75% depending on species and weather conditions. Wilting is usually done directly after the mower or by using a tedder. After spreading the crop, it is common to use a rake to collect the crop before transport or baling. Plants have a dermal and a cuticular tissue protecting the plant from water loss, microbial penetration and digestion. Most of the water disappearing from the plant is through the stomata (Wilson, 1993). After the grass is cut, 30% of the water will disappear through the open stomata (Jones and Harris, 1980), but the stomata close rapidly after the plant is cut because of changes in the humidity status of the plant, thereby increasing the plants resistance to drying considerable. This makes wilting of grass that is not conditioned into a slow process.

Wright et al. (2000) reported a curvilinear relationship between wilting, silage intake and production response in a dataset of 79 comparisons and concluded that wilting rate and the extent of moisture loss was highly correlated with improved silage intake and performance, and that the benefits to the animal was greatest under good wilting conditions. It is known that wilting markedly reduces proteolysis by plant enzymes in the silage (Muck, 1989). If the wilting process is fast, the rate of proteolysis is lower (Anderson, 1983). The reduction in protein solubility by an effective wilting can increase the flow of amino acids to the intestines (Charmley and Veira, 1990) and increase milk yield in lactating dairy cows (Broderick et al., 1993). Charmley (2001) suggested that much of the response in animal

productivity after wilting is caused by an increased utilization of nitrogen, and that one important measure to increase the effect of wilting is through for example use of a tedder to subject it to direct sunlight and remove the outer cuticle to increase the wilting rate.

Use of different silage additives has played an important role in the development of silage making (Wilkinson and Rinne, 2017). Silage additives can be categorized as either (1) fermentation stimulants, (2) fermentation inhibitors, (3) aerobic deterioration inhibitors and (4) nutrients and absorbents (McDonald et al., 1991; Kung et al., 2003). This thesis will focus on fermentation inhibitors, and specifically formic acid.

In the early 20th century, the focus was direct acidification of the crops using a mixture of sulphuric and hydrochloric acid to prevent unwanted fermentation in the silage with damaging consequences in cheese making (Wilkinson and Rinne, 2017). The Finnish biochemist Artturi Ilmari Virtanen who won the Nobel Prize in Chemistry in 1945 for his research and interventions in agriculture and fodder conservation (Nobelprice, 2024) was the driving force behind the use of sulphuric and hydrochloric acid, later known as the AIV method. The use of acidification and improved fermentation quality also reduced DM loss from storage, increased the DM intake and improved the N utilization in ruminants (Wilkinson and Rinne, 2017).

Concerns regarding occupational and animal safety, in addition to the concerns regarding corrosion of machinery by using sulphuric acid which had been the dominating acid for decades, led to the introduction of formic acid (Wilkinson and Rinne, 2017). Straight chained acids like formic, propionic and acrylic acid have additional inhibitory properties against spore forming clostridia in addition to the acidification properties (Woolford, 1978). Formic acid is the acid with the lowest pKa value of the most used silage additives and leads to immediate pH reduction in the crop. This favours lactic acid bacteria and inhibits enterobacteria and aerobe microbes. However, formic acid is not consistently effective against unwanted bacteria, yeast or mould, and the use of formic acid leads to a collapse of the plant cells and increased silage effluent (Kung et al., 2003). It has become more common to use a lower dosage level of formic acid and combine it with benzoic acid or propionic acid to get an antimicrobial effect of the additive (Mo, 2005). Use of formic acid-based additives that restricts fermentation can potentially preserve silage concentrations of WSC compared to silages prepared without additives or with the use of lactic acid bacteria inoculants (Henderson et al., 1972; Bakken et al., 2016). The efficacy of formic acid on animal

performance has been reviewed extensively (Thomas and Thomas, 1985; McDonald et al., 1991). The magnitude of the improvement in animal performance, such as increased dry matter intake, increased average daily weight gain or increased milk production, largely depends on the preservation quality of the untreated silage, with great benefits when the untreated silage is badly preserved (Mo, 2005).

There is little information available on the effect of fermentation quality on CH_4 emission. Therefore, we decided to design an *in vitro* experiment where we investigated the combined effect of herbage species, harvest frequency and fermentation intensity.

1.2 Factors affecting feed intake and milk production

Feed intake is regulated by animal related factors, feed related factors and environmental factors (**Table 1**). It is essential for milk production, animal health and welfare, growth and fetus production that the animal is offered and eats sufficient feed to cover recommended daily intake of nutrients for maintenance and production. This thesis focuses on silage quality, and the emphasis in this chapter will be on feed related factors and especially those related to grass and clover silage that are not described in other parts of the thesis.

Table 1. Factors affecting feed intake (Moderated from Ingvartsen and Kristensen, 2003).

Animal	Feed	Environment
Breed	Species	Feeding frequency
Sex	Morphology	Ad libitum/restricted
Weight	Chemical composition	Eating time
Age	Digestibility	Additives
Growth/Yield	Degradability	Salt
Pregnancy	Passage kinetics	Water
Parity	Particle size	Tied stall/ Loose house
Health	Dry matter content	Daylength/ Light
Welfare	Fermentation products	Temperature
	Palatability	

According to Mertens (1994) between 10 and 40% of the variation in digestible energy intake can be explained by differences in digestibility, while 60 to 90% can be explained by differences in feed intake. There is still much we don't fully understand regarding voluntary feed intake. It is clear that physical factors related to rumen fill is

important, but also metabolic regulation due to rumen pH, plasma glucose, short chain fatty acids like rumen fluid acetate and portal propionate, body temperature and body condition score (fat reserves). Conrad et al. (1964) and Mertens (1994) both suggested that rumen fill was the first limiting factor for feed intake of roughage, and then metabolites. Ellis et al., (2000) emphasized that the supply of aminoacids to the ruminant tissue was of major importance. This contrasts with previous emphasis on energy supply (Van Soest, 1994). In a recent review by Albornoz et al. (2023) the hepatic oxidation theory is described, where the liver plays a central role in sensing the nutrient status, and then sending signals to the brain which results in satiety and feeling of hunger increasing the feed intake.

The maturity of silages and the digestibility of the feed affects silage feed intake (Rinne et al., 2002). Digestibility refers to the part of the feed that is utilized by the animal and not secreted through the faeces (McDonald et al., 2011). Organic matter [dry matter – ash] is often referred to as the part of the feed that can be digested by the animal, and the energy value of the feed can be shown as the organic matter digestibility (OMD). Feed intake normally increase as the OMD increases, which aligns with the retention time in the rumen (McDonald et al., 2011). Dependent on the phenological development stage of the plant, ruminants normally digest 40-50% of the aNDFom in legumes and 60-70% of the aNDFom in grass (Buxton et al., 1995). The proportion of digestible energy from the aNDFom are lower in legumes (20-40%, majority from cell solubles) than in grasses (50-80%, minority from cell solubles) (Buxton and Redfearn, 1997). Degradability of the feed and passage kinetics (K_d) affects dry matter intake. The rate of passage out of the rumen can be affected by both extrinsic and intrinsic factors. Extrinsic factors are related to both the ruminant and the feed (Huhtanen et al., 2006), but intrinsic factors like feed particle size, rate of particle size reduction and gravity properties of the particles are determined by feed type (forage vs. concentrate, forage type and species), stage of maturity, leaf to stem ratio, and harvest frequency (Lund, 2002; Kuoppala et al., 2009; Kuoppala et al., 2010).

1.3 The ruminant digestive system and enteric CH_4 production

Ruminants are herbivore mammals such as cow, goat, sheep, deer, and moose. About 50 million years of evolution adapted these mammals to an environment where they can utilize fibrous feedstuff such as grasses, legumes, bushes, and shrubs (Hackmann & Spain, 2010). The ruminant stomach consists of a four-compartment great enlargement of the

gastrointestinal tract called the forestomach including the rumen, reticulum, omasum, and the abomasum (Sjaastad et al., 2016, **Figure 4**).

The first part of the digestive process is the ingestive chewing and the rumination of the feed boluses which ensures reduction in feed particle size and increased surface to volume ratio of the feed particles for microbial digestion. The rumination and ingestive chewing process typically is accompanied by 150 L/day of saliva production in cows and 10 L/day in sheep. The saliva contains sodium bicarbonate and phosphate buffers. These buffers maintain a ruminal pH of 5.5 to 6.5 under normal conditions. The temperature in the rumen remains close to that of the animal, ranging between 38-42˚C (McDonald et al., 2011). Microbes in the rumen consist of anaerobic members from all the major groups of microorganisms: bacteria, protozoa, archaea, virus, and fungi, where bacteria are the most diverse and abundant group of microbes presented in the rumen (Andersen et al., 2023). The microbes ferment complex structural and non-structural carbohydrates into simple sugars and further into short chained fatty acids (SCFA) which are used as an energy source for the ruminant.

When the feed particles enter the reticulorumen through the oesophagus it is either regurgitated, rechewed and swallowed or it is passed on to the rumen sacs and further to the omasum. The omasum has a large surface area which allows for great water absorption capacity and for absorption of SCFA. Finally, the feed particles enter the abomasum (true stomach) where the feed particles are further degraded to absorbable nutrients by stomach acids and enzymes before entering the small and large intestines (McDonald et al., 2011).

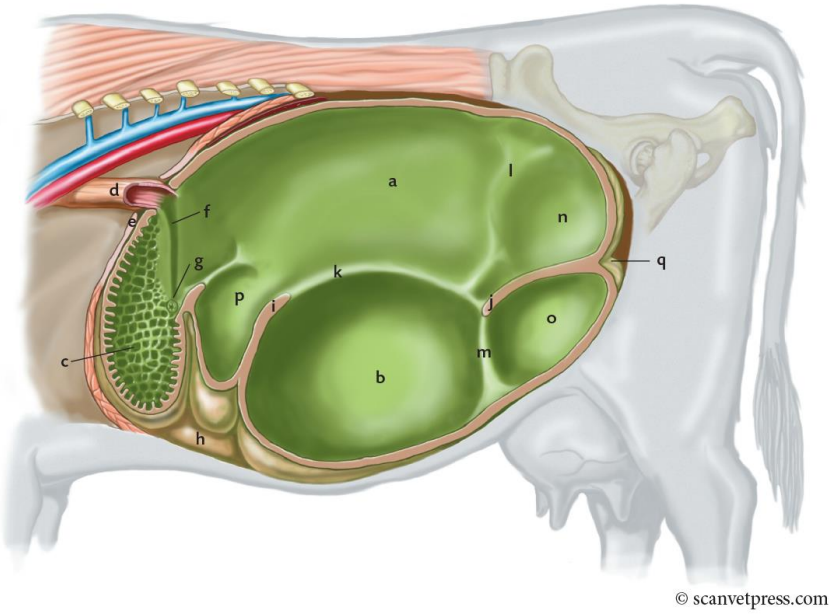

Figure 4. The bovine reticulorumen from the left side. a) dorsal sac, b) ventral sac, c) reticulum, d) esophagus, e) diaphragm, f) esophageal groove, g) reticulo-omasal orifice, h) abomasum, i) cranial pillar, j) caudal pillar, k) longitudinal pillar, l) dorsal coronary pillar, m) ventral coronary pillar, n) dorsocaudal blind sac, o) ventrocaudal blind sac, p) cranial sac, q) caudal transverse groove (Sjaastad et al., 2016).

Methane is a by-product of anaerobic microbial fermentation processes in the gastrointestinal tract of animals. In the process of converting e.g., structured cell wall polymers, sugar or starch to SCFA, the rumen microbes produce dihydrogen (H_2). For the fermentation process to function optimally, reduced cofactors need to be re-oxidized (e.g. NADH to NAD+) which is aligned with the concurrent reduction of CO_2 to CH_4 in a process that consumes H_2 (Hook et al., 2010). The CH_4 gas is then eructated mainly through the mouth (95%) either directly from the rumen or resolved in blood and via the lungs and thereafter out through the mouth. The rest (5%) exits through the anus (Murray et al., 1976). The formation of the SCFA acetate and butyrate generates H_2, which can be utilized by the methanogenic archaea to produce CH_4 (Ranilla et al., 2007). Production of the SCFA propionate on the other hand, will consume H^+ which are associated with the concurrent reduction in CH_4 production (Janssen, 2010).

Previous results show that ruminal fermentation of aNDFom often gives rise to less propionate and more acetate than the ruminal fermentation of starch, water soluble carbohydrates and protein (Janssen, 2010). It has also been reported that fermentation of digestible fiber fractions gives rise to 2.6-fold more CH_4 than the fermentation of digestible CP and digestible nitrogen free extracts (Jentsch et al., 2007). Our previous *in vitro* results (Weiby et al., 2022) showed that WSC was the silage composition variable with greatest positive correlation to CH_4 yield (mL/g OM and mL/g OMD), and that there was a positive association between concentration of WSC and increased rumen molar proportion of acetate and butyrate, and reduced rumen molar proportion of propionate. There are also *in vivo* results showing that feeding perennial ryegrass with increased concentration of WSC increased CH_4 production (MJ/d), but the results were more variable when reporting CH_4 yield or intensity (Ellis et al., 2012).

Grass harvested at a less mature developmental stage increase ruminal degradation of aNDFom fractions (Rinne et al., 2002; Kuoppala et al., 2008, 2010; Randby et al., 2012), which may result in a shift towards more rumen fluid propionate and less acetate (Janssen, 2010). Holtshausen et al., (2012) showed that *in vitro* CH_4 yield (mL/g DM) increased in silages cut at an early vegetative stage compared to a late vegetative stage. However, experiments investigating the effect of grass silage maturity on the ratio between propionate and acetate + butyrate have been inconsistent (Kuoppala et al., 2010; Warner et al., 2016) because it is not only stage of maturity but also silage fermentation characteristics that affects molar proportions of ruminal SCFA. Silage which is extensively fermented with an increased proportion of lactic acid may lead to reduced CH_4 production as lactic acid is metabolized to propionate in the rumen (Huhtanen et al., 2013), which reduce available hydrogen used in CH_4 production.

As farmers are encouraged to reduce CH_4 emissions, changes in feeding regimes are a promising mitigation option (Beauchemin et al., 2020). However, the knowledge in silage production strategies to reduce CH_4 emissions are scarce. Reducing CH_4 emissions in ruminants through changes in feeding regimes and improved silage quality is important not only to reduce the overall climate footprint of the industry, but also to improve the use of national feed resources, increase self-sufficiency and reduce dependency on imported feed.

2. Objectives and hypothesis

The experiments described in this thesis were a part of the larger project Klimagrovfôr. The overall objective of the PhD thesis was to develop strategies in grass silage production that mitigate enteric CH_4 emissions from ruminants. The secondary objectives of this thesis were to:

(1) Identify feed quality parameters and silage fermentation products responsible for variation in *in vitro* CH_4 yield.

(2) Test the effect of grassland harvest frequency, species mixture, wilting and fermentation pattern of grass silages on *in vitro* CH_4 yield.

(3) Investigate the effect of grassland species and harvest frequency for timothy on DMI, daily milk production, digestibility, and enteric CH_4 production in lactating dairy cows.

The above-mentioned objectives were investigated in three different studies, resulting in three papers. The detailed description of objectives and hypotheses for each study is depicted in section 2.1 through section 2.3.

2.1 Study I: Associations among nutrient composition, silage fermentation products, *in vivo* organic matter digestibility, rumen fermentation and *in vitro* methane yield in 78 grass silages

The objective of this study was to identify the most important feed quality parameters and silage fermentation products of diverse grass silages with respect to variation in CH_4 production determined using the *in vitro* method. We expected that the diverse concentrations of nutrients and silage fermentation products would affect *in vitro* CH_4 yield, and that these factors could be used to develop a regional *in vitro* prediction equation for CH_4 yield, measured as CH_4 production *in vitro* expressed relative to OM (CH_4-OM) of the silage incubated and digestible OM in vivo (CH_4-dOM).

2.2 Study II: Effect of grassland cutting frequency, species mixture, wilting and fermentation pattern of grass silages on *in vitro* methane yield

The objective of this study was to test the effect of cutting frequency and growth period (three vs. two cuts per season), crop type (timothy (T3), timothy + red clover (T3/RC3) and perennial ryegrass (RG), wilting (22.5% DM or 37.5% DM) and fermentation pattern (with or without formic acid additive) on *in vitro* CH_4 production using a fully

automated gas *in vitro* system. We hypothesized reduced *in vitro* CH_4 production with (1) less frequent harvesting with longer growth periods, (2) use of ley species with lower WSC concentrations, (3) low crop DM and, (4) extensive silage fermentation.

2.3 Study III: Effect of grassland species and harvest frequency on milk production and enteric methane emissions in dairy cows

The objective of this study was to investigate the effect of grassland species (timothy, perennial ryegrass and red clover) and harvest frequency (three vs. two cuts per season) for timothy on DMI, milk production, digestibility and CH_4 production in lactating dairy cows. We hypothesized that a three-cut system compared to a two-cut system for timothy would increase OM digestibility, and thereby increase DMI and ECM production and reduce CH_4 yield and intensity. Further we hypothesized that timothy would have a lower OM digestibility compared to perennial ryegrass, resulting in greater CH_4 yield and intensity. Lastly, we hypothesized that the aNDFom digestibility and CH_4 intensity would decrease when increasing the dietary proportion of red clover from 0% (T3) to 50% (T3+RC3) and 100% (RC3).

3. Materials and methods

This chapter contains detailed description of some of the central methodological approaches, research materials and experimental designs of study I, II and III. Some additional illustrations, pictures and tables that are not depicted in the papers are shown in this section.

3.1 *In vitro* CH_4 measurement in study I and II

This chapter describes *in vitro* procedures used in study I and II (Ramin and Huhtanen, 2012). The *in vitro* experiment was performed at the Swedish University of Agricultural Sciences, Umeå, Sweden. The handling of animals was approved by the Swedish Ethics Committee on Animal Research (Dnr A 32-16), represented by the Court of Appeal for Northern Norrland, Umeå, and the experiment was carried out in accordance with laws and regulations governing experiments performed with live animals in Sweden.

The silage samples from both experiments were dried at 59°C for 48 h. Samples were ground to pass a 1 mm screen using a Retsch cutting mill with trapezoid sieve holes (Retsch, SM2000, Rheinische, Haan, Germany). Dried and ground samples of 1.00 ± 0.003 g of all grass silage bales were weighed into 250 mL serum bottles (Schott, Mainz, Germany).

Rumen fluid was collected 2 h after morning feeding from two rumen-cannulated Swedish Red cows fed ad libitum a diet consisting of grass silage and concentrate (60:40 on DM basis). In study I rumen fluid was first filtered through two layers of cheesecloth into pre-warmed (39°C) and CO_2 flushed thermos bottles directly after extraction from the rumen of each cow, then equal amounts from each cow were blended and strained through four layers of cheesecloth. In study II rumen fluid was only filtered once, through four layers of cheesecloth and then added to pre-warmed (39°C) and CO_2 flushed thermos bottles. In both studies rumen fluid was added to a buffered mineral solution (Menke and Steingass, 1988) including PeptoneTM (pancreatic digested casein; Merck, Darmstadt, Germany) at 39°C under constant mixing and CO_2 flushing, to give a buffered rumen fluid solution with a rumen fluid:buffer ratio of 1:4 by volume (Ramin and Huhtanen, 2012). Then, 60 mL of buffered rumen fluid was added to each bottle and the bottles were directly placed in a water bath at 39°C under constant agitation. Gas production was measured every 12 min using a fully automated *in vitro* gas system (Gas Production Recorder, GPR-2, Version 1.0 2015, Wageningen UR, **Figure 5**). The amount of headspace gas released from the system through automated valve openings was recorded, and all readings were corrected to normal air pressure (101.3 kPa) (Cone et al., 1996).

The samplings are performed with some minor differences and are described separately. In study I gas samples were taken after 24 h of incubation, as this was for screening purpose. Gas samples were taken from the headspace of each bottle using a gas tight syringe (Hamilton, Bondaduz, Switzerland). Additionally, a 1.5-mL sample of liquid was collected from each bottle at the termination of the 24 h incubation. These procedures were repeated for eight runs in total and all samples were incubated with triplicates of each sample (n = 3 runs/silage). All runs included 36 bottles. In each run, 33 bottles contained forage samples and three bottles contained blanks (i.e., bottles with 60 mL of buffered rumen fluid with no sample included). In study I the 78 silage samples (in triplicate) were randomly allocated to the eight *in vitro* runs, with the same sample never incubated more than once within a run and never in the same bottle.

In study II gas samples were taken every 2, 4, 8, 24, 32 and 48 h from the headspace of each bottle. An inoculant sample (rumen fluid + buffer) of 1.0 mL was collected after 24 and 48 h, and immediately frozen at -18°C until analysis. The 60 silage samples (in triplicates) were randomly allocated to seven *in vitro* runs, and all samples were incubated at least three times (n = 3 runs per silage). All runs had 36 bottles where 30 bottles contained

silage and 4 bottles contained standard hay and 2 bottles contained blanks (i.e., bottles contained only 60 mL buffered rumen fluid).

Figure 5. *In vitro* batch culture at SLU Umeå (photo: Kim Viggo Weiby)

3.2 Measurements of feed intake and milk production in study III

The cows were fed the experimental diets ad libitum. We used 40 feeding bins from Biocontrol (Rakkestad, Norway) and recorded daily individual feed intake at each visit. The feed bins containing the same dietary treatments were placed next to each other, and cows had free access to any feed bin containing the assigned treatment. Feed bins were re-filled with new silage every morning and evening. The feed bins were cleaned Monday and Thursday each week and calibrated every Monday morning. Milk yield was recorded at each visit, and cows had access to the milking robot every 6 h with a maximum of 4 milkings every 24 h. Milk samples were collected from each cow at three consecutive milkings. This was performed during the last seven d in each period. Bronopol (Landteknikk, Økern, Norway) was added to the samples to prevent samples from getting damaged. They were stored at 4°C until analysis was performed within 2 weeks. The cows were weighed (Biocontrol, Rakkestad, Norway) after every milking using a scale that was calibrated before each period.

3.3 *In vivo* CH_4 measurement- Greenfeed system in study III

Measurements of enteric CH_4 and CO_2 described in study III were performed using the Greenfeed system (GF, **Figure 6**). Mass fluxes of enteric CH_4 and CO_2 were measured

using two GF units. All cows had access to both GF units. The barn staff ensured that all animals had a minimum of three visits per d during the last week of each period, and the maximum visit frequency was 5 visits per 24 h. Gas calibrations were conducted once a week, and CO_2 recovery tests was conducted every 2 weeks. The recovery of CO_2 was on average 100 ± 3.3 %. Air filters were cleaned two times per week to ensure airflow above 26 L/s. To ensure the correct head position for 2 min during a visit to the GF units, the cows received 5 drops of 40 g of concentrate with a 40 s interval during the visit. A maximum of 1000 g/d of concentrate was provided in the GF unit. Measurements were transformed from liter to gram using the factor 0.7168 g/L for CH_4 and 1.96 g/L for CO_2. For technical reasons CH_4 and CO_2 data were not recorded from one of the cows.

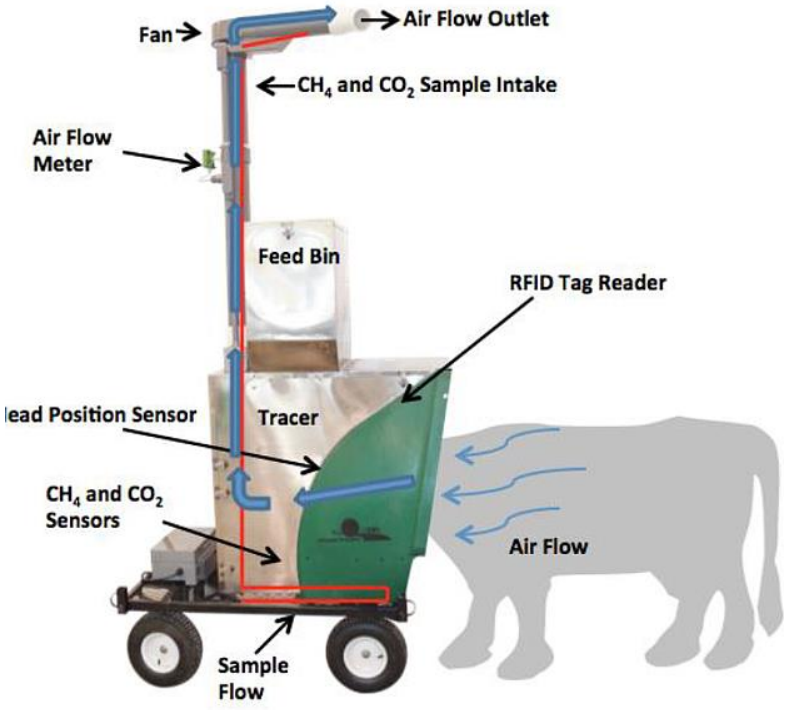

Figure 6. Components of the Automated Head-Chamber System (AHCS, GreenFeed) for measuring CH_4 production in ruminant animals (Hristov et al., 2015).

3.4 *In vivo* apparent digestibility (sheep in study I and cow in study III)

These *in vivo* studies were conducted at the Metabolism Unit of the Norwegian University of Life Sciences (NMBU) in Norway. The experiments were approved by the

Norwegian Ethical Committee on Animal Research and were performed in accordance with regulations controlling live animal experiments in Norway. In vivo apparent OMD of the 78 grass silages in study I was determined according to Åkerlind et al. (2011) (**Figure 7**) using three adult castrated male sheep per grass silage sample. The in vivo study was conducted in 23 runs from May 2017 to December 2019, where 3-5 round bales were tested in each run. The adaptation period was 11 days and each round bale was fed for 21 days. The total collection of faeces was conducted over a period of 10 days, and proportional subsamples of faeces were taken daily, pooled per individual animal and then across animals fed the same test bale, and stored frozen until analysis. Sheep that weighed less than 88 kg daily received 1.0 kg DM of grass silage, and sheep weighing above 88 kg daily received 1.2 kg DM of grass silage. All sheep daily received 10 g of sodium chloride (GC-Rieber, Cort Adelers gate 17, 0254 Oslo) and 35 g of a commercial mixture of vitamins and minerals (VitaMineral Normal Sau, Vilomix, Hensmoveien 30, 3516 Hønefoss, Norway).

Figure 7. *In vivo* apparent digestibility study using castrated adult sheep (photo: NMBU)

In study III we used acid insoluble ash (AIA) as an internal marker in feeds and feces to determine total tract apparent digestibility in dairy cows. Concentration of AIA in the experimental diets was calculated based on the concentration of AIA for the 11 silages and the proportion of these silages in each of the 5 experimental diets in addition to the AIA concentration in concentrate. Fecal output of DM was calculated as total AIA intake from the diet divided by AIA concentration in the feces.

3.5 *In situ* digestibility (cow)

The *in situ* experiment in study III was conducted at the Metabolism Unit of the Norwegian University of Life Sciences (NMBU) in Norway, while the *in situ* experiment in

study II was conducted at the Swedish Agricultural University (SLU) in Sweden. The experiments are performed with some minor differences and are described separately.

Study II, SLU

The experiment was approved by the Swedish ethical committee on Animal Research and performed in accordance with Swedish laws and regulations regarding EU directive 2010/63/EU on animal research. The concentration of iNDF was determined after incubation in 288 h as described by Krizsan et al. (2015). The samples were freeze dried and milled using a Tecator Cyclotec 1093 mill (Foss Tecator AB, Högans, Sweden) and a 2 mm mesh screen. Three ruminal cannulated lactating Nordic red cows were used in the experiment. They were fed a total mixed ration consisting of 60% grass silage and 40% concentrate on DM basis to meet the energy and protein requirements. Cows received the TMR ad libitum 14 days before the start of the experiment. Samples of 2 g of the experimental feed were weighed into polyester bags with 11 µm pore size and a pore area equal to 5% of the total surface area (Sefar Petex 07-11/5-cloth, Sefar AG, Heiden, Switzerland). Organic matter digestibility was calculated from the concentration (g/kg DM) of iNDF and NDFom according to Huhtanen et al. (2013).

Study III, NMBU

The experiment was approved by the Norwegian Ethical Committee on Animal Research and performed in accordance with regulations controlling live animal experiments in Norway. Concentration of iNDF was determined as proportion of NDF remaining in the residue after *in situ* incubation according to the Norfor standard procedure (Åkerlind et al., 2011). The samples were freeze-dried and ground to pass a 1 mm screen using a Retsch cutting mill with trapezoid sieve holes (Retsch, SM200, Rheinische, Haan, Germany). Feed samples of 2 g were added to bags (Sefar Petex 07-11/5-cloth, Sefar AG, Heiden, Switzerland) and intraruminally incubated 288 h according to recommendations of Krizsan et al. (2015) (**Figure 8**). The *in situ* study was conducted using 2 ruminally cannulated Norwegian Red cows fed forage and concentrate (67:33 on DM basis) to meet maintenance energy requirement of the animals. Five bags were incubated into the rumen of each cow, and each sample were incubated into two rumen cannulated cows (e.g 10 bags per sample).

Figure 8. *In situ* incubation study using rumen cannulated Norwegian Red cows (photo: NMBU)

4. Results and discussion

In this section I summarize and compare data from the *in vitro, in situ* and *in vivo* experiment in study I, the *in vitro* and *in situ* experiment in study II and the *in vivo* and *in situ* experiment in study III. The first sub-section "Chemical composition and harvest frequency" discusses data from studies I, II and III, "Species mixture" discusses data from studies II and III, "Silage fermentation intensity" discusses data from studies I and II, and the last sub-section highlights the studies and thesis "Strengths, limitations and practical implementations".

4.1 Chemical composition and harvest frequency (Study I, II & III)

The overall NDFom and iNDF concentration of timothy was greater for the two-cut system than for the three-cut system in both study II and III. In study II the NDFom concentration was 102 g/kg DM greater for the two-cut system compared to the three-cut system, while in study III the NDFom concentration was 78 g/kg DM greater for the two-cut system than for the three-cut system. Concentration of iNDF in timothy was 65 g/kg DM greater for the two-cut system than for the three-cut system of study III, and it was 66 g/kg NDF greater for the two-cut system than for the three-cut system of study II. Increased NDFom and iNDF concentrations for the two-cut system resulted in 7- and 8% lower OMD for the two-cut system compared to the three-cut system in study II and III, respectively.

The greater NDFom and iNDF concentration with a subsequent lower OMD for the two-cut system compared to the three-cut system in both studies (II and III) was expected because the crop was harvested when the plants had reached a more mature phenological stage for the two-cut system. According to Cherney et al. (1993) and Chavez et al. (2006)

harvesting crop at a more mature developmental stage (e.g. less frequent harvest) increase proportion of cell walls and increase the concentration of indigestible lignin in the cell wall structures of the plants with a subsequent reduction in OMD. The effect of less frequent harvest on concentration of NDFom and iNDF as seen in the present studies has also been reported in other experiments on grass and clover silages (Kuoppala et al., 2009; Alstrup et al., 2016). It is also possible that the herbage in the three-cut system had a greater proportion of leaves than in the two-cut system, as the proportion of regrowth was greater for the three-cut system than the two-cut system. The proportion of herbage from regrowth was quite similar for the two present studies. Previous results show that regrowth material contains more leaves (Rinne and Nykänen, 2000; Gustavsson and Martinsson, 2004) and less cell wall carbohydrates (Kuoppala et al., 2008) compared to the spring growth, which ultimately reduce NDFom concentration.

Previous studies show that OMD is lower for the regrowth compared to the spring growth, and Huhtanen et al. (2006) concluded that this was because the regrowth often have an increased iNDF concentration of the cell walls compared to the corresponding spring growth. In addition, regrowth might contain more weeds and dead plant materials with a low digestibility (Kuoppala, 2010). It seems, however, that these factors were not sufficient to overshadow the effect of harvesting the grass at an earlier phenological stage in the present study.

Unexpectedly, silage DMI did not differ between T3 and T2 ($P = 0.16$, **Table 2**) in study III. However, ECM yield was 2.4 kg/d greater ($P < 0.001$) in T3 than T2. This was probably due to the lower aNDFom and iNDF concentration in addition to greater OMD and CP concentration in T3 compared to T2. The concentration of free fatty acids (FFA) in the milk was generally high in this experiment, varying between 1.49 mmol/L (RG) and 1.96 mmol/L (T2). Concentration of FFA is affected by lipolysis in the milk. Lipolysis occurs spontaneously as the enzyme lipoprotein lipase disintegrate the membrane protecting the fat globules (Thomson et al., 2005), and according to TINE, levels above 0.9 mmol/L increase the risk of rancid taste (TINE Medlem, 2024). Treatment T2 had 0.31 mmol/L greater FFA than T3 (1.96 vs. 1.65 mmol/L, $P = 0.03$), and we speculate that the cows receiving the T2 diet had a negative energy balance, which may have increased lipoprotein lipase activity (Thomson et al., 2005).

Our results regarding DMI in early compared to late stage of maturity are not in agreement with other studies. Randby et al. (2010) reported 1.42 kg greater ($P < 0.001$) silage DMI in intact bulls fed early (tillers in stem elongation) vs. later (early heading) harvested timothy, meadow fescue and red clover silage without concentrate from the spring growth. Pang et al. (2021) reported 0.7 kg lower ($P < 0.01$) silage DMI in lactating Nordic Red cows when postponing the first cut and increasing the regrowth interval in timothy and red clover leys (80:20, respectively). The DM concentration was 16% greater (47.4 vs. 31.5%) for the T2 diet than T3 diet, and it was the second cut of the T2 treatment with a DM concentration of 60%, which elevated the total DM concentration of that diet compared to the T3 diet. Increased DM concentration is correlated with increased DMI (Huhtanen et al., 2007), which probably increased DMI for the T2 diet and reducing the effect of differences in chemical composition on ECM yield for T2 vs. T3 diet. In addition, Johansen et al. (2017) found that increased DM concentration in silage increased the amount of amino acids digested in the small intestine due to reduced rumen degradation of feed protein and increased ruminal microbial synthesis. This might also have evened out some of the differences in chemical composition between the T3 and the T2 diet.

In study I we used 78 silage samples to identify the quality attributes of grass silage associated with variation in *in vitro* CH_4 yield. We found that concentration of NDFom and iNDF was negatively correlated with *in vitro* CH_4 yield (mL/g OM) ($r = - 0.63$ and $r = - 0.48$, $P < 0.001$, respectively) and that *in vivo* OMD was positively correlated with *in vitro* CH_4 yield ($r = 0.44$, $P < 0.001$). The negative association between NDFom and iNDF and the positive correlation between OMD and CH_4 yield align with the *in vitro* results of study II where we found that *in vitro* CH_4 production (mL/g OM) was positively correlated ($r = 0.53$, $P < 0.001$) with OMD, and negatively correlated ($r = - 0.54$, $P < 0.001$) with NDF concentration. In study II the main objective was to test the effect of cutting frequency (three vs two cuts per season) on *in vitro* CH_4 production. We found that the two-cut system with a greater concentration of both NDFom and iNDF and a lower OMD reduced *in vitro* CH_4 production (mL/g DM and mL/g OM, $P < 0.001$).

The results in *in vitro* CH_4 production in study I and II were expected. Methane production is the result from rumen methanogens fermenting digestible carbohydrates like cell wall polymers and fructans to SCFA, H_2 and CO_2 (McAllister et al., 1996). Our results are in accordance with Holtshausen et al. (2012) who reported lower *in vitro* CH_4 production (mL and mL/g NDF digested) when more mature grass silages were ensiled compared to less

mature grass. Macome et al. (2018) also found that *in vitro* total gas and CH_4 production (mL/g OM) decreased with advancing maturity of the ensiled grass. The strong correlations between aNDFom and iNDF and CH_4 found in study I indicate that these are two important determinants of the methanogenic potential of silages. Previous *in vivo* studies have shown increased proportions of ruminal acetate and reduced proportions of ruminal butyrate in grass silages with increased aNDFom and iNDF concentrations at ensiling (Rinne et al., 1997, 2002). However, we found no such consistent effect on aNDFom and iNDF concentration and proportions of *in vitro* SCFA in study I. This is in accordance with Holtshausen et al. (2012) who did not see any effect of increased maturity at harvest on *in vitro* rumen fluid proportions of acetate at 24 or 48 h of incubation. However, they reported an increase in rumen fluid proportion of propionate at 48 h of incubation in late maturity grass silage, which might help explain the reduced CH_4 yield (mL and mL/g NDF disappeared) in that study. We speculate that the greater CH_4 yield of less mature grass silages as seen in the two *in vitro* studies was mainly due to increased OMD and amount of substrate fermented in the *in vitro* batch culture system. Johnson and Johnson (1995) argued that there were two primary mechanisms controlling CH_4 production. The first mechanism is the amount of dietary carbohydrates fermented in the rumen, and the second is the available H_2 supply through changes in SCFA production. We speculate that grass silages with greater OMD increased the supply of *in vitro* fermentable carbohydrates and that this might have overshadowed the effect of changes in metabolic H_2 supply through shifting the ratio between propionate: [acetate+butyrate] in the incubated rumen fluid.

However, in study III the more mature silage (T2) had nearly 40 g/kg DM greater WSC concentration than the less mature silage (T3) (97.9 vs. 58.2 g/kg DM, respectively). This was expected, due to a greater DM concentration in T2 vs T3 (47.4 vs. 31.5 % of fresh matter, respectively). In study I we found that WSC was the single silage composition factor contributing most ($r = 0.57$, $P < 0.001$) to the *in vitro* methanogenic potential of silages (mL CH_4/g OM). Silage WSC is quickly metabolised in the rumen fluid. Kellogg and Owen (1969a, b) reported increased butyrate proportion in an *in vivo* study when feeding sucrose. In study I there was a positive correlation ($r = 0.33$, $P < 0.01$) between WSC and molar proportion of butyrate, which probably contributed to increased availability of H^+ (Boadi et al., 2004) and the positive correlation between WSC and CH_4 (mL/g OM). It is possible that the greater CH_4 yield in T2 vs. T3 (38.3 vs. 31.3 g/kg DOM, respectively, $P < 0.001$) at least

partly can be attributed to the increased WSC concentration, in addition to the main explanatory factor which is less ECM yield increasing the CH_4 intensity.

In study I we used forward stepwise regression modelling with Akaike Information Criteria (AIC) as selection criteria to determine if CH_4 could be predicted from grass silage chemical variables. The following explanatory variables were included in descending order: aNDFom ($P < 0.001$, AIC = 130.7), WSC ($P = 0.14$, AIC 106.5), iNDF ($P < 0.01$, AIC = 98.7), propionic acid ($P = 0.34$, AIC = 97.6) and pH ($P = 0.16$, AIC = 97.4).

*Model 1: CH_4-OM (mL CH_4/g OM) = 36.22 – 0.02 × **aNDFom** (g/kg DM) + 0.03 × **WSC** (g/kg DM) – 0.01 × **iNDF** (g/kg aNDFom) + 0.82 × **propionic acid** (g/kg DM) + 0.71 × **pH**. Coefficient of determination (R^2) = 0.65*

In this thesis, I wanted to test if the equation developed in study I was able to predict the CH_4 yield measured in study II (**Figure 9 a, b**). If so, it would be possible to use this equation to predict CH_4 yield potential of silages in general. The model predicted CH_4 yield from the silage samples in study II with a R^2 of 0.37, and the predicted CH_4 yield was consistently lower than the observed CH_4 yield. The low R^2 show that this regression equation cannot be used to precisely predict CH_4 yield from silages in study II as the model failed to explain 63% of the variation in CH_4 emissions in the data from that study.

The CH_4 production in study I varied between 18.9 and 34.1 mL/g OM, with an average of 25.3 mL/g OM and a standard deviation of 2.9. The predicted data are overestimated in lower CH_4 production levels, while it is underestimated in higher CH_4 production levels (**Figure 9 a**). In study II the CH_4 production levels were greater, varying between 27.3 and 40.9 mL/g OM with an average of 33 mL/g OM and a standard deviation of 2.55. The predicted data was consistently overestimated (**Figure 9 b**). As the model was developed based on data from study I which was considerably lower than in study II, this contributed to the low R^2. In a previous study by Lee et al. (2003) they used CH_4 yield data from *in vitro* incubation of different forages to develop CH_4 prediction equations and found that they were able to predict CH_4 yield with a R^2 of 0.99. The reason for the substantially lower R^2 in our studies compared to Lee et al. (2003) may be that both the forage investigated in our study, and the chemical concentration of this forage was very different from what was reported in Lee et al. (2003). In addition, the number of samples was only 15 in the study by Lee et al. (2003) which is probably too low to develop a robust model for the purpose of predicting CH_4 yield. In our studies the number of samples were 78 and 60 for study I and II,

respectively. Other factors of uncertainty in all *in vitro* studies which might affect results, are the rumen fluid and the microorganisms therein, which is greatly affected by both donor animals and experimental feeds, in addition to experimental procedures when extracting the rumen fluid out of the rumen and into the gas *in vitro* system and the buffers used in the procedures (Yáñez-Ruiz et al., 2016).

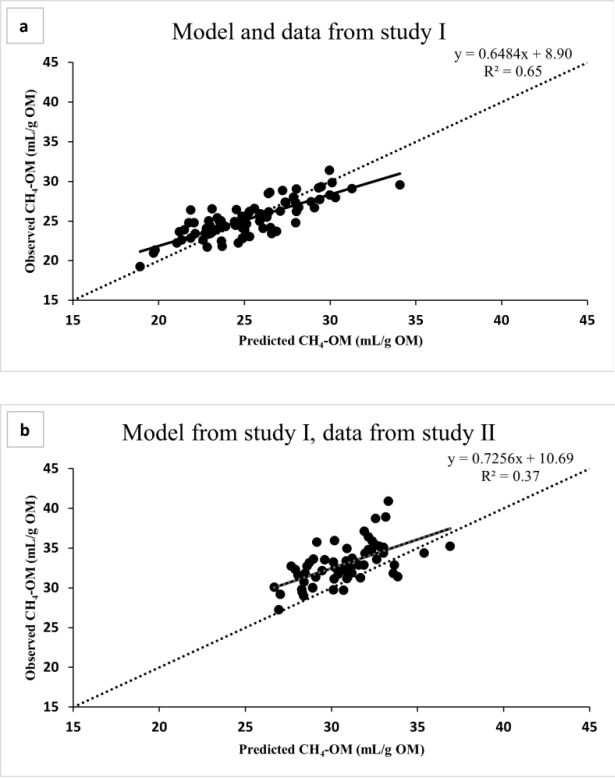

Figure 9 a, b. Relationship between the observed and predicted *in vitro* CH_4 production expressed as CH_4-OM, mL CH_4 per g organic matter, showing the result of regression modelling data from Weiby et al. (2022) (**Figure 9a**), and the same regression model, but data from Weiby et al. (2023) (**Figure 9b**).

4.2 Species mixture (Study II & III)

In this section only study II and III will be included, as study I did not include data on species mixture. Concentrations of NDFom was 38 and 63 g/kg DM lower in RG compared to T3 in studies II and III, respectively. King et al. (2012) reported lower NDFom concentration in RG compared to T harvested from only primary growth. The lower NDFom

concentration was accompanied by a greater DM digestibility, which was not the case in our study III comparing T and RG (77.5 vs 77.0%, respectively, P = 0.67). Concentrations of iNDF in study III was slightly lower in RG compared to T3 (72.2 vs. 76.3 g/kg DM, respectively).

Probably, the most surprising regarding chemical composition of RG in the present studies (II and III) was the elevated iNDF concentrations for the first regrowth. In study II the first regrowth of RG had 133 g/kg NDF greater concentration of iNDF than the spring growth, and in study III the concentrations was 80 g/kg DM greater for the first regrowth compared to the spring growth. This has also been shown in previous experiments with RG in Norway (Østrem et al., 2014). The first regrowth of RG normally has a lower leaf:stem ratio than the spring growth and the second regrowth, because of many new vegetative tillers, which then affects the iNDF concentration (Bakken et al., 2009). The mid-season morphology of RG is affected by the spring harvesting time, where late harvest gives relative higher leaf:stem ratio than early harvest (Hurley et al., 2009).

We also speculate that these changes are related to increased lignification of the cell walls of RG due to high temperatures in the summer months, as also reported in a recent experiment with RG fed to sheep (Garry et al., 2021). The temperature in the period of the first regrowth was between 1.7 and 2.2˚C above the normal temperature in those weeks (calculated from the period 1960-1990, World Meteorology Organization) which might have increased the maturation process and the lignification of the cell walls (Ford et al., 1979) and hence increased the iNDF concentration. The increased concentration of iNDF in RG in both studies reduced OMD, which then resulted in no difference between T and RG. In study II we found that RG had 56 g/kg DM greater WSC concentration compared to T for the fresh grass material (162 vs. 106 g/kg DM respectively, P < 0.001), but when comparing the ensiled grass material, there was no difference (P = 0.160) between RG and T in WSC concentration. In study III RG had a numerically greater WSC concentration compared to T3 (66 g/kg DM). Perennial ryegrass normally has a greater concentration of WSC compared to other grass species, as many perennial ryegrass varieties have been bred for this purpose (Parssons et al., 2011).

Silage DMI was 1.8 kg DM lower (P < 0.001) in RG compared to T3 (**Table 2**). This was a bit surprising as RG normally has a superior feed quality compared to other grasses when harvested at the same developmental stage (Wilkins and Humphreys, 2003; Casler and

Kallenbach, 2007). The lower DMI in RG was probably due to the digestibility of aNDFom which was 3.7% lower in RG compared to T3 (68.4 vs. 72.1%, respectively, P = 0.03). It is possible that the lower digestibility of aNDFom affected the passage rate of the rumen content, thereby increasing the retention time of digesta in the rumen (Huhtanen et al., 2006). Oba and Allen (1999) found that increased digestibility of NDF significantly increased DMI using a dataset of treatment means from 13 sets of forage comparisons in the literature. The lower DMI in RG compared to T3 also resulted in 1.9 kg/d lower ECM yield (27.7 vs 29.6 kg/d, respectively, P < 0.001, **Table 2**). Johansen et al. (2018) found lower ECM yield in italian ryegrass (*Lolium multiflorum* L) than in T (24.3 vs. 26.1 kg ECM, respectively) in a meta-analysis comparing 3 studies with timothy and 9 studies with italian ryegrass. However, this difference was only numerical and not statistically significant.

In study I we found that among all investigated silage composition variables, concentration of WSC had the strongest positive correlation to CH_4 yield (mL/OM and mL/dOM).We speculated that the reason was an increase in available H_2 in the rumen fluid due to increased production of rumen fluid butyrate, which then could be used by the rumen fluid methanogens to produce CH_4 (Boadi et al., 2004). However, there was no difference in CH_4 production (g/d) between T3 and RG (P = 0.46) in study III. Perennial ryegrass had a numerical difference of 66 g/kg DM greater WSC concentration compared to T3 (124 vs. 58.2 g/kg DM respectively). In study II we found no difference in WSC concentration between T3 and RG (P = 0.160). In study III the lactic acid concentration was 6.3 g/kg DM greater in RG than T3 (34.5 vs. 28.2 g/kg DM) and we speculate that much of the residual WSC was lost in the silage fermentation process. Perennial ryegrass obtained a greater CH_4 intensity compared to T3, but that was due to a lower DMI and ECM yield compared to T3, and it seems that concentration of WSC was not the reason for differences in CH_4 emissions.

In legumes it is only the xylem tissue of the cell walls that are lignified, and the other cell wall tissues are almost completely digestible (Wilson and Kennedy, 1996). The NDF concentration is normally lower in legumes compared to grasses (Rinne et al., 2006; Van Dorland et al., 2007; Johansen et al., 2017). Concentration of NDFom was only 36 g/kg DM lower in T3/RC3 compared to T3 in study II, while the concentration was 112 g/kg DM lower in T3/RC3 compared to pure T3 in study III. However, in study II the red clover proportion was very low in first cut, and moderate in second and third cut, which probably also affected the NDFom concentration in study II. However, in study III the actual proportion in the T3/RC3 diet was close to 50/50 as T3 and RC3 was mixed in the mixer wagon before

feeding. In study II there was no difference between T3 and T3/RC3 in iNDF concentration (170 vs 173 g/kg NDFom respectively, P = 0.518), while in study III the iNDF concentration was 5.6 g/kg DM lower in T than T3/RC3 diet (76.3 vs. 81.9 g/kg DM). In study III, OMD of the T3/RC3 diet was 1.7% lower than the pure T3 diet. However, in study II the results were opposite with 1.1% greater OMD for the T3/RC3 diet compared to the pure T3 diet. The differences in OMD were probably affected by the low red clover proportion in the T3/RC3 diet in study II. In addition, the methods for estimating OMD are different for the two studies. In study II we calculated OMD based on concentrations of NDF and iNDF (Huhtanen et al., 2013), while in study III total-tract digestibility of OM was calculated in the experimental diets (n = 5) (including a fixed level of concentrate) based on faecal grab samples using AIA as an internal marker. As the concentration of iNDF for the T3/RC3 diet in study II was not different from the pure T diet, this might explain the unexpected results in OMD for the T3/RC3 diet in study II.

In study III DMI increased when replacing 50% of timothy with red clover in the production trial, but declined when all timothy was replaced with red clover (quadratic effect P < 0.001, **Table 2**). There was a quadratic effect (P = 0.002) of increased proportion of red clover, with lower ECM yield in RC3 than in T3. The ECM yield was numerically greatest for the T3/RC3 diet. The reduced DMI and ECM yield for the pure RC3 diet aligns with the linear decrease in OMD and digestibility of aNDFom with increased proportion of red clover in the diet. Johansen et al. (2017) also reported a linear decrease in OMD and a tendency for a quadratic decrease in NDF digestibility when increasing the proportion of red clover in the diet. The NDF concentration is lower in legumes than in grasses, and legumes are usually, but not always, more lignified which results in a lower digestibility (Buxton and Redfearn, 1997). We did not measure lignin concentration in the current study, but Kriszan et al. (2013) found a tendency (P = 0.09) for greater concentration of acid detergent lignin (ADL) in red clover compared to grasses. We speculate that the regulating factor for DMI in the present study was rather metabolic (SCFA, hormones etc.) than physiologic (rumen fill) (Albornoz et al., 2023) as previous studies show that maximum rumen fill is lower in red clover diets compared to grass silage diets (Bertilsson and Murphy, 2003).

According to Mertens et al. (1985) concentration of NDF should not be below 280 g/kg DM for the rumen microbial fermentation to function optimally, and in the present study the aNDFom concentration for the RC3 diet was only 19 g/kg DM above this level. However, the concentration was still low which resulted in a low aNDFom intake. It is possible that the

low aNDFom concentration in combination with a low aNDFom digestibility for the pure RC3 diet negatively affected both rumen fermentation and microbial synthesis. In addition, the RC3 diet contained 27% more CP than the T3 diet, which resulted in 11% greater CP intake in the RC3 diet than in the T3 diet. The high CP level combined with 11% lower NEl_{20} concentration probably resulted in an imbalance between protein and energy in the rumen (Sinclair et al., 1993). This is supported by a PBV value in RC3 diet twice as high as the level in the T3 diet. We speculate that this imbalance resulted in extra energy costs for detoxification of excess ammonia in the RC3 diet, which ultimately also affected milk production negatively (Reed et al., 2017).

These factors combined resulted in the same amount of milk produced for the T3 compared to the RC3 diet (both 27.1 kg/d, $P = 0.46$), but a linear reduction in milk fat production of 131 g/d (1200 vs 1069 respectively, $P < 0001$) and a quadratic reduction in protein production of 51 g/d (1012 vs 961 g/d respectively, $P < 0.001$), resulting in a linear reduction of 2 kg/d in ECM yield (29.6 vs. 27.6 kg/d respectively, $P = 0.001$). Oba and Allen (1999) evaluated the importance of NDF digestibility in forage on DMI and ECM yield using treatment means from 13 sets reported that a reduction in dietary *in situ* or *in vitro* NDF digestibility from high (62.9%) to low (54.5%) significantly reduced ECM yield (26.3 vs 25.1, $P < 0.0001$) of cows in a dataset of 13 forage comparisons from the literature.

The reduction in milk fat production in RC3 compared to T3 was probably due to a lower ruminal acetate and butyrate and greater ruminal propionate production lowering the rumen pH and then lowering production of precursor for de novo milk fat synthesis (Seymour et al., 2005). The T3/RC3 diet had a greater aNDFom digestibility compared to the pure red clover diet resulting in a greater aNDFom intake and a greater nitrogen efficiency which explains the observed increase in silage DMI and ECM yield in that diet, which is also shown in other studies (Kuoppala et al, 2010; Johansen et al., 2017).

Increased inclusion of red clover from 0 to 100% linearly increased CH_4 production with 19 g/d (476 vs. 495 g/d respectively, $P = 0.05$), CH_4 yield linearly increased with 2.7 g/kg DMI (22.1 vs. 24.8 g/kg DMI respectively, $P < 0.001$) and CH_4 intensity linearly increased with 1.8 g/kg ECM (16.5 vs. 18.3 g/kg ECM respectively, $P < 0.001$, **Table 2**). These results are not in accordance with a recent study (Bica et al., 2022) where they fed red clover diets to cattle from 8-15 months of age. They reported numerically lower *in vivo* CH_4 production in RC diets compared to grass silage diets (122 vs. 133 g/d, $P = 0.1$), however, as

the DMI was similar due to bad silage quality in the RC diet, the CH_4 yield was 3.4 g/kg DMI lower in the RC diet compared to the grass silage diet (17.8 vs. 21.2 g/kg DMI respectively, P = 0.008). Van Dorland et al. (2007) found no difference in DMI, daily milk production, CH_4 production or intensity in diets consisting of 60% perennial ryegrass and 40% red clover. The present results are however supported by the *in vitro* results in study II showing no CH_4 mitigating effect of red clover. It is possible that the inconsistency in literature may be due to differences in forage quality (stage of maturity, fermentation quality, herbage red clover inclusion or presence of tannins) or between animal variations (Knapp et al., 2022). The increased CH_4 yield, and intensity observed in the RC3 diet was probably related to low aNDFom concentration and digestibility of both aNDFom and OM in the RC3 diet as previously described, in combination with imbalance between CP and energy which ultimately lowered DMI and ECM yield, increasing CH_4 yield and intensity.

Table 2. Effect of harvest frequency and grassland species on silage intake, energy corrected milk yield, apparent digestibility and methane (CH_4) emissions (information extracted from tables in study III).

	Experimental diets[1]						Probability[2]			
Item	T3	T2	RG	T3/RC3 50/50	RC3	SEM	T3 vs T2	T3 vs RG	RC3-L	RC3-Q
Silage intake, kg DM/d	15.2	14.5	13.4	16.4	13.9	0.43	0.16	<0.001	0.008	<0.001
ECM, kg/d	29.6	27.2	27.7	30.2	27.6	0.56	<0.001	<0.001	0.001	0.002
Digestibility, %										
OM	77.8	69.6	77.9	76.1	73.5	0.77	<0.001	0.94	0.001	0.63
aNDFom	72.1	61.3	68.4	66.7	52.9	1.18	<0.001	0.03	<0.001	0.02
CH_4										
g/d	476	469	466	510	495	8.68	0.46	0.26	0.05	0.02
g/kg DMI	22.1	22.9	23.4	22.6	24.8	0.58	0.27	0.05	<0.001	0.07
g/kg DOM	31.3	38.3	32.8	32.0	38.4	1.28	<0.001	0.27	<0.001	0.02
g/kg ECM	16.5	17.7	17.4	17.5	18.3	0.39	0.003	0.02	<0.001	0.84

[1]T3 = Timothy 3 cut system, T2 = Timothy 2 cut system, RG = Perennial ryegrass 3 cut system, T3/RC3 = Timothy 3 cut system/red clover 3 cut system, RC3 = Red clover 3 cut system

[2]Probability of treatment effects: T3 vs T2 = Effect of 3 cuts vs. 2 cuts in timothy; T3 vs RG = Effect of 3 cuts in timothy vs. 3 cuts in perennial ryegrass, RC3-L = Linear effect of increasing red clover proportion, RC3-Q = Quadratic effect of increasing red clover proportion

4.3 Silage fermentation intensity (Study I & II)

In this section only study I and II are included, as silage fermentation intensity was not included as a treatment effect in study III.

In study II the concentration of WSC was 25 g/kg DM lower in silage made from herbage wilted to 22.5% than from herbage wilted to 37.5% DM (52 vs. 77 g/kg DM respectively, $P = 0.002$), and concentration of lactic acid was 27 g/kg DM greater in silage wilted to 22.5% DM compared to silage wilted to 37.5% DM (47 vs. 20 g/kg DM respectively, $P < 0.001$). Concentrations of both acetic acid and butyric acid were greater (7 and 4 g/kg DM, respectively, $P < 0.001$) for the less wilted silage compared to the more extensively wilted silage. The greater WSC and lower lactic acid concentration for the more extensively wilted silage compared to the less extensively wilted silage is expected as wilting reduces the activity of all microbes in the silage due to increased osmotic pressure, which restricts fermentation intensity (Charmley, 2001). In addition, wilting normally reduces proteolysis in the silage (Slottner and Bertilsson, 2006) resulting in increased amounts of rumen utilizable protein due to less soluble non protein nitrogen in the silage (Van Vuuren et al., 1990; Tamminga et al., 1991). This was evident in study II as the silage wilted to 37.5% DM had 19 g/kg N lower content of total NH_3-N than silage wilted to 22.5% DM (52 vs. 33 g/kg DM respectively, $P < 0.001$).

Although wilting reduces microbial activity, it seems that especially lactic acid bacteria is more tolerant towards increased DM levels (McDonald et al., 1991) which was evident in study II as it still was acceptable lactic acid concentrations (average 20 g/kg DM) in the silage wilted to 37.5% DM. This is important as lactic acid fermentation reduces the risk of fermentation loss through less useful microbial pathways (McDonald et al., 1991). Fermentation with increased acetic acid is an example of less useful pathways as previous results show that acetic acid levels above 17 g/kg DM reduce DM intake in cattle markedly (Gerlach et al., 2021). In study II wilting reduced acetic acid concentrations with 6.7 g/kg DM (13 vs 6 g/kg DM respectively, $P < 0.001$). As wilting restricts fermentation activity, more organic matter is available for rumen fermentation and the microbial flow out of the rumen increase (Verbic et al., 1999). In addition, less WSC are fermented to lactic acid in the silage. Silages that are extensively fermented with homolactic bacteria often contains very little soluble sugars, but excess of lactic acid and increased levels of acetic, propionic and

butyric acid which can be absorbed directly in the rumen. Lactic acid are metabolized primarily to propionate in the rumen (Charmley, 2001).

In study I we found a strong positive correlation ($r = 0.57$, $P < 0.001$) between concentration of WSC in the silage and *in vitro* CH_4 yield (mL/g organic matter (OM)) in a large data set with very diverse samples. The effect of WSC in increasing CH_4 has also been shown by Ellis et al. (2012) where they used high sugar grasses to investigate the effect of WSC on simulated CH_4 emissions (MJ/d and % of gross energy intake) in cattle using modelling. Based on the results of study I and findings in literature, it was interesting to investigate the effect of wilting on *in vitro* CH_4 production in a controlled field study (Study II). More precisely we wanted to look at fermentation intensity and the role of WSC and lactic acid in affecting CH_4 production. However, in study II we did not find any effect ($P = 0.235$) of wilting level on *in vitro* CH_4 production (mL/g OM), and concentration of WSC only tended ($r = 0.22$, $P < 0.1$) to increase CH_4 production (mL/g OM). Based on the present *in vitro* results and previous studies it seems that the role of WSC in affecting CH_4 production warrants further investigation using *in vivo* techniques. It is also well known that increased concentration of WSC in the silage increase rumen microbial protein synthesis (Jaakola et al., 2006) which might have a positive effect on milk production, thereby reducing CH_4 intensity of the diet.

In study II concentration of WSC was 54 g/kg DM greater in silage preserved with a formic acid additive compared to silage without additive (91 vs. 38 g/kg DM, $P < 0.001$). The effect of additive on silage WSC concentration tended to be stronger (DM by additive interaction, $P = 0.082$) at 22.5% DM compared to 37.5% DM. Use of formic acid in silage production results in an immediate reduction in pH due to the acidification which restricts fermentation of WSC (Saarisalo et al., 2006; Conaghan et al., 2011). Concentration of lactic acid was 21 g/kg DM lower in silage preserved with additive compared to silage preserved without additive (23 vs. 44 g/kg DM respectively, $P < 0.001$) and the effect of additive on silage lactic acid content was stronger on silage made from less wilted herbage then the more wilted silage (DM by additive interaction, $P < 0.001$). The silage prepared with additive had 1.2 mL/g OM (33.6 vs. 32.4 mL/g OM respectively, $P < 0.01$) and 1.6 mL/g DOM greater (46.6 vs 45.0 mL/g DOM respectively, $P < 0.01$) *in vitro* CH_4 production than silage prepared without additive. In silage prepared without additive the readily available WSC in silage is fermented to lactic acid which is metabolized to propionate in the rumen fluid (Huhtanen et al., 2013). The present results are in line with a previous *in vitro* study (Navarro-Villa et al.,

2012) where they demonstrated that the microbial production of propionate consumes H_2 which lower the *in vitro* CH_4 production in the rumen fluid. The metabolism of lactic acid to rumen fluid propionate have also been demonstrated *in vivo* (Counotte et al., 1981; Newbold et al., 1987). The silage prepared with additive had residues of formic acid (12.3 g/kg DM in silage with 22.5% DM and 5.22 g/kg DM in silage with 37.5% DM) which may have increased CH_4 production as shown in other *in vitro* studies where formic acid or formate was added (Kara et al., 2018; He et al., 2019).

The effect of silage additive on CH_4 production per gram OM was dependent upon cut and wilting level, as shown in the three-way interaction ($P = 0.041$) between harvest regime, wilting level and additive (**Figure 10**). The interaction plot shows that for the three-cut system the use of additive generally increased CH_4 production, while for the two-cut system the second cut of silage prepared at 22.5% DM the CH_4 production was reduced by inclusion of additive. The molar concentration of acetate was greater ($P < 0.001$) and the molar proportion of propionate was lower ($P = 0.02$) in silage made from three cut system with additive than two cut system without additive which contributed to a greater CH_4 production. In addition, the residual formic acid in the silage may have increased CH_4 production in silage prepared with additive.

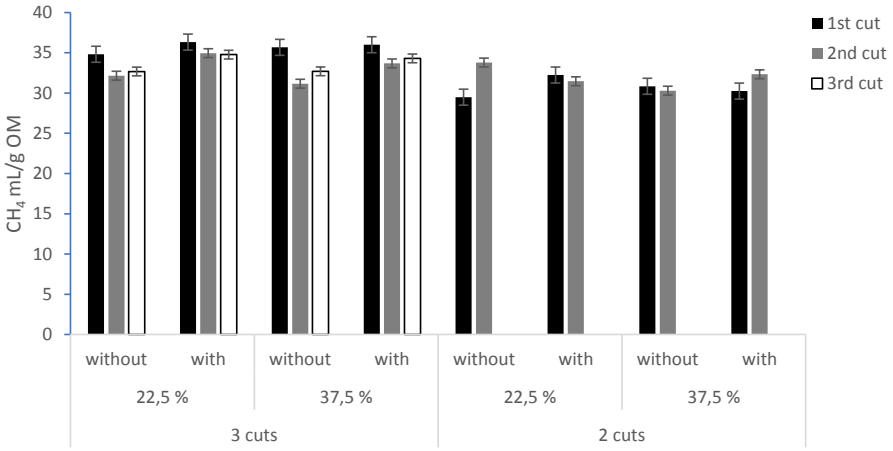

Figure 10. Three-way interaction between harvest regime (two or three cuts per season), wilting levels (22.5% DM or 37.5% DM) and use of additive (with or without

GrasAAT Lacto, formic acid-based additive) on *in vitro* CH_4 production (mL/g OM). Bars represent standard error of the mean (n = 3). Modified figure from Weiby et al. (2023).

4.4 Strengths, limitations and practical implementations

The research presented in this thesis are novel studies providing knowledge and insight into strategies for silage production and enteric CH_4 mitigation. The results may be used by the agricultural extension service to provide new recommendations and advice for farmers. It is imperative for the farming community to get insight into the environmental footprint of agricultural practices, to meet their future obligations in GHG reductions.

Strengths

One important strength in this thesis is that all the studies are sequentially building on each other gaining more knowledge going from study I to study II and lastly to study III. One important strength in study I was the diversity of the chemical composition and the locations from where the samples were collected. In addition, it was an advantage that we performed an *in vivo* total collection trial as these are more robust in determining digestibility compared to using only *in vitro* dry matter disappearance. One of the advantages in study II using a field trial with a split plot design is that we could control the management factors, harvest frequency, species mixture, wilting and use of additives, in the same trial. I would also like to highlight the use of cuts mixed proportionally according to their dry matter yields in study III, which I think is a strength in that study as this is more related to the practical use on farms today. Use of a mix between spring growth silage and regrowth silage in cattle feeding has become more common as farmers use bunker silos and mixer wagons. In a bunker silo the different cuts are placed in horizontal layers in the bunker silo. When extracting the silage from the bunker silo, it is removed in vertical cuts giving a mix of both spring growth and regrowth. When using a mixer wagon it is common to mix e.g. bales from different harvests, often a mix between spring growth and regrowth silage. However, there are few studies investigating mixtures of spring and regrowth silages, although regrowth silages contribute to a large proportion of the silages used in cattle feeding today. Most research are performed using only spring growth or comparing spring growth to first or second regrowth silages. Regrowth silages differ from spring growth silages in chemical composition, fibre digestibility and leaf to stem ratio, and it was important to establish the effect of regrowth silages on CH_4 emissions.

Limitations

Although this thesis and the studies herein present valuable results, I would like to address some perspectives into the limitations of the different studies and thus the thesis. One important limitation in study I is that we do not have information about grassland species, use of silage additives, harvest frequency or any other information about the ley (when the ley was sown, share of different species, weeds etc.) or the exact position of the field (latitude, longitude, meters above sea level etc.). This information was available only for the farm building. Therefore, these factors were excluded as explanation variables in the dataset. Information about grassland species, silage additive, location of the field etc. may have had an impact on the nutritional composition, fermentation quality and consequently on the CH_4 emissions of the silages.

In vitro methods have a high capacity, reduce the number of animals used, are quite cheap, and have proven useful for screening large sets of samples. However, the review article by Yáñez-Ruiz et al. (2016) concluded that *in vitro* studies often overestimate the CH_4 inhibiting properties of additives compared to *in vivo* studies and that donor animals, diet, inoculum collection, substrate, incubation buffer and the procedures used may influence the end result. In addition, the *in vitro* method differs from *in vivo* methods as no absorption takes place in the *in vitro* system which makes the conditions different from continuous systems (e.g. rumen simulation techniques) or *in vivo* methods (e.g. the manually operated closed chamber technique). Overall, there is a risk that the determination of CH_4 production becomes less accurate compared to using *in vivo* techniques like the GF system, chamber technique or tracer methods. In study II, the limitations were confirmed as *in vitro* CH_4 production in T3 was 7.5% greater compared to T2 (31.5 vs 29.3 mL/g DM, $P < 0.001$) while in study III *in vivo* CH_4 production was only 1.5% greater in T3 compared to T2 (476 vs. 469 g/d, $P = 0.46$). Although results are not entirely comparable, it is possible that the *in vitro* CH_4 emissions are overestimated, as suggested by Yáñez-Ruiz et al. (2016). Another limitation with the *in vitro* technique compared to *in vivo* techniques, is that when rumen fluid is removed from the rumen environment, there is a risk that microbes might reduce their activity due to changes in environment (exposure to air, temperature changes, lowering of pH etc.) (Yáñez-Ruiz, 2016). It is also a limitation that we do not have any data on rumen fluid parameters, SCFA proportions or any information on the ruminal microbial community in study III. Having those data would have given us the opportunity to explain the changes in

CH_4 emissions based on the proportion of propionate: [acetate+butyrate] and the availability of H^+ in the rumen fluid.

Practical implementation

In this thesis I wanted to investigate if the CH_4 production and intensity reported in study III was different from what can be predicted using 'TINE Optifôr' based on the models by Nielsen et al. (2013). The feed optimizing system 'TINE Optifôr' are used for balancing feed rations for dairy cows in Norway. The system communicates with the Norwegian Dairy Herd Recording System (NDHRS, TINE SA) where detailed information about each individual cow is registered. In the feed analysis system (FAS) all feed analysis from commercial laboratories (e.g. Eurofins, Ofotlab) are available for farmers. Lastly, all producers of concentrate feeds (e.g. Felleskjøpet Agri, Felleskjøpet Rogaland Agder, Fiskå, Norgesfôr) upload information about chemical composition in each specific concentrate. This enables the extension service and farmers to make detailed feed plans for each individual cow in a herd.

It is possible to predict CH_4 emissions from the feed rations based on basic empirical models in 'TINE Optifôr' (Nielsen et al., 2013):

Model: $CH_4 = 1.23\ (\pm 0.08) \times \mathbf{DMI} - 0.145\ (\pm 0.039) \times \mathbf{FA} + 0.012\ (\pm 0.005) \times \mathbf{NDF}$, $R^2 = 0.75$

The model used was developed from 47 treatment means using 12 different dairy cow experiments. Experiments from Denmark (6), Sweden (3) and Norway (3) were included and provided data on DMI, total fatty acids and NDF concentration in the diet (Nielsen et al., 2013). Silage was based mainly on grass and maize silage, but also one alfalfa silage and one pea-oat silage. Concentrates covered different levels and sources of fat, carbohydrates and CP. Methane was measured using the chamber method and SF_6 method.

In this thesis I have used the chemical composition and DMI of the five experimental silages in Study III, chemical composition and DMI of concentrate, and milk production to calculate the CH_4 production using 'TINE Optifôr' and the model by Nielsen et al. (2013).

Methane production averaged over all treatments were only 3.7% greater (501 vs 483 g/d respectively, **Table 3**) using the prediction in 'TINE Optifôr' compared to the observed data from Study III. Methane production was greater in all treatments using the prediction model, except for diet RC3 where the observed CH_4 production was greatest. As the models

used in 'TINE Optifôr' is based mainly on experiments using a mixture of different grass and legume species, it is probably not well suited to encompass pure species silages, and especially not pure RC. As discussed previously in this thesis, silage made of RC usually have a lower NDF concentration compared to grass silages. The low NDF concentration may have reduced the ability of the equation to precisely predict the CH_4 production and intensity in this experimental diet. It was the T2 diet that had the largest deviation between the predicted and the observed CH_4 production (43 g/d respectively). This is probably because DMI was the explanatory variable with the greatest R^2 (0.66), meaning that this was an important explanatory variable in the model by Nielsen et al. (2013). However, in study 3 we found that DMI was surprisingly high in the T2 diet, and not different from the T3 diet.

Table 3. Methane (CH_4) production and intensity from 'TINE Optifôr' and Study III

Item[1]	CH_4, g per day		CH_4, g per kg ECM	
	TINE Optifôr	Study III	TINE Optifôr	Study III
T3	503	476	17.3	16.5
T2	512	469	18.8	17.7
RG	481	466	17.4	17.4
T3/RC3	533	510	17.6	17.5
RC3	474	495	17.1	18.3
Average	**501**	**483**	**17.6**	**17.5**
SEM	**21.3**	**16.8**	**0.60**	**0.58**

[1]T3 = Timothy 3 cut system, T2 = Timothy 2 cut system, RG = Perennial ryegrass 3 cut system, T3/RC3 = Timothy 3 cut system/red clover 3 cut system, RC3 = Red clover 3 cut system

It is reassuring that the model in TINE Optifôr can predict CH_4 production and intensity with only minor differences to what we observed in study III. It is possible to include the results from study III in the model, to improve the prediction accuracy for different grass and legume species and harvest regimes.

The results from this project will be included in the revision of the agreement between the government and the farmers union in reducing GHG from the agricultural sector ("Landbrukets Klimaplan"). The present results are important as they provide estimates of which emission reductions that can be expected when implementing mitigation strategies in grass silage production in Norway.

5. Conclusions and future perspectives

The main goal for this PhD project was to develop strategies for silage production to mitigate enteric CH_4 emissions from ruminants. This is crucially important for a more sustainable future in ruminant production systems.

In conclusion, our results from study I showed that greater WSC and OMD, and lower NDFom and iNDF concentrations in grass silages are associated with greater *in vitro* CH_4 yield. We also found that regression models can be used to predict CH_4 yield as mL/g OM with a coefficient of determination (R^2) of 0.65 using aNDFom, WSC, iNDF, propionic acid, and pH as explanatory variables. Study II showed that less frequent harvesting and extensive silage fermentation reduce *in vitro* CH_4 production. The effect of harvest frequency was mainly due to increased aNDFom and iNDF concentration and reduced OMD in the two-cut system compared to the three-cut system. The effect of extensive silage fermentation was due to increased concentration of lactic acid increasing the rumen fluid molar proportion of propionate and hence reducing the CH_4 production. We also speculate that residual formic acid increased CH_4 production in silage prepared with formic acid additive. We found that CH_4 production was lower in timothy than in red clover, probably due to differences in total substrate availability for the methanogens. In study III we found that changing harvest frequency for timothy from two to three harvests per season did not affect CH_4 production or yield, but CH_4 intensity was reduced. Replacing T3 with RG and increased inclusion rate of red clover both increased CH_4 yield and intensity.

Future research should aim to elucidate the *in vivo* effect of formic acid additive on enteric CH_4 emissions in ruminants fed grass and legume silages wilted to different DM levels. It is possible that increased lactic acid in the silage when not using additive increase the propionate production in the rumen, reducing H^+ availability and CH_4 production. Study II confirmed that silage prepared with formic acid additive increased *in vitro* CH_4 production by 3.7% compared to those silages prepared without formic acid. We expect that using silage additive increase DMI and ECM yield compared to not using additive, which should contribute to reduced CH_4 yield and intensity. However, it is uncertain if this positive effect "overshadows" the increased CH_4 production observed *in vitro* in study II.

Although the present study clearly shows reduced CH_4 yield and intensity with increased harvest frequency (3 vs 2 cuts per season), it is possible that the increased use of commercial fertilizer, diesel, plastic, and more frequent grassland renewal due to more

intensive production systems might increase CO_2 and N_2O emissions and thereby offset the beneficial CH_4 reductions. It is also possible that the return of investments is too low to justify the proposed changes from two to three cuts. These research questions are part of another work package in the "Klimagrovfôr project" and will hopefully be answered in future research.

The results of this thesis are important to enable farmers and the dairy and meat industry to meet their obligations in reducing CH_4 emissions and thereby fulfil agreements with policymakers and governments. In addition, reducing CH_4 emissions through improved silage quality helps to improve the use of national feed resources, self-sufficiency and thereby reduce dependency on imported feed.

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Associations among nutrient concentration, silage fermentation products, *in vivo* organic matter digestibility, rumen fermentation and *in vitro* methane yield in 78 grass silages

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ABSTRACT

Grass-clover silage constitutes a large part of ruminant diets in Northern and Western Europe, but the impact of silage quality on methane (CH_4) production is largely unknown. This study was conducted to identify the quality attributes of grass silage associated with variation in CH_4 yield. We expected that silage nutrient concentrations and silage fermentation products would affect CH_4 yield, and that these factors could be used to predict the methanogenic potential of the silages. Round bales (n = 78) of grass and grass-clover silage from 37 farms in Norway were sampled, incubated, and screened for *in vitro* CH_4 yield, *i.e.* CH_4 production expressed on the basis of incubated organic matter (CH_4-OM) and digestible OM (CH_4-dOM) using sheep. Concentration of indigestible neutral detergent fiber (iNDF) was quantified using the *in situ* technique. The data were subjected to correlation and principal component analyses. Stepwise multiple regression was used to model methanogenic potential of silages. Among all investigated silage composition variables, neutral detergent fiber (aNDFom) and water-soluble carbohydrate (WSC) concentrations obtained the greatest correlations to CH_4-OM ($r = -0.63$ and $r = 0.57$, respectively, $P < 0.001$), while concentration of iNDF negatively correlated with CH_4-OM ($r = -0.48$, $P < 0.001$). *In vivo* organic matter digestibility (OMD) and concentration of ammonia-N (NH_3-N) in silages were also correlated to CH_4-OM ($r = 0.44$ and $r = -0.32$, $P < 0.001$ and $P < 0.01$, respectively). The stepwise regression using CH_4-OM as response variable included aNDFom, WSC, iNDF, silage propionic acid and pH in descending order. The stepwise regression using CH_4-dOM as response

Abbreviations: AIC, Akaike Information Criterion; aNDFom, ash corrected neutral detergent fiber; CH_4, methane; CO_2, carbon dioxide; CV, coefficient of variation; DM, dry matter; dNDF, digestible aNDFom; ECM, energy corrected milk; H_2, hydrogen; iNDF, indigestible neutral detergent fiber; NH_3-N, ammonia-nitrogen; NMBU, Norwegian University of Life Science; OM, organic matter; OMD, organic matter digestibility; PC, principal component; PCA, principal component analysis; R^2, coefficient of determination; SCFA, short-chain fatty acids; WSC, water soluble carbohydrates.

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variable included WSC, aNDFom and iNDF in descending order. Among *in vitro* rumen short chain fatty acids (SCFA), molar proportion of butyrate was the most prominent in increasing CH_4-OM and CH_4-dOM ($r = 0.23$ and $r = 0.36$, $P < 0.05$ and $P < 0.01$, respectively), while molar proportion of propionate was the most prominent SCFA in reducing CH_4-OM and CH_4-dOM ($r = -0.23$ and $r = -0.26$, respectively, $P < 0.05$). Regression models that account for silage quality attributes can be used to predict CH_4 yield from silages with a coefficient of determination (R^2) between 0.33 (CH_4-dOM) and 0.65 (CH_4-OM). In conclusion, concentration of WSC increased *in vitro* CH_4-OM and CH_4-dOM, while concentration of aNDFom and iNDF decreased CH_4-OM and CH_4-dOM in grass silages.

1. Introduction

Grass and grass-clover silage are predominant forages in Northern and Western Europe and hence constitute a large part of ruminant diets. In Norway, multispecies swards based on perennial grasses such as timothy (*Phleum pratense L.*) and meadow fescue (*Festuca pratensis Huds.*) combined with the legume red clover (*Trifolium pratense L.*), are the most common species due to their agronomic suitability for the climatic conditions (Steinshamn et al., 2016). Grass silages show large variations in feed quality, intake and performance in cattle because of differences in botanical composition (Thomas et al., 1981), stage of maturity (Steen, 1984) and ensiling quality (Krizsan and Randby, 2007).

The emission of greenhouse gases from the global agricultural sector has received increased attention over the last decade and is estimated at 5.2–5.8 Gt carbon dioxide (CO_2) equivalents per year in 2010, or 10–12% of global anthropogenic emissions. Between 1.9 and 2.1 Gt CO_2 equivalents of the total agricultural greenhouse gas emissions arises from enteric methane (CH_4) emissions predominantly from ruminants (IPCC, 2014). The methanogens play a vital role in the rumen ecosystem by converting excess hydrogen (H_2) and CO_2 into CH_4, which allows microbial fermentation of nutrients to short-chain fatty acids (SCFA) to function optimally (Hook et al., 2010).

Fibrous plant material such as grass silage is an important source of fermentable carbohydrates for ruminants, and in this process, methanogens produce enteric CH_4. *In vitro* studies (Holtshausen et al., 2012) have shown increased CH_4 yield (mL/g dry matter (DM) disappeared, mL/g neutral detergent fiber (aNDFom) disappeared) in silages cut at early compared to late stage of maturity. Regrowth grass has greater proportion of vegetative material compared to primary growth grass (Kuoppala et al., 2010), but also greater concentration of indigestible aNDFom (iNDF). As a result, cows fed primary growth grass silages had greater feed intake and milk production compared to cows fed silages made from regrowth grass (Kuoppala et al., 2008). Therefore, enteric CH_4 emissions (per unit of DM intake or milk production) are usually lower in cows fed silages cut at an early, compared to late stage of maturity (Brask et al., 2013; Warner et al., 2016, 2017).

Manipulation of SCFA production is an effective strategy to reduce CH_4 production. The stoichiometric ratio between different SCFA and enteric CH_4 emissions depends upon feed chemical composition, DM intake and digestibility of the diet (Johnson et al., 1995; Hristov et al., 2013). It is well established that there is a negative correlation between the amount of CH_4 produced in the rumen and the ratio of propionate:[acetate+butyrate] (Janssen, 2010), because production of acetate and butyrate generates H_2, which increases CH_4 production in the rumen. The production of propionate on the other hand, consumes H_2, thereby decreasing CH_4 production (Boadi et al., 2004). According to Janssen (2010) the ruminal fermentation of aNDFom in feed gives less propionate than the non-aNDFom fraction [mainly protein, starch and water-soluble carbohydrates (WSC)]. Jentsch et al. (2007) reported that the CH_4 production rate from the digestible fiber fraction was 2.6-fold greater than that from digestible crude protein and digestible nitrogen free extracts, respectively. However, results are inconsistent. Ellis et al. (2012) found that feeding ryegrass with increased concentration of WSC increased CH_4 production (MJ/day), although results were more variable when CH_4 was expressed per kg milk or per kg DM intake. On the other hand, harvesting at an early phenological plant stage increases the ruminal degradation of aNDFom (Rinne et al., 2002; Kuoppala et al., 2008, 2010; Randby et al., 2012), which may increase the proportion of propionate in the fermentation end products (Janssen, 2010). The WSC in the harvested forage is subjected to fermentation during ensiling, with lactic acid as the major fermentation end-product in well preserved silage. Lactic acid is further fermented to propionate in the rumen (Huhtanen et al., 2013). Therefore, it is likely that silages with high concentrations of lactic acid yield less enteric CH_4 than restricted fermented silages.

Early maturity silage with a more rapidly fermentable aNDFom fraction, and a greater non-aNDFom fraction compared to late maturity silage, may change SCFA proportion from acetate towards propionate and reduce CH_4 production. However, the results from experiments with cattle studying the effect of grass silage maturity on the propionate:[acetate+butyrate] ratio in the rumen have been inconsistent (Kuoppala et al., 2010; Warner et al., 2016). It appears that not only stage of maturity at harvest, but also silage fermentation characteristics may affect ruminal SCFA, and the complexity of these interacting factors may contribute to the lack of consistency.

The aim of this study was to identify the most important feed quality parameters and silage fermentation products of diverse grass silages with respect to variation in CH_4 production determined using the *in vitro* method. We expected that the diverse concentrations of nutrients and silage fermentation products would affect *in vitro* CH_4 yield, and that these factors could be used to develop a regional *in vitro* prediction equation for CH_4 yield, measured as CH_4 production *in vitro* expressed relative to OM (CH_4-OM) of the silage incubated and digestible OM *in vivo* (CH_4-dOM).

2. Materials and methods

The study used *in vitro*, *in situ* and *in vivo* techniques. Grass silage samples were screened for CH_4 production using the batch culture technique (Ramin and Huhtanen, 2012). *In vivo* organic matter digestibility (OMD) and *in situ* digestible aNDFom were measured using the methods described by Åkerlind et al. (2011) and concentration of indigestible aNDFom was determined *in situ* (NorFor 2011; Krizsan et al., 2015). The *in vitro* experiment was performed at the Swedish University of Agricultural Sciences, Umeå, Sweden. The handling of animals was approved by the Swedish Ethics Committee on Animal Research (Dnr A 32–16), represented by the Court of Appeal for Northern Norrland, Umeå, and the experiment was carried out in accordance with laws and regulations governing experiments performed with live animals in Sweden. The *in situ* and *in vivo* studies were conducted at the Metabolism Unit of the Norwegian University of Life Sciences (NMBU) in Norway. The experiments were approved by the Norwegian Ethical Committee on Animal Research. These experiments were done in accordance with regulations controlling live animal experiments in Norway.

2.1. Selection and sampling of grass silages

In total 78 round bales of grass and grass-clover silages (referred to herein as grass silage) from 37 farms (Supplementary Fig. S1) were sampled from 58°32'39″ N, 5°41'08″ E in the south of Norway to 69°13'21″ N, 19°14'17″ E in the north of Norway, with the farms positioned from 5 to 530 m above sea level. The silage bales were made in 2016 and 2017, and the harvest window was 71 days for the first cut, 70 days for the second cut and 30 days for the third cut (Table 1).

The silage bales were selected using the feed analysis system database (Volden, 2011), which contains results of feed analysis (near infrared reflectance spectroscopy and wet chemistry) for Norwegian farms. The bales were selected to obtain substantial variation in DM, aNDFom, crude protein, WSC concentration and digestibility. In addition, the round bales collected represented a variety in botanical composition typical of grass silages in Norway, *i.e.* mixtures of timothy (*Phleum pratense L.*), meadow fescue (*Festuca pratensis Huds.*), red clover (*Trifolium pratense L.*), and perennial ryegrass (*Lolium perenne L.*). To obtain a large variation in the dataset, grass silages of pure ryegrass, pure timothy or timothy with a large inclusion of red clover were also selected. The selection of round bales represented the use of different types of silage additives, including additives that stimulated or restricted fermentation, as well as grass silage bales without silage additives.

The bales were transported to the Metabolism unit at NMBU in Ås, where each bale was opened and homogenized for approximately 15 min in a mixer wagon (Siloking, Kverneland Duo 1814, 18 m^3, 84529 Tittmoning, Germany). Each bale was then sampled and retained for use in the study.

2.2. In vitro incubation of grass silage samples

The silage samples were dried at 59 °C for 48 h. Samples were ground to pass a 1 mm screen using a Retch cutting mill with trapezoid sieve holes (Retsch, SM2000, Rheinische, Haan, Germany). Dried and ground samples of 1.00 ± 0.003 g of all grass silage bales were weighed into 250 mL serum bottles (Schott, Mainz, Germany). Rumen fluid was collected 2 h after morning feeding from two rumen-cannulated Swedish Red cows fed *ad libitum* a diet consisting of grass silage and concentrate (60:40 on DM basis). The rumen fluid was filtered through two layers of cheesecloth into pre-warmed (39 °C) and CO_2 flushed thermos bottles directly after extraction from the rumen of each cow. Equal amounts from each cow were blended, strained through four layers of cheesecloth, and added to a buffered mineral solution (Menke and Steingass, 1988) including Peptone™ (pancreatic digested casein; Merck, Darmstadt, Germany) at 39 °C under constant mixing and CO_2 flushing, to give a buffered rumen fluid solution with a rumen fluid:buffer ratio of 1:4 by volume (Ramin and Huhtanen, 2012). Then, 60 mL of buffered rumen fluid was added to each bottle and the bottles were directly placed in a water bath at 39 °C under constant agitation. Gas production was measured every 12 min using a fully automated *in vitro* gas system (Gas Production Recorder, GPR-2, Version 1.0 2015, Wageningen UR). The amount of headspace gas released from the system through automated valve openings was recorded, and all readings were corrected to normal air pressure (101.3 kPa) (Cone et al., 1996). Gas samples were taken after 24 h of incubation from the headspace of each bottle using a gas tight syringe (Hamilton, Bondaduz, Switzerland). Additionally, a 1.5-mL sample of liquid was collected from each bottle at the termination of the 24 h incubation and immediately frozen at −20 °C. These procedures were repeated for eight runs in total and all samples were incubated with triplicates of each sample (n = 3 runs/silage). All runs included 36 bottles. In each run, 33 bottles contained forage samples and three bottles contained blanks (*i.e.*, bottles with 60 mL of buffered rumen fluid with no sample included). The 78 silage samples (in triplicate) were randomly allocated to the 8 *in vitro* runs, with the same sample never incubated more than once within a run and never in the same bottle.

Table 1
Description of the grass silage samples and farms.

	Average	Minimum	Maximum
Harvest date 1st cut (n = 38)	June 22nd	May 24th	July 31st
Harvest date 2nd cut (n = 32)	August 13th	July 15th	September 23rd
Harvest date 3rd cut (n = 8)	September 5th	August 20th	September 19th
Farm position (latitude, longitude)	62°06' N, 10°29' E	58°32' N, 5°41' E	69°13' N, 19°14' E
Farm topography (meters above sea level)	147	5	530

2.3. In situ and in vivo studies

Concentration of iNDF was determined as proportion of NDF remaining in the residue after *in situ* incubation according to the Norfor standard procedure (Åkerlind et al., 2011). The samples were freeze-dried and ground to pass a 1 mm screen using a Retsch cutting mill with trapezoid sieve holes (Retsch, SM200, Rheinische, Haan, Germany). Feed samples of 2 g were added to bags (Sefar Petex 07–11/5-cloth, Sefar AG, Heiden, Switzerland) and intraruminally incubated 288 h according to recommendations of Krizsan et al. (2015). The *in situ* study was conducted using 2 ruminally cannulated Norwegian Red cows fed forage and concentrate (67:33 on DM basis) to meet maintenance energy requirement of the animals. Five bags were incubated into the rumen of each cow, and each sample were incubated into two rumen cannulated cows (e.g 10 bags per sample). *In vivo* apparent OMD of the 78 grass silages was determined according to Åkerlind et al. (2011) using three adult castrated male sheep per grass silage sample. The *in vivo* study was conducted in 23 runs from May 2017 to December 2019, where 3–5 round bales were tested in each run. The adaptation period was 11 days and each round bale was fed for 21 days. The total collection of faeces was conducted over a period of 10 days, and proportional subsamples of faeces were taken daily, pooled per individual animal and then across animals fed the same test bale, and stored frozen until analysis. Sheep that weighed less than 88 kg daily received 1.0 kg DM of grass silage, and sheep weighing above 88 kg daily received 1.2 kg DM of grass silage. All sheep daily received 10 g of sodium chloride (GC-Rieber, Cort Adelers gate 17, 0254 Oslo) and 35 g of a commercial mixture of vitamins and minerals (VitaMineral Normal Sau, Vilomix, Hensmoveien 30, 3516 Hønefoss, Norway).

2.4. Laboratory analyses

Fresh feed samples for analyses of fermentation parameters and *in vivo* OMD were collected and frozen at −20 °C. Feed and faecal samples were oven-dried at 59 °C for > 48 h and ground to pass a 1-mm screen using a Retsch cutting mill with trapezoid sieve holes (Retsch, SM200, Rheinische, Haan, Germany) prior to chemical analysis of feed and faeces samples and *in vitro* incubation of feed samples.

The DM content of the pre-dried samples was determined by further oven-drying for 16 h at 105°C and ash was determined at 550 °C for a minimum of 4 h. The aNDFom concentration was determined with the Ankom^{200} Fiber Analyzer (Ankom Technology, Macedon NY 14502, USA) using sodium sulfite, heat-stable α-amylase, with ash correction (AOAC, 1995; method 2002.04). Total nitrogen was analyzed on a Kjeltec™ 8400 (Foss, Hillerød, Denmark) using 95% sulfuric acid and a Cu-catalyst (AOAC method 968.06). Crude fat was analyzed using an ASE® 350 Accelerated Solvent Extractor (Nerliens Mezanski, Oslo, Norway). For determination of WSC, carbohydrates were extracted in 0.05 M Na-acetate buffer. Sucrose and fructans were hydrolyzed with 0.074 M H_2SO_4 in 90 °C for 70 min. Monosaccharides were further converted to glucose-6-phosphate and fructose-6-phospate by an enzymatic method using a kit (K-FRUGL, Megazyme, Wicklow, Ireland). The concentrations were determined spectrophotometrically by the increase in absorbance of NADPH at 340 nm. Fresh samples of the bales were analyzed for NH_3-N, pH, organic acids and ethanol as described by Randby et al. (2010). Oven DM concentrations of the grass silages were corrected for volatile losses according to the NorFor DM determination method (Åkerlind et al., 2011). Faeces were analyzed for concentrations of DM, ash and aNDFom for calculation of OMD and aNDFom digestibility (dNDF).

The CH_4 concentration in gas samples taken from the headspace of each *in vitro* bottle after 24 h of incubation was measured according to Ramin and Huhtanen (2012) by injecting 0.2 mL of gas into a Varian Star 3400 CX gas chromatograph (Varian Analytical Instruments, Walnut Creek, California, USA) equipped with a thermal conductivity detector. Gases were separated using a 1.8 m long stainless-steel column packed with Haysept T (80–100 mesh) and argon as a carrier gas. The flow rate was 32 mL/min and oven temperature was 32 °C. Injector and detector temperatures were set to 110 °C and 135 °C, respectively. For calibration of the gas chromatograph, a mixture of CO_2 and CH_4 (100 mmol CO_2/mol CH_4) was used (Aga Gas AB, Sundbyberg, Sweden). Peaks were identified by comparison with the calibration gas. Samples of liquid from *in vitro* batch culture were thawed and analyzed for concentrations of SCFA and NH_3-N. Concentrations of SCFA in the liquid samples were analysed using a Waters Alliance 2795 UPLC system (Waters, Milford, Massachusetts, USA) equipped with an ultraviolet detector as described by Puhakka et al. (2016). Concentrations of NH_3-N was determined using a method provided by Seal Analytical (Method no. G-102–93 multitest MT7) using an Autoanalyzer 3 (SEAL Analytical Ltd., Mequon, Wisconsin, USA).

2.5. Calculations

In vivo OMD was calculated as: (OM consumed (g) - OM excreted in faeces (g))/OM consumed (g). The three observations per bale were averaged before statistical analysis. In situ dNDF (g/kg aNDFom) was calculated as: (aNDFom (g/kg DM) – iNDF (g/kg DM)) * 1000 /aNDFom (g/kg DM). The molar proportions of individual SCFA were calculated related to total SCFA. Total *in vitro* SCFA production was calculated according to the following equation:

Total SCFA (mmol/L) = ($\sum$ individual SCFA concentration (mmol/L) – mean of blank SCFA (mmol/L)) × 0.06 L (*i.e.*, fraction of buffered rumen fluid).

Total gas production was calculated by subtracting mean blank gas production from sample gas production. Methane production was predicted from CH_4 concentration and total gas production measured *in vitro* as described by Ramin and Huhtanen (2012) using a dynamic, mechanistic two-compartment rumen model:

$CH_4 = 265 \times CH_4$ concentration + total gas production × CH_4 concentration × 0.55,

where CH_4 is in mL, 265 is the total headspace volume (mL), CH_4 concentration is in %, total gas production is in mL and 0.55 is the ratio of CH_4 concentration in outflow gas to headspace volume. A mean retention time of 50 h (20 h in the first compartment and 30 h

in the second compartment) corresponding to the maintenance level of feed intake was used in model simulations.

The CH_4 production (mL) was converted to CH_4 yield on the basis of OM of the silage incubated and digestible OM (dOM), respectively:

CH_4-OM (mL/g OM) = CH_4 (mL) / OM (g) and

CH_4-dOM (mL/g dOM) = CH_4 (mL/kg OM) / *in vivo* dOM (g/kg OM).

2.6. Statistical analyses

Data for CH_4 yield (mL/g DM) were subjected to analysis of variance using the MIXED procedure in SAS version 9.4 (SAS Institute Inc., Cary, NC) according to the model:

$Y_{ijk} = \mu + T_i + R_j + B_k + E_{ijk}$, where Y_{ijk} is the dependent variable, μ is the overall mean, T_i is the fixed effect of grass silage ($i = 78$), R_j is the fixed effect of run ($j = 8$), B_k is the random effect of bottle ($k = 36$), and E_{ijk} represents the random residual error. Run was considered as fixed effect because the run effect is standardized regarding system, rumen fluid, diet and cows. Bottles were considered as random effect because the precalibration of each bottle revealed differences in the gas volume leaving each bottle upon opening of the valve and therefore bottles were randomized between each run. Differences were considered statistically significant when $P < 0.05$, and trends were apparent when $0.05 \leq P < 0.10$.

The statistical correlation analysis for grass silage parameters and rumen fermentation variables was performed using the statistical software R (R Core Team, 2020). Pearson correlation coefficients were calculated to determine relationships between the individual grass silage or rumen fermentation variables and CH_4-OM or CH_4-dOM. A similar approach was used to determine correlations between CH_4-dOM and grass silage variables within different cuts. Principal component analysis (PCA) was performed using the procedure prcomp in R (scale=TRUE), and grass silage variables from the correlation analysis that were significant or tended to be significant ($P < 0.1$) were included in the analysis, as well as crude protein and crude fat because of their great relevance in cattle nutrition and the potential mitigating effect of crude fat on CH_4 yield.

To determine whether CH_4-OM and CH_4-dOM could be predicted from grass silage variables, a forward stepwise multiple regression approach was performed using the stepwise procedure in R (direction=forward). Akaike Information Criterion (AIC) was used as a selection criterion, and new variables were included in the model if AIC was reduced after inclusion. Although it was of great interest to obtain a large variety in botanical composition of the silage round bales, the collected data were incomplete and botanical composition was therefore excluded as a variable in the dataset.

3. Results

3.1. Chemical composition of the grass silages

There was substantial variation in the DM concentration, nutritive value, silage fermentation products and *in vitro* CH_4 yield of the grass silages as intended (Table 2). The silage fermentation products were among the traits with greatest coefficient of variation (CV) (butyric acid> formic acid> propionic acid> acetic acid> ethanol> lactic acid). Concentration of WSC also obtained a large CV, with the lowest WSC concentration being almost zero. Concentration of iNDF varied with a CV of about 30%, and the CV of aNDFom, CH_4-dOM and CH_4-OM were smaller with about 10%.

Table 2
Chemical composition, *in vivo* digestibility and *in vitro* methane yield of the 78 grass silage round bales collected from farms in Norway.

Trait	Mean	Minimum	Maximum	SD	CV (%)
Dry matter (g/kg wet weight)	372	179	705	123	32.9
Organic matter (g/kg DM)	925	856	960	18.1	1.96
Neutral detergent fiber (g/kg DM)	537	408	665	57.9	10.8
In situ indigestible aNDFom (g/kg aNDFom)	198	109	422	57.5	29.0
In situ digestible aNDFom (g/kg aNDFom)	802	578	891	57.5	7.17
Crude protein (g/kg DM)	139	77.2	230	31.3	22.5
Crude fat (g/kg DM)	25.2	13.7	46.2	5.88	23.3
Water soluble carbohydrates (g/kg DM)	42.6	0.32	137	36.8	86.3
Lactic acid (g/kg DM)	31.9	2.00	101	22.5	70.7
Acetic acid (g/kg DM)	8.41	2.00	40.0	7.08	84.2
Propionic acid (g/kg DM)	0.47	0.10	2.50	0.44	93.7
Butyric acid (g/kg DM)	0.92	0.01	12.6	2.29	248
Formic acid (g/kg DM)	2.49	0.00	14.0	3.49	140
pH	4.58	3.90	5.90	0.44	9.61
Ethanol (g/kg DM)	7.98	0.50	36.9	6.46	81.0
Ammonia-nitrogen (g/kg nitrogen)	114	42.0	220	35.0	30.6
In vivo OMD (g/kg OM)	733	590	832	54.4	7.42
CH_4-OM (mL/g OM)	25.3	18.9	34.1	2.93	11.6
CH_4-dOM (mL/g dOM)	34.6	26.0	48.4	3.71	10.7

aNDFom: Neutral detergent fiber, OM: Organic matter, OMD: *In vivo* organic matter digestibility (g/kg OM), CH_4-OM (mL/g OM): mL methane/g OM; CH_4-dOM (mL/g dOM): (mL methane /kg OM) / (g digestible OM/kg OM).

Table 3
Pearson correlation coefficients between grass silage chemical composition, silage fermentation quality, *in vivo* digestibility and *in vitro* methane yield (n = 78 round bales).

g/kg DM	aNDFom	iNDF, g/kg aNDFom	Crude Protein	Crude Fat	WSC	Formic acid	Acetic acid	Propionic acid	Butyric acid	Lactic acid	Ethanol	NH_3-N, g/kg N	pH	OMD, g/kg OM	dNDF, g/kg aNDFom	CH_4-OM, mL/g OM
iNDF, g/kg aNDFom	0.26*															
Crude protein	-0.46***	-0.11														
Crude fat	-0.33**	-0.14	0.66***													
WSC	-0.27*	-0.32**	-0.33**	-0.40***												
Formic acid	0.00	0.15	0.18	-0.05	0.00											
Acetic acid	-0.07	0.03	0.37**	0.42***	-0.28*	0.14										
Propionic acid	0.03	0.06	0.13	0.08	-0.07	0.33**	0.54***									
Butyric acid	0.20†	0.07	-0.19†	-0.14	-0.23*	0.01	0.18	0.09								
Lactic acid	-0.23*	-0.06	0.13	0.31**	-0.22†	-0.01	0.59***	0.14	0.08							
Ethanol	-0.01	0.03	-0.35**	-0.17	-0.02	-0.14	0.00	0.04	0.03	0.18						
NH_3-N, g/kg N	0.02	0.17	0.39***	0.58***	-0.69***	-0.12	0.50***	0.15	0.33**	0.24*	0.09					
pH	0.06	-0.22†	0.07	-0.17	0.28*	-0.15	-0.12	-0.04	0.10	-0.53***	-0.36**	-0.13				
OMD, g/kg OM	-0.51***	-0.67***	0.37***	0.27*	0.19†	0.00	-0.06	-0.02	-0.20†	0.06	0.04	-0.05	0.14			
dNDF, g/kg aNDFom	-0.26*	-1.00***	0.11	0.14	0.32**	-0.15	-0.03	-0.06	-0.07	-0.06	-0.03	-0.17	0.22†	0.67***		
CH_4-OM, mL/g OM	-0.63***	-0.48***	0.11	-0.02	0.57***	0.03	-0.03	0.07	-0.14	0.00	0.03	-0.32**	0.22†	0.44***	0.48***	
CH_4-dOM, mL/g dOM	-0.32**	-0.06	-0.15	-0.21†	0.49***	0.02	0.00	0.07	-0.02	-0.05	-0.01	-0.32**	0.15	-0.24*	0.06	0.76***

aNDFom: Neutral detergent fiber; dNDF: *in situ* digestible aNDFom; iNDF: *in situ* indigestible fiber; OM: organic matter; OMD: *In vivo* organic matter digestibility (g/kg OM); WSC: Water soluble carbohydrates; CH_4 -OM (mL/g OM): mL methane/g OM; CH_4-dOM (mL/g dOM): (mL methane /kg OM) / (g digestible OM/kg OM)
As dNDF is calculated as aNDFom – iNDF, the correlation between dNDF (g/kg aNDFom) and iNDF (g/kg aNDFom) is −1.
†P < 0.1.* P < 0.05.** P < 0.01.*** P < 0.001.

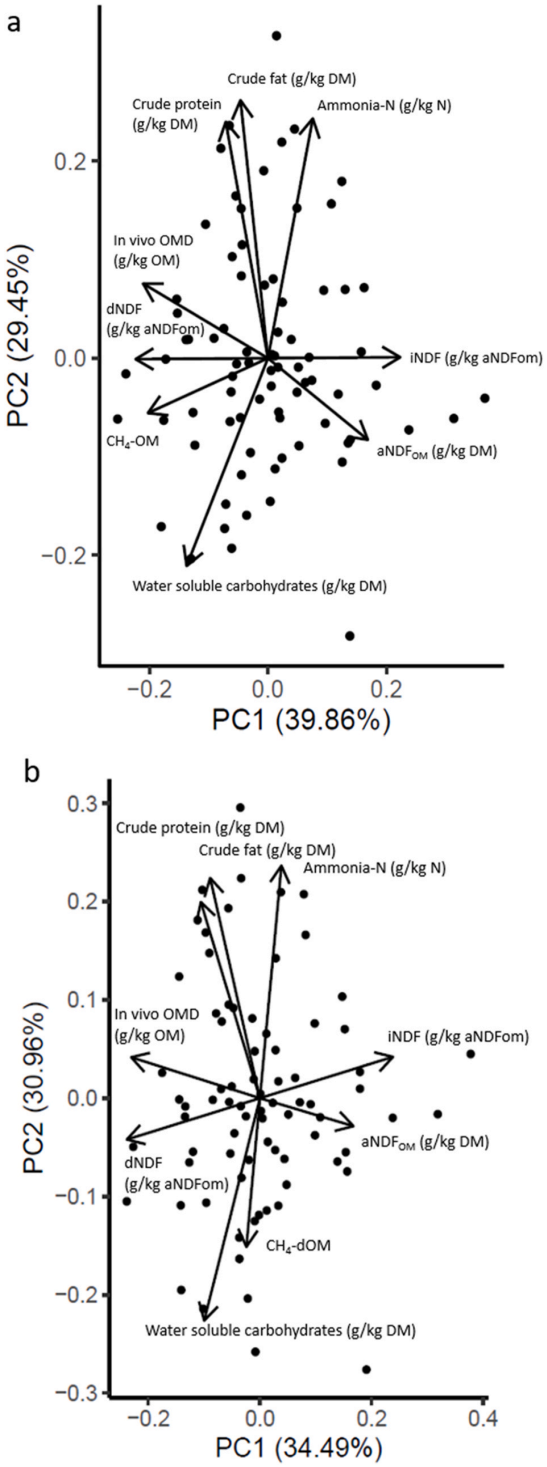

Fig. 1. Principal component analysis (PCA) biplot showing the relationship between grass silage composition variables (g/kg DM), *in vivo* digestibility of organic matter (OMD) and *in situ* digestible aNDFom (dNDF) with methane production expressed on the basis of OM and dOM as a) methane yield CH_4-OM (mL/g OM): mL methane/g OM or b) CH_4-dOM (mL/g dOM): (mL methane /kg OM) / (g digestible OM/kg OM). Principal

component 1 (PC1) and principal component 2 (PC2) explained 69% (a) and 65% (b) of the variance in the data. The dots show each round bale (PC-score), and the arrows show the loadings of each vector. The further away the vectors are from a PC origin (arrow length), the more influence they have on that PC. A small angle between different vectors (*e.g.*, WSC and CH_4-dOM) indicate positive correlation and a large angle (*e.g.*, iNDF and dNDF concentration) indicate negative correlation. A 90° angle between the vectors indicate low correlation (*e.g.* CH_4-OM and crude protein concentration).

3.2. Correlations between different grass silage composition factors

Among all investigated grass silage composition factors, aNDFom concentration had the greatest correlation to CH_4-OM (r = - 0.63, $P < 0.001$, Table 3), but also iNDF and dNDF concentration were moderately correlated with CH_4-OM (r = −0.48 and r = 0.48 respectively, $P < 0.001$). The results also showed a strong positive correlation between the concentration of WSC and CH_4-OM (r = 0.57, $P < 0.001$). Methane yield (mL/g OM) was positively correlated with OMD (r = 0.44, $P < 0.001$) and dNDF (r = 0.48, $P < 0.001$), but negatively correlated with NH_3-N (r = −0.32, $P < 0.01$). The correlation between the pH of the grass silages and CH_4-OM only tended to be significant ($P < 0.10$). There was no correlation between any of the other silage fermentation products and CH_4-OM or CH_4-dOM. When CH_4 was expressed per dOM, the greatest correlation obtained was between CH_4-dOM and WSC (r = 0.49, $P < 0.001$). However, the correlation between CH_4-dOM and aNDFom concentration in grass silages was less pronounced (r = −0.32, $P < 0.01$) compared to when CH_4 yield was expressed as CH_4-OM (r = −0.63, $P < 0.001$). There was no correlation between iNDF or dNDF and CH_4-dOM. CH_4-dOM tended to decrease when concentration of crude fat increased (r = −0.21, $P < 0.1$). The correlation between concentration of NH_3-N and CH_4-dOM was the same as for CH_4-OM (r = −0.32, $P < 0.01$). The greatest correlation coefficient obtained

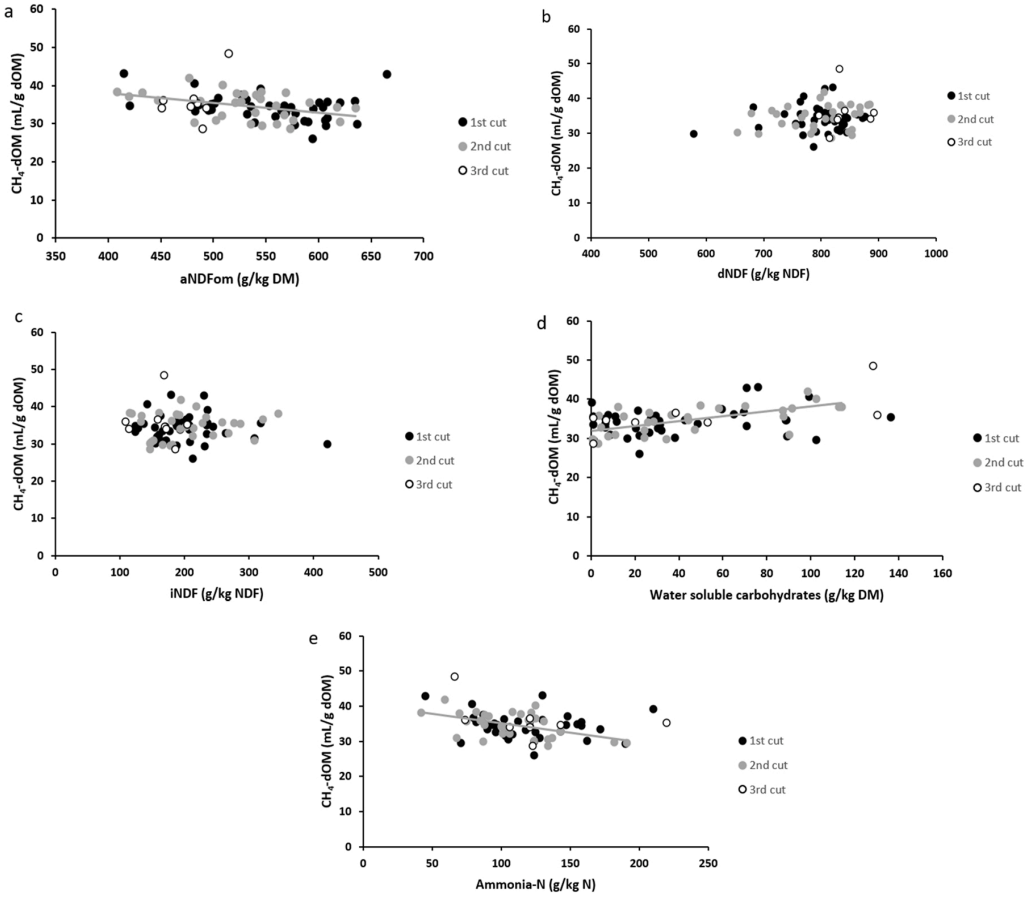

Fig. 2. Relationships of methane production (mL) on the basis of digestible organic matter (dOM, g dOM), *i.e.*, methane yield (CH_4-dOM), from first, second and third cut grass silages with concentrations of a) aNDFom (g/kg DM), b) digestible aNDFom (g/kg aNDFom), c) *in situ* indigestible aNDFom (g/kg aNDFom), d) water soluble carbohydrates (g/kg DM) and e) ammonia nitrogen (g/kg N). Black trendline indicates significant ($P < 0.05$) relationship in 1st cut, gray trendline indicates significant ($P < 0.05$) relationship in 2nd cut and dotted black trendline indicates significant ($P < 0.05$) relationship in 3rd cut.

in the dataset was between concentration of WSC and NH_3-N ($r = -0.69$, $P < 0.001$), and increased concentration of either aNDFom or iNDF was associated with a low *in vivo* OMD ($r = -0.51$ and $r = -0.67$ respectively, $P < 0.001$).

3.3. Principal component analyses of the different grass silage composition factors and in vitro CH_4 yield and comparison with correlation analysis

The result of the PCA was in line with the correlation analysis. The further away the vectors are from a principal component (PC) origin (arrow length), the more they influence that PC. Grass silage characteristics with longer arrows (*e.g.* WSC) explained the PC more than shorter arrows (*e.g.* dNDF). The large angle between CH_4-OM or CH_4-dOM and crude protein or crude fat concentration indicated a weak relationship to CH_4 yield. The grass silage samples positioned close to CH_4-OM or CH_4-dOM in the biplot have a great methanogenic potential, and those positioned orthogonally have a small methanogenic potential. Principal component 1 (PC1) and principal component 2 (PC2) explained 69% of the variation in the dataset for CH_4-OM (40% and 29% for PC1 and PC2, respectively) (Fig. 1a). For CH_4-dOM, the combination of PC1 and PC2 explained 65% of the variation in the dataset (34% and 31% for PC1 and PC2, respectively) (Fig. 1b). Grass silage characteristics positioned close to CH_4-OM in the PCA biplot (Fig. 1a), such as concentrations of dNDF and WSC, were positively correlated to CH_4-OM. For CH_4-dOM (Fig. 1b) the distance to dNDF is larger compared to CH_4-OM and dNDF in Fig. 1a, which is in line with the correlation result (Table 3). Further, the distance between CH_4-dOM and WSC was very small (Fig. 1b) which is in line with the large positive correlation presented in Section 3.2.

3.4. Effect of cut number on the relationship between chemical composition and CH_4-dOM

The decrease in CH_4-dOM with increasing aNDFom concentration was only significant for second cut silages ($r = -0.41$, $P < 0.05$, Fig. 2a), although the relationship tended to be significant also in the first cut ($r = -0.31$, $P < 0.1$). The correlation between CH_4-dOM and dNDF or iNDF concentration was not significant for any of the cuts. The increase in CH_4-dOM with increasing concentration of WSC was only significant in second cut grass silages ($r = 0.64$, $P < 0.05$). The reduction in CH_4-dOM as the concentration of NH_3-N increased was only significant for second cut silages ($r = -0.51$, $P < 0.05$).

3.5. Results of the stepwise forward regression modeling

The stepwise forward regression analysis for CH_4-OM (Model 1) included the following explanatory variables in descending order: aNDFom ($P < 0.001$, AIC = 130.7), WSC ($P = 0.14$, AIC = 106.5), iNDF ($P < 0.01$, AIC = 98.7), propionic acid ($P = 0.34$, AIC = 97.6)

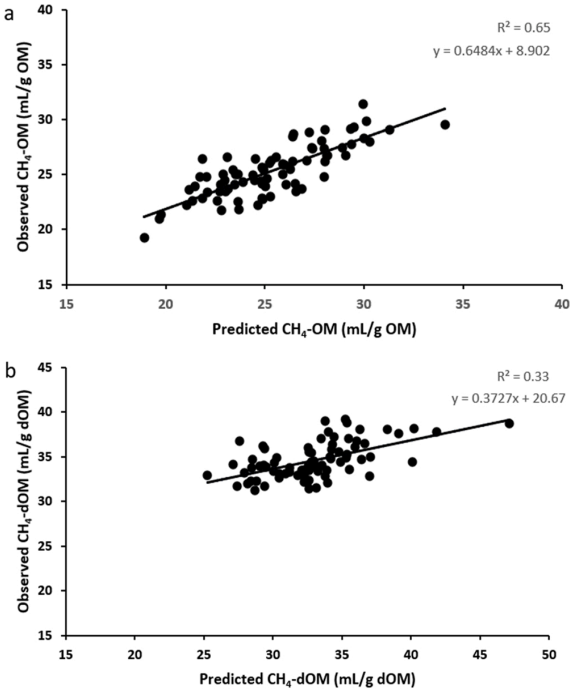

Fig. 3. Relationship between the observed and predicted *in vitro* methane production expressed on the basis of organic matter (OM) and digested OM (dOM), as a) CH_4-OM, mL methane/g OM using Model 1 and b) CH_4-dOM, mL methane/g dOM using model 2.

and pH (P = 0.16, AIC = 97.4).

Model 1: CH_4-OM (mL CH_4/g OM) = 36.22–0.02 × aNDFom (g/kg DM) + 0.03 × WSC (g/kg DM) – 0.01 × iNDF (g/kg aNDFom) + 0.82 × propionic acid (g/kg DM) + 0.71 × pH. Coefficient of determination (R^2) = 0.65.

The OMD was excluded in the stepwise forward regression analysis for CH_4-dOM (Model 2). The analysis included the following explanatory variables in descending order: WSC (P = 0.27, AIC = 187.5), aNDFom (P < 0.01, AIC = 185.7) and iNDF (P = 0.31, AIC = 185.5).

Model 2: CH_4-dOM (mL CH_4/g dOM) = 38.38 + 0.05 × WSC (g/kg DM) – 0.01 × aNDFom (g/kg DM) + 0.01 × iNDF (g/kg aNDFom) (R^2 = 0.33)Fig. 3.

3.6. Correlation between in vitro rumen fermentation characteristics, CH_4 yield and grass silage parameters

Increased molar proportion of butyrate increased CH_4-dOM (r = 0.36, P < 0.001, Table 4), but the effect was less pronounced when expressed as CH_4-OM (r = 0.23, P < 0.05). Increasing molar proportion of propionate was associated with a reduction in CH_4-dOM (r = −0.26, P < 0.05), but the effect was slightly less with CH_4-OM (r = −0.23, P < 0.05). Increased molar proportion of acetate tended to be associated with increased CH_4-OM and CH_4-dOM (r = 0.19 and r = 0.20 respectively, P < 0.10), and increased ratio between acetate and propionate was associated with increased CH_4-OM and CH_4-dOM (r = 0.25 and r = 0.26 respectively, P < 0.05). *In vitro* rumen fermentation characteristics are depicted in Supplementary Table S1.

The WSC concentration was the variable with greatest influence on *in vitro* rumen fermentation characteristics. The WSC concentration was negatively correlated to *in vitro* NH_3 (r = −0.50, P < 0.001) and molar proportion of propionate (r = −0.34, P < 0.01), but positively correlated to molar proportion of acetate (r = 0.39, P < 0.001), molar proportion of butyrate (r = 0.33, P < 0.01) and the ratio between molar proportion of acetate and propionate (C_2:C_3) (r = 0.40, P < 0.001).

Increased molar proportion of acetate was negatively correlated to molar proportion of propionate (r = −0.83, P < 0.001), butyrate (r = −0.28, P < 0.05), iso-butyrate (r = −0.53, P < 0.001), valerate (r = −0.52, P < 0.001), iso-valerate (r = −0.33, P < 0.01) and hexanoate (r = −0.25, P < 0.05). When the *in vitro* molar proportion of propionate increased, the molar proportion of valerate also increased (r = 0.24, P < 0.05). *In vitro* NH_3 concentration was positively correlated to molar proportion of iso-valerate (r = 0.86, P < 0.001), iso-butyrate (r = 0.82, P < 0.001) and valerate (r = 0.71, P < 0.001), but was negatively correlated to molar proportion of acetate (r = −0.26, P < 0.05).

3.7. Principal component analysis of in vitro ruminal SCFA and NH_3 concentrations and in vitro CH_4 yield

According to the PCA analysis 61% of the total variation in the dataset was explained by the two first principal components (38% and 22% respectively; Fig. 4a, b). Methane yield expressed as CH_4-OM or CH_4-dOM did not explain a significant portion of the total variation in the dataset, as indicated by the short length of the arrows. However, CH_4 yield was positively correlated to both acetate molar proportion and the acetate: propionate ratio, and negatively correlated to propionate molar proportion. Propionate and acetate

Table 4
Pearson correlation between *in vitro* rumen fermentation characteristics, methane (CH_4) yield and grass silage parameters (n = 78 round bales).

	NH_3 (mmol/L)	Total SCFA (mmol/l) and molar proportions (mmol/mol) in incubated rumen fluid							
		Total SCFA	Acetate	Propionate	Butyrate	Iso-butyrate	Valerate	Iso-valerate	Hexanoate	C_2:C_3
Total SCFA (mmol/L)	-0.17									
Molar proportions (mmol/mol)										
Acetate (C_2)	-0.26*	0.26*								
Propionate (C_3)	-0.08	-0.04	-0.83***							
Butyrate	0.00	-0.21†	-0.28*	-0.09						
Iso- butyrate	0.82***	-0.35**	-0.53***	0.15	0.00					
Valerate	0.71***	-0.23*	-0.52***	0.24*	-0.15	0.85***				
Iso- valerate	0.86***	-0.31**	-0.33**	-0.06	0.00	0.93***	0.75***			
Hexanoate	-0.07	-0.22†	-0.25*	-0.01	0.26*	0.11	-0.04	0.03		
C_2:C_3	-0.05	0.15	0.93***	-0.97***	-0.04	-0.30**	-0.36***	-0.10	-0.11	
Grass silage parameters										
aNDFom (g/kg DM)	-0.11	0.02	0.15	-0.14	-0.13	-0.04	-0.22†	-0.05	0.34**	0.13
dNDF (g/kg aNDFom)	-0.13	0.21†	0.08	-0.01	-0.09	-0.19†	-0.02	-0.26*	0.15	0.06
iNDF (g/kg aNDFom)	0.13	-0.21†	-0.08	0.01	0.09	0.19†	0.02	0.26*	-0.15	-0.06
WSC (g/kg DM)	-0.50***	0.03	0.39***	-0.34**	0.33**	-0.57***	-0.48***	-0.58***	0.00	0.40***
CH_4-OM (mL/g OM)	-0.10	0.14	0.19†	-0.23*	0.23*	-0.16	-0.06	-0.19†	-0.18	0.25*
CH_4-dOM (mL/g dOM)	-0.13	0.00	0.20†	-0.26*	0.36**	-0.17	-0.19†	-0.22†	-0.13	0.26*

C_2, acetate; C_3, propionate; aNDFom, neutral detergent fiber; iNDF, indigestible neutral detergent fiber; NH_3, ammonia, OM, organic matter; WSC, water-soluble carbohydrates; CH_4-OM (mL/g OM): mL methane/g OM; CH_4-dOM (mL/g dOM): (mL methane /kg OM) / (g digestible OM/kg OM).
† P < 0.1 * P < 0.05. ** P < 0.01. *** P < 0.001.

molar proportions and the ratio between the two SCFA were identified as very important factors in explaining the total variation in the dataset unlike for molar proportions of butyrate and hexanoate which had very short arrows.

4. Discussion

In this study CH_4 yield was expressed as CH_4-OM (mL/g OM) because silages largely differed in OM concentration, the main determinant of CH_4 yield. Further, it was important to express CH_4 yield as CH_4-dOM (mL/g dOM) to explain factors within the

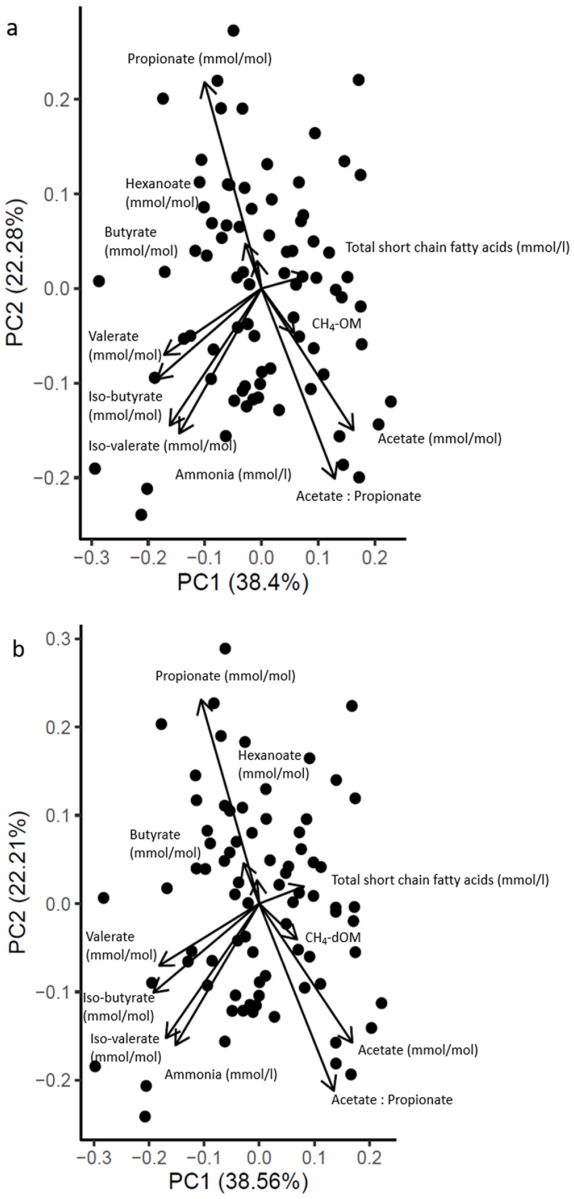

Fig. 4. Principal component analysis biplot showing the relationship between methane production expressed on the basis of organic matter (OM) or digestible OM (dOM) as methane yield CH_4-OM and CH_4-dOM, respectively, and rumen fermentation characteristics. Principal component (PC) 1 and 2 explained 61% of the variation in the dataset. The dots show each round bale (pc-score) and the arrows show the loadings of each vector. The further away the vector is from a PC origin (arrow length), the greater the influence on that PC. A small angle between two vectors indicates a positive correlation, and a large angle indicates a negative correlation. An 90° angle indicates low correlation.

digestible OM that affect CH_4 production. We were successful in obtaining a large variation in DM, aNDFom, crude protein, WSC concentration and digestibility as depicted in Table 2. This study showed that grass silage nutrients and fermentation products affected CH_4-OM and CH_4-dOM as expected, with 1.8-fold and 1.85-fold difference respectively, between the greatest and lowest CH_4-OM (34 *vs* 19 mL/g OM) and CH_4-dOM (48 *vs* 26 mL/g dOM). This large range in CH_4 yield was partly explained by differences among silages in concentrations of aNDFom, dNDF, iNDF, WSC, NH_3-N, propionic acid and pH of the silages, in addition to differences in *in vivo* OMD.

4.1. Relationship between aNDFom and iNDF concentration in grass silages and CH_4 yield

The observed negative associations between CH_4 yield and concentrations of aNDFom and iNDF were in accordance with our expectations and other *in vitro* studies on grass silage (Holtshausen et al., 2012; Macome et al., 2018), and the strong correlations indicate that aNDFom and iNDF concentrations are two major determinants of the methanogenic potential of grass silages. Thus, increased aNDFom and iNDF concentrations in grass silage are associated with reduced *in vitro* CH_4 yield. The importance of aNDFom and iNDF are further strengthened by inclusion as significant explanatory variables in both CH_4 yield regression models.

Previous *in vivo* experiments have shown greater proportions of ruminal acetate and lower proportions of ruminal butyrate in grass silages with high compared to low concentration of aNDFom and iNDF at ensiling (Rinne et al., 1997, 2002). However, we found no consistent effect of silage aNDFom and iNDF concentration and the proportions of SCFA, and hence the lack of effect of SCFA on CH_4 yield. Holtshausen et al. (2012) showed that increased maturity at harvest had no significant effect on *in vitro* molar proportion of acetate at 24 or 48 h of incubation. But surprisingly, increased maturity at harvest gave greater molar proportion of propionate at 48 h of incubation, which might explain the reduced CH_4 production and yield (mL and mL/g NDF disappeared) in that experiment. Their finding is not in accordance with the present study, as we did not find significant correlations between aNDFom or iNDF concentrations of the grass silages and molar proportions of ruminal acetate, propionate or butyrate in the rumen fluid. It is possible that the greater CH_4 yield of less mature grass silages was partly due to the non-aNDFom fraction (mainly WSC) as suggested by Holtshausen et al. (2012), as grass harvested at an earlier stage of maturity usually has a greater concentration of WSC compared with more mature grass (Randby et al., 2012). In addition, Johnson and Johnson (1995) argued that the two primary mechanisms regulating CH_4 yield are: 1) the amount of dietary carbohydrates fermented in the rumen fluid, and 2) the available H_2 supply through changes in SCFA production. It is possible that grass silages with greater OMD increased the supply of *in vitro* fermentable carbohydrates, which overshadowed the effect of changed metabolic H_2 supply due to changes in the ratio between propionate: [acetate+butyrate] in the incubated rumen fluid.

4.2. Relationship between WSC concentration in grass silages and methane yield

It has been reported that molar proportion of ruminal propionate increases at the expense of acetate as WSC concentration in silage increases (Lee et al., 2003b; Purcell et al., 2014; Rivero et al., 2020), which may lower CH_4 yield. However, our results showed the opposite effect; increased concentration of WSC in grass silages was associated with increased molar proportion of acetate and butyrate at the expense of propionate molar proportions.

Type of WSC fermented in the rumen affect rumen SCFA profile (Sutton, 1968, 1969; Czerkawski and Breckenridge, 1969) and potentially CH_4 yield. Kellogg and Owen (1969a,b) reported increased butyrate proportion in rumen fluid *in vivo* when feeding sucrose, and in contrast to propionate, butyrate production is known to increase CH_4 formation in the rumen because it generates H_2 which is used by methanogens to produce CH_4 (Boadi et al., 2004). Others have reported no such effect of feeding sucrose (Sannes et al., 2002; Broderick et al., 2008; Penner and Oba, 2009) or even a tendency for a decrease in rumen butyrate (McCormick et al., 2001). Børsting et al. (2020) reported greater H_2 production and greater CH_4 yield per kg DM intake and per kg energy corrected milk (ECM) when feeding a diet supplemented with sugar from molasses compared to a diet supplemented with starch from wheat, which supports the association between WSC and CH_4 yield as was found in the present study. In the present study, ruminal butyrate was the single SCFA with the greatest correlation to CH_4-dOM, which might partly explain the positive correlation between WSC and CH_4-dOM. Molar proportion of acetate obtained a lower correlation to both CH_4-OM and CH_4-dOM compared to molar proportion of butyrate, although the correlation to CH_4-OM and CH_4-dOM tended to be significant. Ellis et al. (2012) modeled the effect of feeding grasses high in WSC concentration on *in vivo* CH_4 yield (relative to gross energy intake) and found that simulated CH_4 yield increased in grasses high in WSC concentration, which is in accordance with our *in vitro* results.

The ensiling process depends on forage WSC concentration, DM concentration at ensiling, buffering capacity, and the use or type and dosing level of silage additives. Extensive fermentation of WSC during ensiling results in increased concentrations of lactic acid in grass silages (Huhtanen et al., 2013), which is supported by the tendency for a negative association between silage WSC and silage lactic acid concentration as was found in the present study. There is limited information on *in vitro* CH_4 yield as affected by silage fermentation products in the literature, although it is well known that lactic acid in grass silage is subjected to fermentation in the rumen, with propionate as end product (Chamberlain et al., 1983; Jaakkola and Huhtanen, 1992; Huhtanen et al., 2013). Our study showed a negative correlation between molar proportion of propionate and CH_4-OM or CH_4-dOM. Despite the strong correlation between WSC and CH_4-OM and CH_4-dOM, there was no correlation between lactic acid in grass silage and CH_4-OM or CH_4-dOM, which suggests that silage sugar concentration and rumen SCFA production have a greater impact on CH_4 yield than the fermentation profile due to ensiling of grass.

4.3. Relationship between OMD of grass silages and CH_4 yield

The positive correlation between *in vivo* OMD and CH_4-OM corresponds to the results of Holtshausen et al. (2012) who found that *in vitro* CH_4 yield (mL CH_4/g DM disappeared) decreased when grass was ensiled at increasing maturity with reduced *in vitro* DM disappearance. The present study is also in accordance with previous *in vivo* results using respiration chambers showing that increased digestibility of feeds leads to greater CH_4 production (Blaxter and Clapperton, 1965). These results were later confirmed by Ramin and Huhtanen (2013) who developed *in vivo* CH_4 prediction equations based on 52 published papers and found that increased digestibility at maintenance level increased CH_4 yield per unit of gross energy or DM intake. Jonker et al. (2016) reported a similar effect for beef cattle fed fresh pasture. We speculate that the positive correlation observed in our study between *in vivo* OMD and *in vitro* CH_4-OM relates to a greater amount of fermentable substrate in the rumen fluid when OMD increases. The positive correlation between *in vivo* OMD and CH_4-OM corresponds to the negative correlation between iNDF and CH_4-OM and further to the negative correlation between iNDF and WSC indicating that highly digestible grass silage with low iNDF concentrations provides greater amounts of highly fermentable carbohydrates (*e.g.* WSC) to the rumen microbiota. Despite the positive correlation between OMD and CH_4-OM ($r = 0.44$, $P < 0.001$), OMD was not included as a significant explanatory variable in the prediction of CH_4-OM (model 1) likely because of the co-linearity with the other significant explanatory variables (aNDFom, WSC, iNDF). Correlations only indicate associations between two variables, whereas regression analysis reveals how multiple variables interact. Thus, increased OMD did not cause a direct increase in CH_4-OM, although there was a positive correlation between the two variables.

4.4. Predicting methane yield based on regression modeling

Regression modeling can be used to predict enteric CH_4 yield by ruminants, as confirmed in the present study using forward step by step regression analyses. Results from the regression analyses deviated from correlation analyses because the latter only consider the relationship between two variables whereas regression analyses consider multiple variables and interactions between these. The review by Yáñez-Ruiz et al. (2016) indicated that it is possible to obtain a high R^2 when comparing *in vitro* and *in vivo* CH_4 measurements when these are conducted simultaneously and using the same diets, but that the R^2 depends on diet tested, animal species, adaptation period and *in vitro* and *in vivo* methods applied. Few *in vitro* studies have developed prediction equations to *estimate* CH_4 yield from forages. Lee et al. (2003a) used CH_4 yield data from *in vitro* incubation (24 h) of alfalfa hay, rice straw and orchard grass hay to develop CH_4 prediction equations and found that increased concentration of crude protein and crude fiber increased CH_4 yield, while increased concentration of nitrogen free extracts reduced CH_4 yield (mL/0.2 g DM) ($R^2 = 0.99$). Both aNDFom and WSC were included in the prediction model of Lee et al. (2003a) and in the present models. However, the results are contradictory as we found a negative relationship between aNDFom and CH_4 yield, and a positive relationship between WSC and CH_4 yield, which is opposite to Lee et al. (2003a). Our study is not completely comparable to Lee et al. (2003a) because that study did not measure NDF or WSC, but instead reported crude fiber and nitrogen-free extracts. Additionally, the contradictory results for the effects of these variables might in part be explained by the low crude fiber concentrations in the study by Lee et al. (2003a) which were not greater than 34% and the small range in nitrogen-free extracts of 44–45%. The number of observations was 78 in our study compared to only 15 observations (5 samples per forage type) in the analysis of Lee et al. (2003a). The present study obtained a high R^2 when plotting the relationship between observed and predicted CH_4-OM ($R^2 = 0.65$), but the R^2 was substantially lower for CH_4-dOM ($R^2 = 0.33$). The CH_4-dOM is largely underestimated at high observed CH_4-dOM which might be explained by differences in nutrient concentrations of higher compared to lower digestible grass silages.

4.5. Implications for grass silage production

Our study showed that greater WSC and lower aNDFom and iNDF concentrations in grass silages are associated with greater *in vitro* CH_4 yield, with CH_4 production expressed relative to the composition of the forage incubated *in vitro* (CH_4-OM). Thus, as farmers implement production practices such as earlier harvest (which influences concentration of aNDFom, iNDF and WSC) and choice of botanical composition (use of species with greater content of WSC) to improve digestibility and animal performance, CH_4 production potential per kilogram of forage DM consumed may also increase. Expressing CH_4 yield relative to dOM to account for the variability in digestibility revealed similar relationships between nutritional quality and CH_4 yield. We recognize that the relationship between *in vitro* CH_4 yield of grass silages and nutritional quality variables reported in the study must be confirmed *in vivo* along with animal production. However, in commercial feeding operations, low *in vitro* CH_4 yielding silages characterized by lower WSC and greater aNDFom and iNDF concentrations would be expected to lower ECM production in dairy cows and average daily gain in youngstock and thereby unfavorable increase CH_4 emission intensity (CH_4/kg ECM, CH_4/kg average daily gain). Thus, there appears to be a contradiction between selecting forages that have low *in vitro* CH_4 yield, and those that support high levels of animal production and low CH_4 intensity.

CRediT authorship contribution statement

Kim Viggo Weiby: Writing – original draft, Writing – review & editing, Investigation, Methodology, Software, Formal analysis, Visualization. **Sophie J. Krizsan:** Resources, Writing – review & editing, Investigation. **Margrete Eknæs:** Writing – review & editing, Supervision, Methodology. **Angela Schwarm:** Writing – review & editing, Supervision, Methodology. **Anne Cathrine Whist:** Writing – review & editing, Supervision. **Ingunn Schei:** Investigation, Writing – review & editing. **Håvard Steinshamn:** Writing – review &

editing. **Peter Lund:** Writing – review & editing. **Karen A. Beauchemin:** Writing – review & editing. **Ingjerd Dønnem:** Conceptualization, Formal analysis, Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors do not have any conflict of interest and this submission has been done upon agreement of all the co-authors.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anifeedsci.2022.115249.

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Paper II

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Effect of grassland cutting frequency, species mixture, wilting and fermentation pattern of grass silages on in vitro methane yield

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Mitigating enteric methane (CH_4) emissions is crucial as ruminants account for 5% of global greenhouse gas emissions. We hypothesised that less frequent harvesting, use of crops with lower WSC concentration, ensiling at low crop dry matter (DM) and extensive lactic acid fermentation would reduce in vitro CH_4 production. Timothy (T), timothy + red clover mixture (T + RC) or perennial ryegrass (RG), cut either two or three times per season, was wilted to 22.5% or 37.5% DM and ensiled with or without formic acid-based additive. Silages were analysed for chemical composition and fermentation products. In vitro CH_4 production was measured using an automated gas in vitro system. Methane production was, on average, 2.8 mL/g OM lower in the two-cut system than in the three-cut system ($P < 0.001$), and 1.9 mL/g OM lower in T than in RG ($P < 0.001$). Silage DM did not affect CH_4 production ($P = 0.235$), but formic acid increased CH_4 production by 1.2 mL/g OM compared to the untreated silage ($P = 0.003$). In conclusion, less frequent harvesting and extensive silage fermentation reduce in vitro CH_4 production, while RG in comparison to T resulted in higher production of CH_4.

Global warming caused by increased concentrations of greenhouse gases (GHGs) in the atmosphere is a major threat to the planet[1]. Food systems contribute up to 30% of global GHG emissions[2], and methane (CH_4) from ruminant production systems contributes to 5% of global GHG emissions[3,4]. Methane is 20 times as potent greenhouse gas as carbon dioxide (CO_2), and its contributing share to global warming is increasing[5]. Enteric CH_4 is produced by the removal of excess hydrogen (H_2) and CO_2, which results from the ruminal fermentation of feed carbohydrates, such as cell wall polymers, fructans, and starch, into volatile fatty acids (VFA)[6]. Therefore, finding a means to reduce enteric CH_4 is crucial.

Forages such as grass and grass-clover silage (hereafter grass silage) constitute a large part of ruminant diets in Northern and Western Europe, as well as in Northern America. In Norway, ruminant production systems are located at approximately 58° to 71°N and within the coastal and alpine parts of the country. The growing degree-days, defined as accumulated mean temperature above 5 °C, is between 700 and 1200 °C, and the annual precipitation ranges from less than 300 mm to 4000 mm. Hence, climatic conditions for herbage production vary greatly[7]. Agricultural practices like cutting frequency, use of different species mixtures, wilting and use of silage additives also vary partly according to the climatic conditions.

In vitro studies have shown that advanced maturity of the forage used in the ensilage with decreased organic matter digestibility (OMD) at harvest resulted in a linear decrease in in vitro CH_4 production per unit of feed dry matter (DM) incubated but increased CH4 out per g DM digested[8]. Purcell et al.[9] found no difference between grass species in in vitro CH_4 production (mL per gram of DM incubated). Genotypes of perennial ryegrass have been bred for high concentration of water-soluble carbohydrates (WSC) as measure to improve animal performance[10], and such high sugar grasses are more prone to display extensive lactic acid fermentation during the ensiling process[11]. The readily available WSC in grass silage is subjected to fermentation, where lactic acid is the major end product in well-fermented silage. In the rumen, lactic acid is transformed into propionate[12], and

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it is therefore possible that silages with high concentrations of lactic acid produce lower amounts of enteric CH_4 compared to restrictively fermented silages. On the other hand, a recent study[13] showed that increased concentrations of WSC in grass and grass-clover silage increase in vitro CH_4 production possibly due to increased butyrate and acetate concentrations in the rumen fluid.

Navarro-Villa et al.[14] found that perennial ryegrass had greater in vitro CH_4 production (mL/g DM incubated) compared to red clover (*Trifolium pratense* L.) cultivars. Red clover contains less fibre (NDF) compared to perennial ryegrass[15]. Reduced NDF concentration might increase the propionate:acetate ratio in in vitro rumen fluid and reduce CH_4 production, as feed with less fibre gives higher H_2 concentration, more propionate and therefore less CH_4 as propionate formation competes with methanogenesis for H_2 in rumen[16,17]. Plant secondary compounds, such as condensed tannins or polyphenol oxidase, may have a specific lowering effect on enteric CH_4 production[18,19], but results are not unequivocal, as some in vivo studies show no effect of red clover on CH_4 production[20].

Wilting grass during silage production reduces water activity with immediate reduction in microbial activity and fermentation intensity during preservation[21]. Elevated DM concentration and reduced fermentation intensity in silage retain more WSC in the silage[22,23]. In addition, the use of formic acid-based additives that restrict fermentation can potentially preserve silage concentrations of WSC compared to silages prepared without additives or with the use of lactic acid bacteria inoculants[24,25].

Although the above-cited studies indicate that forage species, harvest frequency, wilting and use of additives affect CH_4 production, to the best of our knowledge no attempts have been made to compare combined effects of these factors. Therefore, the aim of this study was to test the effect of cutting frequency, growth period, crop type, wilting and fermentation pattern on in vitro CH_4 production using a fully automated gas in vitro system. We hypothesised that (1) less frequent harvesting with longer growth periods, (2) use of ley species with lower WSC concentrations, (3) low crop DM and (4) extensive silage fermentation reduce in vitro CH_4 production.

Results

Dry matter production, clover proportion and mean stage by count. Total annual DM yield was, on average, 7% greater in the two-cut system compared to the three-cut system ($P<0.001$, Table 1). Timothy (T) obtained 16% less DM yield compared to perennial ryegrass (RG) across different harvest systems ($P<0.001$), but 10% greater DM yield compared to the timothy red clover mixture (T + RC) ($P<0.001$).

In the three-cut system, the first, second and third cuts accounted for 47%, 33% and 20% of the total annual DM yield across the different species mixture, respectively, while the first and second cuts accounted for 67% and 33% of the total annual DM yield, respectively, in the two-cut system.

The mean stage by count for T in the three-cut system was 2.69 and 2.72 in the first and second cuts, respectively, while it was 2.97 and 1.98 in the first and second cuts of the two-cut system, respectively. For RG, the mean stage by count in the three-cut system was 2.47 and 1.96 in the first and second cuts, respectively and 2.65 and 2.21 for the first and second cuts, respectively, in the two-cut system.

Chemical characteristics of fresh and wilted materials. The concentration of WSC in fresh herbage was 33 g/kg DM greater in the three-cut system than in the two-cut system (124 vs. 91 g/kg DM respectively, $P<0.001$, Table S1), while crude protein (CP) content was 27 g/kg DM greater in the three-cut system than in the two-cut system (132 vs. 105 g/kg DM respectively, $P<0.001$). The NDFom concentration was 109 g/DM lower in the two-cut system than in the three-cut system (492 vs. 601 g/kg DM respectively, $P<0.001$). The concentration of WSC was 56 g/kg DM greater in RG compared to T (162 vs. 106 g/kg DM respectively, $P<0.001$) but there was no difference between T and T + RC ($P=0.306$). The CP concentration was 10 g/kg DM lower in RG than in T (113 vs. 123 g/kg DM respectively, $P=0.01$), but there was no effect of including red clover on the CP concentration of the fresh herbage ($P=0.264$).

NDFom concentration was 60 g/kg DM lower in RG than in T (508 vs. 568 g/kg DM respectively, $P<0.001$), and T + RC had 38 g/kg DM lower NDFom concentration than T (530 vs. 568 g/kg DM, $P<0.001$). RG had a 22 g/kg DM lower NDFom than T + RC (508 vs. 530 g/kg DM, $P=0.006$). In wilted herbage, concentrations of WSC were 13 g/DM greater in the two-cut system than in the three-cut system (119 vs. 106 g/kg DM respectively,

		Three cuts			Two cuts				*P* value							
	Harvest	T	T + RC	RG	T	T + RC	RG	SEM	Cut	Crop	Cut × crop	C1	C2	C3	C4	C5
DM yield, g/m^2	1st	465	458	602	729	740	837	21.72	< 0.001	< 0.001	0.023	< 0.001	< 0.001	0.215	< 0.001	< 0.001
	2nd	370	263	452	419	331	404									
	3rd	198	196	240												
	Total	1034	917	1294	1148	1071	1240	28.63	0.006	< 0.001	0.004	–	–	–	< 0.001	0.003

Table 1. Effect of Cut (1–5 where 1,3 and 5 is the first, second and third cut in the three-cut system, and 2 and 4 is the first and second cut in the two-cut system, respectively) and crop (T, timothy; T + RC, timothy + red clover; RG, perennial ryegrass) on dry matter yield per cut and total annual yield (n = 3). SEM is the standard error of the means. C1 is the contrast three versus two cuts per season, overall; C2 is the contrast three versus two cuts per season in the 1st cut; C3 is the contrast three versus two cuts per season in the 2nd cut; C4 is the contrast T versus RG; C5 is contrast T versus T + RC.

$P < 0.001$, Table S2), while CP concentrations were 34 g/kg DM greater in the three-cut system than in the two-cut system (134 vs. 99.6 g/kg DM respectively, $P < 0.001$).

NDFom concentrations were 74 g/kg DM greater in the two-cut system than in the three-cut system (579 vs. 505 g/kg DM respectively, $P < 0.001$). Concentrations of WSC in wilted herbage were 36 g/kg DM greater in RG than in T (136 vs. 99.8 g/kg DM respectively, $P < 0.001$), but there was no difference between T and T + RC ($P = 0.856$). Concentrations of CP were 10 g/kg DM greater in T than in RG (123 vs. 113 g/kg DM respectively, $P = 0.001$), but there was no difference in CP concentrations between T and T + RC ($P = 0.635$). Concentrations of NDFom were 56 g/kg DM greater in T than in RG (566 vs. 510 g/kg DM respectively, $P < 0.001$), and T + RC was 37 g/kg DM lower in NDFom compared to T (566 vs. 529 g/kg DM respectively, $P < 0.001$).

Wilting had no effect on the concentration of ash, CP or NDFom (Table S3). The concentration of soluble CP as proportion of total CP increased with wilting. Wilting rate had an inconsistent effect on the concentration of WSC; it had no effect in T + RC and RG but increased the WSC concentration in T (Table S3).

Effect of cut and crop types on silage chemical constituents and fermentation characteristics. The average NDFom concentration was 102 g/kg DM lower in the three-cut system compared to the two-cut system (451 vs. 553 g/kg DM respectively, $P < 0.001$, Table 2), and harvesting at a later stage of maturity resulted in 92 and 69 g/kg DM greater NDFom concentration in the first and second cut of the two-cut system compared to the three-cut system, respectively ($P < 0.001$).

The NDFom concentration was 38 g/kg DM lower in RG compared to T (478 vs. 516 g/kg DM respectively, $P < 0.001$), and 36 g/kg DM lower in T + RC compared to T (480 vs. 516 g/kg DM respectively, $P < 0.001$). The iNDF concentration was 66 g/kg NDFom lower across all cuts in the three-cut system compared to the two-cut system (151 vs. 217 g/kg NDFom respectively, $P < 0.001$).

Perennial ryegrass had 19 g/kg NDFom greater iNDF concentrations than T across all cuts (189 vs. 170 g/kg NDFom respectively, $P < 0.001$), but there was no significant difference between T and T + RC (170 vs. 173 g/kg NDFom respectively, $P = 0.518$). OMD was 7 percent point greater across all cuts in the three-cut system compared to the two-cut system (75 vs. 68%, respectively, $P < 0.001$). Postponing the first and second cuts resulted in

		Three cuts			Two cuts				*P* value							
	Cut	T	T + RC	RG	T	T + RC	RG	SEM	Cut	Crop	Cut × crop	C1	C2	C3	C4	C5
DM, g/kg	1st	31.1	31.9	29.3	35.2	37.3	34.8	4.666	0.481	0.918	0.999	0.200	0.198	0.916	0.724	0.994
	2nd	34.3	36.2	33.1	35.3	32.7	34.4									
	3rd	31.0	28.8	29.9	–	–	–									
Organic matter, g/kg DM	1st	930	932	924	942	945	932	0.762	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	2nd	930	923	929	940	930	918									
	3rd	931	911	911	–	–	–									
NDFom, g/kg DM	1st	508	501	425	576	586	543	8.958	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	2nd	487	429	487	562	515	535									
	3rd	449	370	400	–	–	–									
iNDF, g/kg NDFom	1st	119	112	121	230	220	246	6.268	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.011	< 0.001	0.518
	2nd	155	159	254	204	200	205									
	3rd	143	174	121	–	–	–									
OMD, %	1st	75.3	75.9	77.3	65.8	66.2	66.1	0.464	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.859	< 0.001
	2nd	73.7	75.3	67.9	68.1	70.1	69.0									
	3rd	75.5	76.3	77.9	–	–	–									
CP, g/kg DM	1st	147	126	121	103	89.3	95.4	1.709	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.008
	2nd	141	144	124	106	109	113									
	3rd	113	158	112	–	–	–									
sCP, g/kg CP	1st	790	755	781	580	557	721	24.85	< 0.001	< 0.001	0.012	0.002	< 0.001	0.181	< 0.001	0.002
	2nd	582	538	704	596	486	660									
	3rd	510	459	664	–	–	–									
WSC, g/kg DM	1st	37.6	46.4	91.5	60.4	58.0	83.8	21.78	0.639	0.160	0.641	0.961	0.619	0.649	0.218	0.502
	2nd	41.8	56.0	64.8	56.9	51.8	78.3									
	3rd	113	50.8	77.1	–	–	–									

Table 2. Effect of Cut (1–5 where 1,3 and 5 is the first, second and third cut in the three-cut system, and 2 and 4 is the first and second cut in the two-cut system) and crop (T, Timothy; T + RC, Timothy + red clover; RG, perennial ryegrass) on silage feed quality parameters averaged across DM and additive treatments (n = 4). DM, dry matter; NDFom, neutral detergent fibre; iNDF, indigestible NDF; OMD, organic matter digestibility; CP, crude protein; sCP, soluble crude protein; WSC, water-soluble carbohydrates. C1 is the contrast three versus two cuts per season overall; C2 is the contrast three versus two cuts per season, 1st cut; C3 is the contrast three versus two cuts per season, 2nd cut; C4 is the contrast T versus RG; C5 is contrast T versus T + RC.

10 and 3 percent point lower OMD in the first and second cuts of the two-cut system compared to the three-cut system, respectively ($P < 0.001$).

There was no significant difference in OMD between T and RG (71.7 vs. 71.6%, respectively, $P = 0.859$), but 1.1 percent point greater OMD in T + RC compared to T (71.7% vs. 72.8%, respectively, $P < 0.001$). The concentration of WSC was not affected by either the harvest system ($P = 0.961$) or species mixture ($P = 0.160$). Silage concentration of lactic acid was, on average, 21 g/kg DM greater across all cuts in the three-cut system compared to the two-cut system (42 vs. 21 g/kg DM respectively, $P = 0.001$, Table 3). However, there was no significant difference between T and RG or between T and T + RC ($P = 0.631$ and $P = 0.254$, respectively).

Silage concentration of lactic and acetic acid was 20.5 (41.7 vs. 21.2 g/kg DM, respectively, $P = 0.001$) and 4.65 g/kg DM (11.3 vs. 6.61 g/kg DM respectively, $P = 0.011$) greater across all cuts in the three-cut system than in the two-cut system, respectively, but there was no difference between the different species ($P = 0.515$ for lactic acid and $P = 0.262$ for acetic acid). The concentration of propionic acid in the silage was 0.41 g/kg DM greater in the three-cut system than in the two-cut system (1.27 vs. 0.86 g/kg DM respectively, $P < 0.001$), but there was no difference between different species ($P = 0.251$). Concentrations of butyric acid and ammonia nitrogen (NH_3-N) were not different between either harvest systems ($P = 0.305$ and $P = 0.220$, respectively) or species mixture ($P = 0.673$ and $P = 0.360$, respectively). Silage pH (5.0 vs 4.1, $P < 0.001$) and ethanol concentration (5.0 vs 1.4 g/kg DM, $P < 0.001$) were on average higher in the second cut of the two-cut than in the three-cut system, but did not differ between species mixtures ($P = 0.499$ and $P = 0.374$, respectively).

Effect of wilting and the use of silage additives on silage chemical constituents and fermentation characteristics. The concentration of NDFom was 20 g/kg DM greater in silage made from herbage wilted to 37.5% DM compared to 22.5% DM (501 vs. 482 g/kg DM respectively, $P = 0.040$, Table 4), while there was no effect of silage additive on NDFom concentration ($P = 0.398$). Concentrations of ash, iNDF, OMD, CP or sCP were not affected by wilting level or silage additive. Concentrations of WSC were 25 g/kg DM greater in silage made from wilted herbage compared to unwilted herbage (77 vs. 52 g/kg DM respectively, $P = 0.002$) and 54 g/kg DM greater in silage preserved with additive than without (91 vs. 38 g/kg DM, $P < 0.001$, Table 4).

		Three cuts			Two cuts				*P* value							
	Cut	T	T + RC	RG	T	T + RC	RG	SEM	Cut	Crop	Cut × crop	C1	C2	C3	C4	C5
Lactic acid, g/kg DM	1st	35.6	33.3	38.8	22.6	18.9	23.3	11.31	0.007	0.515	0.889	0.001	0.129	0.104	0.631	0.254
	2nd	33.3	40.7	34.2	15.7	24.9	21.6									
	3rd	40.6	71.3	47.2	–	–	–									
Acetic acid, g/kg DM	1st	7.44	9.95	17.0	5.89	5.44	7.91	3.319	0.069	0.262	0.831	0.011	0.068	0.396	0.111	0.273
	2nd	8.09	9.38	9.90	5.71	7.77	6.92									
	3rd	10.3	16.6	12.7	–	–	–									
Propionic acid, g/kg DM	1st	2.35	1.93	1.99	1.00	0.88	1.47	0.199	< 0.001	0.251	0.392	< 0.001	< 0.001	0.037	0.583	0.104
	2nd	1.26	0.76	0.82	0.56	0.64	0.60									
	3rd	0.81	0.73	0.75	–	–	–									
Butyric acid, g/kg DM	1st	7.32	8.91	8.09	3.25	3.20	5.87	1.986	0.048	0.673	0.889	0.305	0.018	0.458	0.849	0.512
	2nd	3.37	2.58	3.89	6.03	4.19	3.25									
	3rd	5.76	2.71	5.84	–	–	–									
Ethanol, g/kg DM	1st	2.44	3.08	1.94	2.18	1.63	1.71	0.700	< 0.001	0.374	0.964	< 0.001	0.268	< 0.001	0.486	0.478
	2nd	1.28	1.74	1.29	5.27	5.11	4.51									
	3rd	1.46	2.67	1.64	–	–	–									
Formic acid, g/kg DM	1st	5.17	5.79	6.61	4.16	3.58	3.74	2.995	0.374	0.970	1.000	0.065	0.411	0.157	0.934	0.808
	2nd	5.72	5.35	5.01	1.47	2.69	1.37									
	3rd	5.57	6.99	6.15	–	–	–									
pH	1st	4.42	4.50	4.24	4.22	4.41	4.31	0.193	< 0.001	0.499	0.939	< 0.001	0.639	< 0.001	0.496	0.243
	2nd	4.04	4.21	4.06	4.80	4.98	5.21									
	3rd	4.08	4.19	4.16	–	–	–									
NH_3, g/kg total N	1st	45.4	40.6	38.3	39.1	29.6	36.0	8.173	0.016	0.360	0.919	0.220	0.336	0.061	0.444	0.505
	2nd	33.1	31.9	46.8	56.9	50.4	64.3									
	3rd	38.9	43.6	47.9	–	–	–									

Table 3. Effect of Cut (1–5 where 1, 3 and 5 is the first, second and third cut in the three-cut system, and 2 and 4 is the first and second cut in the two-cut system) and crop (T, Timothy; T + RC, Timothy + red clover; RG, perennial ryegrass) on silage fermentation characteristics averaged across DM and additive treatments (n = 4). C1 is the contrast three versus two cuts per season overall; C2 is the contrast three versus two cuts per season, 1st cut; C3 is the contrast three versus two cuts per season, 2nd cut; C4 is the contrast T versus RG; C5 is contrast T versus T + RC.

	22.5% DM		37.5% DM			*P* value						
	Without	With	Without	With	SEM	Cut	DM	Cut × DM	Add	Cut × Add	DM × Add	Cut × DM × Add
DM, g/kg	250	253	405	414	4.952	< 0.001	< 0.001	< 0.001	0.240	0.807	0.541	0.980
Organic matter, g/kg DM	929	928	928	928	2.126	< 0.001	0.778	0.994	0.834	0.997	0.780	0.999
NDFom, g/kg DM	481	483	510	493	9.187	< 0.001	0.040	0.976	0.398	0.967	0.313	0.789
iNDF, g/kg NDFom	179	183	179	170	7.767	< 0.001	0.434	0.813	0.731	0.998	0.411	0.952
OMD, %	72.3	71.9	71.3	72.4	0.526	< 0.001	0.623	0.975	0.452	0.995	0.183	0.707
CP, g/kg DM	119	119	121	121	3.816	< 0.001	0.606	0.995	0.946	0.930	0.975	0.999
sCP, g/kg CP	647	609	623	624	22.24	< 0.001	0.859	0.655	0.405	0.195	0.389	0.731
WSC, g/kg DM	18.7	85.7	56.8	97.0	7.513	0.243	0.002	0.505	< 0.001	0.381	0.082	0.050

Table 4. Effect of wilting rate (target 22.5 or 37.5% DM) and silage additive (without or with) on silage feed quality parameters averaged across cuts and crop types (n = 15). DM, dry matter; NDFom, neutral detergent fibre; iNDF, indigestible NDF; OMD, organic matter digestibility; CP, crude protein; sCP, soluble crude protein; WSC, water-soluble carbohydrates. Cuts are numbered 1, 3 and 5 for the three consecutive cuts in the three-cut system and 2 and 4 for 1st and 2nd cut in the two-cut system, respectively. Cut × DM: Interaction between cut and dry matter; Add, Silage additive (with or without); Cut × Add: Interaction between cut and Add; DM × Add, interaction between DM and Add; Cut × DM × Add, three-way interaction between Cut, DM and Add.

The effect of additives on silage WSC content tended to be stronger at low compared to high wilting levels (DM by additive interaction, $P = 0.082$). Concentrations of lactic acid were, on average, 26 g/kg DM lower in silage made from herbage wilted to 37.5% DM compared to 22.5% DM (20 vs. 47 g/kg DM respectively, $P < 0.001$, Table 5) and 22 g/kg DM lower in silage preserved with additive than without (23 vs. 44 g/kg DM respectively, $P < 0.001$, Table 5). The effect of additive on silage lactic acid content was stronger in silage made from less wilted herbage than more extendedly wilted herbage (DM × Add, $P < 0.001$) and depended on cut (three-way interaction $P = 0.025$).

The effect of additive on silage lactic acid concentration was relatively stronger in the second and third cuts than in the first cut of the less wilted herbage of the three-cut system. Silage acetic acid concentrations were on average 6.7 g/DM greater in the silage wilted to 22.5% DM than in the silage wilted to 37.5% (13 vs. 6 g/kg DM respectively, $P < 0.001$), and 7 g/kg DM greater in silage preserved without silage additive than with additive (13 vs. 6 g/kg DM respectively, $P < 0.001$).

Concentrations of butyric acid in silage were, on average, 4 g/kg DM greater in low DM silages than in silages wilted to 37.5% (7 vs. 3 g/kg DM respectively, $P < 0.001$), and 2 g/kg DM greater in silage without additive compared to silage with additive (6 vs. 4 g/kg DM respectively, $P < 0.001$). Concentrations of NH_3-N were 19 g/kg DM greater in low DM silages compared to silages wilted to 37.5% DM (52 vs. 33 g/kg DM respectively, $P < 0.001$), and 16 g/kg DM greater in silages without additive compared to silages with additive (51 vs. 35 g/kg DM respectively, $P < 0.001$).

Effect of cutting system and crop type on in vitro rumen fermentation characteristics, in vitro total gas and CH_4 production, and fractional rate of gas production. The molar proportion of acetate in rumen fluid was, on average, 0.014 mmol/mmol greater in silage made from the two-cut system than

	22.5% DM		37.5% DM			*P* value						
	Without	With	Without	With	SEM	Cut	DM	Cut × DM	Add	Cut × Add	DM × Add	Cut × DM × Add
Lactic acid, g/kg DM	61.7	31.4	26.9	13.9	2.475	< 0.001	< 0.001	0.183	< 0.001	0.003	0.001	0.025
Acetic acid, g/kg DM	17.7	7.83	8.70	3.41	0.861	< 0.001	< 0.001	0.493	< 0.001	0.022	0.012	0.290
Propionic acid, g/kg DM	1.54	0.98	0.99	0.91	0.079	< 0.001	< 0.001	0.619	< 0.001	0.094	0.005	0.637
Butyric acid, g/kg DM	8.50	5.64	3.95	1.71	0.705	< 0.01	< 0.001	0.345	< 0.001	0.140	0.662	0.534
Ethanol, g/kg DM	3.44	2.61	2.70	1.36	0.254	< 0.001	< 0.001	0.433	< 0.001	0.003	0.319	0.720
Formic acid, g/kg DM	0.70	12.3	0.28	5.22	0.377	< 0.001	< 0.001	0.505	< 0.001	< 0.001	< 0.001	0.821
pH	4.17	4.14	4.51	4.73	0.050	< 0.001	< 0.001	< 0.001	0.050	0.312	0.021	0.002
NH_3, g/kg total N	63.4	41.3	38.0	28.8	2.102	< 0.001	< 0.001	0.020	< 0.001	0.877	0.004	0.854

Table 5. Effect of wilting rate (target 22.5% or 37.5% DM) and silage additive (without or with) on silage fermentation characteristics averaged across cuts and crop types (n = 15). Cuts are numbered 1, 3 and 5 for the three consecutive cuts in the three-cut system and 2 and 4 for 1st and 2nd cut in the two-cut system, respectively. Cut × DM: Interaction between cut and dry matter; Add, Silage additive (with or without), Cut × Add: Interaction between cut and Add, DM × Add: interaction between DM and Add, Cut × DM × Add, three-way interaction between Cut, DM and Add.

the three-cut system (0.618 vs. 0.632 mmol/mmol respectively, $P < 0.001$, Table 6), but the molar proportion of propionate did not differ between harvest systems ($P = 0.547$). Consequently, the acetate:propionate ratio was on average greater in the rumen fluid incubated with silages from the two-cut than three-cut system (3.07 vs. 2.98, $P = 0.034$). The molar proportion of butyrate was 0.007 mmol/mmol greater in the three-cut system compared to the two-cut system (0.104 vs. 0.097 mmol/mmol respectively, $P < 0.001$).

The molar proportion of acetate was 0.01 mmol/mmol greater in rumen fluid where silage made from T was incubated compared to RG (0.625 vs. 0.615 mmol/mmol respectively, $P < 0.001$) and 0.05 mmol/mmol greater

		Three cuts			Two cuts				*P* value[1]							
	Cut	T	T + RC	RG	T	T + RC	RG	SEM	Cut	Crop	Cut × crop	C1	C2	C3	C4	C5
Total VFA, mmol/L	1st	146	145	145	135	131	132	2.4	< 0.001	0.346	0.030	< 0.001	< 0.001	0.219	0.421	0.500
	2nd	139	140	135	138	134	133									
	3rd	132	144	138												
Molar Proportions																
Acetate, mmol/mmol	1st	0.621	0.628	0.600	0.634	0.636	0.619	0.003	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.001	< 0.001	0.007
	2nd	0.625	0.628	0.614	0.630	0.636	0.635									
	3rd	0.617	0.625	0.606												
Propionate, mmol/mmol	1st	0.200	0.201	0.221	0.205	0.207	0.216	0.003	< 0.001	< 0.001	0.009	0.547	0.534	0.979	< 0.001	0.986
	2nd	0.202	0.199	0.215	0.205	0.204	0.206									
	3rd	0.207	0.209	0.223												
Butyrate, mmol/mmol	1st	0.100	0.100	0.111	0.098	0.095	0.100	0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.015	< 0.001
	2nd	0.104	0.104	0.103	0.099	0.097	0.094									
	3rd	0.107	0.100	0.108												
Acetate:Propionate	1st	3.10	3.13	2.72	3.12	3.09	2.88	0.053	< 0.001	< 0.001	0.016	0.034	0.469	0.238	< 0.001	0.445
	2nd	3.10	3.16	2.87	3.10	3.14	3.09									
	3rd	2.99	3.01	2.73												
CH_4, mL/ g DM	1st	32.2	31.4	35.8	28.2	27.5	30.7	0.79	< 0.001	0.005	0.003	< 0.001	< 0.001	0.353	0.003	0.815
	2nd	30.1	31.2	30.2	29.8	30.4	29.1									
	3rd	30.3	30.7	31.6												
CH_4, mL/g OM	1st	34.6	33.7	38.7	30.0	29.1	32.9	0.86	< 0.001	< 0.001	0.002	< 0.001	< 0.001	0.299	< 0.001	0.460
	2nd	32.4	33.8	32.5	31.7	32.7	31.7									
	3rd	32.6	33.7	34.7												
CH_4, mL/g DOM	1st	46.0	44.4	50.1	45.4	44.0	49.7	1.17	0.004	< 0.001	0.017	0.309	0.711	0.309	< 0.001	0.827
	2nd	43.9	45.0	48.0	46.5	46.7	45.9									
	3rd	43.2	44.2	44.5												
Total gas, mL/g DM	1st	194	196	204	178	176	186	4.1	< 0.001	0.226	0.264	< 0.001	< 0.001	0.268	0.183	0.732
	2nd	183	176	180	175	178	174									
	3rd	190	190	190												
Total gas, mL/g OM	1st	208	210	221	189	186	200	4.4	< 0.001	0.032	0.219	< 0.001	< 0.001	0.217	0.014	0.751
	2nd	197	191	194	186	192	190									
	3rd	204	209	209												
Total gas, mL/g DOM	1st	277	277	285	285	281	301	5.9	< 0.001	0.002	0.007	0.047	0.087	0.462	0.008	0.365
	2nd	268	254	286	272	274	274									
	3rd	270	274	267												
CH_4, ml/L of total gas	1st	165	159	177	162	157	166	4.8	0.019	0.261	0.082	0.924	0.270	0.920	0.129	0.855
	2nd	164	178	168	172	171	168									
	3rd	160	160	165												
Fractional rate of gas production, /h	1st	0.058	0.061	0.064	0.058	0.055	0.060	0.0012	< 0.001	0.065	< 0.001	< 0.001	0.012	0.003	0.035	0.058
	2nd	0.058	0.061	0.059	0.056	0.056	0.055									
	3rd	0.060	0.064	0.061												

Table 6. Effect of Cut (1–5 where 1, 3 and 5 is the first, second and third cut in the three-cut system, and 2 and 4 is the first and second cut in the two-cut system) and crop (T, Timothy; T + RC, Timothy + red clover; RG, perennial ryegrass) on ensiled herbage in vitro rumen fermentation characteristics (total volatile fatty acids (VFA) and acids as molar proportion of total VFA), in vitro total gas CH_4 production, and fractional rate of gas production (n = 4). C1 is the contrast three versus two cuts per season, overall; C2 is the contrast three versus two cuts per season, 1st cut; C3 is the contrast three versus two cuts per season, 2nd cut; C4 is the contrast T versus RG; C5 is contrast T versus T + RC. DM, dry matter; OM, organic matter, DOM, digestible organic matter.

in rumen fluid from T + RC than T (0.631 vs 0.625 mmol/mmol, respectively, $P = 0.008$, Table 6). There was no difference in the molar proportion of propionate between T and T + RC ($P = 0.986$). However, the molar proportion of propionate was 0.012 mmol/mmol greater in rumen fluid with RG than with T ($P < 0.001$), and the acetate:propionate ratio was greater in rumen fluid with T than RG (3.08 vs 2.86, $P < 0.001$). The molar proportion of butyrate was 0.001 mmol greater in RG compared to T ($P = 0.015$). Timothy resulted in a 0.003 mmol greater butyrate proportion compared to T + RC ($P < 0.001$).

CH_4 production was, on average, 2.2 ml/g DM and 2.8 mL/g OM greater in silage made from the three-cut systems compared to the two-cut systems (31.5 vs 29.3 mL/g DM and 34.1 vs. 31.3 mL/g OM respectively, $P < 0.001$, Table 6). The first cut taken at a later stage of maturity reduced CH_4 production by 4.6 ml/g DM and 5 mL/g OM compared to an earlier stage of maturity (30.6 vs. 35.7 mL/g OM respectively, $P < 0.001$), but there was no difference between harvest systems in the second cut ($P > 0.2$). CH_4 production expressed per g digestible organic matter (DOM) was not affected by cutting system ($P > 0.1$, Table 6).

CH_4 production was, on average, 1.3 ml/g DM, 1.9 mL/g OM and 2.6 mL/g DOM greater in silage from RG than T (31.5 vs. 30.1 mL/g DM, 34.1 vs. 32.2 mL/g OM, and 47.6 vs. 45.0 mL/g DOM, respectively, $P < 0.01$), but there was no difference between T and T + RC ($P > 0.4$).

Total gas production was, on average, 6 mL/g DM and 14 mL/g OM greater in silage from the three-cut system than the two-cut system (189 vs. 178 mL/g DM and 204 vs. 190 mL/g OM respectively, $P < 0.001$), but total gas produced per g DOM was on average greater in the two-cut system than in the three- cut systems (281 vs 273 ml/g DOM). Total gas production was 5 mL/g OM and 8 mL/g DOM greater in RG compared to T (202 vs. 197 mL/g OM and 283 vs 275 g/ DOM, respectively, $P < 0.05$). However, there was no difference between T and T + RC in total gas production ($P > 0.3$). There was no significant effect of cutting system or crop type on CH_4 production relative to total gas production (Table 6).

The fractional rate of gas production was 0.004 units greater in the three-cut system compared to the two-cut system (0.061 vs. 0.057/h respectively, $P < 0.001$), and 0.002 units greater in RG compared to T (0.060 vs. 0.058/h, respectively, $P = 0.035$).

Effect of wilting and use of silage additive on in vitro total gas and CH_4 production, VFA production, and fractional rate of gas production. Rumen fluid molar proportion of acetate was 0.01 mmol/mmol greater from the incubation with wilted grass silage than the less wilted grass silage (0.628 vs. 0.618 mmol/mmol respectively, $P < 0.001$, Table 7), and silage preserved with additive also increased the molar proportion of acetate in the less wilted silage ($P = 0.002$). The molar proportion of propionate in rumen fluid was 0.01 mmol/mmol greater with the less wilted than with more extensively wilted grass silage (0.212 vs. 0.204 mmol/mmol respectively, $P < 0.001$), and the rumen fluid molar proportion of propionate was 0.01 mmol/mmol greater with grass silage preserved without additive than with additive (0.211 vs. 0.205 mmol/mmol respectively, $P < 0.001$). The acetate:propionate ratio in the rumen fluid increased both with wilting rate (2.93 vs. 3.10, $P < 0.001$) and with the use of additive (2.95 vs. 3.08, $P < 0.001$), but the effect of additive was stronger at 22.5% DM than at 37.5% DM as indicated by wilting rate by additive use interaction ($P = 0.007$, Table 7).

	22.5% DM		37.5%DM			*P* value						
	Without	With	Without	With	SEM	Cut	DM	Cut × DM	Add	Cut × Add	DM × Add	Cut × DM × Add
Total VFA, mmol/L	139	138	136	138	1.3	< 0.001	0.235	0.085	0.604	0.608	0.109	0.514
Molar Proportions												
Acetate, mmol/mmol	0.611	0.625	0.626	0.630	0.00	< 0.001	< 0.001	0.839	< 0.001	< 0.001	0.002	0.248
Propionate. mmol/mmol	0.217	0.207	0.205	0.202	0.001	< 0.001	< 0.001	0.923	< 0.001	0.023	0.027	0.400
Butyrate, mmol/mmol	0.103	0.102	0.100	0.100	0.001	< 0.001	0.002	0.687	0.490	0.665	0.512	0.381
Acetate:Propionate	2.83	3.03	3.06	3.13	0.028	< 0.001	< 0.001	0.850	< 0.001	0.007	0.019	0.447
CH_4, ml/g DM	30.3	31.5	29.8	30.9	0.47	< 0.001	0.214	0.694	0.002	0.273	0.852	0.041
CH_4, mL/g OM	32.6	34.0	32.1	33.3	1.11	< 0.001	0.235	0.721	0.003	0.272	0.817	0.056
CH_4, mL/g DOM	45.1	47.2	45.1	46.0	0.72	0.005	0.318	0.493	0.007	0.455	0.266	0.183
Total gas, mL/g DM	186	188	181	184	2.6	< 0.001	0.019	0.333	0.130	0.155	0.978	0.060
Total gas, mL/g OM	200	203	195	198	2.9	< 0.001	0.025	0.359	0.143	0.185	0.931	0.076
Total gas, mL/g DOM	276	282	273	273	4.1	< 0.001	0.052	0.282	0.316	0.230	0.255	0.282
CH_4, ml/L of total gas	163	169	164	169	2.7	0.031	0.750	0.362	0.033	0.374	0.838	0.774
Fractional rate of gas production, /h	0.060	0.060	0.057	0.060	0.0006	< 0.001	0.005	0.390	0.002	< 0.001	0.120	0.003

Table 7. Effect of wilting rate (target 22.5% or 37.5% DM) and silage additive (without or with) on silage in vitro rumen fermentation characteristics (total volatile fatty acids (VFA) and acids as molar proportion of total VFA), in vitro total gas and CH_4 production, and coefficient of degradation (n = 15). Cuts are numbered 1, 3 and 5 for the three consecutive cuts in the three-cut system and 2 and 4 for 1st and 2nd cuts in the two-cut system, respectively. Cut × DM: Interaction between cut and dry matter; Add, Silage additive (with or without); Cut × Add: Interaction between cut and Add; DM × Add, interaction between DM and Add; Cut × DM × Add, three-way interaction between Cut, DM and Add. DM, dry matter; OM, organic matter, DOM, digestible organic matter.

There was no effect of herbage wilting rate on in vitro CH_4 production (mL/g DM, mL/g OM, mL/g DOM, $P > 0.2$), but the use of silage additive increased in vitro CH_4 production 1.2 mL/g DM, 1.2 mL/g OM, and 1.6 mL/g DOM compared to silage preserved without additive (31.2 vs 30.0 mL/g DM, 33.6 vs. 32.4 mL/g OM, and 46.6 vs. 45.0 mL/g DOM, respectively, $P < 0.01$). The effect of silage additive on CH_4 production per g DM depended on cut and wilting rate, as indicated by a three-way interaction effect between cut, wilting rate and additive ($P = 0.041$). The interaction is illustrated in Fig. 1 showing that use of the additive generally increased CH_4 production in the three-cut system but reduced CH_4 production in the silage produced at the low wilting level of the second cut. Total gas production was not affected by using silage additive ($P > 0.1$), but the less wilted silage (22.5% DM) resulted in 4 mL/g DM and OM more gas production than the silage preserved at 37.5% DM (Table 7, $P < 0.05$). CH_4 production of total gas production was 4.9 ml/L higher with the use of additive (168 vs 164 ml/L, $P = 0.033$, Table 7). Fractional rate of gas production was greater on low than high wilting rate (0.059 vs. 0.058/h respectively, $P = 0.005$) and greater with additive than without additive (0.060 vs. 0.058/h respectively, $P = 0.002$). However, the additive effect depended on both cutting system and wilting level, as indicated by the significant three-way interaction ($P = 0.003$). The use of additive increased the fractional rate of gas production in the three-cut system but decreased the rate in the second cut of the two-cut system (figures not shown).

Correlations between grass silage parameters and in vitro CH_4 production. The quantity of CH_4 produced per g DM and OM incubated was positively associated with silage OMD ($r = 0.47$ and 0.53, respectively, $P < 0.001$, Table S4), formic acid concentration ($r = 0.34$ and 0.35, respectively, $P < 0.01$), and tended to be positively associated with silage WSC concentration ($r = 0.21$ and 0.22, respectively, $P < 0.10$). CH_4 produced per g OM incubated was also positively associated with silage CP concentration ($r = 0.28$, $P < 0.05$) and CH_4 produced per g DM tended to correlate with silage CP ($r = 0.23$, $P < 0.10$). Both CH_4 produced per g DM and per g OM were negatively associated with silage NDFom concentration ($r = -0.45$ and -0.54, respectively, $P < 0.001$). The CH_4 produced per g DOM incubated was negatively associated with silage OMD ($r = -0.31$. $P < 0.05$), CP ($r = -0.26$, $P < 0.05$), and lactic acid concentrations ($r = -0.35$, $P < 0.01$), but tended to be positively associated with silage WSC concentration ($r = 0.23$, $P < 0.10$).

Discussion

Higher total DM yield (Table 1) in the two-cut than in the three-cut system is in accordance with other studies[26,27]. Prolonged harvest interval with increased maturity of the plant in a two-cut system increases the proportion of cell wall structures with greater concentrations of aNDFom and total DM[28,29].

Perennial ryegrass had a greater total DM yield than both T and T + RC. This was in accordance with a previous study investigating the difference between RG and T in harvest systems with four cuts per season, where T yielded more than RG only in the first cut[30]. Field trials of similar species and cultivars harvested annually two and three times showed similar DM yields for T and RG across two production years[31].

The greater NDFom and iNDF concentration in the two-cut system compared to the three-cut system was a result of harvesting the crop at a more mature phenological stage in the two-cut system, with an increased

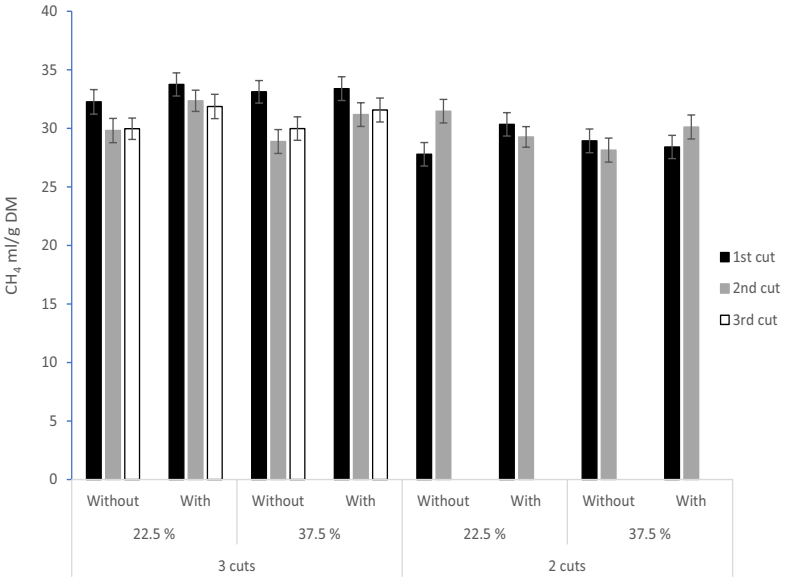

Figure 1. Three-way interaction between harvest regime (2 or 3 cuts per season), wilting levels (22.5% or 37.5% DM) and additive use (with or without formic acid-based additive) on in vitro methane production (mL/g DM). Bars represent standard error of the mean (n = 3).

proportion of cell walls and accumulation of indigestible lignin in the cell wall structures[28,29]. The effect of increased growth stage on increased silage concentration of NDFom, iNDF and reduced concentration of CP and reduced OMD, as seen in the present study, has also been reported in previous experiments on grass and clover silages[32,33].

The lower fibre (aNDFom) concentration in RG compared to T in the present study has also been reported from a study in Ireland[34]. However, the greater iNDF concentration in RG, particularly of the second cut in the three-cut system, relative to both T and T + RC was surprising, especially since legumes like red clover normally have a greater iNDF:aNDFom ratio compared to grasses[15,35]. However, an increase in iNDF concentration of RG regrowth related to herbage mass has also been reported by Garry et al.[36]. The increased iNDF concentration of RG reduced OMD, which ultimately resulted in no difference between T and RG in OMD. The greater OMD in T + RC compared to T was expected, as the cell wall (NDF) concentration is normally lower in legumes compared to grasses and inhibition of cell wall (NDF) digestion by lignification with maturity is stronger in grasses than in legumes[37,38].

The concentration of WSC was greatest and the concentration of lactic acid was lowest in silage wilted to 37.5% DM and treated with formic acid, which is in accordance with previous studies[24,39,40]. The addition of formic acid reduces pH by immediate acidification and restricts fermentation of WSC[39,41], and wilting reduces microbial activity in the silage, thereby restricting fermentation intensity[21].

The fermentation quality of the silages was in general more affected by the use of additives and wilting levels than harvest systems or species. Judged by the concentration of lactic acid, acetic acid, propionic acid, ethanol, NH_3-N, and pH of the silages, the fermentation quality was acceptable, with generally low levels of fermentation products compared to other studies in grass and legume silage[42–44]. However, the butyric acid levels were high in silage with 22.5% DM and without additives (on average 8.5 g/kg DM, Table 5), which is not uncommon in silages without the use of chemical additives that prevent clostridia[45,46]. Higher concentration of ethanol in the second cut in the two-cut system (Table 3) than in the other cuts is difficult to explain. Usually, high concentrations of ethanol are associated with high numbers of yeasts[44], which we did not record. However, even the highest ethanol concentrations observed in the current study are within levels regarded as typical (5—10 g/kg DM) for grass and legume silages[44]. The formation of NH_3-N during ensiling is a result of degradation of plant protein caused by plant enzymes and proteolytic microbes like Clostrida. We have no records of the epiphytic flora or activity of plant proteases, but it is found that both plant proteases and epiphytic microbiota is affected by growth stage[47,48]. It has been reported higher protease activity and higher content of NH3-N in silage made from grass harvested at more mature growth stage than early[47]. The reduction of NH_3-N concentration as a proportion of total-N in the silage with wilting and use of the formic acid-based additive is a consequence of restricted fermentation[42,44]. There were no association between silage ethanol and butyric acid concentration, NH_3-N or ethanol concentration and in vitro CH_4 production (Table S4).

The greater in vitro CH_4 production observed for silage made from the three-cut system compared to the two-cut system coincides with greater OMD and lower aNDFom and iNDF concentrations. This is because CH_4 is an end product from the rumen bacteria fermentation of digestible carbohydrates, like cell wall polymers and fructans, to VFA, H_2 and CO_2[49]. Holtshausen et al.[50] also reported increased in vitro CH_4 production (mL and mL/g NDF digested) when grass was ensiled from material harvested at early maturity, but no difference between grass maturity stages when CH_4 production was expressed per g dry matter disappearance. We did not measure dry matter or organic matter disappearance in our in vitro cultures, but CH_4 production per g digestible organic matter was not affected by maturity stage (Table 6) and as such in line with Holtshausen et al.[50].

The acetate:propionate ratio in the rumen fluid was greater with silages made from the two-cut system than in the three-cut system, because of greater acetate production. It is well established that changes in ruminal VFA production towards a higher acetate:propionate ratio might increase CH_4 production as acetate production generates H_2 which is converted to CH_4 by the methanogen microbiota[16]. However, in the present study, the greater acetate:propionate ratio in the two-cut system did not coincide with greater CH_4 production. According to Johnson and Johnson[51], there are two primary mechanisms regulating CH_4 production, 1) the total amount of fermentable carbohydrates in the rumen and 2) changes in H_2 supply through changes in VFA production. We speculate that the primary mechanism behind higher CH_4 production in the three-cut silages in the present study was the amount of fermentable substrates, as indicated by greater OMD, giving higher total rumen fluid VFA production but only small effects on the molar proportions of actetate and propionate.

The on average greater in vitro CH_4 production (mL CH_4/g OM) from RG silages compared to that from T in the present study is in accordance with Purcell et al.[9], who also observed greater in vitro CH_4 production (mL/g DM incubated) in RG compared to T. Although the direction of rumen fluid fermentation in the present study was more methanogenic in T compared to RG, with a greater acetate:propionate ratio, the rate and extent of in vitro fermentation in RG was greater, as shown by the total gas (mL/g OM and DOM) and fractional rate of gas production (Table 6). This was likely a result of a greater total substrate availability, H_2 production and CH_4 production in RG compared to T. In addition, T had a greater NDFom concentration compared to RG.

A previous study showed that a greater NDFom concentration resulted in lower CH_4 production[13], which might explain why CH_4 production was lower in T than RG in the present experiment. It has been shown that diets with red clover reduced in vivo CH_4 production compared to diets with grass in cattle (17.8 vs 21.2 g/kg DM intake, respectively)[52]. However, in the present study, we did not observe lower CH_4 production in T + RC compared to T. This supports earlier findings showing no such effect in diets with 60/40 perennial ryegrass and clover[20]. The inconsistency in the literature may be due to differences in forage quality, chemical composition, or herbage red clover proportion.

The silage produced without additive had a greater lactic acid concentration, resulting in a greater concentration of propionate when incubated in rumen fluid and was less methanogenic than silage produced with a formic acid-based additive. This is in line with a previous in vitro study that also demonstrated that propionic

acid production in rumen fluid consumes H_2 resulting in a lower in vitro CH_4 output[53]. In vivo studies have also shown that lactic acid in silage is transformed into propionic acid in the rumen[54,55]. In addition, the silages produced with the additive contained residual formic acid (12.3 and 5.2 g/DM, Table 5), which may have contributed to higher CH_4 production as demonstrated in other in vitro fermentation studies where increasing levels of formic acid or formate were added to the substrate[56,57]. This is supported by the positive association between CH_4 production (ml/g DM and mL/g OM) and silage formic acid concentration in the correlation analysis of the present study (Table S4). Stronger effect of the formic acid-based additive on CH_4 production in the three- than in the two-cut system and from the less wilted silage in the three-cut system (Fig. 1) is likely due to greater increase in rumen fluid acetate relative to propionate production (Table 7) and higher residual formic acid concentration (Table 5).

Previous studies have shown that increased DM concentration and reduced fermentation intensity in grass silage retain more WSC in the silage[22,23]. The present study also showed that DM levels affected WSC concentration. However, as we found no effect of wilting level and DM concentration on CH_4 production, the role of WSC in affecting CH_4 production was probably not as prominent as reported in other studies[11,13]. However, the correlation analysis indicated a tendency ($P < 0.10$) for a positive relationship between CH_4 production and silage WSC concentration (Table S4).

This study showed that less frequent harvesting, extensive silage fermentation in the absence of silage additives, and the use of T as a grass species reduced in vitro CH_4 production, while the use of formic acid based additive increased in vitro CH_4 production. We recognise that these results must be confirmed in vivo along with animal production data.

In conclusion, our results confirmed the hypothesis that less frequent harvest and extensive silage fermentation reduce in vitro CH_4 production. The effect of harvest frequency was mainly due to increased NDFom and iNDF concentration and reduced OMD in the two-cut system compared to the three-cut system, implying reduced amount of fermentable substrate available for rumen microorganisms. The effect of extensive silage fermentation was caused by an increased lactic acid production in the silage, increased rumen fluid propionate production and ultimately reduced CH_4 production. Although we found that CH_4 production was lower in T than in RG, this was probably not due to differences in WSC concentration but rather due to differences in the total substrate availability. Lastly, our results do not support the hypothesis that restricted lactic acid fermentation by wilting the crop before ensiling increases in vitro CH_4 production, but that restricting silage fermentation by use of formic acid as an additive increase CH_4 production most likely due to residual formic acid. Wilting resulted in a higher content of WSC, but there was no direct effect of DM level on in vitro CH_4 production.

Methods

Experimental design. Silages were made from three crops: pure timothy (T; *Phleum pratense* L., cv. 'Liljeros'), timothy and red clover mixture (T + RC; mixture of 85% timothy, cv. 'Liljeros' and 15% red clover, *Trifolium pratense* L., cv. 'Gandalf', based on seed weight) and pure perennial ryegrass (RG; *Lolium perenne* L., cv. 'Figgjo'), harvested two (H2) or three (H1) times per season. After harvest, the crop was wilted to two different dry matter levels, target was 225 and 375 g DM/kg, and fermented with a formic acid-based additive, or without additive, and later analysed for chemical composition, fermentation products and in vitro and in situ characteristics. The design was factorial with two harvesting systems × three crops × two DM levels × two additive treatments. The field layout was a split plot design with a harvest regime on main plots and crop on sub-plots. There were four field replicates of all wilting rates and additive combinations within the harvests. Silages made from replicate 1–3 were used for further analysis, and silages made from replicate 4 were used as spare samples.

Establishment of ley. The field trial was established on a medium sandy soil with high organic matter content (10.7% loss of ignition), pH of 5.9 and medium levels of plant available P (P-AL = 7.6 mg/100 dry soil) and K (K-AL = 7.9 mg/100 g dry soil). The crops were sown at a seeding rate of 25 kg ha^{-1} for T, 20 kg ha^{-1} timothy and 5 kg ha^{-1} clover in T + RC, and 35 kg ha^{-1} perennial ryegrass in RG, in four replicated blocks on 22 May 2019 at the Norwegian Institute of Bioeconomy Research, Fureneset (61°17.6′ N, 5°2.9′ E; elevation 30 m a.s.l.). Just before sowing, 4 hl ha^{-1} of lime, 35 tonnes ha^{-1} of cattle slurry + 60 kg N in NPK 18-3-15 was applied. In the spring of the first production year (year 2020), H1 plots received 150 kg N ha^{-1} in spring, 100 kg N ha^{-1} after the first cut, and 30 kg N ha^{-1} after the second cut, while the H2 plots received 160 kg N ha^{-1} in spring and 100 kg N ha^{-1} after the first cut. The clover plots (T + RC) received 50% of the N amount applied to the grass plots (T and RG). No weed control was needed.

The experiment was performed in accordance with all relevant institutional, national, and international guidelines and regulations for experimental research and field studies on plants/plant materials, such as the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora. The research did not involve rare or endangered species of fauna or flora, or species at risk of extinction. Timothy, red clover and perennial ryegrass are common species used in grassland cultivation in Norway; they are not protected species under national conservation laws and no permissions or licenses are required for the cultivation.

Harvest. The crop was cut to a stubble height of 8 cm using a Haldrup grass harvester (J. Haldrup a/s, Løgstør, Denmark / Haldrup GmbH, Ilshofen, Germany). In the experimental year (2020), the first cut was taken on 2 June (H1) and 16 June (H2), the second on 14 July (H1) and 11 August (H2) and the third on 1 September (H1). The phenological development stage of timothy at harvest was determined as the mean stage by count[58]. At harvest, a grab sample was taken from each grass clover plot (T + RC) and frozen for later hand separation into clover and grass fractions. The samples were dried at 60° for 48 h and weighed. The red clover proportion (% DM

yield) in the three-cut system of T + RC was 6%, 12% and 44% in the first, second and third cuts, respectively and 1% and 18% in the first and second cuts, respectively, of the two-cut system.

Wilting and preservation. Approximately 2 kg of fresh crop from each plot were sampled at harvest and were frozen, while another 15 kg of fresh crop material was put into plastic mesh containers (6 containers with approximately 2.5 kg in each), weighed and moved indoors, where the crop from three boxes were force dried, in ambient temperature, to target level of 225 g DM/kg and the other three to target level of 375 g/kg DM. Target DM was verified by weighing the boxes regularly and final DM was determined by freeze drying. The wilted crop was chopped to lengths of 1–2 cm using a Hans-Ulrich Hege Saatzuchtmaschinen (Hohebuck, Waldenburg, Germany). From each of the two wilting levels, three chopped samples were taken and weighed to contain approximately 350 g DM each. One sample was frozen, while the two others were preserved as silage in evacuated and sealed polyethylene bags (Magic Vac IL VERO Scottvuoto, Flaem Nuova SpA., Brescia, Italy). Each bag was subjected to vacuum (− 1 bar) for about 60 s using a LAVA V300 Premium (Bad Saulgau, Germany). The control treatment (C) received no additive, while the other (G) received 4 ml/kg GrasAAT Lacto (Addcon Gmbh, Bitterfeld-Wolfen, Germany), containing 57%–67% formic acid, 14%–18% sodium formate, 1%–2% lactose. All bags were stored in a dark room with ambient temperature for 3 months. Thereafter, the bags were frozen at − 20 °C until further preparation, chemical analysis, and in vitro gas testing.

Sample preparation and chemical analysis. The fresh (n = 45) and wilted samples (n = 90) were lyophilised and milled using a Tecator Cyclotec 1093 mill (Foss Tecator AB. Högans. Sweden), 1 mm mesh screen and split in two, where one subsample was analysed chemically at the Swedish University of Agricultural Sciences (SLU, Umeå). The DM concentration was determined by oven drying at 105 °C for 16 h, and ash concentration was determined by combustion of the dried samples at 500 °C for 4 h[59]. CP concentration was calculated from the nitrogen concentration (N × 6.25) measured by the Kjeldahl method[60], using a 2520 digestor, Kjeltec 8400 analyser unit and 8460 sampler unit (all from Foss Analytical, Hillerød, Denmark). The ash-corrected neutral detergent fibre (NDFom)concentration was determined with the filter bag technique in an Ankom200 Fiber analyser (Ankom Technology Corp., Macedon, NY) using a heat stable α- amylase and sodium sulfite[61]. The analyses are presented in Tables S1–S3.

The other subsample of fresh or wilted samples was analysed at LabTek, Norwegian University of Life Sciences (NMBU) for DM (104 °C, ISO 6496), WSC according to Randby et al.[62] and buffer soluble CP (sCP) according to Licitra et al.[63]. The analyses are presented in Tables S1–S3.

The frozen silages were split into two subsamples. One subsample was sent frozen to the Swedish University of Agricultural Sciences (SLU, Ultuna) and analysed for pH (Metrhom, Herisau, Switzerland), NH_3-N[64], lactic acid, acetic acid, propionic acid, 2,3-butandiol and ethanol using high-performance liquid chromatography[65]. The analyses are presented in Tables 3 and 5.

The other silage subsample was lyophilised and split into three subsamples. Two subsamples were milled using a Tecator Cyclotec 1093 mill (Foss Tecator AB. Högans. Sweden), a 1 mm mesh screen; one was stored frozen as a spare sample without any processing, while the second was merged with the two other field replicates of the same treatment and stored frozen in a sealed plastic bag. The merged samples were split into two samples; one was used in the in vitro gas test at SLU Umeå, and analysed for DM, ash, nitrogen, and NDFom as described above for fresh and wilted samples. The analyses are presented in Tables 2 and 4.

The other merged sample was analysed for DM (60 and 104 °C), WSC and sCP at LabTek (NMBU) as described above for fresh and wilted material, and presented in Tables 2 and 4. The third freeze-dried silage subsample was milled using a Tecator Cyclotec 1093 mill (Foss Tecator AB, Högans, Sweden), a 2 mm mesh screen, merged with the two other field replicates of the same treatment for determination of indigestible NDF (iNDF) at SLU, Umeå.

In situ and in vitro measurements. All experimental procedures involving animals were approved by the Swedish Ethics Committee on Animal Research (Umeå, Sweden) and in accordance with Swedish laws and regulations regarding EU Directive 2010/63/EU on animal research. The iNDF concentration of the samples was determined as NDF after 288 h in situ rumen incubation, as described by Krizsan et al.[66], using three ruminal cannulated lactating Nordic Red cows. The cows were fed for at least 14 days before in situ incubation a total mixed ration consisting of grass silage and a concentrate mixture (0.6:0.4 on a DM basis) ad libitum, which covered the animal's energy and protein requirement. Samples of 2 g were weighed into polyester bags with 11 µm pores and a pore area equal to 5% of the total surface area (Sefar Petex 07–11/5-cloth, Sefar AG, Heiden, Switzerland). Organic matter digestibility (OMD, g/g) was calculated from concentrations (g/kg DM) of iNDF and NDFom according to Huhtanen et al.[12]:

$$\mathrm{OMD} = 0.882 - 0.00121 \times \mathrm{iNDF} - 0.00011 \times \mathrm{NDF}$$

Data on OMD and iNDF is presented in Tables 2 and 4.

Rumen fluid for the in vitro gas trial was collected approximately 2 h after morning feeding from two fistulated Nordic Red cows fed the same diet as described for the in situ measurements. The rumen fluid was kept in 2 steel thermoses that had been prewarmed and flushed with CO_2 to ensure an anaerobic environment. The pH value of the rumen fluid (mean 6.27, standard deviation 0.12) was recorded (744 pH Meter; Metrohm Ltd., Herisau, Switzerland) before it was filtered through four layers of cheesecloth into a measuring cylinder continuously flushed with CO_2.

A total of 483 mL of rumen fluid was transferred through a funnel into another measuring cylinder containing 483 mL of buffer solution mixed with micro- and macro minerals, as described by Menke and Steingass[67], at 39 °C under constant stirring and continuous flushing with CO_2.

Feed samples were incubated in 60 mL of buffered rumen fluid and placed in a water bath at 39 °C, with continuous agitation for 48 h. The in vitro gas production experiment was conducted using a fully automated gas production technique described by Cone et al.[68], in which the total gas volume was automatically recorded at 0.2-h intervals and corrected for normal atmospheric pressure (101.3 kPa).

Gas samples for in vitro CH_4 determination were sampled every 2, 4, 8, 24, 32 and 48 h from each bottle using a gas tight syringe (Hamilton, Bonaduz, Switzerland). The concentration of CH_4 was determined by injecting 0.2 mL of gas into a Star 3400 (CX series) gas chromatograph (Varian Chromatography, USA) equipped with a thermal conductivity detector (TCD)[69]. After 24 and 48 h of incubation, one ML of rumen fluid was collected from the bottles, mixed with 200 µl of 22 M formic acid and stored at − 18 °C until analysis.

The concentration of VFA in the rumen fluid was determined using high-performance liquid chromatography (HPLC), and the acids were separated using a packet ReproGel H column (Ammerbuch, Germany). They were further detected with an RI 2414 detector (Waters Assoc, USA). These procedures were repeated in a total of seven runs and all samples were incubated at least three times (n = 3 runs/silage). All runs included 36 bottles; in each run, 30 bottles contained silage samples, four bottles contained standard hay and two bottles contained blanks (i.e., bottles contained only 60 mL buffered rumen fluid). The 60 silage samples (in triplicates) were randomly allocated to the seven in vitro runs and the same sample was never incubated more than once within a run and never in the same bottle. The analyses are presented in Tables 6 and 7.

Statistical analyses. The data analysis of the constituents in fresh, wilted and ensiled forages were derived from linear mixed-effects models using the procedure GLIMMIX in SAS (Version 9.4, SAS Institute Inc., Cary, NC, USA).

The constituents in fresh and wilted material constituents were modelled with cut (numbered chronological from 1 to 5, where 1, 3 and 5 is the first, second and third cut in the three cuts system and 2 and 4 is the first and second cut in the two cuts system), crop (T, T + RC or RG), wilting level (225 or 375 g/kg DM) and their interactions as fixed effects and field replicate (1–3) as random effects.

In order to test the effect of harvest system and species mixture on the constituents in ensiled material, cut (1–5), seed mixture (T, T + RC or RG) and their interactions were treated as fixed effects, and silage additive (without or with GrasAAT Lacto) and wilting level (225 or 375 g/kg DM) as random effects. The effects of silage additive and wilting were tested with cut (1–5), silage additive (without or with GrasAAT Lacto), wilting level (225 or 375 g/kg DM) and their interactions as fixed effects and species mixture as random effect. The data for total gas production (mL/g OM), CH_4 production (mL/g OM), fractional rate of gas production (/h) and VFA (mmol/L in vitro fluid) were analysed using the same model as for silage constituents but included in addition the fixed effect of run (1–7) and the random effect of bottle (1–36).

The effect of the harvest system (two or three cuts per season) across seasons and within cuts and the separation of crops were tested using orthogonal contrasts. Tukey's test was used for pairwise comparisons of means. Significance of effects were declared at $P \leq 0.05$ and trends $0.05 < P \leq 0.10$.

Residual normality was assessed using plots = residual panel option in GLIMMIX, with no data showing deviation from normal distribution.

Pearson correlation coefficients were calculated to determine relationships between the individual silage's chemical composition, fermentation parameters and CH_4 production using the procedure CORR in SAS.

Significance of effects were declared at $P \leq 0.05$ and trends $0.05 < P \leq 0.10$.

Data availability

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

H.S. initiated the study. H.S., K.V.W., L.Ø., I.D., M.E., and S.J.K. designed the study. K.V.W., L.Ø., and H.S. conducted field trial and the silage preparation. S.J.K. performed the gas in vitro trial. K.V.W. and H.S. analysed the results and wrote the main manuscript text. All authors read and approved the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Paper III

Interpretive Summary: Effect of changes in silage quality on enteric methane emissions in dairy cows. *Weiby et al.* Dairy farmers need to reduce their environmental impact and changes in silage quality can mitigate methane emissions. This study aimed to quantify the effect of grassland species and harvest frequency on enteric methane emissions in dairy cows. Timothy obtained 5.2% lower methane intensity compared to perennial ryegrass and increasing red clover proportion in the diet from 0 to 100% linearly increased methane intensity 9.8%. Changing from two to three cuts per season in timothy reduced methane intensity 6.8%.

Effects of grassland species and harvest frequency on milk production and enteric methane emission in dairy cows

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ABSTRACT

Methane emission from ruminant production systems account for 5% of global greenhouse gas emissions, however the impact of forage management remains unclear. The aim of this study was to quantify the effect of grassland species and harvest frequency on feed intake, milk production, and methane (CH_4) emission in dairy cows. We hypothesized that more frequent harvesting, use of grass species with greater organic matter digestibility and legumes with lower NDFom concentration would increase silage dry matter intake and milk yield and thereby decrease CH_4 yield and intensity.

Forty Norwegian Red cows (15 primiparous and 25 multiparous) in early- to mid-lactation (102 ± 21.2 DIM; mean ± SD), weighing 584 ± 79 kg and yielding 30.2 ± 6.0 kg of milk/d were blocked according to parity, days in milk and body weight and within block randomly allocated to five treatments in a cyclic changeover design comprised of four 21-d periods (14 d of adaptation, 7 d of recording and sampling). The five treatment diets evaluated silages produced from timothy (*Phleum pratense* L.) in a three-cut system (T3), timothy in a two-cut system (T2), perennial ryegrass (*Lolium perenne* L.) in a three-cut system (PR3), red clover (*Trifolium pratense* L.) in a three-cut system (RC3) and a mix of T3 and RC3 (50:50 on DM basis) (T3/RC3). The proportion of DM from spring growth, first regrowth and second regrowth in the different treatment diets was based on each cut's share of the total DM yield over the season. Mass fluxes of enteric CH_4 and CO_2 were measured using two Greenfeed units, and the cows were offered the experimental diets *ad libitum* in forty electronic feeding bins designed to measure feed intake of each cow. Milk yield was recorded in the milking robot at each visit, and milk samples were collected from the cows at three consecutive milkings during the last 7 d of each period. Cows were weighed after each milking, and total tract digestibility of each diet was estimated using acid insoluble ash as internal marker in fecal grab samples. The data were analysed using the MIXED procedure of

SAS with block, period and treatment as fixed effects and animal within block as random effect. Silage and total DMI did not differ between T3 and T2 diets, but total DMI was lower for PR3 than for T3. There was a quadratically effect of increased proportion of red clover, with lower intakes of RC3 than of T3.

Energy corrected milk (ECM) yield was lower for T2 than T3, and for PR3 compared with T3. There was a quadratically effect of increased proportion of red clover, with lower ECM yield in RC3 than in T3. Organic matter digestibility was lower for T2 compared with T3, but it did not differ between T3 and PR3. Including red clover in the diet linearly decreased organic matter digestibility. Methane production (g/d) did not differ between T3 and T2, but CH_4 intensity (g/kg ECM) was greater for T2 than for T3. There was no difference between T3 and PR3 for CH_4 production but yield and intensity were greater for PR3 than T3. Including red clover in the diet linearly increased CH_4 production, yield and intensity with numerically greatest intensity in the 100% red clover diet.

In conclusion, changing harvesting frequency for timothy from two to three harvests per season did not affect CH_4 production or yield, but CH_4 intensity was reduced. Replacing timothy with perennial ryegrass and increased inclusion rate of red clover both increased CH_4 yield and intensity.

Key words: Enteric methane, timothy, red clover, perennial ryegrass, greenfeed system.

INTRODUCTION

Greenhouse gas (GHG) emissions from agriculture and animal husbandry have become increasingly important over the last decade; global food systems contribute up to 34% of global anthropogenic GHG emissions and enteric methane (CH_4) emissions from

78 ruminant production systems account for 5% of global GHG emissions (Vermeulen et al.,
79 2012; Crippa et al., 2021). Methane has a warming potential in the atmosphere 28 times
80 greater than that of carbon dioxide (CO_2) when compared over a 100-year period (IPCC,
81 2019), and concentration of CH_4 in the atmosphere is rapidly increasing (Saunois et al.,
82 2016).

83 Methane is produced in the rumen as a byproduct of feed fermentation. Cell wall
84 polymers, sugars and starch are converted to volatile fatty acids (VFA) with CH_4 being
85 produced as a final endproduct. The CH_4 gas is then eructated through the mouth and nostrils.
86 As farmers strive to reduce CH_4 emissions, changes in feeding regimes are a promising
87 mitigation option (Beauchemin et al., 2020). In Northern and Western Europe, as well as in
88 North America, silages based on grass and grass-clover mixtures constitute a large part of
89 dairy cow diets. Feeding silages for herbage harvested earlier by using a three-cut system
90 (i.e., plants harvested at vegetative stage) compared to silages prepared from a two-cut
91 system (i.e., plants harvested at more mature stage) may increase CH_4 production, expressed
92 as grams per day, but reduce CH_4 yield (g/kg dry matter intake (DMI)) and intensity (g/kg
93 energy corrected milk (ECM)) if DMI and milk production increase (Warner et al., 2016).
94 Harvesting at a more vegetative stage promotes greater organic matter (OM) digestibility and
95 DMI of silages due to lower ash corrected neutral detergent fiber (aNDFom) and indigestible
96 fiber (iNDF) concentrations (Warner et al., 2017; Macome et al., 2018). It is also possible that
97 more frequent harvest may alter VFA concentrations towards less acetate and butyrate and
98 more propionate, which would reduce hydrogen (H_2) availability for CH_4 formation (Janssen,
99 2010). However, studies examining the relationship between feeding silages prepared from
100 grass harvested at different maturity stages are inconsistent (Kuoppala et al., 2010; Warner et
101 al., 2016).

Few studies report enteric CH_4 production for dairy cows fed different grassland species. A recent study showed lower CH_4 yield (g/kg DMI) for heifers fed red clover compared to perennial ryegrass due to a greater DMI of the red clover silage (Parnian-Khajehdizaj et al., 2023). Red clover has a lower NDF concentration compared to most grasses resulting in a faster ruminal passage rate and greater DMI compared to grasses (Kuoppala, 2010; Johansen, 2017), which may result in lower CH_4 yield in red clover-based diets. Waghorn et al. (2002) also reported lower CH_4 yield for sheep fed legumes rather than grass, which was attributed to lower aNDFom concentration and greater DMI. Plant secondary compounds such as condensed tannins and polyphenol oxidase in red clover may have a direct effect on lowering enteric CH_4 production (Meale et al., 2012; Loza et al., 2021). However, not all studies confirm this effect (Van Dorland et al., 2007; Storlien 2014). Previous studies have shown greater OMD and hence a greater DMI and ECM yield in perennial ryegrass compared to timothy (reference) which might suggest a lower CH_4 yield and intensity. However, our *in vitro* study showed greater CH_4 production (mL/g OM) for perennial ryegrass compared to timothy (Weiby et al., 2023), but results have yet to be confirmed *in vivo*.

Previous studies have focused on how maturity stage in spring growth or in different cuts affects animal production and CH_4 (Warner et al., 2017; Pang et al., 2021). In Norway, climatic conditions limits harvest to either two or three times per season and the first and second regrowth constitutes a substantial part of the yearly total dry matter (DM) yield. The first and second regrowth often differs from the spring growth both in quality and quantity. In practice it is common to mix silages from different harvests before feeding, either by mixing round bales from different cuts at feeding or by placing the materials from the various cuts in layers in the same bunker silo. Herbage yield and silage feed quality differs when silage is prepared from regrowth after an early or late cut. Mixing silages proportional to the yield of each species and cut gives a more representative feed of the crop type; however, to our

127 knowledge, this approach has not been used when evaluating effects of species and cutting
128 frequency on feed intake, enteric CH_4 and milk production.

129 Farmers are being directed to reduce GHG emissions for their cows; however, the
130 impact of forage management is unclear (Beauchemin et al., 2020). Therefore, the objective
131 in this study was to investigate the effects of grassland species (timothy, perennial ryegrass,
132 and red clover) and cutting frequency (two vs. three cuts per season) for timothy on DMI,
133 milk production, digestibility, and CH_4 production in lactating dairy cows. We hypothesized
134 that a three-cut system compared to a two-cut system for timothy would increase OM
135 digestibility, and thereby increase DMI and ECM production and reduce CH_4 yield and
136 intensity. Further we hypothesised that timothy would have a lower OM digestibility
137 compared to perennial ryegrass, resulting in greater CH_4 yield and intensity. Lastly, we
138 hypothesized that the diet aNDFom concentration and CH_4 intensity would decrease when
139 increasing the red clover proportion in the silage from 0% to 100%.

140 ## MATERIALS AND METHODS

141 The experiment was conducted at the Livestock Production Research Centre and the
142 Metabolism Unit at the Norwegian University of Life Sciences, Ås, Norway. Care and
143 handling of animals complied with laws and regulations controlling experiments on live
144 animals in Norway, under the supervision of the Norwegian Animal Research Authority.

145 ### *Experimental Design*

146 The experiment was conducted from January 17 to April 10, 2022, and was designed
147 as a cyclic changeover experiment (Davis and Hall, 1969) with 40 cows fed five treatment
148 diets in four 21-d periods. Each period consisted of 14 d adaptation to the experimental diets
149 and 7 d of recording and sampling (recording week). The five treatment diets evaluated
150 silages produced from timothy (*Phleum pratense* L.) in a three-cut system (T3), timothy in a

two-cut system (T2), perennial ryegrass (*Lolium perenne* L.) in a three-cut system (PR3), red clover (*Trifolium pratense* L.) in a three-cut system (RC3) and a mix of T3 and RC3 (50:50 on DM basis) (T3/RC3). The proportion of DM from each cutting was based on each cut's share of the total DM yield over the season. For T3, the silage was comprised of 45% DM from spring growth (first cutting), 30% DM from first regrowth (second cutting) and 25% DM from second regrowth (third cutting). For T2, the proportions were 64% and 36% from spring growth and first regrowth, respectively. For PR3, the proportions were 47, 29 and 24% from spring growth, first and second regrowth, respectively, while for RC3 the proportions were 46, 31 and 23% from spring growth, first and second regrowth, respectively. Finally, for T3/RC3 the proportions were 22 and 24% for T3 and RC3 from the spring growth, 14 and 16% for T3 and RC3 from the first regrowth and 12% for T3 and RC3 from the second regrowth.

The cows were assigned to eight blocks according to parity, DIM and BW. There were 3 blocks of primiparous cows and 5 blocks of multiparous cows, with 5 cows within a block. The cows within each block were randomly assigned to a pre-defined sequence of diets, where each cow followed its own unique sequence of four diets during the four experimental periods to minimize the effect of the diet sequence.

Establishment of Grassland and Silage Management

In the spring of 2020, fields were established with pure timothy (*Phleum pretense* 'Grindstad', Felleskjøpet Agri SA, Lillestrøm, Norway), perennial ryegrass (*Lolium perenne* 'Spire surfôr pluss 100', Felleskjøpet Agri SA, Lillestrøm, Norway), and red clover (*Trifolium pratense* 'Lea', Felleskjøpet Agri SA, Lillestrøm, Norway). Spring barley was sown as cover crop in all fields and harvested in early August. In 2021 the grass leys were fertilized with both cattle manure and a compound fertilizer (Yara Opti NS, Yara AS, Oslo,

Norway), whereas the red clover ley (RC3) only received cattle manure. The T3 ley received 300 kg N per ha (138 kg/ha in spring, 102 kg/ha after spring growth and 60 kg/ha after first regrowth), the T2 ley received 240 kg N per ha (140 kg/ha in spring and 100 kg/ha after spring growth), PR3 ley received 260 kg N per ha (80 kg/ha in spring, 100 kg/ha after spring growth and 80 kg/ha after first regrowth) and RC3 received 30 kg N per ha (30 kg/ha only in spring).

A total of 11 different silages were produced in the summer of 2021 (**Table S1**), and the harvest date was decided based on phenological development stage of timothy, determined as mean stage by count (MSC) (Moore et al., 1991). The spring growth of both T3 and PR3 was harvested at MSC of 2.9-3.0, equivalent to early heading stage. The first regrowth was harvested at 450 degree-days after spring growth. Degree-days were defined as accumulated daily mean temperature above 0 °C. The spring growth of RC3 was harvested at MSC 1.6-1.8, and first regrowth was harvested 450 degree-days after spring growth. The spring growth of T2 was harvested at MSC 3.3, and first regrowth was harvested 790 degree-days after spring growth.

The spring growth of the three-cut silages was taken between 1st and 3rd of June, first regrowth was taken between 7th and 9th of July and the second regrowth was harvested on the 30th and 31st of August. The spring growth of the two-cut silage was taken on the 15th and 16th of June and the first regrowth on the 19th and 20th of August. The grass was cut using a Kverneland 3632 FT/FN disk mower with grass conditioner in the front and a Kverneland 5087M disc mower without grass conditioner in the rear (Kverneland Group Operation Norway, Klepp, Norway). Both spring growth and second regrowth of all treatments were wilted for 10-24 h. The second regrowth of T3 and PR3 was wilted for 5 h, and RC3 was wilted for 24 h. After wilting, the grass was raked using a Kverneland 9590 C Hydro rake (Kverneland Group, Klepp, Norway), and baled using a combined baler and

wrapper with fixed chamber, with a total of 20 knives in the pickup unit (Orkel HIQ Smartbaler, Fannrem, Norway). A silage additive containing 62% formic acid, 16% sodium formate and 1.5% lactose (GrasAAT Lacto, Addcon Nordic AS, Sweden) was added during baling. The dosing rate of the additive was based on the weight of the bales and was 4.7 L/t fresh herbage for T3, 4.6 L/t herbage for PR3 and RC3 and 5.1 L/t herbage for T2. All bales were covered in 10 layers of plastic wrapping (Triowrap Loop, 750 mm width, 0.025 mm thickness, 1700 m length, Trioworld, Smålandsstenar, Sweden) before storing outdoor for a minimum of four months.

Animals and Feeding

Forty Norwegian Red cows (15 primiparous and 25 multiparous) in early- to mid-lactation at the start of the experiment (102 ± 21.2 DIM; mean ± SD), weighing 584 ± 79 kg and yielding 30.2 ± 6.0 kg of milk/d were used in the experiment. The cows were maintained in a loose housing system and offered the experimental diets *ad libitum*. The primiparous cows received (as-fed basis) 6 kg/d and multiparous cows received 9 kg/d of commercial concentrate (Drøv Energirik, Norgesfôr, Oslo, Norway) offered in the milking robot (Delaval, Tumba, Sweden) and Greenfeed system (GF) (C-Lock Inc., Rapid City, SD). Concentrate supplementation level was calculated using the Nordic Feed evaluation system (NorFor; Volden, 2011) based on the best forage quality, which was set to a low fixed dietary proportion for all treatments to ensure maximum forage intake. The cows had free access to drinking water and salt (Saltstein SP Red Rock, Strand Unikorn, Norway). Before the start of the experiment, there was a preparation period of two weeks during which the cows were fed a mixture of spring growths from treatments T2, PR3 and RC3 to adapt the cows to using the feed bins. For each treatment, the silages from the various cuts were added to the mixing wagon (Siloking, Kverneland Duo 1814, 18 m^3, 84529 Tittmoning, Germany) in the appropriate proportions and mixed for approximately 20 min. Mixing was done two times per

week and each tonne of silage (as-is basis) was treated with 2 L of silage additive containing 60% propionic acid and 40% sodium lignosulfonate (Ensil Fullfôr, Felleskjøpet Agri, Lillestrøm, Norway) to prevent heating and degradation. A commercial vitamin and mineral mix (Vitamineral Normal, Vilomix, Hønefoss, Norway) was added to the mixing wagon to provide 50 g/d per cow, with treatment T2 also provided with 100 g/d of urea (G. C. Rieber Salt AS, Oslo, Norway) to meet metabolizable protein requirements.

Recording and Sampling

The cows were offered the experimental diets *ad libitum* in 40 feeding bins that recorded individual feed intake at each visit (Biocontrol, Rakkestad, Norway). Feed bins containing the same treatment were placed next to each other, and cows could visit any bin containing the assigned diet treatment. The bins were re-filled every morning and evening and cleaned Monday and Thursday each week. The bins were calibrated every Monday morning. Milk yield was recorded at each visit to the milking robot and cows had access every 6 h with a maximum of 4 milkings every 24 h. Milk samples were collected from each cow at three consecutive milkings during the last 7 d in each period. Bronopol (Landteknikk, Økern, Norway) was added to the samples, which were stored at 4°C until analysis within 2 weeks. The cows were weighed (Biocontrol, Rakkestad, Norway) after every milking using a scale that was calibrated before each period.

During harvest every 10^{th} silage bale was weighed, and a core sample of fresh grass was collected with a hand-held drill for analysis of DM concentration. This was used to calculate DM yield and adjust for dietary proportion of each cutting on a DM basis. In the recording week, a sample from each of the 11 silages was collected before each mixing and stored at -20°C. The 11 samples were pooled within period before chemical analysis, except samples for iNDF analysis where all four periods were pooled to one sample.

Every Monday, Wednesday and Friday, a sample of the dietary treatment was collected for DM analysis immediately after filling and from different locations inside the feed bins of each of the 5 treatments. The samples were stored at -20°C until analysis. In addition, a sample of the concentrate was collected once a week and stored at -20°C. The samples were pooled for each period before chemical analysis.

Mass fluxes of enteric CH_4 and CO_2 were measured using two GF units. All cows had access to both GF units. The barn staff ensured that all animals had a minimum of three visits per d during the last week of each period, and the maximum visit frequency was 5 visits per 24 h. Gas calibrations were conducted once a week, and CO_2 recovery tests was conducted every 2 weeks. The recovery of CO_2 was on average 100 ± 3.3 %. Air filters were cleaned two times per week to ensure airflow above 26 L/s. To ensure the correct head position for 2 min during a visit to the GF units, the cows received 5 drops of 40 g of concentrate with a 40 s interval during the visit. A maximum of 1000 g/d of concentrate was provided in the GF unit. Measurements were transformed from liters to grams using the factor 0.7168 g/L for CH_4 and 1.96 g/L for CO_2. For technical reasons CH_4 and CO_2 data were not recorded from one cow.

Total tract digestibility was estimated using acid insoluble ash (AIA) in the feed as an internal marker (Van Keulen and Young, 1977). For this purpose, fecal spot samples were collected from 20 multiparous cows in 4 blocks at 6:00 am and 3:30 pm on three consecutive days during the last week of each period, and frozen immediately at -20°C. At the end of each period, the samples were thawed and pooled within cow and period.

Chemical Analysis

The fresh herbage samples and silage treatment diets (n=5) were oven-dried for 16 h at 105 °C and weighed warm to obtain DM concentration. This DM measurement was used to calculate daily DMI of silage after correction for volatile losses according to the Norfor method (Åkerlind et al. 2011). The silages (n=11) and fecal samples were freeze-dried, equilibrated to room humidity overnight, and milled to pass a 1.0 mm screen (Retsch GmbH cutting mill, Haan, Germany) prior to analyses of DM, crude ash, AIA, total nitrogen, soluble crude protein (sCP), aNDFom, water-soluble carbohydrates (WSC) (not for fecal samples) and crude fat.

Dry matter concentration was determined by drying the samples in an oven for 4 h at 103 °C. Crude ash concentration was determined using incineration at 550 °C for 4 h. The AIA concentration was determined according to Van Keulen and Young (1977) using the 2N HCL procedure. Crude protein (CP) concentration was calculated from the total nitrogen concentration (N × 6.25) and determined using a Kjeltec^{TM} 8400 (Foss, Hillerød, Denmark) with 95 % sulfuric acid and a Cu-catalyst (AOAC method 2001.11; AOAC 2002). Soluble CP was analysed according to Licitra et al. (1996) and defined as the difference between the total CP fraction and the insoluble CP fraction. Soluble CP was analysed using borate-phosphate buffer (pH 6.7-6.8) and sodium azide 10% solution. Concentration of aNDFom was determined using the Ankom^{200} Fiber Analyzer (Ankom Technology, Macedon NY 14502, USA) using sodium sulphite, heat stable α-amylase, with ash correction (AOAC, 1995; method 2002.04). Concentration of WSC was determined using extraction in 0.05 M Na-acetate buffer. Sucrose and fructans were hydrolyzed with 0.074 M H_2SO_4 for 70 min in 90 °C. The monosaccharides were converted to glucose-6-phosphate and fructose-6-phosphate using a kit with an enzymatic method (K-FRUGL, Megazyme, Wicklow, Ireland). Concentrations were determined spectrophotometrically by the absorbance of NADPH at 340

298 nm. Crude fat was analysed using accelerated solvent extraction with Dionex^{TM} ASE^{TM} 350
299 Accelerated Solvent Extractor (Thermo Scientific, Waltham, USA).

300 Fresh silages were analysed for pH (Metrhom, Herisau, Switzerland) and fermentation
301 products. The ammonia-nitrogen (NH_3-N) was analysed according to Broderick and Kang
302 (1980), and lactic acid, propionic acid, acetic acid, 2,3-butandiol and ethanol were analysed
303 using high performance liquid chromatography (Ericson and Andre, 2010).

304 Concentration of iNDF was calculated as the proportion of NDF remaining in the
305 residue after *in situ* incubation according to Åkerlind et al. (2011). Samples were freeze dried
306 and ground to pass a 1 mm screen (Retsch, SM200, Rheinische, Haan, Germany). Two g of
307 silage was added to bags (Saatifil PES 12/16, Saatitech S.p.A., Veniano, Como, Italy) and
308 intraruminally incubated for 288 h. The *in situ* study was conducted using two ruminal
309 cannulated, dry Norwegian Red cows fed a diet consisting of forage and concentrate (67:33
310 on DM basis) and a CP level above 120 g/kg DM to meet maintenance energy and protein
311 requirements of the animals.

312 Samples of concentrate were analysed for DM, crude ash, CP, aNDFom, WSC and
313 AIA according to the methods used for silage and fecal samples. In addition, concentrate
314 samples were analysed for concentration of starch which was determined by an enzymatic
315 method using α-amylase and amyloglucosidase (Megazyme, Wicklow, Ireland).

316 Milk samples were analysed at TINE SA in Heimdal, Norway, for concentrations of
317 fat, true protein, lactose, urea N and free fatty acids (FFA) and somatic cell count, using a
318 combination of flow cytometer and a Fourier transform spectrometer (Bentley FTS/FCM
319 Combisystem, Minnesota, USA).

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Calculations and Statistical Analysis

Chemical composition and feeding values of the experimental diets (n = 5) were calculated from the proportion of each silage in the diets and their respective analysis. Digestible OM (DOM) of the silages (n=11) was calculated from OM concentration and OM digestibility (OMD), which was estimated from the concentration of iNDF and NDFom (Huhtanen et al., 2013). The concentration of net energy for lactation was based on 20 kg DMI (NEL_{20}) and metabolizable protein content, expressed as amino acids absorbed in the small intestine (AAT), and protein balance in the rumen (PBV) was calculated according to NorFor as described by Volden (2011). The ECM yield was calculated according to Sjaunja et al. (1991). Feed efficiency was calculated as ECM yield divided by DMI. Total-tract apparent digestibility of nutrients was calculated using AIA as an internal marker in feeds and feces. Concentration of AIA in experimental diets was calculated based on concentration of AIA in each of the 11 silages and proportion of these silages in each diet and the concentration of AIA in concentrate. Fecal output of DM was calculated as total AIA intake from the diet divided by AIA concentration in feces. Fecal output of total nitrogen and aNDFom were calculated using estimated total fecal DM output and determined fecal concentration of total nitrogen and aNDFom.

Daily CH_4 emissions from each cow was calculated by averaging the CH_4 flux at each individual visit to the GF system over the 7 days of measurement. Only visits where the cows had the correct head position into the GF and visits that lasted for more than 3 minutes were used. Cows that did not visit the GF units were followed into one of the GF units to make sure all cows had a minimum of three visits in the period from 06.00 am to 18.00 pm every day. Animals with missing data in one or more days during the 7 d period was excluded from the analysis.

346 The data (5 cows × 8 blocks × 4 periods = 160 observations, with 32 cows for each of
347 the 5 treatments) were analysed using the MIXED procedure of SAS (SAS Inc. 2002-2003,
348 Release 9.4; SAS Institute Inc., Cary, NC) and the following model:

349 $Y_{ijklm} = \mu + B_i + A_j(B_i) + P_k + T_l + E_{ijklm,}$

350 where Y_{ijklm} is the dependant variable and μ is the mean for all observations, B_i is the effect
351 of block I, $A_j(B_i)$is the effect of animal j within block i, P_k is the effect of period k, T_l is the
352 effect of treatment l, and E_{ijklm} is the normally distributed random residual error with
353 expected mean of zero and constant variance. All terms were considered fixed, except for
354 Aj(Bi), which was considered random. Based on the Bayesian information criteria in fit
355 statistics, a variance component was used by including the repeated statement for animal
356 within block in each period. There was no significant carry over effects (the effect of diet in
357 the previous diet). Interaction P_k x T_l was only significant for apparent digestibility data and
358 was removed from the model of the other variables. Residual normality was assessed using
359 plots = residual panel option in the MIXED procedure, with no data showing deviation from
360 normal distribution. Least square means and their standard errors are presented in tables. The
361 contrast function was used to test the effect of cutting system of timothy (T3 vs T2) and the
362 effect of grassland species, where the comparisons were timothy vs. perennial ryegrass (T3 vs
363 PR3), and linear and quadratic effects of increasing the proportion of red clover (RC3-L and
364 RC3-Q). Statistical significance between treatments was declared at $P \leq 0.05$ and tendency at
365 $P \leq 0.1$.

366 # RESULTS

367 ## *Experimental diets*

368 The chemical composition and fermentation profiles of the eleven silages and one
369 concentrate are indicated in **Table S2**. The results focus on the experimental diets produced

370 from these silages. Dry matter concentration was as high as 47% for diet T2 and around 30%
371 for T3, PR3, T3/RC3, and RC3 (**Table 1**). Crude ash concentration was 8% for T3, 6% for T2
372 and about 10% for PR3, T3/RC3, and RC3. Concentrations of aNDFom and iNDF were 7.8
373 and 6.5% lower for T3 than for T2, respectively, and concentration of CP was 2.6% greater
374 for T3 than for T2. Concentration of WSC was 4% lower for T3 than for the T2 diet.
375 Compared to T3 and PR3, concentration of aNDFom for RC3 were 21.4 and 15.1% lower,
376 respectively, and concentration of iNDF were 1.1 and 1.5% greater for RC3, respectively. The
377 RC3 diet had 4.2 and 5.4% greater CP concentration compared to T3 and PR3, respectively.
378 Concentration of WSC was greatest for PR3, with 6.6 and 9.1% greater concentration
379 compared to T3 and RC3, respectively. Replacing 50% of timothy with red clover (T3/RC3)
380 reduced aNDFom concentration with 11.2% and increased iNDF concentration with 0.6%,
381 compared to the pure T3 diet. Concentration of CP was 2.2% greater when including red
382 clover in the diet, but concentration of WSC was slightly reduced. The silage fermentation
383 parameters measured (VFA, NH3-N, pH) indicate that all silages were well preserved.

384 The NEL_{20} was 0.9 MJ/kg DM greater for the T3 than for the T2 diet, and it was 0.3
385 MJ/kg DM greater for the T3 diet compared to the mixed T3/RC3 diet. The concentration of
386 NEL_{20} was 5.9 MJ/kg DM for PR3 and 6.0 MJ/kg DM for RC3, and it was 0.3 and 0.2 MJ/kg
387 DM greater for T3 compared to PR3 and RC3, respectively. The PBV was lowest for the T2
388 diet and greatest for the T3/RC3 diet. The AAT was similar among diets.

389 ***Intake, milk production, and digestibility***

390 The silage intake and total DMI did not differ ($P > 0.10$) between T3 and T2 diets
391 (**Table 2**). However, silage DMI was 1.8 kg DM greater ($P < 0.001$) for T3 than for PR3 and
392 there was a linear effect ($P = 0.008$) of increased proportion of red clover. However, no
393 benefits of the 100% red clover diet were obtained, leading to a significant quadratic effect (*P*

394 < 0.001) of red clover inclusion. Intake of aNDFom was 746 g/d greater ($P = 0.001$) for T2
395 than for T3 diet, and it was 1786 g/d greater ($P < 0.001$) for the T3 diet than for PR3 diet. The
396 aNDFom intake was 3657 g/d lower in RC3 than in T3, and 2479 g/d lower in RC3 than in
397 T3/RC3, resulting in a negative linear and quadratic effect (both $P < 0.001$) of red clover
398 inclusion on aNDFom intake. Intake of CP was 874 g/d greater ($P < 0.001$) for the T3 diet
399 than for the T2 diet, and it was 454 g/d greater ($P < 0.001$) for the T3 than for the PR3 diet.
400 Intake of CP increased with 566 g/d when mixing timothy with red clover silage but
401 decreased with 176 g/d when silage red clover proportion in the diet was increased from 50 to
402 100%, resulting in a significant quadratic effect of red clover proportion in the diet (P <
403 0001). The intake of NEL_{20} was 17.5 MJ/d greater ($P < 0.001$) for the T3 diet compared to
404 the T2 diet, and it was 16.9 MJ/d greater ($P < 0.001$) for T3 than for the PR3 diet. Including
405 red clover in the diet resulted in a linear effect ($P < 0.001$) on the NEL_{20} intake. Milk, fat,
406 protein and lactose yield were greater ($P < 0.001$) for the T3 diet than for the T2 diets, and it
407 was greater ($P < 0.001$) for the T3 than for the PR3 diet (**Table 2**). There was a quadratic
408 effect on milk ($P < 0.001$), fat ($P = 0.01$), protein ($P < 0.001$) and lactose ($P < 0.001$) yield,
409 with maximum yield observed for the T3/RC3 diet.

410 Energy corrected milk (ECM) yield was 2.4 kg/d greater ($P < 0.001$) for the T3 than
411 for the T2 diet, and it was 1.9 kg/d greater ($P < 0.001$) for T3 diet than for the PR3 diet. There
412 was a linear ($P = 0.001$) and quadratic ($P = 0.002$) effect on ECM yield on increasing the
413 dietary proportion of red clover, with numerically greatest ECM yield of 30.2 kg/d with
414 T3/RC3. However, increasing red clover proportion in the diet from 50% to 100%, reduced
415 ECM yield with 2.6 kg/d. Feed conversion efficiency (ECM/DMI) and BW of the animals did
416 not differ ($P > 0.10$) between the different diets in this experiment (**Table 2**). Nitrogen
417 efficiency was 36 g/kg greater ($P < 0.001$) for the T3 diet than for the T2 diet, and it was 21
418 g/kg greater ($P = 0.002$) for the PR3 than for T3 diet. Nitrogen efficiency linearly decreased

($P < 0.001$) as the proportion of red clover in the diet increased. The T3 diet tended ($P = 0.09$) to increase milk urea concentration compared to the T2 diet, and milk urea concentration was 0.4 mmol/L greater ($P = 0.02$) for T3 than for PR3 diet. Including red clover in the diet linearly ($P < 0.001$) and quadratically ($P = 0.05$) increased milk urea concentration. Free fatty acid (FFA) concentration in milk was 0.3 mmol/L lower ($P = 0.03$) in T3 compared to T2 diets, but none of the other diets differed significantly in milk FFA. No effect of treatment on milk somatic cell count was observed ($P > 0.10$).

Organic matter digestibility was 8.2 g/kg greater ($P < 0.001$) for T3 than for T2, and aNDFom digestibility was 10.8 g/kg greater ($P < 0.001$) for the T3 diet than for T2 diet (**Table 3**). There was no difference ($P = 0.67$) between T3 and PR3 in OMD but including red clover in the diet linearly ($P = 0.001$) decreased OMD. Digestibility of aNDFom was 3.7 g/kg greater ($P = 0.03$) for T3 diet than for PR3 diet and including red clover linearly ($P < 0.001$) and quadratically ($P = 0.02$) reduced aNDFom digestibility.

Gas emissions

There was no difference between T3 and the T2 ($P = 0.46$), or between T3 and PR3 ($P = 0.26$) in total CH_4 production (g/d) (**Table 4**). Including red clover in the diet quadratically increased ($P = 0.02$) CH_4 production, with numerically greatest CH_4 production in the T3/RC3 diet.

There was no difference ($P = 0.27$) in CH_4 yield between T3 and T2 diets, but CH_4 yield was 1.3 g/kg DMI lower ($P = 0.05$) for the T3 diet than for the PR3 diet and including red clover in the diet linearly increased ($P < 0.001$) CH_4 yield.

There was 7.0 g/kg DOM greater ($P < 0.001$) CH_4 yield in the T2 diet compared to the T3 diet, but there was no difference ($P = 0.27$) between T3 and PR3. Including red clover linearly ($P < 0.001$) increased CH_4 yield.

Compared to the T3 diet, methane intensity was 1.2 g/kg ECM greater ($P = 0.003$) for the T2 diet, and 0.9 g/kg ECM greater ($P = 0.02$) for the PR3 diet. Including red clover linearly increased ($P < 0.001$) CH_4 intensity with the numerically greatest intensity for the 100% red clover diet (RC3). There was no difference between T3 and T2 ($P = 0.46$), or between T3 and PR3 ($P = 0.22$), in methane intensity in g/kg BW. Including red clover in the diet tended ($P < 0.1$) to linearly and quadratically increase CH_4 intensity in g/kg BW with the numerically greatest intensity for the T3/RC3 diet. Daily CO_2 production was greater ($P < 0.001$) in cows fed T3 than T2, and consequently the CH_4/CO_2 ratio was 2.1 g/kg lower ($P < 0.001$) for the T3 diet than the T2 diet. There was no difference ($P = 0.85$) between T3 and PR3 in CH_4/CO_2 ratio but including red clover in the diet linearly increased ($P < 0.001$) the CH_4/CO_2 ratio.

DISCUSSION

Effect of harvest frequency and grassland species on diet composition

Both the spring growth and the first regrowth included in the T3 diet were harvested at an earlier stage of maturity than those included in the T2 diet, and this had a major impact on concentrations of aNDFom, iNDF and CP, as well as OMD and energy content of the diets. The observed increases in aNDFom and iNDF concentrations, and the simultaneous reduction in CP concentration when reducing the harvest frequency from for timothy has been reported by Kuoppala et al. (2008). These authors reported a greater increase in aNDFom and iNDF in spring growth than in the first regrowth when postponing the harvest (Kuoppala et al., 2008). As the plant matures, the leaf to stem ratio changes, and the proportion of cell wall compared with proportion of cell content increases (Chaves et al, 2006), accounting for the increase in aNDFom and iNDF concentration in the silages included in T2 in the present study. The concentration of aNDFom was greater in T3 than in

PR3, with the level in PR3 being very similar to the results by Purcell et al. (2012). In the present study the iNDF concentration of PR3 diet was greater than expected due to a high iNDF concentration in the second cut of PR3. We speculate that the high temperatures in early July 2021 increased the maturation process which increased lignification (Ford et al., 1979) and hence iNDF concentration, especially in PR3.

Red clover (R3) had a numerically lower aNDFom, but a greater iNDF concentration compared to T3. This aligns with previous studies comparing grasses and legumes (Van Dorland et al., 2007; Johansen et al., 2017). Only the xylem vascular tissue is lignified in legumes which causes the cell walls in this tissue to be completely indigestible, whereas there is no lignin in other tissues which makes these cell walls almost completely digestible (Wilson and Kennedy, 1996). This explains why the potential digestible NDF in our study was lower in the red clover (R3) than in the grasses (T3, PR3).

Effect of harvest frequency and grassland species on digestibility, dry matter intake and milk production

The greater OMD in T3 compared to T2 was expected and has also been shown in other studies using silage from spring growth investigating the effect of grass maturity on OMD (Rinne et al., 1997; Randby et al., 2010). In young and less mature grass, the plant cell wall and lignin concentrations are lower (Cherney et al., 1993) with subsequent increase in OMD (Chaves et al., 2006). However, we did not observe differences in silage DMI or total DMI between T3 and T2. These results are not in agreement with other studies. Randby et al. (2010) reported increased DMI of silage in early compared to late stage of maturity when intact bulls were fed a timothy, meadow fescue and red clover diet from the spring growth. Pang et al. (2021) also reported increased DMI in early compared to late harvested timothy and red clover silage. Although we did not observe differences in DMI, the lower aNDFom

concentration and greater OMD resulted as expected in greater ECM production for T3 compared to T2. Pang et al. (2021) also found that reducing the regrowth interval increased ECM yield in a timothy clover (80:20) diet. In particular, the second cut silage in the T2 diet had a high DM concentration, which elevated the total DM concentration of that diet compared to the T3 diet. As increased DM concentration is correlated with increased DMI (Huhtanen et al., 2007), this would have a positive effect on the total intake and ECM yield of cows fed the T2 diet, thereby reducing the effects of differences in chemical composition on milk production for the T2 and T3 diets.

Unexpectedly, the T3 diet did not differ from the PR3 diet in OMD, but the cows produced more ECM when fed the T3 diet. This was probably due to a greater digestibility of aNDFom and hence a greater DMI in the T3 diet than in the PR3 diet. The summer temperature was high (data not shown), especially the week before the first regrowth was harvested. The T3 and PR3 silages were cut at almost the same date (**Table S1**). However, it seems like the lignification and maturation process were faster in PR3 than in T3 resulting in a greater iNDF concentration especially in the first regrowth of PR3. Other studies have also reported a greater iNDF concentration in the first regrowth of perennial ryegrass compared to timothy (Østrem et al., 2014; Weiby et al., 2023). In addition, the first regrowth of PR3 normally have more stems than leaves compared to the spring growth and the second regrowth due to many new vegetative tillers (Bakken et al., 2009). This affects the iNDF concentration of PR3 as the first and second regrowth constituted more than half of the total DM in the mix. The morphology of PR3 during mid-summer is affected by harvesting time in the spring. Late harvest gives relative higher leaf:stem ratio than early harvest (Hurley et al., 2009). We also speculate that increased iNDF concentration of PR3 is related to increased lignification of the cell walls during summer months due to high temperatures, as also reported in a previous experiment with RG fed to sheep (Garry et al., 2021). The temperature

was between 1.7 and 2.2˚C above the normal temperature for this area in the period of the first regrowth (World Meteorology Organization) which might have increased the maturation process and the lignification of the cell walls (Ford et al., 1979) and hence increased the iNDF concentration. Dry matter intake and ECM yield was lowest in RC3 diets, which aligns with the observed linear decrease in digestibility of both OM and aNDFom with increasing proportion of red clover in the diet. Reduced OMD with increased red clover proportion has also been reported in other studies with Holstein cows (Moorby et al., 2009; Johansen et al., 2017). However, in the study by Moorby et al., (2009) they reported linear increase in both forage DMI and milk yield with increasing inclusion of red clover, although they observed a decrease in milk yield going from 66% to 100% red clover, which aligns with the present results as ECM yield was decreased going from 50% to 100% red clover inclusion. Legumes contain less fiber compared to grasses, but legumes are normally more lignified which results in a lower digestibility (Buxton and Redfearn, 1997). Lignin was not measured in the current study, but Kriszan et al. (2013) found a tendency ($P = 0.09$) for greater concentration of acid detergent lignin (ADL) in red clover compared to grasses. Previous results also show that maximum rumen fill is lower in red clover diets compared to grasses, which indicates a metabolic rather than a physiologic regulation of the intake (Bertilsson and Murphy, 2003). Differences in ECM yield is closely connected to intake of DOM. The aNDFom concentration was 21% lower, but the aNDFom digestibility was 19% lower in the RC3 compared to the T3 diet. According to Mertens et al., (1985) the concentration of NDF in the diet should not be below 280 g/kg DM for an optimal rumen fermentation. In the RC3 diet the aNDFom concentration was 19 g/kg DM above this level, but the concentration was still low, leading to a low aNDFom intake. The low aNDFom intake in combination with low NDFom digestibility probably gave a negative effect on rumen fermentation and rumen microbial synthesis. This resulted in equal amount of milk production in T3 compared to the

RC3 diet (both 27.1 kg/d, P = 0.46). However, there was a linear reduction in the production of milk fat of 131 g/d (1200 vs 1069 respectively, P < 0001) and a quadratic reduction in the production of milk protein of 51 g/d (1012 vs 961 g/d respectively, P < 0.001). This resulted in a linear reduction of 2 kg/d in ECM yield (29.6 vs. 27.6 kg/d respectively, P = 0.001). Oba and Allen (1999) reported that when NDF digestibility changed from high to low, this significantly reduced ECM yield (26.3 vs 25.1, P < 0.0001) of cows in a comparison of 13 datasets of forage from the literature. The latter probably due to lower ruminal acetate and butyrate and greater ruminal propionate production lowering milk fat synthesis (Seymour et al., 2005). The T3/RC3 diet had 14% increase in aNDFom digestibility compared to RC3 diet resulting in greater aNDFom intake and greater nitrogen efficiency. This probably explains the observed increase in silage DMI and ECM yield as also shown by Kuoppala et al. (2010) and Johansen et al. (2017).

The RC3 diet contained 27% more CP than the T3 diet, which entailed 11% greater CP intake in the RC3 diet than in the T3 diet. However, the NEl_{20} concentration was 11% lower in the RC3 diet than the T3 diet, which probably led to an imbalance between protein and energy in the rumen (Sinclair et al., 1993). This is supported by the twice as high PBV value in RC3 compared to T3. It is possible that this imbalance has incurred extra energy costs for detoxification of excess ammonia in the RC3 diet, which ultimately also affected milk production negatively (Reed et al., 2017).

Effect of harvest frequency and grassland species on gas emissions

In contrast to our hypothesis, increased harvest frequency and thereby reduced herbage maturity had no effect on CH_4 production or CH_4 yield, possibly due to unexpectedly no difference in DMI between T3 and T2 treatments. Previous studies (Johnsen and Johnsen, 1995; Hristov et al., 2013) concluded that DMI is the most important factor regulating CH_4

565 production in dairy cows. In addition, aNDFom concentration is one of the major chemical
566 components determining CH_4 yield both *in vitro* (Weiby et al., 2022) and *in vivo* (Jentsch et
567 al., 2007). Increased aNDFom concentration gives rise to more acetate (Rinne et al., 1997)
568 which in turn increases H_2 availability and CH_4 formation in the rumen (Janssen, 2010). The
569 difference between T3 and T2 in aNDFom concentration in the present study was minor, only
570 78 g/kg DM. This might partially explain the lack of difference in CH_4 production between
571 the two treatments. It is possible that mixing silages from each crop proportional to yield at
572 each harvest before feeding lessened the effect of maturity stage of the spring growth
573 compared to other studies that evaluated the effect of maturity on CH_4 production by using
574 forage harvested at each cutting separately.

575 The increase in OM digestibility led to increased ECM production and hence a lower
576 CH_4 emission intensity in timothy harvested three compared to two times per season, which
577 was expected and is consistent with other studies (Warner et al., 2016; Pang et al., 2021). This
578 is reflected in the 22.4% lower CH_4 yield (g/kg DOM) in the T3 diet compared to T2 diet due
579 to a greater proportion of DOM. Shorter regrowth interval due to increased harvest frequency
580 reduced CH_4 intensity because feeding more digestible silages improved ECM yield (Warner
581 et al., 2016). Although our previous *in vitro* studies show a positive association between
582 OMD of silages and CH_4 yield (Weiby et al., 2022; Weiby et al., 2023), we speculate that the
583 8% increase in OMD from T2 to T3 diet in the present study was not enough to increase the
584 CH_4 production, although ECM yield increased resulting in a reduced CH_4 intensity of the T3
585 diet.

586 There was no difference in CH_4 production (g/d) between T3 and PR3 treatments.
587 Unexpectedly, CH_4 yield, and intensity were greater for PR3 than for T3 treatment, due to
588 greater DMI and ECM production. These differences were probably due to a very high iNDF
589 concentration especially in the second cut (constituting 30% of the diet) for the PR3 treatment

(**Table S2**), lowering potential digestible NDF concentration and DMI. In addition, the greater digestibility of aNDFom for the T3 diet may have increased rate of particulate passage (Bosch et al., 1992) from the rumen leading to an increase in DMI and ECM production compared to the PR3 diet. Previous results show that digestibility and fermentation quality have a great impact on DMI (Rinne et al., 1997; Huhtanen et al., 2002; Huhtanen et al., 2007) which again affects ECM yield (Martin and Sauvant, 2002; Hristov et al., 2005). In the present study the OMD was not different between PR3 and T3, but the PR3 was slightly more intensive fermented, and this may have negatively affected DMI of the PR3 diet.

The observed linear increase in CH_4 yield with increased inclusion of red clover are opposite to a recent *in vivo* study on red clover diets fed to cattle from 8-15 months of age (Bica et al., 2022). In that study they reported numerically lower CH_4 production in red clover diets compared to grass silage diets (122 vs. 133 g/d, P = 0.1), but because of similar DMI due to inferior fermentation quality in red clover silage, the CH_4 yield was 3.4 g/kg DMI lower in red clover silage compared to grass silage (17.8 vs. 21.2 g/kg DMI respectively, P = 0.008). Van Dorland et al. (2007) reported no difference in DMI, daily milk production, CH_4 production or intensity in diets with 60/40 perennial ryegrass and red clover. These results are not in accordance with the present results as we found linear increase in both DMI and ECM yield in the T3/RC3 (50/50) diet. However, as the daily CH_4 production was 34 g/d greater in the T3/RC3 diet compared to the T3 diet, the CH_4 yield increased accordingly. It is possible that the inconsistency in literature may be due to differences in forage quality (stage of maturity, fermentation quality, herbage red clover inclusion or presence of tannins) or between animal variations (Knapp et al., 2014). The positive effect on both DMI and ECM production when including 50% red clover in the diet disappeared when exceeding this level of inclusion. The increased CH_4 yield and intensity observed in the RC3 diet was probably

615 related to low digestibility of both aNDFom and OM. The low digestibility may have led to
616 unfavourable conditions for microbial synthesis and a surplus of ammonia in the rumen,
617 lowered DMI and ECM yield, thereby increasing CH_4 yield and intensity.

618 ## CONCLUSIONS

619 This study showed that increasing harvesting frequency from two to three harvests per season
620 did not affect DMI, but as grass harvested at an earlier phenological developmental stage
621 obtained a greater OMD, the ECM yield and hence CH_4 yield (g/kg DOM) and CH_4 intensity
622 (g/kg ECM) was lower in dairy cows receiving the less mature T3 diet. Replacing T3 with
623 PR3 increased CH4 yield and intensity and increasing the inclusion rate of RC from 0% to
624 100% linearly increased CH_4 production, yield, and intensity. In conclusion, as farmers are
625 being directed to reduce their enteric CH_4 emissions, this study show it is a viable strategy to
626 mitigate enteric CH_4 emissions in dairy cows by increasing harvest frequency and to use
627 timothy rather than perennial ryegrass or pure red clover silage in the diet.

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816 **Table 1.** Chemical composition and nutritive values of experimental forage diets and concentrate

Experimental diets[1]																
Treatment	T3			T2		PR3			T3/RC3 50/50			RC3			Concentrate
Cut number	1.	2.	3.	1.	2.	1.	2.	3.	1.	2.	3.	1.	2.	3.	
Proportion, %[2]	45	30	25	64	36	47	29	24	22/24	14/16	12/12	46	31	23	
Chemical composition[3], % of DM															
DM (% of fresh matter)	31.5			47.4		32.5			29.8			28.4			86.2
OM	91.9			93.8		89.7			90.3			88.9			72.9
CP[2]	15.8			13.2*		14.6			18.0			20.0			18.9
aNDFom	51.3			59.1		45.0			40.1			29.9			17.5
iNDF	7.63			14.1		7.22			8.19			8.74			
pdNDF	43.7			45.0		37.8			31.9			21.2			
WSC	5.82			9.79		12.4			4.48			3.29			6.93
Fermentation quality[4], g/kg DM															
Lactic acid	28.2			10.0		34.5			46.3			62.4			
Acetic acid	3.30			0.95		3.76			8.15			12.49			
Propionic acid	0.81			0.35		2.57			1.33			1.80			
Butyric acid	0.59			0.35		1.25			0.63			0.66			
NH_3-N, g/kg N	42.9			21.9		52.2			47.9			52.4			
pH	4.50			4.82		4.68			4.46			4.43			
Nutritive value[5]															
NEL_{20}, MJ/kg DM	6.2			5.3		5.9			6.0			5.9			6.57
PBV, g/kg DM	35			-9		27			56			75			14

AAT, g/kg DM	79	75	76	78	77	124

[1]T3 = Timothy 3 cut system, T2 = Timothy 2 cut system, PR3 = Perennial ryegrass 3 cut system, T3/RC3 = Timothy 3 cut system/red clover 3 cut system, RC3 = Red clover 3 cut system,
[2]Proportion (%) from spring growth, first regrowth and second regrowth used in the experimental diets
[3]iNDF = indigestible neutral detergent fiber, pdNDF = potential digestible neutral detergent fiber, WSC = water soluble carbohydrates
[4]NH_3-N = ammonia nitrogen
[5]PBV = protein balance in the rumen, AAT = amino acids absorbed in the intestines
*Added urea (100 gram/d)

Table 2. Effect of harvest frequency and grassland species on feed intake, performance and feed efficiency.

	Experimental diets[1]						Probability[3]			
Item[2]	T3	T2	PR3	T3/RC3	RC3	SEM	T3 vs T2	T3 vs PR3	RC3-L	RC3-Q
Intake, kg/d										
Silage, DM	15.2	14.5	13.4	16.4	13.9	0.43	0.16	<0.001	0.008	<0.001
Concentrate, DM	6.60	6.62	6.62	6.69	6.62					
Total DM	21.8	21.2	20.0	23.2	20.6	0.43	0.17	<0.001	0.01	<0.001
CP, g/d	3650	2776	3196	4217	4041	69.6	<0.001	<0.001	<0.001	<0.001
aNDFom, g/d	8957	9703	7171	7779	5300	202	0.001	<0.001	<0.001	<0.001
NEL_{20}, MJ/d	139.1	121.6	122.2	142.9	125.7	2.52	<0.001	<0.001	<0.001	0.17
Milk yield (kg/d)	27.1	25.6	26.0	28.8	27.1	0.61	<0.001	<0.001	0.46	0.001
ECM (kg/d)	29.6	27.2	27.7	30.2	27.6	0.56	<0.001	<0.001	0.001	0.002

Milk constituents										
Fat, %	4.37	4.28	4.28	4.19	3.97	0.07	0.19	0.17	<0.001	0.64
Protein, %	3.67	3.68	3.69	3.61	3.55	0.04	0.60	0.39	<0.001	0.99
Lactose, %	4.69	4.68	4.73	4.78	4.81	0.02	0.61	0.02	<0.001	0.06
Fat, g/d	1200	1092	1111	1201	1069	25.4	<0.001	<0.001	<0.001	0.01
Protein, g/d	1012	939	955	1036	961	18.1	<0.001	<0.001	0.08	<0.001
Lactose, g/d	1298	1199	1230	1375	1305	29.3	<0.001	<0.001	0.32	<0.001
Urea, mmol/L	3.46	3.21	3.11	4.19	4.77	0.13	0.09	0.02	<0.001	0.05
FFA, mmol/L	1.65	1.96	1.49	1.59	1.60	0.17	0.03	0.28	0.98	0.79
Cell count, ×1000/mL	190	148	186	77.5	88.1	61.4	0.58	0.96	0.19	0.52
BW, kg	604	599	601	603	598	8.75	0.29	0.50	0.12	0.69
FE, kg/kg	1.37	1.32	1.39	1.32	1.37	0.03	0.15	0.53	0.96	0.17
NE, g/kg	279	243	300	249	242	5.85	<0.001	0.002	<0.001	0.05

[1]T3 = Timothy 3 cut system, T2 = Timothy 2 cut system, PR3 = Perennial ryegrass 3 cut system, T3/RC3 = Timothy 3 cut system/red clover 3 cut system, RC3 = Red clover 3 cut system,

[2] FFA = Free fatty acids, FE = Feed efficiency, calculated as kg ECM/kg dry matter intake, NE = Nitrogen efficiency, calculated as milk N output/feed N intake

[3]Probability of treatment effects: T3 vs T2 = Effect of 3 cuts vs. 2 cuts in timothy; T3 vs PR3 = Effect of 3 cuts in timothy vs. 3 cuts in perennial ryegrass, RC3-L = Linear effect of increasing red clover proportion (0%, 50%, 100% in T3, T3/RC3, RC3), RC3-Q = Quadratic effect of increasing red clover proportion (T3, T3/RC3, RC3)

Table 3. Effect of harvest frequency and grassland species on apparent digestibility of the experimental diets and on the fecal output.

	Experimental diets[1]						Probability[2]			
Item	T3	T2	PR3	T3/RC3 50/50	RC3	SEM	T3 vs T2	T3 vs PR3	RC3-L	RC3-Q
Digestibility, %										
DM	77.5	69.9	77.0	75.4	72.1	0.77	<0.001	0.67	<0.001	0.54
OM	77.8	69.6	77.9	76.1	73.5	0.77	<0.001	0.94	0.001	0.63
CP	75.4	66.2	70.4	71.0	66.1	0.98	<0.001	0.002	<0.001	0.82
aNDFom	72.1	61.3	68.4	66.7	52.9	1.18	<0.001	0.03	<0.001	0.02
Fecal output										
DM, kg/d	5.3	6.7	4.8	6.2	6.2	0.30	<0.001	0.19	0.22	0.27
CP, g/d	983	982	1007	1340	1485	59.1	0.99	0.74	<0.001	0.23

[1]T3 = Timothy 3 cut system, T2 = Timothy 2 cut system, PR3 = Perennial ryegrass 3 cut system, T3/RC3 = Timothy 3 cut system/red clover 3 cut system, RC3 = Red clover 3 cut system

[2]Probability of treatment effects: T3 vs T2 = Effect of 3 cuts vs. 2 cuts in timothy; T3 vs PR3 = Effect of 3 cuts in timothy vs. 3 cuts in perennial ryegrass, RC3-L = Linear effect of increasing red clover proportion, RC3-Q = Quadratic effect of increasing red clover proportion

847 **Table 4.** Effect of harvest frequency and grassland species on methane (CH_4), and carbon dioxide (CO_2) emissions

	Experimental diets[1]						Probability[2]			
Item[2]	T3	T2	PR3	T3/RC3 50/50	RC3	SEM	T3 vs T2	T3 vs PR3	RC3-L	RC3-Q
CH_4										
g/d	476	469	466	510	495	8.68	0.46	0.26	0.05	0.02
g/kg DMI	22.1	22.9	23.4	22.6	24.8	0.58	0.27	0.05	<0.001	0.07
g/kg DOM	31.3	38.3	32.8	32.0	38.4	1.28	<0.001	0.27	<0.001	0.02
g/kg ECM	16.5	17.7	17.4	17.5	18.3	0.39	0.003	0.02	<0.001	0.84
g/100 kg BW	80	79	78	84	83	0.02	0.46	0.22	0.07	0.05
CO_2										
g/d	12764	11920	12450	13199	12688	167.3	<0.001	0.04	0.83	0.008
g/kg DMI	594.9	580.7	526.6	585.8	637.8	12.8	0.34	0.04	0.002	0.009
g/kg ECM	444.3	449.5	468.5	455.2	472.2	9.0	0.52	0.003	0.006	0.67
g/100 kg BW	2140	2000	2090	2200	2140	0.3	<0.001	0.06	0.87	0.04
CH_4/CO_2, g/kg	37.2	39.3	37.3	38.5	38.8	0.4	<0.001	0.85	<0.001	0.2

848
849 [1]T3 = Timothy 3 cut system, T2 = Timothy 2 cut system, PR3 = Perennial ryegrass 3 cut system, T3/RC3 = Timothy 3 cut system/red clover 3
850 cut system, RC3 = Red clover 3 cut system
851 [2]Probability of treatment effects: T3 vs T2 = Effect of 3 cuts vs. 2 cuts in timothy; T3 vs PR3 = Effect of 3 cuts in timothy vs. 3 cuts in perennial
852 ryegrass, RC3-L = Linear effect of increasing red clover proportion, RC3-Q = Quadratic effect of increasing red clover proportion
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854

Supplementary material

Table S1. Cutting date and phenological development stage in 11 different silage qualities.

Silage[1]	Cutting date	Phenological stage
T3		
Spring growth	June 2nd to June 3rd	2.9-3.0 MSC[2]
First regrowth	July 8th to July 9th	450 daytime degrees after spring growth
Second regrowth	August 31st	
T2		
Spring growth	June 15th to June 16th	3.3 MSC
First regrowth	August 19th to August 20th	790 daytime degrees after spring growth
PR3		
Spring growth	June 1st to June 3rd	2.9-3.0 MSC
First regrowth	July 7th to July 8th	450 daytime degrees after spring growth
Second regrowth	August 30th	
RC3		
Spring growth	June 2nd to June 3rd	1.6-1.8 MSC
First regrowth	July 8th to July 9th	450 daytime degrees after spring growth
Second regrowth	August 30th to August 31st	

[1]T3 = Timothy 3 cut system, T2 = Timothy 2 cut system, PR3 = Perennial ryegrass 3 cut system, RC3 = Red clover 3 cut system, T3/RC3 = Timothy 3 cut system/red clover 3 cut system

[2]MSC = Mean stage by count

865 **Table S2.** Chemical composition and fermentation profiles in 11 silage qualities and in concentrate.

	Grassland species and cut number[1]										
	T3			T2		PR3			RC3		
Item	1.	2.	3.	1.	2.	1.	2.	3.	1.	2.	3.
Cutting date	June 2	June 8	August 31	June 15	August 19	June 1	June 7	August 30	June 2	June 8	August 30
MSC[2]	2.9-3.0			3.3		2.9-3.0			1.6-1.8		
DM, g/kg	264 ± 0.58	362 ± 0.83	353 ± 4.54	401 ± 3.59	603 ± 2.64	346 ± 3.01	307 ± 1.75	304 ± 3.07	244 ± 0.79	272 ± 1.47	379 ± 2.98
Chemical composition, g/kg DM[3]											
OM	917 ± 1.07	919 ± 2.18	922 ± 2.60	931 ± 1.69	949 ± 0.99	911 ± 1.75	895 ± 4.64	873 ± 3.14	891 ± 3.69	887 ± 2.37	888 ± 5.19
CP	164 ± 16.8	177 ± 5.44	130 ± 3.40	116 ± 3.35	77.3 ± 3.24	130 ± 7.79	153 ± 8.06	167 ± 9.75	212 ± 1.46	195 ± 8.87	181 ± 6.08
sCP	127 ± 12.6	91.8 ± 0.91	69.4 ± 0.33	67.0 ± 3.67	31.7 ± 2.60	98.2 ± 6.15	106 ± 3.21	105 ± 7.29	121 ± 6.30	92.8 ± 11.3	85.6 ± 6.85
Crude fat	40.0 ± 2.74	37.1 ± 3.48	33.4 ± 2.54	28.0 ± 1.07	21.5 ± 4.06	27.1 ± 2.43	40.0 ± 3.66	46.1 ± 2.36	29.1 ± 2.95	23.2 ± 2.88	17.5 ± 0.88
NDFom	520 ± 17.4	510 ± 11.9	502 ± 6.78	608 ± 2.91	560 ± 2.28	430 ± 10.1	506 ± 13.8	424 ± 3.87	279 ± 13.1	325 ± 20.6	304 ± 10.9
iNDF	63.6 ± 0.00	90.0 ± 0.00	83.3 ± 0.00	135 ± 0.00	151 ± 0.00	43.4 ± 0.00	123 ± 0.00	67.5 ± 0.00	72.6 ± 0.00	94.3 ± 0.00	108 ± 0.00

WSC	34.2 ± 5.89	56.9 ± 0.99	104.8 ± 6.30	57.0 ± 9.41	170 ± 14.0	193 ± 20.7	67.5 ± 10.4	55.9 ± 19.1	27.0 ± 8.35	28.0 ± 4.96	51.04 ± 8.17
Starch											
Fermentation quality, g/kg DM											
Lactic acid	37.3 ± 7.33	20.3 ± 2.01	20.7 ± 8.92	14.9 ± 4.61	1.30 ± 0.26	30.2 ± 9.76	28.7 ± 6.32	49.9 ± 17.2	86.6 ± 22.7	59.0 ± 18.9	19.5 ± 14.7
Acetic acid	4.3 ± 1.17	2.6 ± 0.24	2.4 ± 1.34	1.3 ± 0.14	0.4 ± 0.20	3.30 ± 0.34	3.5 ± 0.62	5 ± 2.03	16 ± 3.30	12 ± 2.14	4.8 ± 1.58
Butyric acid	0.7 ± 0.02	0.5 ± 0.02	0.5 ± 0.09	0.4 ± 0.05	0.2 ± 0.02	0.6 ± 0.06	2.8 ± 1.21	0.8 ± 0.36	0.8 ± 0.03	0.7 ± 0.05	0.5 ± 0.04
Ethanol	8.5 ± 1.02	3.4 ± 0.26	4.1 ± 1.08	5.8 ± 1.20	2.8 ± 0.61	21 ± 4.61	6.7 ± 0.95	5.6 ± 1.50	5.8 ± 1.45	4.8 ± 1.10	3.3 ± 0.70
Ammonium-N, g/kg of N	52.0 ± 6.77	33.2 ± 2.00	37.6 ± 12.7	28.4 ± 8.52	10.4 ± 2.52	41.6 ± 9.25	58.4 ± 5.31	65.8 ± 16.38	51.3 ± 4.84	56.0 ± 5.20	49.8 ± 10.58
pH	4.3 ± 0.06	4.5 ± 0.02	4.8 ± 0.17	4.5 ± 0.09	5.3 ± 0.22	4.6 ± 0.21	4.6 ± 0.09	5.0 ± 0.17	4.2 ± 0.09	4.4 ± 0.14	5.0 ± 0.10
Feeding values[4]											
OMD, %	74.8 ± 0.19	71.7 ± 0.13	72.6 ± 0.07	65.2 ± 0.07	63.8 ± 0.03	78.2 ± 0.11	67.7 ± 0.15	75.4 ± 0.04	76.3 ± 0.14	73.2 ± 0.23	71.8 ± 0.12
DOM, g/kg DM	686 ± 1.13	659 ± 1.71	669 ± 1.30	607 ± 0.93	605 ± 0.75	713 ± 1.97	606 ± 2.83	658 ± 2.32	680 ± 2.93	649 ± 3.17	638 ± 2.86
NEl, MJ/kg DM	6.37	6.17	6.04	5.39	5.10	6.17	5.47	6.03	6.13	5.73	5.48

PBV, g/kg DM	37	53	11	0	-26	8	43	46	83	72	62
AAT, g/kg DM	81	78	77	77	70	79	70	77	82	74	73

[1]T3 = Timothy 3 cut system, T2 = Timothy 2 cut system, PR3 = Perennial ryegrass 3 cut system, T3/RC3 = Timothy 3 cut system/red clover 3 cut system, RC3 = Red clover 3 cut system

[2]MSC = Mean stage by count

[3]iNDF = indigestible neutral detergent fiber, WSC = water soluble carbohydrates

[4]OMD = organic matter digestibility, DOM = digestible organic matter, PBV = protein balance in the rumen, AAT = amino acids absorbed in the intestines

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