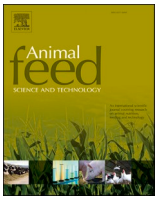
Animal Feed Science and Technology 285 (2022) 115249

Contents lists available at ScienceDirect

Animal Feed Science and Technology

journal homepage: www.elsevier.com/locate/anifeedsci



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

Kim Viggo Weiby [a,b,*], Sophie J. Krizsan [c], Margrete Eknæs [a], Angela Schwarm [a], Anne Cathrine Whist [b], Ingunn Schei [b], Håvard Steinshamn [d], Peter Lund [e], Karen A. Beauchemin [f], Ingjerd Dønnem [a]

[a] Norwegian University of Life Sciences, Department of Animal and Aquacultural Sciences, P.O. Box 5003, NO-1432 Ås, Norway
[b] TINE SA, Langbakken 20, 1430 Ås, Norway
[c] Swedish University of Agricultural Sciences, Department of Agricultural Research for Northern Sweden, SE-901 83 Umeå, Sweden
[d] Norwegian Institute of Bioeconomy Research (NIBIO), Division of Food Production and Society, Department of Grassland and Livestock, Gunnars veg 6, N-6630 Tingvoll, Norway
[e] Aarhus University, Department of Animal Science, AU Foulum, Blichers Allé 20, DK-8830 Tjele, Denmark
[f] Agriculture and Agri-Food Canada, Lethbridge Research and Development Centre, 5403 1st Avenue South, Lethbridge, Alberta T1J 4B1, Canada

ARTICLE INFO

Keywords:
Grass silage
Indigestible neutral detergent fiber
Methane production
Neutral detergent fiber
Water soluble carbohydrates

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.

* Corresponding author at: Norwegian University of Life Sciences, Department of Animal and Aquacultural Sciences, P.O. Box 5003, NO-1432 Ås, Norway.
E-mail addresses: kim.weiby@nmbu.no, kim.viggo.weiby@tine.no (K.V. Weiby).

https://doi.org/10.1016/j.anifeedsci.2022.115249
Received 24 September 2021; Received in revised form 9 February 2022; Accepted 10 February 2022
Available online 12 February 2022

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.

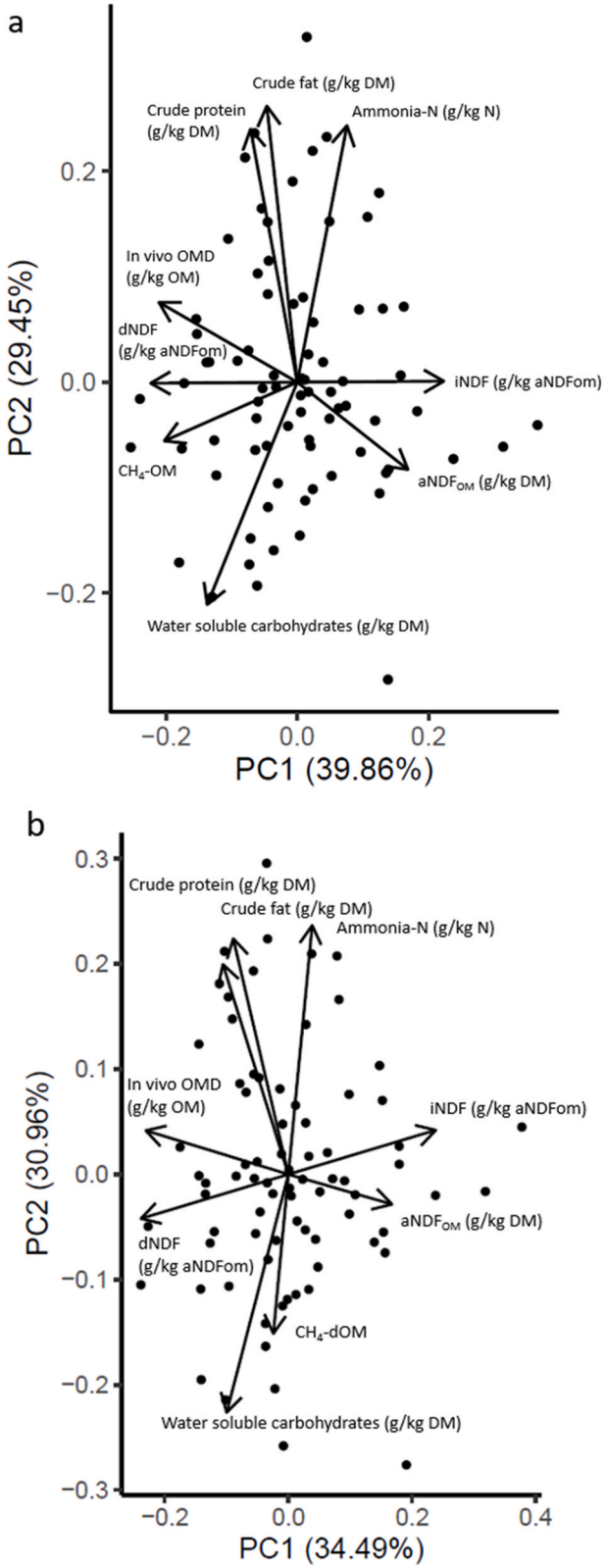


Fig. 1. Principal component analysis (PCA) biplot showing the relationship between grass silage composition variables (g/kg DM), *in vivo* digestibility of organic matter (OMD) and *in situ* digestible aNDFom (dNDF) with methane production expressed on the basis of OM and dOM as a) methane yield CH_4-OM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

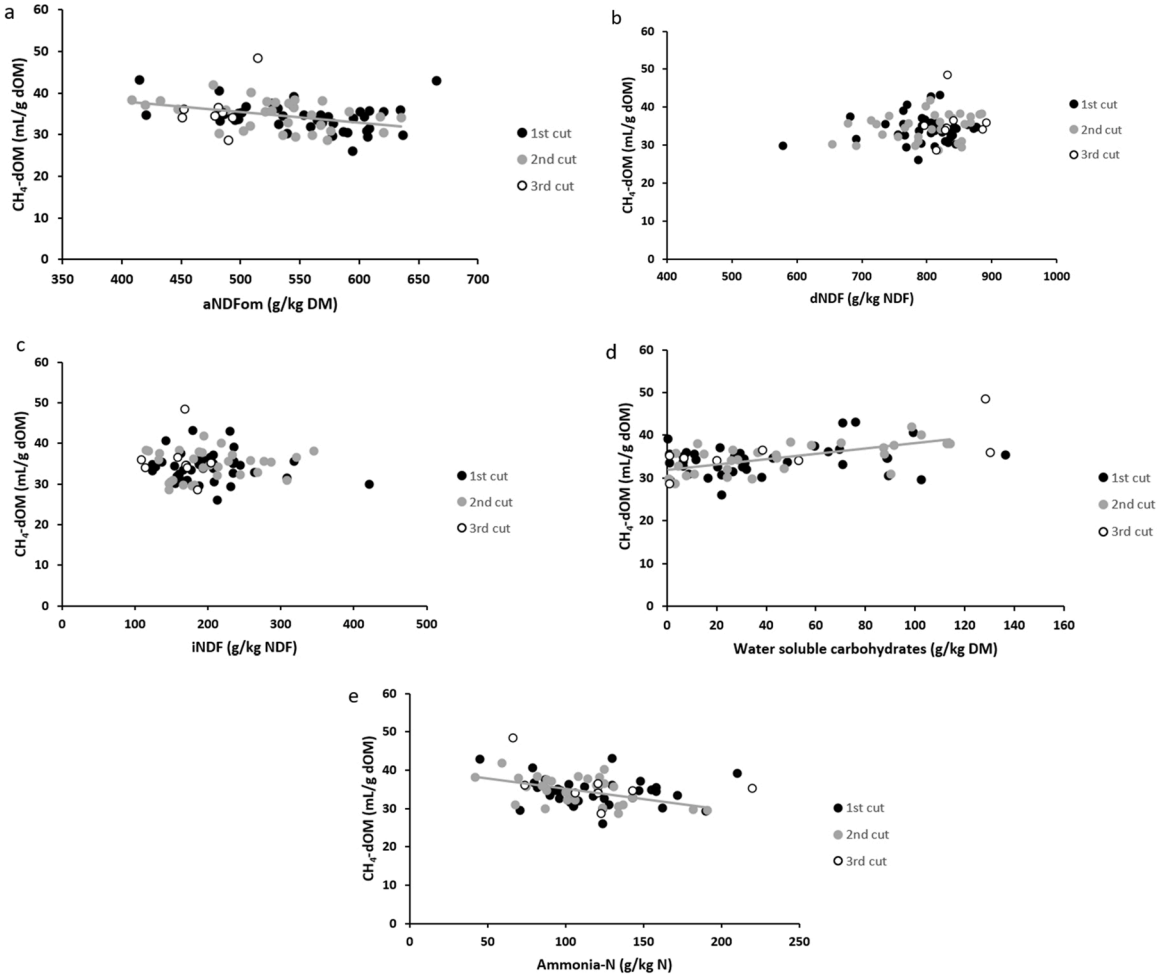


Fig. 2. Relationships of methane production (mL) on the basis of digestible organic matter (dOM, g dOM), *i.e.*, methane yield (CH_4-dOM), from first, second and third cut grass silages with concentrations of a) aNDFom (g/kg DM), b) digestible aNDFom (g/kg aNDFom), c) *in situ* indigestible aNDFom (g/kg 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)

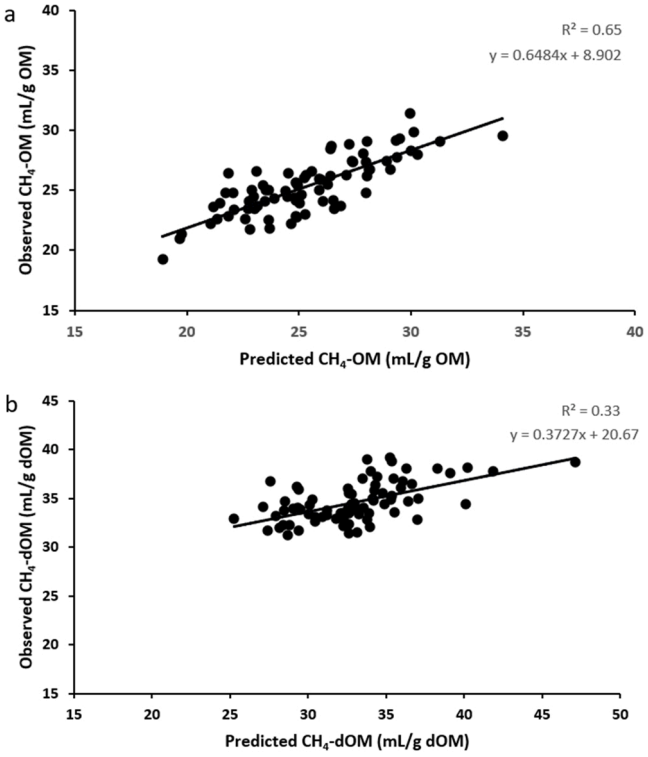


Fig. 3. Relationship between the observed and predicted *in vitro* methane production expressed on the basis of organic matter (OM) and digested OM (dOM), as a) CH_4-OM, mL methane/g OM using Model 1 and b) CH_4-dOM, mL methane/g dOM using model 2.

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

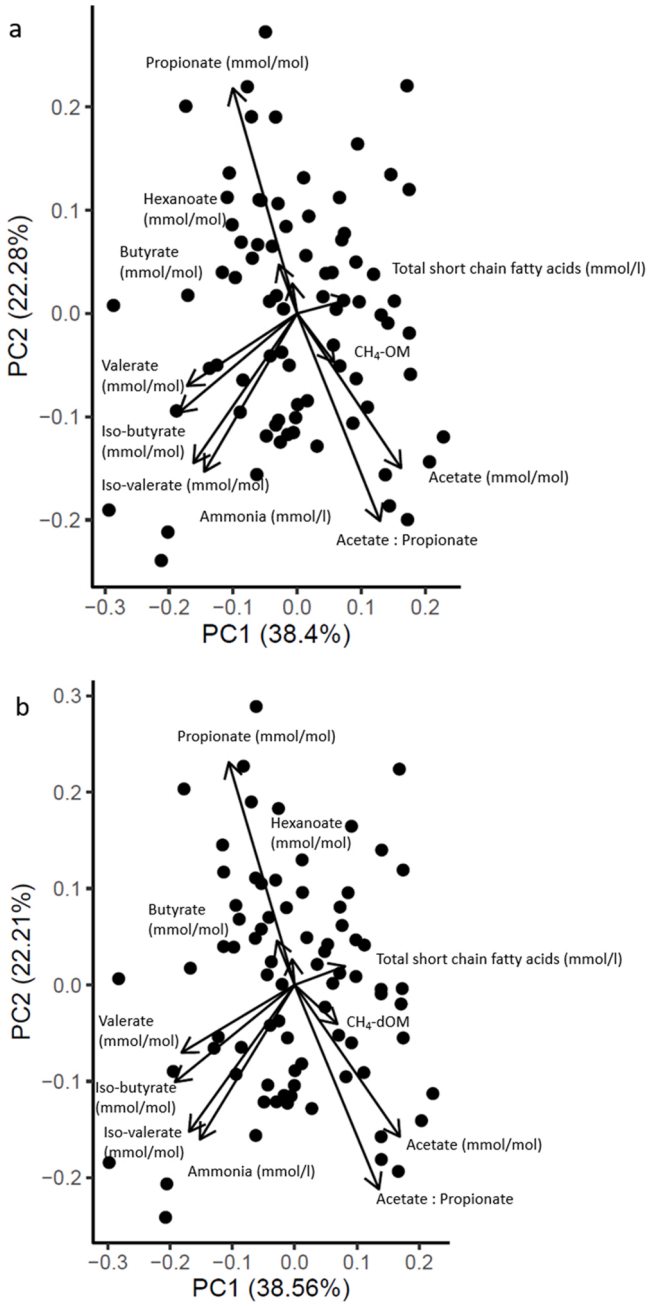


Fig. 4. Principal component analysis biplot showing the relationship between methane production expressed on the basis of organic matter (OM) or digestible OM (dOM) as methane yield CH_4-OM and CH_4-dOM, respectively, and rumen fermentation characteristics. Principal component (PC) 1 and 2 explained 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.

Acknowledgments

We would like to thank the 37 farmers and TINE SA for providing the silage samples for this experiment, and the staff at the Metabolism Unit at NMBU for both handling all the samples and performing the *in situ* and *in vivo* digestibility trial. We would also like to thank the staff at SLU for performing the *in vitro* screening of the grass silage samples. This study was a part of the project "Strategies in grass silage production to mitigate enteric CH_4 emissions from ruminants", financed by the Foundation for Research Levy on Agricultural Products, Norway, the Agricultural Agreement Research Fund, Norway, and TINE BA, Norway, through signed contract by the Research Council of Norway, Norway (Grant number 295207/E50).

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.

References

Åkerlind, M., Weisbjerg, M., Eriksson, T., Tøgersen, R., Udén, P., Ólafsson, B.L., Harstad, O.M., Volden, H., 2011. Feed analyses and digestion methods. In: Volden, H. (Ed.), NorFor-The Nordic Feed Evaluation System. Wageningen Academic Publishers, Wageningen, The Netherlands, pp. 41–54.

AOAC, 1995. Official Methods of Analysis. Association of Official Analytical Chemists, Arlington, VA, USA.

Blaxter, K.L., Clapperton, J.L., 1965. Prediction of the amount of methane produced by ruminants. Br. J. Nutr. 19, 511–522. https://doi.org/10.1079/bjn19650046.

Boadi, D., Benchaar, C., Chiquette, J., Massé, D., 2004. Mitigation strategies to reduce enteric methane emissions from dairy cows: update review. Can. J. Anim. Sci. 84, 319–335. https://doi.org/10.4141/A03-109.

Børsting, C.F., Brask, M., Hellwing, A.L.F., Wejsbjerg, M.R., Lund, P., 2020. Enteric methane emission and digestion in dairy cows fed wheat or molasses. J. Dairy Sci. 103, 1448–1462. https://doi.org/10.3168/jds.2019-16655.

Brask, M., Lund, P., Hellwing, A.L.F., Poulsen, M., Wejsbjerg, M.R., 2013. Enteric methane production, digestibility and rumen fermentation in dairy cows fed different forages with and without rapeseed fat supplementation. Anim. Feed. Sci. Technol. 184, 67–79. https://doi.org/10.1016/j.anifeedsci.2013.06.006.

Broderick, G.A., Luchini, N.D., Reynal, S.M., Varga, G.A., Ishler, V.A., 2008. Effect on production of replacing dietary starch with sucrose in lactating dairy cows. J. Dairy Sci. 91, 4801–4810. https://doi.org/10.3168/jds.2008-1480.

Chamberlain, D.C., Thomas, P.C., Anderson, F.J., 1983. Volatile fatty acid proportions and lactic acid metabolism in the rumen in sheep and cattle receiving silage diets. J. Agric. Sci. 101, 47–58. https://doi.org/10.1017/S0021859600036352.

Cone, J.W., Van Gelder, A.H., Visscher, G.J.W., Oudshoorn, L., 1996. Influence of rumen fluid and substrate concentration on fermentation kinetics measured with a fully automated time related gas production apparatus. Anim. Feed Sci. Technol. 61, 113–128. https://doi.org/10.1016/0377-8401(96)00950-9.

Czerkawski, J.W., Breckenridge, G., 1969. Fermentation of various soluble carbohydrates by rumen micro-organisms with particular reference to methane production. Br. J. Nutr. 23, 925–937. https://doi.org/10.1079/BJN19690104.

Ellis, J.L., Dijkstra, J., France, J., Parsons, A.J., Edwards, G.R., Rasmussen, S., Kebreab, E., Bannink, A., 2012. Effect of high-sugar grasses on methane emissions simulated using a dynamic model. J. Dairy Sci. 95, 272–285. https://doi.org/10.3168/jds.2011-4385.

Holtshausen, L., Liestøl, S.-O., Nes, S.K., Beauchemin, K.A., Harstad, O.M., McAllister, T.A., 2012. Effect of maturity at harvest on in vitro methane production from ensiled grass. Acta Agric. Scand., Sect. A Animal Sci. 62, 40–45. https://doi.org/10.1080/09064702.2012.671846.

Hook, S.E., Wright, A.D.G., McBride, B.W., 2010. Methanogens: methane producers of the rumen and mitigation strategies. Archaea 11. https://doi.org/10.1155/2010/945785.

Hristov, A.N., Oh, J., Lee, C., Meinen, R., Montes, F., Ott, T., Firkins, J., Rotz, A., Dell, C., Adesogan, A., Yang, W., Tricarico, J., Kebreab, E., Waghorn, G., Dijkstra, J., Oosting, S., 2013. Mitigation of greenhouse gas emissions in livestock production. In: Gerber, Pierre J., Henderson, Benjamin, Makkar, Harinder, P.S. (Eds.), A Review of Technical Options for Non-CO2 Emissions. FAO Animal Production and Health Paper No. 177. Food and Agriculture Organization of the United Nations: Rome, Italy. ⟨http://www.fao.org/docrep/018/i3288e/i3288e.pdf⟩.

Huhtanen, P., Jaakkola, S., Nousiainen, J., 2013. An overview of silage research in Finland: from ensiling innovation to advances in dairy cow feeding. Agric. Food Sci. 22, 35–56. https://doi.org/10.23986/afsci.6632.

IPCC, 2014. IPCC Fifth Assessment Report (AR5). ⟨https://www.ipcc.ch/report/ar5/wg3/⟩. (Accessed 15 November 2020).

Jaakkola, S., Huhtanen, P., 1992. Rumen fermentation and microbial protein synthesis in cattle given increasing levels of lactic acid with grass silage based diets. J. Agric. Sci. 119, 411–418. https://doi.org/10.1017/S0021859600012259.

Janssen, P.H., 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. Anim. Feed Sci. Technol. 160, 1–22. https://doi.org/10.1016/j.anifeedsci.2010.07.002.

Jentsch, W., Schweigel, M., Weissbach, F., Scholze, H., Pitroff, W., Derno, M., 2007. Methane production in cattle calculated by the nutrient composition of the diet. Arch. Anim. Nutr. 61, 10–19. https://doi.org/10.1080/17450390601106580.

Johnson, K.A., Johnson, D.E., 1995. Methane emissions from cattle. J. Anim. Sci. 73, 2483–2492. https://doi.org/10.2527/1995.7382483x.

Jonker, A., Muetzel, S., Molano, G., Pacheco, D., 2016. Effect of fresh pasture forage quality, feeding level and supplementation on methane emissions from growing beef cattle. Anim. Prod. Sci. 56, 1714–1721. https://doi.org/10.1071/AN15022.

Kellogg, D.W., Owen, F.G., 1969a. Relation of ration sucrose level and grain content to lactation performance and rumen fermentation. J. Dairy Sci. 52, 657–662. https://doi.org/10.3168/jds.S0022-0302(69)86624-5.

Kellogg, D.W., Owen, F.G., 1969b. Alterations of in vitro rumen fermentation patterns with various levels of sucrose and cellulose. J. Dairy Sci. 52, 1458–1460. https://doi.org/10.3168/jds.S0022-0302(69)86775-5.

Krizsan, S.J., Randby, Å.T., 2007. The effect of fermentation quality on the voluntary intake of grass silage by growing cattle fed silage as the sole feed. Anim. Feed Sci. Technol. 85, 984–996. https://doi.org/10.2527/jas.2005-587.

Krizsan, S.J., Rinne, M., Nyholm, L., Huhtanen, P., 2015. New recommendations for the ruminal in situ determination of indigestible neutral detergent fibre. Anim. Feed Sci. Technol. 205, 31–41. https://doi.org/10.1016/j.anifeedsci.2015.04.008.

Kuoppala, K., Rinne, M., Nousiainen, J., Huhtanen, P., 2008. The effect of cutting time of grass silage in primary growth and regrowth and the interactions between silage quality and concentrate level on milk production of dairy cows. Livest. Sci. 116, 171–182. https://doi.org/10.1016/j.livsci.2007.10.002.

Kuoppala, K., Rinne, M., Ahvenjärvi, S., Nousiainen, J., Huhtanen, P., 2010. The effect of harvesting strategy of grass silage on digestion and nutrient supply in dairy cows. J. Dairy Sci. 93, 3253–3263. https://doi.org/10.3168/jds.2009-3013.

Lee, H.J., Lee, S.C., Kim, J.D., Oh, Y.G., Kim, B.K., Kim, C.W., Kim, K.J., 2003a. Methane production potential of feed ingredients as measured by in vitro gas test. Asian-Aust. J. Anim. Sci. 16, 1143–1150. https://doi.org/10.5713/ajas.2003.1143.

Lee, M.R.F., Merry, R.J., Davies, D.R., Moorby, J.M., Humphreys, M.O., Theodorou, M.K., MacRae, J.C., Scollan, N.D., 2003b. Effect of increasing availability of water-soluble carbohydrates on in vitro rumen fermentation. Anim. Feed Sci. Technol. 104, 59–70. https://doi.org/10.1016/S0377-8401(02)00319-X.

Macome, F.M., Pellikaan, W.F., Hendriks, W.H., Warner, D., Schonewille, J.T., Cone, J.W., 2018. In vitro gas and methane production in rumen fluid from dairy cows fed grass silages differing in plant maturity, compared to in vivo data. J. Anim. Physiol. Anim. Nutr. 102, 843–852. https://doi.org/10.1111/jpn.12898.

McCormick, M.E., Redfearn, D.D., Ward, J.D., Blouin, D.C., 2001. Effect of protein source and soluble carbohydrate addition on rumen fermentation and lactation performance of Holstein cows. J. Dairy Sci. 84, 1686–1697. https://doi.org/10.3168/jds.S0022-0302(01)74604-8.

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

Penner, G.B., Oba, M., 2009. Increasing dietary sugar concentration may improve dry matter intake, ruminal fermentation, and productivity of dairy cows in the postpartum phase of the transition period. J. Dairy Sci. 92, 3341–3353. https://doi.org/10.3168/jds.2008-1977.

Puhakka, L., Jaakkola, S., Simpura, I., Kokkonen, T., Vanhatalo, A., 2016. Effects of replacing rapeseed meal with fava bean at 2 concentrate crude protein levels on feed intake, nutrient digestion, and milk production in cows fed grass silage-based diets. J. Dairy Sci. 99, 7993–8006. https://doi.org/10.3168/jds.2016-10925.

Purcell, P.J., Boland, T.M., O`Kiely, P., 2014. The effect of water-soluble carbohydrate concentration and type on in vitro rumen methane output of perennial ryegrass determined using a 24-hour batch-culture gas production technique. Ir. J. Agric. Res. 53, 21–36. ⟨https://www.jstor.org/stable/24369733⟩.

R Core Team, 2020. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. ⟨https://www.R-project.org/⟩.

Ramin, M., Huhtanen, P., 2012. Development of an in vitro method for determination of methane production kinetics using a fully automated in vitro gas system—a modelling approach. Anim. Feed Sci. Technol. 174, 190–200. https://doi.org/10.1016/j.anifeedsci.2012.03.008.

Ramin, M., Huhtanen, P., 2013. Development of equations for predicting methane emissions from ruminants. J. Dairy Sci. 96, 2476–2493. https://doi.org/10.3168/jds.2012-6095.

Randby, Å.T., Nørgaard, P., Weisbjerg, M.R., 2010. Effect of increasing plant maturity in timothy-dominated grass silage on the performance of growing/finishing Norwegian Red bulls. Grass Forage Sci. 65, 273–286. https://doi.org/10.1111/j.1365-2494.2010.00745.x.

Randby, Å.T., Weisbjerg, M.R., Nørgaard, P., Heringstad, B., 2012. Early lactation feed intake and milk yield responses of dairy cows offered grass silages harvested at early maturity stages. J. Dairy Sci. 95, 304–317. https://doi.org/10.3168/jds.2011-4454.

Rinne, M., Jaakkola, S., Huhtanen, P., 1997. Grass maturity effects on cattle fed silage-based diets. 1. Organic matter digestion, rumen fermentation and nitrogen utilization. Anim. Feed Sci. Technol. 67, 1–17. https://doi.org/10.1016/S0377-8401(96)01141-8.

Rinne, M., Huhtanen, P., Jaakkola, S., 2002. Digestive processes of dairy cows fed silages harvested at four stages of grass maturity. J. Anim. Sci. 80, 1986–1998. https://doi.org/10.2527/2002.8071986x.

Rivero, M.J., Keim, J.P., Balocchi, O.A., Lee, M.R.F., 2020. In vitro fermentation patterns and methane output of perennial ryegrass differing in water-soluble carbohydrate and nitrogen concentrations. Animals 10, 1076. https://doi.org/10.3390/ani10061076.

Sannes, R.A., Messman, M.A., Vagnoni, D.B., 2002. Form of rumen-degradable carbohydrate and nitrogen on microbial synthesis and protein efficiency of dairy cows. J. Dairy Sci. 85, 900–908. https://doi.org/10.3168/jds.s0022-0302(02)74148-9.

Steen, R., 1984. A comparison of two-cut and three-cut systems of silage making for beef cattle using two cultivars of perennial ryegrass. Anim. Sci. 38, 171–179. https://doi.org/10.1017/S0003356100002166.

Steinshamn, H., Nesheim, L., Bakken, A.K., 2016. Grassland production in Norway. In: Höglind, M., Bakken, A.K., Hovstad, K.A., Kallioniemi, E., Riley, H., Steinshamn, H., Østrem, L. (Eds.), The Multiple Roles of Grassland in the European Bioeconomy. European Grassland Federation EGF, Wageningen. In: ⟨https://www.europeangrassland.org/fileadmin/documents/Infos/Printed_Matter/Proceedings/EGF2016.pdf⟩.

Sutton, J.D., 1968. The fermentation of soluble carbohydrates in rumen contents of cows fed diets containing a large proportion of hay. Br. J. Nutr. 22, 689–712. https://doi.org/10.1079/BJN19680080.

Sutton, J.D., 1969. The fermentation of soluble carbohydrates in rumen contents of cows given diets containing a large proportion of flaked maize. Br. J. Nutr. 23, 567–583. https://doi.org/10.1079/BJN19690065.

Thomas, C., Gibbs, B.G., Tayler, J.C., 1981. Beef production from silage. 2. The performance of beef cattle given silages of either perennial ryegrass or red clover. Anim. Prod. 32, 149–153. https://doi.org/10.1017/S0003356100024934.

Volden, H., Rokkjær, H.D., Göran, A., Åkerlind, M., 2011. The Norfor IT system, ration formulation and optimization. In: Volden, H. (Ed.), Norfor-The Nordic Feed Evaluation System. Wageningen Academic Publishers, Wageningen, The Netherlands, pp. 167–179.

Warner, D., Hatew, B., Podesta, S.C., Klop, G., Van Gastelen, S., Van Laar, H., Dijkstra, J., Bannink, A., 2016. Effects of nitrogen fertilisation rate and maturity of grass silage on methane emission by lactating dairy cows. Animal 10, 34–43. https://doi.org/10.1017/S1751731115001640.

Warner, D., Bannink, A., Hatew, B., Van Laar, H., Dijkstra, J., 2017. Effects of grass silage quality and level of feed intake on enteric methane production in lactating dairy cows. J. Anim. Sci. 95, 3687–3699. https://doi.org/10.2527/jas.2017.1459.

Yáñez-Ruiz, D.R., Bannink, A., Dijkstra, J., Kebreab, E., Morgavi, D.P., O`Kiely, P., Reynolds, C.K., Schwarm, A., Shingfield, K.J., Yu, Z., Hristov, A.N., 2016. Design, implementation and interpretation of in vitro batch culture experiments to assess enteric methane mitigation in ruminants-a review. Anim. Feed Sci. Technol. 216, 1–18. https://doi.org/10.1016/j.anifeedsci.2016.03.016.