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Utility of an *in vitro* test with rumen fluid from slaughtered cattle for capturing variation in methane emission potential between cattle types and with age

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Abstract: Differences in methane emission between cattle types and 273 to 4903 day old cattle were evaluated. Rumen content from 59 beef and 32 dairy cattle, collected at an abattoir, was used for *in vitro* incubation with a forage-based diet alone or supplemented with extruded linseed. Incubating rumen fluid from dairy cows yielded lower methane/g diet dry matter incubated (-5.5%), methane/total gas ratios (-8.5%) and ammonia concentrations (-38%) than beef cattle. Linseed decreased methane yield (-6.5%), total gas production (-3.6%) and methane/total gas ratio (-2.7%). High emitters responded slightly more to linseed than low emitters. Lower methane yield (-15%), total gas (-11%) and ammonia concentrations (-20%), and a trend for lower methane/total gas ratios (-5.2%) were found in Limousin × Brown Swiss compared to Limousin × Holstein. In beef cattle, methane yield increased (up to 40 ml/g dry matter) with age in a linear manner, whereas in dairy and beef animals the relationship was curvilinear (high in young and old animals) concerning methane/total gas ratio and ammonia concentration (trend in beef). In conclusion, rumen fluid from slaughtered animals may be useful to study variations in methane emission from different cattle types.

Key words: breed, beef, dairy, fermentation, environment.

Abbreviations: CH₄, methane; CO₂, carbon dioxide; GHG, greenhouse gas; FOAG, Federal Office for Agriculture; L×B, Limousin × Brown Swiss; L×H, Limousin × Holstein; L×S, Limousin × Swiss Fleckvieh; BS, Brown Swiss; HF, Holstein Friesian; SF, Swiss Fleckvieh; DM, dry matter; NDF, neutral detergent fiber; N, nitrogen; T, cattle type respectively breed type; D, diet type; T×D, interaction; R, run; A and A², linear and squared transformed; D×A, interaction of age and diet type; AIC, Akaike information criterion; SE, standard error

INTRODUCTION

The increasing awareness of global warming caused considerable research attention to develop effective and easy-to-apply approaches for abating greenhouse gas (GHG) emissions. Beef and dairy production have been estimated to account for 60% of the total emissions of livestock production, with methane (CH_4) emitted as the end product of enteric fermentation being a major contributor of GHGs (Slade et al. 2016). Various CH_4 mitigation strategies have been investigated, among them nutritional changes, breeding for low CH_4 emission, rumen manipulation and management (Cottle et al. 2011; Wanapat et al. 2013).

Variation among animals of different or within the same breed in CH_4 emission has been reported, making it possible to decrease CH_4 emission by selectively breeding livestock that transform nutrients more efficiently into performance or which have inherently a lower CH_4 yield and emission intensity (Cottle et al. 2011; De Haas et al. 2011; Pickering et al. 2015). As an alternative to nutritional strategies, genetic improvement or selection thus could produce cumulative and permanent outcomes in CH_4 mitigation. However, the success of this approach is only given with cost-effective technologies to estimate or assess the potential CH_4 yield of individuals (Wall et al. 2010). Pickering et al. (2015) reviewed evidence showing that both CH_4 emission and production (g/day) are heritable and repeatable. A first step towards lower emitting cattle could make use of cattle type differences, in case there are any.

Recently, Grandl et al. (2016) reported that CH_4 yields (g/kg of DM intake) and emission intensities (g/kg of energy-corrected milk yield) were related to age in dairy cows aged between 3 and 10 years. This was manifested in a curvilinear relationship where CH_4 yield first increased from weaning at about 10 months of age to cows with several lactations from approximately 15 to 23 g/kg of DM intake and then decreased in older cows to approximately 15 g/kg of DM intake. Investigating rumen fluid obtained from the abattoir would yield a

dataset of animals of varying age as well, allowing to further follow up the relationship of methanogenesis and the animal's age.

The techniques currently in use for measuring enteric CH₄ emission from individual animals include continuous measurement techniques such as static and portable respiration chambers as well as the sulfur hexafluoride tracer technique. Furthermore, techniques based on short-term measurements of CH₄ concentration in exhaled air like automated head chambers, sniffers and hand-held laser CH₄ detectors are in use (Hammond et al. 2016). Short-term measurement techniques are suitable for the screening of a large number of animals and thus might be useful for genetic evaluation and further establishing age effects (Pickering et al. 2015). Indirect traits related to CH₄ emission are also a promising way to practice selection of low CH₄ emitting animals (Roehe et al. 2016; Van Lingen et al. 2014). The proxy currently considered most promising is predicting CH₄ emission by the way of an equation based on milk mid-infrared spectra (Vanlierde et al. 2015), an approach which is, however, restricted to lactating animals. Roehe et al. (2016) suggested that archaeal abundance in the ruminal digesta was under bovine host genetic control, which could be used to genetically predict and select animals with lower CH₄ emission. Meanwhile the archaeal abundance in the ruminal contents collected post-mortem could also possibly be used as a proxy to predict CH₄ emission from cattle (Wallace et al. 2014). Gill et al. (2011) suggested faecal archaeol, a compound specific to archaea, as a proxy to be measured in feces, but, apart from the time-consuming analysis, the accuracy to which CH₄ emissions are predicted is limited (Schwarm et al. 2015).

An approach to assess differences between (groups of) animals in CH₄ emission potential not yet considered would consist of building on *in vitro* ruminal fermentation techniques. Considering animal welfare and the cost of management of maintaining rumen cannulated animals used for *in vitro* fermentation research, it has been reported and suggested that rumen

fluid from slaughtered animals might be used as an alternative for *in vitro* fermentation studies (Basalan et al. 2011; Mohamed and Chaudhry 2008; Yáñez-Ruiz et al. 2016). In the present context, this would have the additional advantage that it gives access to a large number of individuals from different breed types, where therefore not only data on *in vitro* digestibility but, with the same approach, also on CH₄ emission of known individuals could be obtained.

The aim of the present study was to evaluate the utility of a medium-throughput *in vitro* test to detect differences in CH₄ emission between cattle types and cattle of different age using rumen fluid collected at the abattoir from slaughtered animals. With this approach, we investigated in detail whether it is possible to detect (1) a difference in CH₄ emission between beef and dairy cows and different crossbred (beef) or breed (dairy) types, and (2) to establish a relationship between CH₄ emission and age in beef cattle or dairy cows. (3) Finally, a linseed supplementation challenge was included to show whether the response to linseed in CH₄ emission differs between low and high CH₄ emitters and animals of different age and whether it might be suitable for facilitating differentiation between animals and cattle types. Linseed was chosen out of various nutritional approaches as it was repeatedly demonstrated to have the potential to abate CH₄ production from ruminants (e.g. Martin et al. (2016)).

MATERIALS AND METHODS

Collection of Rumen Contents from Slaughtered Cattle and their Characterization

The rumen contents from a total of 103 cattle originating from 70 farms were collected on 13 different days at the abattoir of Zurich. This structure of the dataset obtained therefore minimized potential farm effects. Samples of rumen content were collected from random animals slaughtered on these days within minutes after slaughter. For that purpose, the rumen, passing on a conveyer, was cut open and content was collected from different locations within

the rumen. From one aliquot of the rumen content, the rumen fluid was squeezed immediately into a pre-warmed bottle (filled to the top without headspace) to yield approx. 250 ml. Another aliquot (approx. 200 g) of rumen content was placed into an aluminum container for the later analysis whereof fiber content served as one indicator of the quality of the diet the sampled animals had received last, as diet was otherwise unknown. The rumen fluid samples were kept warm (in a thermobox filled with hand-warm water) and airtight until being processed within 1 h after collection.

Animal identification numbers were recorded. These numbers were retrospectively used to obtain detailed information on breed type, gender and age of the animals through the online database “Agate” of FOAG. Based on this, five animals were excluded because they were far outside of the normal age range of their category. As only seven animals were non-lactating dairy heifers, this category was excluded as well. The remaining 91 animals included 59 beef cattle (beef crossbred cattle of both genders and purebred dairy breed bulls of a maximal age of 600 d), as well as 32 dairy cows. In the sample only entire males were present. The age range covered a span from 273 to 584 days in the beef cattle, and from 1,021 to 4,903 days in the dairy cows. Beef crossbred and dairy breed type comparisons were performed only with types which were represented by at least four animals. This reduced the beef cattle datasets to 28 individuals (12 Limousin $\times$ Brown Swiss (L $\times$ B), four Limousin $\times$ Holstein (L $\times$ H) and 12 Limousin $\times$ Swiss Fleckvieh (L $\times$ S)). The remaining dairy cow datasets contained 26 individuals (11 Brown Swiss (BS), five Holstein Friesian (HF) and ten Swiss Fleckvieh (SF)).

***In Vitro* Fermentation Procedure**

The processing of the rumen fluid is described in this section; the processing of the rumen content is described in the section “Laboratory Analyses”. In the laboratory, the rumen fluid was strained through four layers of gauze (1 mm pore size). An approximate volume of 20 ml

of rumen fluid was analyzed directly for ammonia concentration and pH, and another portion of 100 ml rumen fluid was diluted with preheated (39°C), reduced Menke buffer (Menke and Steingass 1988) in a ratio of 33:67 (v/v) whereof 30 ml were incubated anaerobically in rotating syringes (as described by Soliva and Hess (2007)) at 39°C for 24 h. The syringes were either filled with rumen fluid/buffer mixture alone (as a blank) or together with 200 mg dry matter (DM) of a basal diet. The basal diet consisted of grass hay and dairy concentrate (UFA 142, Herzogenbuchsee, Switzerland) in a ratio of 80:20 (w/w). Hay and concentrate had been ground with a centrifugal mill through a 0.75 and 1.0 mm screen, respectively. As analyzed, the basal diet had contents of organic matter, neutral detergent fiber (NDF), crude protein and ether extract of 912, 457, 150 and 28 g/kg DM, respectively. The rumen fluid of each individual was also tested with this basal diet plus additional linseed (*Linum usitatissimum*) in an amount equivalent to 35 g ether extract/kg dietary DM. For this purpose, extruded linseed (TradiLin 135; Trinova AG, Wangen, Switzerland) was used consisting of a mixture of linseed and mill by-products in a ratio of 60:40; this is currently a common form of use on farm. It contained, per kg of DM, 957, 320, 181 and 298 g organic matter, NDF, crude protein and ether extract, respectively. The extruded linseed was not further ground, as granularity was already very fine with a particle size distribution (mm) of > 3.1, 2.0-3.1, 1.0-2.0, 0.5-1.0 and < 0.5 in proportions (% of total) of 13, 16, 38, 24 and 9, respectively. Basal diet without and with extra linseed were incubated in duplicate each per cow. The results of the duplicate measurements were averaged before statistical analysis. The total volumes in the syringes were recorded from the calibrated scale before and after 24 h of incubation. Ammonia concentration and pH were measured in the undiluted rumen fluid as well as in the incubation fluid after the incubation was stopped by decanting the fluid into tubes. Meanwhile the fermentation gas was left in the syringe for quantification of CH₄.

Laboratory Analysis

Basal diet and extruded linseed were analyzed for contents of dry matter and organic matter (TGA 701, Leco Corporation, St Joseph, Michigan, USA; AOAC index no. 942.05; AOAC, 1997), nitrogen (N) (TruMac CN, Leco Corporation, St. Joseph, Michigan, USA; AOAC index no. 968.06), NDF by using heat stable α -amylase and being expressed exclusive of residual ash (Fibertec System M 1020 Hot Extractor and 1021 Cold Extractor; Tecator, Höganäs, Sweden), and ether extract (extraction System B-811, Büchi, Flawil, Switzerland; AOAC (AOAC) index no. 963.15). The DM of the rumen content was determined by drying at 105 °C for 48 h. After grinding samples of dried rumen content through a 1-mm screen with a centrifugal mill (Model ZM1, Retsch GmbH, Haan, Germany), the NDF of the rumen content was determined according to Van Soest et al. (1991) using an Ankom 2000 Fibre Analyser (Ankom Technology, Macedon, NY, USA) with a heat-stable α -amylase and expressed exclusive of residual ash. Ammonia concentration and pH in rumen fluid and incubation fluid were measured with the respective glass electrodes (6.0506.100 and 6.0204.100 Metrohm, Herisau, Switzerland) connected to a digital pH meter (model 713 and 632, respectively; Metrohm). Fermentation gas was obtained by a gastight syringe (Hamilton, Bonaduz, Switzerland) through a rubber cap from the fermentation syringes. The CH₄ concentration was analyzed by a gas chromatograph (6890N, Agilent Technologies, Wilmington, DE, USA) equipped with a flame ionization detector.

Statistical Analysis

The gas production of the blank, ranging from 7 to 60 ml, was subtracted from the gas production recorded with the test samples to obtain the net amount of gas produced. Data were analyzed using the Mixed procedure of SAS (version 9.3; SAS Institute Inc., Cary, NC,

USA). At first, descriptive statistics was performed. Then the 54 datasets from the six selected crossbred and breed types were subjected to analysis of variance using the model

$$Y_{ijkl} = \mu + T_i + D_j + R_k + T \times D_{ij} + \varepsilon_{ijkl}$$

where Y_{ijkl} = individual observation of the respective trait, μ = overall mean, T = cattle type respectively breed type, D = diet type and $T \times D$ = interaction, all considered as fixed effects, R = run (equivalent to slaughter date) considered as random effect, and ε_{ijkl} = random residual. Multiple comparisons among means were made using the PDIFF option and adjusted by Tukey's method. Differences at a level of $P < 0.05$ were considered significant and at $0.05 \leq P < 0.10$ as a tendency. Finally, within the two major beef crossbred types ($L \times B$ and $L \times S$) and the two main dairy breed types (BS and SF), cattle were classified into low emitters and high emitters based on the ratio of CH_4 /total gas production from *in vitro* fermentation of the basal diet alone. The thresholds for classification as low and high emitters within each cattle breed were set as values being lower or higher than the respective median regarding to CH_4 /total gas ratio, for beef cattle and dairy cows, respectively. These data were evaluated by Student's *t*-test.

In order to characterize the relationship between diet type and age of cattle for traits describing rumen fermentation and CH_4 emission, regression analysis was performed by the REG procedure of SAS with the full model

$$Y_{ijkl} = \mu + \beta_i D + \beta_j A + \beta_k A^2 + \beta_l (D \times A) + \varepsilon_{ijkl}$$

where Y_{ijkl} = individual observation of the respective trait, μ = overall mean, $\beta_{i...l}$ = regression coefficients of the fixed effects diet type (without and with linseed), age (A and A^2 , linear and squared transformed), the interaction of age and diet type ($D \times A$), and ε_{ijkl} = random residual. Here data from all 91 cattle were included. Including age both as linear and age squared term allowed modeling nonlinear relationships of the traits with age. Eventually, the models for each trait with the lowest Akaike information criterion (AIC) were chosen which

included D and either A or A² or both. The equations are given together with the unadjusted R², standard errors (SE) as well as significance levels for individual coefficients and the overall equation in a table. The plots were produced by SigmaPlot 12 (Systat Software GmbH, Germany).

RESULTS

Characteristics of the Rumen Contents of the Slaughtered Animals as collected

The pH range measured in the fresh rumen fluid from beef cattle was from 5.3 to 6.7, which was smaller than that from 5.2 to 6.9 found in the dairy cows (Table 1). On the contrary, the range in ammonia concentration found in the fresh rumen fluid from beef cattle (0.7 to 40.0 mmol/l) was larger than that found in the dairy cows (1.5 to 18.8 mmol/l). The DM in the rumen content was covering a range from 98 to 177 g/kg and was quite similar in beef cattle and dairy cows. Also the NDF contents were similar on average (about 600 g/kg DM) though with a slightly lower span of the range for the dairy cows.

Cattle and Diet Type Effects on *in vitro* Fermentation traits

The gas production of the blank (ml) amounted to 30 ± 12 (mean $\pm$ SD, $n = 103$) and was higher in beef and dairy males ($n = 59$, range: 18 to 60, mean $\pm$ SD: 36 ± 9.8) than in dairy cows ($n = 32$, range: 7.0 to 39, mean $\pm$ SD: 21 ± 8.3). Concerning pH, no effects of cattle type, i.e. beef cattle (crossbreds only) vs. dairy cows, and diet type (+/- linseed) were found, and there was also a lack of cattle type effect in total gas yield (gas amount produced per unit of diet DM) (Table 2). The incubation of the rumen fluid from the dairy cows produced lower ($P < 0.05$) ammonia concentrations, CH₄ yields (ml per unit of diet DM) and CH₄/total gas ratios than that of beef cattle. Compared to control, the supplementation of linseed decreased ($P < 0.05$) total gas yield, CH₄ yield and proportion of CH₄ in the total gas. Within different

crossbred types of beef cattle, the incubation of rumen fluid from L×B resulted in lower ($P < 0.05$) ammonia concentration, CH₄ yield, total gas yield and CH₄/total gas ratio ($P < 0.10$) compared to the crossbred type L×H. No differences were found between L×B and L×S, whereas the latter differed ($P < 0.05$) from L×H in ammonia concentration. Within different breed types of dairy cows, the incubation of rumen fluid from BS resulted in a higher pH ($P < 0.05$) and ammonia concentration ($P < 0.10$) compared to HF and SF, respectively. No significant difference between dairy cow breeds in total gas, CH₄ yield and the ratio of CH₄/total gas were found. Compared to control, supplementing linseed decreased total gas ($P < 0.10$) and CH₄ yield ($P < 0.05$) across beef cattle crossbreds, and reduced ($P < 0.05$) CH₄ yield and ratio of CH₄/total gas across dairy cow breeds. There was no interaction between diet type and cattle type in any of the variables determined.

Response of Low and High Emitters in *in vitro* CH₄ Production to Supplementary Linseed

Compared to control, supplementing linseed decreased ($P < 0.05$) CH₄/total gas ratios from high emitters of all breeds and crossbreds and from low emitters of BS (Figure 1). In the low emitters of L×B crossbreds there was a similar trend ($P < 0.10$). The response to linseed differed ($P < 0.05$) between low and high emitters in L×S, with the greater response found in the high emitters.

Relationship between Cattle Age and *in vitro* Rumen Fermentation and CH₄ Emission

In beef cattle and dairy cows, total gas and CH₄ yields, CH₄/total gas ratio, ammonia concentration and pH (beef cattle only) were related ($P < 0.05$; $P < 0.10$ for pH and ammonia concentration in beef cattle) to age of the cattle (Table 3, Figures 2 and 3). The relationship between age and CH₄ yield was positive and linear for beef cattle. It was curvilinear for dairy

cows with higher values for young and old dairy cows. For both beef cattle and dairy cows, relationships ($P < 0.05$) similar in shape to that found for CH_4 yield in dairy cows were observed regarding the CH_4 /total gas ratio and ammonia concentration ($P < 0.10$ in beef; pH as well). The shapes of the regression curves were similar without and with linseed supplementation and not significantly different.

DISCUSSION

We determined variation in CH_4 emission from fermenting the same feed in rumen fluid from beef cattle and dairy cow types and from different beef crossbred and dairy breeds, with an additional view on potential age-related changes. The dataset comprised 91 individuals in an age range from < 1 yr to > 13 yr from various cattle types. Methane data were given either per unit of DM supplied (CH_4 yield) or as a proportion of total gas, with the latter indicating changes in CH_4 yield per unit of digestible organic matter reflected by total gas production in Hohenheim Gas Test (Menke and Steingass 1988). Both variables are related to the anticipated CH_4 emission intensity per unit of milk or meat produced by these animals, the real target variable for achieving abatement at similar food production level.

Differences between Beef cattle and Dairy cows in Rumen Fermentation and Methane Emissions

One of the differences noted in the present *in vitro* fermentation test was that the incubation of rumen fluid from beef cattle resulted in a higher CH_4 emission both when expressed per DM (+5.8%) or as a proportion of total gas (+9.3) than that of dairy cows. There are limited published papers comparing directly the level of CH_4 emission between beef cattle and dairy cows. Yan et al. (2009) discussed that higher CH_4 emission levels can be expected in beef cattle vs. dairy cows based on their lower feed quality and lower feeding

level. In addition, due to the lower relative feed intake, beef cattle, compared to dairy cows, are characterized by longer rumen retention time of the feed allowing a greater amount of CH₄ to be produced by the rumen microbes (Yan et al. 2009). Boadi and Wittenberg (2002) reported no significant difference in CH₄ emission between Holstein dairy and Charolais × Simmenthal beef. This might be explained by the same age (12 month), body weight (310 kg), diet and dry matter intake (8.3 kg/d) of the two groups in that study. This is different from the present study where the dairy cows included were mature (minimum age, 2.8 yr), whereas beef cattle were not older than 1.6 yr. The stage of development of the rumen differs between growing and mature animals as does their diet type, which usually also differs between beef and dairy animals. This causes rumen physiology and composition of the microbial population to differ as well. This could explain the difference in CH₄ emission, and in ammonia concentration, between beef and dairy cows observed in the present study.

Nevertheless, the finding of higher CH₄ emission in beef compared to dairy cattle seems puzzling. Swiss beef cattle diets are, different from other countries (Yan et al. 2009), typically richer in starch than Swiss dairy cow diets. In beef fattening often maize silage is the preferred forage and cereal-based concentrate is supplemented, whereas grass and conserved grass are predominant and concentrate levels are typically rather low in Swiss dairy cow systems. Methane formation from starch is much lower than that from fiber, because fibrolytic bacteria produce more acetate and hydrogen while starch-degrading bacteria produce more propionate and less hydrogen (Demeyer 1991). However, the composition of the diet offered to the slaughtered animals was unknown. The rumen content obtained from beef cattle had a higher maximal NDF content than that of the dairy cows consistent with the findings in CH₄ level. This may have been because young bulls from dairy breeds, possibly getting diets with rather low energy and high fiber contents, were included in this evaluation although they were excluded for the beef vs. dairy comparison (n = 28 vs. 26, Table 2). The higher gas production

of the blank (rumen fluid/buffer mixture without basal diet) in beef and dairy males compared to dairy cows (36 ± 9.8 vs. 21 ± 8.3 ml) might be explained by the difference in diet regime. Similar to batch culture experiments where rumen fluid is collected in the morning before feeding (from the living animal), the slaughtered animals in the present study likely have had no access to feed for several hours. The duration of feed withdrawal is expected to differ in a random manner and thus might have increased the variability of the results because time of rumen sampling relative to feeding has an effect on microbial diversity and activity (Yáñez-Ruiz et al. 2016).

With respect to ammonia concentration, the results were unexpected as beef cattle, having higher levels than dairy cows both before and after incubation, typically receive diets lower in protein content than dairy cows especially in the finishing period.

Differences between and within Breed Types in Rumen Fermentation and Methane Emissions

In the present study, differences in CH_4 emission were observed among crossbred types of beef cattle, but not among dairy cow breeds. Compared with L×H, L×B produced less ammonia (-20%), CH_4 (-15%, ml/g DM) and total gas (-11%) and exhibited a lower ratio of CH_4 /total gas (-5.2%). The L×S were quite similar to the L×H but variables did not always differ significantly from L×B. Since all crossbreds were Limousin sired, the differences, if genetically determined, are likely to have originated mainly from differences among the dam breeds and less so from within-Limousin breed differences. In this respect, the BS dams apparently had a favorable influence in environmental terms. However, no such differences were found between Brown Swiss cows on the one hand and HF as well as SF cows on the other hand. Therefore, it cannot be excluded that (unknown) diet type differences might have

contributed to this although NDF content of the rumen content did not differ among crossbred types (data not shown).

The CH₄/total gas ratio (ml/l) varied from 153 to 212 (183 ± 13.0 ; mean $\pm$ standard deviation) between individuals, and grouping into low and high emitters resulted in averages of 181 ± 7.2 vs. 197 ± 7.6 for beef and 167 ± 8.2 vs. 185 ± 4.2 for dairy cows a variation which gives rise to the hope for the feasibility of a future selection of low emitters. De Haas et al. (2011) reported that, based on existing genetic variation, a reduction in CH₄ emission in the order of 11 to 26 % is theoretically possible. Genotypic differences in methanogenesis can either be explained by direct effects (Goopy et al. 2014) like different digesta passage times and rumen volumes, but also the rumen environment might be changed. The other contributing factors are indirect effects such as a better feed conversion efficiency as found by Hegarty et al. (2007) in beef cattle with low residual feed intake. These cattle had a lower daily CH₄ production. From the heritability assessments made by Pinares-Patiño et al. (2013), it can be concluded that about half of the inheritance of enteric CH₄ emissions can be explained by direct effects, the other half by effects on feed efficiency. The *in vitro* test introduced in the present study is only useful to identify differences based on shifts manifesting in changes of microbial populations but differences mediated by host animal-dependent traits like passage rate, rumen volumes and feed conversion efficiency may not be captured.

Response of different Cattle Types in CH₄ Emission to Linseed Supplementation

Lipids have been shown to be among the diet components with the highest potential to decrease CH₄ emission. Their effect is mediated through diminishing the activity of methanogens and protozoa, through the hydrogenation of fatty acids in case of lipids rich in unsaturated fatty acids and, to some extent, through decreasing ruminal fiber digestibility (reviewed by Beauchemin et al. (2008)). For sustainable ruminant production it is decisive that

the proportionate decrease in CH_4 emission by nutritional means is greater than the proportionate decrease in digestibility. Therefore, comparisons of CH_4 emission measurements (*in vitro* and *in vivo*) must be made using a relative scale, e.g. in relation to (digested) dry matter (intake), rather than comparing absolute daily CH_4 emissions. Especially linseed has been studied extensively with respect to the potential to abate CH_4 production from dairy cows (most recently by Martin et al. (2016) and growing-fattening animals (Machmüller et al. 2000)). The mitigating effect is variable depending on the type of diet (Chung et al. 2011), the doses supplemented and the way linseed is presented (Martin et al. 2010; Martin et al. 2008). In the present study, the incubation of extruded linseed supplemented at a level of 35 g lipids/kg dietary DM overall decreased CH_4 yield and CH_4 /total gas ratio by 6% and 3%, respectively, which is lower than the expected reduction calculated from the equation developed by Beauchemin et al. (2008) that CH_4 g/kg DM intake should have decreased by 20% with this level of addition of supplemental fat.

The main focus of the present approach was to test whether a CH_4 abating feed supplement would promote cattle type differences to get more pronounced. The underlying hypothesis (not tested in the present study) is that cattle types or group of animals differ in the number and diversity of rumen microbial species as well as their proportions and activities, and therefore would vary in their response to a feed supplement. However, there were no significant interactions between cattle type and linseed effects. Also concerning ammonia concentration in incubation liquid no such interaction was found. We performed a closer look at the response of the two crossbred types and two dairy breeds which were represented by higher animal numbers in the dataset by grouping them retrospectively according to the ratio of CH_4 yield to total gas from the control diet. The CH_4 mitigating effect of linseed supply apparently was more pronounced in cattle with high CH_4 producing ability. For application purpose a differentiation of low emitters with the linseed challenge would have been more

useful. Overall it thus seems that linseed is only weakly helpful in enhancing potential cattle type differences in CH₄/total gas ratio.

Variation with Age of Cattle in *in vitro* Rumen Fermentation and Methane Emission

The age variation was consistent across cattle types regarding CH₄/total gas ratio and ammonia concentration, but not for CH₄ yield and total gas production. Most CH₄ dependent variables first showed a decline at medium ages of the respective age range followed by an increase with greater age. One exception was CH₄ yield with its linear increase in beef cattle, a finding consistent with Grandl et al. (2016) in heifers (0.5 yr to almost 3 yr of age). However, the increase with age was not as high and starting at a clearly higher level in the present study. Across a large age range (up to 3500 d), Grandl et al. (2016) also found an age-dependent shape in CH₄ yield in cows; however, this with a maximum at medium age (around 1500 to 2000 d), which is opposite to the result in the present study (minimum occurring around 2000 to 3000 d). The study of Grandl et al. (2016) and the present study are not completely comparable, because the former was an *in vivo* study with CH₄ emissions assessed in respiration chamber. Whereas in the present test age-related changes were assessed in the rumen environment only and influences of, for instance, changing rumen size or ruminal retention time could not be accounted for. Other factors changing with age like chewing behavior as well as feed efficiency, found to be also important explanatory variables for the age effects in the *in vivo* study of Grandl et al. (2016), could not be simulated. This could explain the differences in the findings. Like with the cattle types, the incubation of rumen fluid supplemented with linseed did not interact with the age effects. Therefore, the mitigating effect of linseed on CH₄ emission seems to be independent from the age of cattle.

The relationship between incubation fluid ammonia and age of beef and dairy cows had a similar curvilinear shape as that in CH₄. It is unclear if the dietary crude protein content varied

accordingly between age classes or if the maximum ability to absorb the ammonia by rumen first increased with the age of cattle and decreased afterwards. The latter might reflect the rhythm of the development of rumen function and digestive physiological properties along with age.

CONCLUSIONS

The present study provided the first indications for a potential usefulness of rumen fluid from slaughtered cattle to detect differences in CH₄ emission of cattle types and age by an *in vitro* test. The advantage of this test is that it is comparably fast (rumen fluid from about 50 animals could be incubated at once) and quite inexpensive once the equipment is available. The test is not yet sensitive enough to be used as a tool to follow genotypic differences in CH₄ yield, especially not for individuals. Another limitation of the test investigated in the present study is that only genetic differences in the rumen environment can be detected and no other animal-specific variables, and that there is the confounding factor of the unknown diet the animal received before slaughter. Improvements may be obtained by recording rumen mass and getting additional knowledge about the diet the slaughtered individuals received. This might be accomplished by integrating diet data in the animal database or by farm identification or both. Using this information would also allow to consider the effects of farm of origin. The test could be also useful to determine age-dependent changes in the ruminal environment as far as CH₄ and ammonia formation is concerned, but lacks the potential to cover overall relevant age-dependent changes taking place in the animals. Linseed supplementation apparently is only a weak means to promote differentiation between animals and cattle types of different CH₄/total gas ratios.

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Table 1. Properties of the rumen fluid and the rumen content as obtained from the slaughtered cattle.

Item	Cattle type ^a	n	Mean	SD	Min	Max
Rumen fluid						
pH	All cattle	91	5.99	0.408	5.24	6.86
	Beef cattle	59	6.01	0.394	5.30	6.67
	Dairy cows	32	5.95	0.437	5.24	6.86
Ammonia, mmol/l	All cattle	83	8.84	6.650	0.67	39.95
	Beef cattle	59	9.49	7.134	0.67	39.95
	Dairy cows	24	7.23	5.053	1.47	18.79
Rumen content						
Dry matter (DM), g/kg	All cattle	91	147	16.0	98	177
	Beef cattle	59	152	12.9	108	177
	Dairy cows	32	138	17.5	98.2	169
Neutral detergent fiber, g/kg DM	All cattle	91	602	33.3	519	710
	Beef cattle	59	602	35.6	519	710
	Dairy cows	32	601	29.0	550	653

Note: SD, standard deviation; Min, minimum; Max, maximum.

^aAge range: Beef cattle (273-584 days); dairy cows (1021-4903 days).

Table 2. Fermentation characteristics after 24 h *in vitro* incubation of rumen fluid harvested from different crossbred types of beef cattle (young purebred bulls of dairy breed excluded) and different breeds of dairy cows at the abattoir together with either control feed or control feed supplemented with linseed.

Group ^a	n	pH	Ammonia, mmol/l	Total gas, ml/g dry matter	CH ₄ , ml/g dry matter	CH ₄ /total gas, ml/l
All cattle						
Beef cattle	28	6.63	31.7	213	39.9	188
Dairy cows	26	6.58	19.5	219	37.7	172
Control	54	6.62	25.8	220	40.2	183
Linseed	54	6.59	26.0	212	37.6	178
SEM		0.015	1.10	1.9	0.44	1.3
<i>P</i> -values						
Cattle type		0.13	<0.001	0.32	0.018	<0.001
Diet type		0.18	0.96	0.028	<0.001	0.001
Cattle × diet		0.80	0.68	0.45	0.66	0.52
Beef cattle						
Limousin × Brown Swiss	12	6.63	28.9b	206b	38.0b	184(b)
Limousin × Holstein	4	6.52	36.0a	231a	44.8a	194(a)
Limousin × Swiss Fleckvieh	12	6.67	33.0b	213ab	40.3ab	189(ab)
Control	28	6.65	31.4	218	41.4	190
Linseed	28	6.62	32.0	207	38.5	185
SEM		0.021	1.53	3.1	0.65	1.4
<i>P</i> -values						
Crossbred type		0.31	0.015	0.043	0.003	0.071
Diet type		0.52	0.78	0.056	0.015	0.18
Crossbred × diet		0.67	0.98	0.89	0.93	0.89
Dairy cows						
Brown Swiss	11	6.60a	21.3(a)	220	37.6	171
Holstein Friesian	5	6.52b	20.1(ab)	222	39.0	175
Swiss Fleckvieh	10	6.58ab	17.4(b)	216	37.1	172
Control	26	6.59	19.9	221	38.8	176
Linseed	26	6.57	19.2	216	36.6	169
SEM		0.020	0.99	2.2	0.53	1.6
<i>P</i> -values						
Breed type		0.007	0.079	0.66	0.40	0.95
Diet type		0.38	0.55	0.11	0.009	0.008
Breed × diet		0.99	0.67	0.32	0.52	0.88

Note: Means in the same column within category (beef cattle, dairy cows) sharing no common superscript are significantly different ($P < 0.05$). Superscripts in brackets indicate a trend of a difference among means ($P < 0.10$).

^aValues of each (cross) breed type are given as the average of the control and linseed treatment because the interaction of breed and diet was not significant.

Table 3. Regression coefficients for variables depending on age and diet type (without and with linseed) for beef and dairy cattle.

Item	Intercept	Age	Age × age	Diet type	Age × diet	R ²	SE	CV	P-value
Beef cattle (n=59)									
pH	7.65*	-4.34×10 ⁻³ *	4.31×10 ⁻⁶ #	-0.0352	— ^a	0.107	0.160	2.42	0.005
Ammonia, mmol/l	105*	-0.309*	3.14×10 ⁻⁴ #	0.408	—	0.078	11.6	35.9	0.026
Total gas, ml/g dry matter	59.1	0.676*	-6.88×10 ⁻⁴ *	-9.19*	—	0.125	23.1	10.8	0.002
Methane emission, ml/g dry matter	34.8*	0.0148*	—	-2.27*	—	0.085	5.13	12.88	0.006
ml/l of total gas	297*	-0.516*	5.93×10 ⁻⁴ *	-2.67	—	0.106	11.7	6.28	0.005
Dairy cows (n=32)									
pH	6.60*	—	-8.75×10 ⁻¹⁰	-0.0195	—	0.007	0.140	2.12	0.81
Ammonia, mmol/l	43.5*	-0.0190*	3.37×10 ⁻⁶ *	0.316	—	0.280	6.45	30.8	<0.001
Total gas, ml/g dry matter	235*	-5.07×10 ⁻³ *	—	-7.85#	—	0.138	17.2	7.89	0.011
Methane emission ml/g dry matter	48.7*	-7.35×10 ⁻³ *	1.21×10 ⁻⁶ *	-2.47*	—	0.252	3.38	8.93	<0.001
ml/l of total gas	193*	-0.0164*	3.26×10 ⁻⁶ *	-5.39#	—	0.196	11.1	6.36	0.004

Note: CV, coefficient of variation; R², coefficient of determination; SE, standard error; * $P < 0.05$; # $P < 0.10$.

^aVariable not included in the final regression model.

Figure Captions

Figure 1. Inhibition of methane (CH_4) production by linseed supplementation in *in vitro* incubation in low and high CH_4 emitters (i.e. lower or higher than the median of CH_4 /total gas ratios within breed type without linseed for beef cattle and dairy cows, respectively) depending on cattle type. * $P < 0.05$, # $P < 0.10$ describe differences between control and linseed supplementation in low or high methane emitters. P -values given in the figure describe differences between low and high CH_4 emitters within cattle type ($n = 5-6$).

Figure 2. Observed values and prediction lines for regression fits for beef cattle for rumen fermentation and methane emissions measured without (Control, gray symbol and line) and with linseed supplementation (black symbol and line) depending on age. Predictions were illustrated by solid lines if the regression coefficients for both age and diet type were significant at $P < 0.05$. Only one prediction line was drawn in case of $P \geq 0.10$ for diet type. Dashed lines were drawn for $0.05 \leq P < 0.10$ for age-squared term. Regression equations are given in Table 3.

Figure 3. Observed values and prediction lines for regression fits for dairy cows for rumen fermentation and methane emissions measured without (Control, gray symbol and line) and with linseed supplementation (black symbol and line) depending on age. Predictions were illustrated by solid lines if the regression coefficients for both age and diet type were significant at $P < 0.05$. Only one prediction line was drawn in case of $P \geq 0.10$ for diet type. Dotted lines were drawn for $0.05 \leq P < 0.10$ for diet type. Regression equations are given in Table 3.

Figure 1.

Figure 2.

Figure 3.