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Immediate effect of *Acacia mearnsii* tannins on methane emissions and milk fatty acid profiles of dairy cows

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*Abbreviations:* AA, arachidonic acid (C20:4 n-3);ADFom, ash-free acid detergent fiber; ALA, α-linolenic acid (C18:3 n-3); aNDFom, ash-free neutral detergent fiber; BH, biohydrogenation; CT, condensed tannins; DHA, docosahexaenoic acid (C22:6 n-3); DM, dry matter; DMI, dry matter intake; ECM, energy-corrected milk; EPA, eicosapentaenoic acid (C20:5 n-3); Eq, equation; FA, fatty acid; FAME, FA methyl ester; Im, methane emission intensity; MFA, milk fatty acids; MUFA, mono-unsaturated fatty acid; Pm, methane production; PUFA, polyunsaturated fatty acids; RC, respiration chamber; RMSPE, root means square of prediction error; Ym, methane yield

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A B S T R A C T

The effects of dietary supplements for modifying cattle digestion and metabolism are typically measured after one or more weeks of adaptation. Consequently, how quickly the effects occur remains unknown. The long-term efficacy of *Acacia mearnssii* bark tannins (Acacia) on methane mitigation has been previously demonstrated. The present study, therefore, investigated the time it took for the extract to affect methane emissions and milk fatty acid profiles. Twenty lactating Brown Swiss dairy cows, categorized as 10 low- and 10 high methane emitters (average difference in methane yield: 0.10 of total), were housed in respiration chambers for 4 days. A control diet consisting of a mixed ration supplemented with grass pellets and concentrate pellets was fed initially (Day 0). The original pellets were then replaced with pellets containing 141 g Acacia/kg, providing 30 g Acacia/kg of dietary dry matter (DM) (Days 1-3). Methane emissions were measured every 10 min and gas chromatography was used to analyze individual fatty acids from daily milk samples. A significant decline in methane production was detected 20 min after starting supplementation, with methane production (g/day) and methane yield (g/kg DM intake) decreasing in a linear fashion from Day 0 to Day 3 by up to proportionately 0.18 and 0.16, respectively. Additionally, changes in proportions of various milk fatty acids occurred within 3 days of Acacia feeding. Using stepwise multiple regression analysis, several milk fatty acids were identified as being related to methane emissions. Applicable equations from the literature also showed relationships to methane emissions of high and low emitters as well as to the Acacia diet effect on methane emissions. The equations with close relationships all included minor and non-dietary milk fatty acids like odd-chain fatty acids. These specific fatty acids originate from rumen microbial activity. In conclusion, *A. mearnsii* extract is an immediately acting methane mitigating supplement. Certain milk fatty acids are related to methane emission in dairy cows and may assist in determining whether and when tanniferous supplements will act against enteric methane formation.

*Keywords:* Supplement, methanogenesis, milk fat, rumen, cattle

**1. Introduction**

Methane is a potent greenhouse gas and its emissions from ruminant husbandry substantially contribute to climate change. Among the dietary means for mitigating enteric methane formation in ruminants is supplementation with plant secondary constituents, especially tannins. Tannins are chemically heterogeneous polyphenols (Patra and Saxena, 2010) including hydrolyzable tannins (molecular weight 500 to 3000 g/mol) and condensed tannins (CT; molecular weight up to 20,000 g/mol). Tannins are known for the direct and indirect effects they exert on rumen microbes, with potential to lower dietary protein degradability in the rumen, decrease methanogenesis and biohydrogenation (BH) of unsaturated fatty acids (FA; e.g., Makkar, 2003), and promote microbial formation of non-dietary FA (*iso/anteiso* and uneven chain FA; Cabiddu et al., 2009). However, to date only a few tannin sources have been found to be highly effective methane mitigators *in vivo*. These included tannins from *Acacia mearnsii, Castaneva sativa, Hedysarum coronarium, Lespedeza cuneata* and *striata, Lotus corniculatus* and *Terminalia chebula* (reviewed by Patra and Saxena, 2010). Additionally, *in vitro* results are often not repeatable *in vivo*, or are lower in magnitude (Patra and Saxena, 2010). The tannin-rich extract from the bark of *A. mearnsii* has repeatedly proven effective in *in vivo* methane mitigation (Alves et al., 2017; Carulla et al., 2005; Grainger et al., 2009; Staerfl et al., 2012). In this context, Staerfl et al. (2012) showed that there was no adaptation of microbes involved in ruminal methanogenensis over 11 months of feeding this tannin source to beef cattle. Tannins from *A. mearnsii* were also demonstrated *in vitro* by Khiaosa-Ard et al. (2009) to partially terminate the last step of the BH of polyunsaturated FA (PUFA) and to enrich BH intermediates, especially C18:1 t11 (vaccenic acid). This FA can be transformed to C18:2 c9 t11 (rumenic acid) in the rumen and particularly in the milk (Buccioni et al., 2015). Tannins may also protect some PUFA from being subjected to BH at all (e.g., Bucconi et al., 2015; Henke et al., 2017). In one study (Aprianita et al., 2014) only weak effects of *A. mearnsii* on proportions of the FA in milk typically affected by BH (C18:1 t11, C18:2 c9 t11, and C18:3 n-3) were found. Other researchers (Benchaar and Chouinard, 2009; Dschaak et al., 2011) also found only a minor impact of other tannin sources on the milk fatty acid (MFA) profile. Studies on digestion and metabolism are typically carried out after a longer adaption period has been carried out. Accordingly, this happens when any time trends have equilibrated. Therefore, it has remained unexplored how quickly *A. mearnsii* tannins influence rumen microbes, and thus methane formation and the MFA profile.

The concomitant modification of methanogenesis and the milk FA profile indicates that there might be a relationship with cows’ methane emissions and the dietary effects on methane. Several authors have shown a moderate relationship between MFA and methane emissions (Bougouin et al., 2019; Engelke et al., 2018 and 2019; Rico et al., 2016; van Gastelen et al., 2017 and 2018; van Lingen et al., 2014). Such a relationship could be useful in confirming or disproving the methane mitigating effect of a tannin-based supplement by analyzing the MFA profile.

The goal of the current study was to obtain more information about the efficiency of the extract of *A. mearnsii*, particularly concerning the time needed to start mitigation of methanogenesis in the rumen and to determine whether there is a concomitant change in the MFA profile of the cows. A respiration chamber (RC) experiment with dairy cows was conducted to thoroughly test the following hypotheses. (i) There is a response to the *A. mearnsii* tannin extract in enteric methane production within the first 3 days of supplementation. (ii) There is a response to the *A. mearnsii* tannin extract in MFA. (iii) The responses in methane and MFA are similar in cows having an inherently low or high methane production. (iv) There is a close relationship between the dairy cows’ methane emissions and the proportions of those MFA which are modified by rumen microbes. (v) Proportions of certain MFA can be used to confirm a methane mitigating effect of *A. mearnsii*.

**2. Material and methods**

*2.1. Experimental protocol and diets*

The experiment was conducted from November 2017 to April 2018 at the research station AgroVet-Strickhof (Eschikon, Lindau, Switzerland) after being approved by the veterinary office of the Swiss Canton of Zurich (license no. ZH050/17). Each cow stayed there for 23 days. From a total of 30 Brown Swiss dairy cows kept for a different experimental purpose, 20 cows were selected for the present study. The herd was divided into two groups according to their measured methane yield on the first day in the RC (category ‘High’ = 10 cows, category ‘Low’ = 10 cows) based on the results of methane (g/kg dry matter intake) measured in RC on Day 20, i.e. after 19 days of feeding the same diet. Each of the 20 cows originated from a different commercial farm. At the start of the experiment, the cows were, on average, 241 ± 30 days in milk (mean ± standard deviation), weighed 653 ± 48 kg, and yielded 19.8 ± 3.2 kg/day of energy-corrected milk (ECM; i.e., milk yield (kg) × [(0.038 × fat (g/kg) + 0.024 × protein (g/kg) + 0.017 × lactose (g/kg)]/3.14; Agroscope, 2019).

Each of the 20 cows was adapted to the same diet (with ad libitum access) and management (tie-stall barn, swing-over milking system) for 19 days and cows were subsequently kept in the RC for 4 days. Cows were investigated simultaneously, in blocks of four, in the four available RC. On the first day in the RC (Day 0) the cows received the same diet (control) as received in the preceding 19 days. This was a total mixed ration proportionately composed on a dry matter (DM) basis of corn silage, 0.55; grass silage, 0.38; grass hay, 0.02; and dairy protein concentrate (UFA-250, UFA, Sursee, Switzerland), 0.05 (Table 1). In separate troughs, cows received 125 g grass pellets and 250 g of a pelleted energy-rich concentrate (UFA-243, UFA, Sursee, Switzerland), per kilogram of milk produced. The energy-rich concentrate was included to meet the requirements for maintenance and milk yield (Agroscope, 2019).

From the second day in the RC onward (Days 1 to 3), the grass pellets and concentrate pellets were replaced with pellets containing an extract from the bark of *Acacia mearnsii* (Weibull Black, TANAC S.A., Montenegro, Brazil) providing the Acacia diet*.* The batch of *A. mearnsii* extract used was analyzed and contained 75.1, 17.9 and 60.8 g/kg total tannins, CT and hydrolysable tannins, respectively. These pellets contained (g/kg DM): *A. mearnsii* extract, 141; maize, 153; wheat, 133; soybean meal, 433; rumen-protected fat, 50; molasses, 25; CaCO3, 25; Ca(H2PO4)2, 11.5; MgO, 12.5; NaCl, 8; mineral mix, 5.The Acacia pellets were allocated to each individual cow in amounts equivalent to 30 g Acacia extract/kg of dietary DM. The Acacia pellets were 4.5 mm in diameter (Kahl 40 PS, Amandus Kahl GmbH & Co, Reinbeck, Germany) and were produced with steam (Bühler AG, Uzwil, Switzerland) at < 60°C. For both the control and Acacia diets, one third of the pellets were fed at 0600 h, 1000 h, and 1700 h. All cows received 50 g NaCl and 100 g vitamin-mineral mix daily during the morning feeding. The latter contained (per kg) 160 g Ca, 80 g P, 100 g Mg, 32 g Na, 10 g S, 8.0 mg Zn, 4.0 mg Mn, 1.0 mg Cu, 30 mg Se, 100 mg I, 30 mg Co, 1,200,000 IU vitamin A, 150 g biotin, 200,000 IU vitamin D3, and 3,000 mg vitamin E.

*2.2. Data recording and sampling*

Feed intake was recorded from the day before until the end of the RC stay by weighing feeds offered and leftover at each feeding event. Pellets (energy concentrate, grass, and tannin) were always consumed completely. Forage samples were taken once per week and pellet samples three times during the entire experiment. Samples were dried at 60°C and ground to 1 mm using either a cutting or a centrifugal mill. Milking took place at 0550 h and 1645 h. Milk amounts were recorded on a scale (ID2 Multirange, Mettler-Toledo, Greifensee, Switzerland). Samples from each milking were collected. Part of the milk was conserved with Bronopol and the other part was pooled proportionally to the milk amount and frozen at -20°C.

Individual cows’ methane emissions were measured in four RC (No Pollution, Industrial Systems, Edinburgh, United Kingdom) recently installed at the research station. The cows were familiarized with the RC in the first part of the 19-day adaptation period by stays in RC of several hours. The RC were 4.75 m wide, 3.25 m deep, and 2.5 m tall (38.0 m3). They were sealed with rubber and fitted with one large back door for animal entrance and a smaller front door with safety closing devices. The tie stalls were 255 × 150 cm and were equipped with water troughs and feed bins mounted on a scale. The RC were opened twice daily at the same time for milking and feeding, which took approximately 20 min. Subsequently, re-establishing the chamber equilibrium in methane concentration needed 40 to 60 min. For that reason, methane data from these time periods (40 to 60 min) were not used but were interpolated from values obtained before and after this period. By contrast, the start of Acacia feeding at 10.00 h on Day 1 was accomplished by entering the chambers for about 5 s and putting the pellets onto the basal diet. Thus a decline in CH4 concentration could be avoided and the short-term response to the Acacia feeding could be measured without bias. Fresh air was supplied through a common duct through two shutters (SPI-F160, Systemair AB, Buchs ZH, Switzerland; and LM 230, BELIMO Automation AG, Hinwil, Switzerland). The RC were maintained at 16°C at a relative humidity of 60%. Spent air was removed at 19.0 to 23.0 L/s (equivalent to an air exchange of about 12 times/h) with an extraction fan (K06 – MAS Blower, FPZ Blower Technology, Concorezzo, Italy), coupled with a frequency controller (VLT 3.3 Kw, HWAC Drive, Danfoss, Offenbach, Germany) for maintaining airflow. The RC were kept under slight negative pressure.

In five measurement intervals per 10-min cycle, methane concentration in the air exiting each of the four RC and in the fresh air collected at a distant site was measured with an MGA 3500 (ADC Gas Analysis, Hertfordshire, UK). This resulted in measurement intervals of 2 min each, the time needed for concentration in the analyzer to stabilize. Zero and standard gas calibration was conducted immediately before and after each experimental run. The gas analyzer calibration was initiated manually, but the calibration process was performed automatically. First, pure N2 was applied. Then a first standard gas mixture (proportionately 0.001 H2 and 0.999 N2) was delivered for 3 min until the H2 level stabilized, followed by applying only N2 for 3 min again. The second standard gas mixture (proportionately 0.0008 methane, 0.209 O2, 0.004 CO2, and 0.786 N2) was delivered to let the instrument return to its normal operating range. A recovery test (total calibration) for methane was performed three times per RC throughout the experiment. Methane (0.999 of total) was injected at 0.35 L/min via a tube outside the wall for 4 h until the measured concentration reached a plateau. The flow rate was controlled by a Sierra mass flow controller (MC-5SLPM-RD, Alicat Scientific, Tucson, AZ, USA). Calibration of the RC and gas analyzer provided a calibration factor for methane. The average recoveries in the four RC were 88, 88, 90, and 89%. Data were adjusted by the recoveries.

*2.3. Laboratory analyses*

Feeds were analyzed according to standard protocols (AOAC, 1995). A thermogravimetric device (TGA-701, Leco St. Joseph, MI, USA; AOAC index no. 942.5) was used for DM and total ash, with organic matter being the difference between them. Crude protein was determined as 6.25 × N obtained with a C/N analyzer (Type TruMac CN, Leco Coperation, St. Joseph, MI; AOAC index no. 968.06). Ether extract was assessed with a Soxhlet extraction apparatus B-811 (Büchi Flawil, Switzerland). Starch contents were analyzed using a polarimeter and following method 7.2.1 of VDLUFA (2012). Ash-corrected contents of neutral (aNDFom; AOAC index no. 2002.04) and acid detergent fiber (ADFom; AOAC index no. 973.1) were analyzed using the Fibertherm FT 12 (Gehardt, Königswinter, Germany). Heat-stable α-amylase (Sigma-Aldrich, St. Louis, USA) was used for aNDFom analysis. Determination of lignin(sa) in feed samples was performed sequentially after ADFom analysis by incubation in sulfuric acid (72%) for 3 h.

Total extractable phenols (TEP), CT and non-tannin phenols were analyzed in the feeds following Makkar (2003) with modifications described by Jayanegara et al. (2011). A double beam spectrometer was used for quantification (UV-6300, VWR international, Radnor, PA, USA) at 725 nm. The TEP and non-tannin phenol contents were expressed as gallic acid equivalents and the difference between the two was assumed to be total tannins. The CT were expressed as leucocyanidin equivalents. Hydrolyzable tannins were computed as the difference between total tannins and CT.

The method used for FA analysis in feed items and milk is described in detail in Ineichen et al. (2019). Briefly, the FA were extracted from the feeds by using a solvent extractor (ASE 200, Dionex Corporation, Sunnyvale, CA, USA) and a hexane:propane-2-ol mixture (3:2 v/v). They were transformed to FA methyl esters (FAME) according to the IUPAC (1987) method 2.301. Cleaning was done as described by Wettstein et al. (2001). The FAME were separated on a gas chromatograph (model HP 6890 equipped with a FID detector, Hewlett Packard, Palo Alto, CA, USA) equipped with a CP7421 column (200 m × 0.25 mm, 0.25 µm; Varian Inc., Darmstadt, Germany). In the milk, FA were analyzed from one pooled sample per cow per day. Sodium methylate was used for cold transesterification to FAME (Suter et al., 1997). The Bronopol-conserved milk was analyzed for fat, protein, and lactose contents using a Milkoscan FT6000 (Foss Electric, Hillerød, Denmark) at SuisseLab (Zollikofen, Switzerland).

*2.4. Statistical analysis*

Analysis of variance was conducted with a linear mixed model using R version 3.3.1 (R Core Team, 2017) with methane emission category (low vs. high emitters), day in RC (Days 0 to 3), and the interaction of both as fixed effects. Experimental run was considered as random effects. Dietary aNDFom content was considered as a covariate when significant. The ‘nlme’ R-package was used (Pinheiro et al., 2017), with the individual cows representing the experimental units. The Shapiro-Wilk test was used to assess data normality and Bartlett’s procedure was used to assess homogeneity of variance. Tukey’s procedure was used for multiple comparisons among Day means, considering *P* < 0.05 significant. Results were presented as least square means and standard errors of the mean. In addition, linear, quadratic, and cubic effects of day in RC were evaluated using the ‘contrasts’ function in R. Methane production was compared for the first 12 × 10 min bouts of Day 0 (control diet) with Day 1 (Acacia diet) using repeated measures analysis of variance.

In order to relate methane measured in RC with MFA profile, regression equations were developed. These included those of methane production (Pm; g/day; Equations (Eq) 1, 2, 6, 11 to 16), methane yield (Ym, g/kg DMI; Eq 3, 8, 10), and methane emission intensity (Im, g/kg ECM; Eq 4, 5, 7, 9). For these calculations, values for methane and MFA from the available 80 datasets (20 cows, 4 days) were used and linear regression models were applied to identify the MFA which might be primarily responsible for methane emission variation. A limitation of this approach was that the within-cow data were not completely independent from each other. Still there was quite a variation across days in methane emission and MFA which justified this approach. For the multivariate regression analysis with individual MFA as the independent variable and methane as dependent variable, the ‘lm’ (linear model) procedure in R version 3.3.1 (R Core Team, 2018) was used applying a stepwise procedure. The *P-*value for MFA to enter or stay in the model was set to 0.10. The final equations were selected based on the minimum Akaike’s information criterion. The selected models were shown to be free of multicollinearity using the ‘fmsb’ R-package (Nakazawa, 2018; variation inflation factor > 10). Bias correction factors Cb (‘epiR’ package in R; Stevenson et al., 2018), concordance correlation coefficients (CCC), and root mean square prediction error (RSMPE) provided accuracy measures for the equations.

In a second approach, regression equations published by other authors were tested for their accuracy to calculate the measured methane emissions from the MFA of our dataset. The regression equations tested were:

Equation from Dijkstra et al. (2011) developed from 50 observations and ten diets:

CH4 (g/kg DMI) = 24.6 + 8.74 × C17:0-*anteiso* – 1.97 × C18:1 t10 & t11 – 9.09 × C18:1 c11 + 5.07 × C18:1 c13: (Eq 5)

Equations from Van Gastelen et al. (2017) developed from 29 observations and four diets:

CH4 (g/day) = 211.2 + 50.4 × C4:0 + 77.7 × C14:1 c9 – 82.0 × C18:1 t11 (Eq 6)

CH4 (g/kg DMI) = 27.2 – 7.0 × C18:2 c9, t11 (Eq 7)

CH4 (g/kg fat-protein-corrected milk yield) = 16.5 + 24.6 × C15:0-*iso* – 15.5 × C17:0 + 52.4 × C22:0   
(Eq 8)

Equations from Van Lingen et al. (2014) developed from 146 observations and 30 diets:

CH4 (g/kg DMI) = 23.39 + 9.74 × C16:0-*iso* – 1.06 × C18:1 t10 & t11 – 1.75 × C18:2 c9 (Eq 9)

CH4 (g/kg fat-protein-corrected milk yield) = 21.13 – 1.38 × C4:0 + 8.53 × C16:0-*iso* – 0.22 × C18:1 c9 – 0.59 × C18:1 t10 & t11 (Eq 10)

Equations from Engelke et al. (2019) developed from 20 observations and four diets:

CH4 (L/d) = 651.2 – 274.4 × 18:1 c11 (Eq 11)

CH4 (L/d) = 841.0 – 11.4 × ∑ of mono-unsaturated FA (MUFA) (Eq 12)

CH4 (L/d) = 118.2 + 25.8 × DMI – 57.2 × 18:1 t9 (Eq 13)

CH4 (L/d) = 271.8 + 24.3 × DMI – 6.1 × ∑ MUFA (Eq 14)

CH4 (L/d) = 201.7 + 14.2 × ECM – 66.9 × 18:1 t9 (Eq 15)

CH4 (L/d) = 390.7 + 13.1 × ECM – 7.6 × ∑ MUFA (Eq 16)

These emissions calculated with the equations were subjected to analysis of variance as described above. The regression models published by Rico et al. (2016) and van Gastelen et al. (2018) were not applicable because they included distinct MFA, which we could not quantified with our GC analysis technique.

**3. Results**

*3.1. Composition of the experimental diets*

The chemical composition of the control diet and the Acacia diet was mostly similar (Table 2). There were some differences in fatty acid proportion; however, these likely resulted from the ingredient composition of the Acacia pellets (other than the *A. mearnsii* extract itself) and control pellets (grass and concentrate). These items (Table 1) were also responsible for some differences in proximate contents between the diets (Table 2). The extract contained twice the content of TEP, which was exclusively due to tannins (both CT and hydrolyzable tannins).

*3.2. Performance and milk composition*

Overall, there was no difference between emission categories (High, Low) in DMI, milk yield, and milk gross composition (fat, protein, and lactose; Table 3). Moreover, there was no change with day in RC in these variables, no significant contrasts, and no significant interaction with category.

Categories only differed in a few MFA (Table 4). The milk fat of the Low cows had lower (*P* < 0.05) proportions of C13:0-*iso* C14:0-*iso*, C16:0-*anteiso*, C15:1, C22:5 *n-3*, and C22:6 *n-3* than that of the High cows. There was also a trend (*P* < 0.10) for higher C18:1 t11 proportions in the Low cows. Along with days in the RC, as determined either by multiple comparisons among means or by contrast analysis, or both, several FA showed a linear change with days. Some FA also responded in a non-linear (quadratic, cubic contrasts) manner or had non-linear components. This was the case with the C12:0-*iso*, C16:0-*iso*,and C12:1 proportions,which were higher (*P* < 0.05) on Day 1 than some or all other days. Although contrasts only showed linear declines (*P* < 0.05) in proportions of some FA, in several cases values were either significantly (C12:0, C14:0-*iso*, C18:0 c10, C18:1 t12, C20:1 n-9, and C22:6 n-3; *P* < 0.05) or numerically (C6:0, C10:0, C15:0, C22:0, C18:1 t8, C18:2 c9 t11, and C18:3 n-6) highest on Day 1 (i.e., the first day of *A. mearnsii* supplementation) compared to Days 2 and 3 and, less pronounced, Day 0. In the proportions of some other FA (C18:1 n-9, C18:2 n-6, C18:2 c9 c15, and C20:3 n-6), the same observation in the opposite direction was made. A linear increase (*P* < 0.05) with day was found with C18:0, c19:1 t9, and C18:2 c9, t11 & t8, c12. Nonsystematic variations among days were registered with C18:1 c14 & t16. Category × Day interactions occurred only in C18:3 n-6 and C22:6 n-3.

*3.3. Methane emissions measured with respiration chambers and calculated by regressions with milk fatty acids*

The cows categorized as low emitters had lower Pm and Ym than the cows categorized as high emitters (proportionately by 0.04 and 0.10 of total, respectively; *P* < 0.05), whereas Im did not differ (Table 5). Our own developed equation discriminated (*P* < 0.05) between Low and High cows in Pm and Ym as well. None of the investigated equations from the literature yielded corresponding category differences. The regressions developed from the current dataset included four useful models, two for Pm, one for Ym, and one for Im (Table 6). The regression model of Pm based on MFA alone had slightly lower adjusted R2, CCC, and a higher RMSPE than that including DMI as an additional input variable. Adjusted R2 and CCC were lower for the best fitting regression for Ym and Im than for Pm. The selected FA were quite different for Pm compared to the FA selected for Ym and Im, with smaller differences between Ym and Im

The measured Pm was highest on Day 0, followed by Day 1, and lowest on Days 2 and 3 (*P* < 0.05) decreasing to 0.82 of the Day 0 Pm value. This day-to-day decline in Pm was also obvious from the within-day evolution where diurnal differences were particularly obvious at times with peak emissions (Fig. 1A). On closer inspection (Fig. 1B), Pm declined shortly after feeding the Acacia diet for the first time on Day 1 compared to the same time on Day 0, showing a significant difference (*P* < 0.05) in eight of the 12 measurements made in 10 min periods. In Ym, the decline was only significant (*P* < 0.05) between Day 0 vs. Days 2 and 3 (Table 5). In two of three methane variables, Pm and Ym the decline with progressing days was linear overall (contrast, *P* < 0.05) eventually amounting to -0.18 of the initial methane production. Our MFA-based regressions showed the methane decline with days in RC in the case of Pm (MFA without and with DMI; Eq 1-2) but not Ym (Eq 3), as shown in the linear contrasts (*P* < 0.05) and partly in the multiple comparisons among means. Equations 9 and 10 successfully described the declines in Ym and Im, but had both a linear and a non-linear component (cubic contrast, *P* < 0.05). With the other equations derived from the literature, no significant changes with day were calculated in either Pm, Ym, or Im and there were no category × Day interactions in the measured or calculated methane emissions.

**4. Discussion**

*4.1. Short-term effects of the* Acacia mearnsii *tannin extract on methanogenesis*

In recent years, tannins have been found to be useful phytochemicals for modulating rumen microbial fermentation in a beneficial way (Mueller‐Harvey, 2006). However, it is challenging to find a balance between a sufficiently high concentration to generate an effect without impairing, for instance, feed intake and nutrient utilization (Kumar and Vaithiyanathan, 1990). Moreover, the carbon footprint of the tannin supplement must be smaller than the gain by its use. The *A. mearnsii* product as used at a level of 30 g/kg (equivalent to 15 g total tannins/kg as analyzed) by Staerfl et al. (2012) proved to fulfill both requirements. The DMI remained unchanged from the day before the cows were put into the RC (16.0 kg DM) and changed only minimally afterwards (16.1, 15.8, 15.8, and 15.6 kg DM, on Days 0-3, respectively). This indicates that the methane decline on Day 1 was not a delayed response to a lower feed intake on Day 0. *A. mearnsii* reduced methane formation, supporting the results of previous ruminant studies (cf. introduction). Linear declines from Day 0 to Day 3 were not only found for Pm but also for Ym and Im, which is important because declines in Pm, even with intake and performance unchanged, could result from lower digestibility. The most important result was the immediate response in methane production after feeding *A. mearnsii* (confirming hypothesis i). The animals were observed to start eating immediately after being provided with the Acacia diet. This quick response would only be possible when the tannins acted directly upon rumen microbes involved in methanogenesis (methanogens and protozoa, or fiber degrading bacteria, or all of them; Patra and Saxena, 2011). The decline in methane formation progressed with days of exposure suggesting that the decline in Ym to 0.16 of initial after 3 days of exposure to *A. mearnsii* did not reach the maximum level of mitigation. This is consistent with the decline in methane (Pm and Ym) following *A. mearnsii* ingestion described by Alves et al. (2017) and Grainger et al. (2009) in dairy cows and by Staerfl et al. (2012) in beef cattle. In contrast, feeding 30 g *A. mearnsii* extract/kg diet did not decrease the CH4 level in the short- or long-term in a study using a photoacoutsic system for measuring CH4 emission at the herd level (Schmithausen et al., 2018). Given the lack of interactions between category and Day, the present results demonstrated that *A. mearnsii* was similarly effective in both low and high emitters (confirming hypothesis iii). Therefore, dietary interventions like the one tested seem efficient even in herds with already low methane emission potential.

*4.2. Short-term effects of the* Acacia mearnsii *tannin extract on milk fatty acid profile*

Several authors have shown that tannins at least partially inhibit the ruminal BH of PUFA (Buccioni et al., 2015, Khiaosa-Ard et al., 2019, Vasta et al., 2010). This is of dietary relevance as milk fat may contain more nutritionally relevant MFA, such as C18:3 n-3 and C18:2 n-6, with an unchanged dietary supply (Henke et al., 2017). However, in the short-term (3 days), *A. mearnsii* had no clear effect on the proportions of these MFA, consistent with the results reported by Aprianita et al. (2014). The FA influenced were those associated with *de novo* synthesis by the rumen microbes or the mammary gland supplied with short-chain FA from the rumen. For instance, FA such as C12:0 and C14:0 are synthesized de novo in the mammary gland from ruminal butyrate and acetate produced by fibrolytic bacteria (Bernard et al., 2008); but these two MFA may also originate from dietary C12:0 and C14:0 (Patra, 2013; Van Lingen et al., 2014). We observed an increase of C14:0 proportion in the milk fat from Day 0 to Day 1, which might be the result of a higher C14:0 content in the Acacia diet (0.14 vs. 0.09 g/kg dietary DM when considering ether extract equivalent to total FA). However, C14:0 decreased with time of exposure to *A. mearnsii* (i.e., from Day 1 to Day 3). Henke et al. (2017) also showed a reduced C14:0 concentration when offering 30 g quebracho tannins/kg DM to dairy cows. This suggests a decline in fiber digestibility with the tannins, as described by Carulla et al. (2005) and Staerfl et al. (2012). Because reliable digestibility data need several days of collection under a constant feeding regimen, no such data were available in the present study. Supporting this assumption, C12:0-*iso*, C14:0-*iso*, and C16:0-*iso*, which are FA prevalent in fibrolytic bacteria (Vlaeminck et al., 2006), decreased in a linear and partly cubic manner with the Acacia diet. An anti-protozoal activity (Patra and Saxena, 2011) and an inhibitory action against enzyme activity (Horigome et al., 1988; Il Oh and Hoff, 1986) may provide a mode of action for tannins to modify dietary FA and produce new FA by affecting rumen microbes and their activity. This could be mediated by binding to the microbial enzymes resulting in decreased microbial attachment to feed particles (Morales and Ungerfeld, 2015). One of our specific findings was that the main change in the FA profile (especially the increase in minor FA) happened on Day 1 after of *A. mearnsii* supplementation (precisely: on average 17 h later in the milk composited from the following evening and morning milking) which confirming hypothesis ii. Thereafter, values declined to levels below control values. This suggests a certain and quick adaptation of the rumen microbes directly or indirectly involved in lipid metabolism.

*4.3. Relationships between milk fatty acid proportions and methane emissions of dairy cows*

Direct methane measurements are difficult to conduct under farm conditions; therefore, an MFA-based calculation would facilitate methane emission estimation on farms (Dijkstra et al., 2011, 2016; Rico et al., 2016). The MFA profile mirrors changes in absorbed FA as affected by ruminal metabolism, including lipolysis, BH, and microbial FA synthesis (Fievez et al., 2012). If some of the same microbes are also involved in methanogenesis or are concomitantly affected by external influences, there would be a relationship between MFA and methane emission. Accordingly, attempts to relate MFA and methane emission started about a decade ago (Chilliard et al., 2009; Mohammed et al., 2011). Indeed, proportions of the short- and medium-chain FA in milk (up to C14 or C16) seem to have a close relationship to methane emission (Chilliard et al., 2009; Engelke et al., 2019; Rico et al., 2016; van Gastelen and Dijkstra, 2016; van Lingen et al., 2014). The medium-chain FA are mainly derived from *de novo* synthesis in the mammary gland from ruminal acetate and butyrate, which themselves are positively related to enteric methane production (Bernard et al., 2008). C15:0 and C17:0 are originating from *de novo* synthesis from ruminal propionate, which is negatively related to methane emission (French et al., 2012) and C17:1. Milk C17:1 comes from mammary desaturation of C17:0 (Fievez et al., 2012). Indeed, in the current study, we observed a significant negative slope of the C17:1 by means of all regression models developed from the present data. The *iso*-FA are generally prevalent in fibrolytic bacteria (Montoya et al., 2011; Vlaeminck et al., 2006). Hence, a positive relationship between *iso*-MFA and methane emission (Pm, Ym) is expected (Chilliard et al., 2009). Amylolytic bacteria, which are negatively associated with enteric methane production, are known for their *anteiso*-FA (Fievez et al., 2012). However, in the current study, there was a positive relationship of C17 *anteiso* with methane emission. When supplementing diets with elevated levels of PUFA, these FA and their BH intermediates in milk obtained from ruminal BH should be negatively associated with methane emission (Patra, 2013). However, the PUFA content of the present experimental diets was too low to be effective here. This dichotomy shows that care is needed when interpreting results from studies where oil was supplemented (e.g., Chilliard et al., 2009; Mohammed et al., 2011), because this confounds the effects on methane emission and MFA.

Generally, the relationship between MFA and methane emissions appears to be only moderate, especially when only a single FA is used in the regression equation. Though significant, the relationship calculated for Ym in the present study was less close compared to those described by van Gastelen et al. (2017) and van Lingen et al. (2014) using data from a large variation in forage. When adding DMI as an explanatory variable, Rico et al. (2016) found a clearly higher relationship to Pm (*R*2 = 0.80) than we found in the current study. However, including DMI also improved the R2 of the regression in our study, and we achieved a lower RMSPE in Pm than Rico et al. (2016), indicating our equation’s better accuracy. Engelke et al. (2019) also showed that R2 of the equations increases by including DMI or ECM yield. The results thus partially confirm hypothesis iv, that certain MFA proportions are related to the dairy cows’ methane emissions.

The regression equations derived in the present study allowed to discriminate between categories of low or high emitting cows. This was also the case with the equations of van Lingen et al. (2014). Castro-Montoya et al. (2017) measured daily enteric methane emission with RC and classified cows into three categories according to their daily Pm (high, medium, low). These authors reported that the milk fat of the high emitting cow category had lower proportions of iso-C17:0, C18:0-*iso*, C17:1 *c9*, and C17:0. In addition, they concluded that it is possible to exclude observations from extreme categories by the MFA, whereas the MFA are less suitable to correctly identifying individual low emitters. We observed that the low emitters were categorized by lower proportions of C13:0-*iso* and C14:0-*iso* in milk fat, suggesting that these cows may have a lower prevalence of fibrolytic bacteria or lower fiber degrading activity in the rumen, associated with lower methane formation. This would be consistent with the reported shorter digesta retention time and smaller rumen size of low emitting sheep (Goopy et al., 2014).

A further important use of relationships of MFA and methane emission is to confirm whether dietary measures applied on the farm or in an experiment are effective. This approach likely excludes lipid-based dietary interventions, because of confounding with dietary FA transferred directly to the milk or after modification in the rumen. However, it might be applicable with dietary interventions based on plant secondary compounds like tannins. Indeed, it was possible to recover with our own regression equations the linear decrease, at least in Pm and Im (confirming hypothesis v). Additionally, the equations of van Lingen et al. (2014) also mirrored the Acacia effect. Their regression analysis was based on data from 30 different diets. As in farm practice, DMI data are much harder to obtain than MFA, regression equations including DMI are not useful for that purpose.

**5. Conclusions**

Dairy cows’ response in methane formation to *A. mearnsii* tannins was immediate (i.e., within the first hour after starting the supplementation). Given its known long-term efficacy, and that it is produced in large quantities for the leather industry, the *A. mearnsii* tannin extract represents a promising dietary measure for methane mitigation. Within the first day, the proportions of several fatty acids in milk fat were also significantly modified by *A. mearnsii* supplementation. The lack of interactions by cow category (low vs. high methane emitters) and day (control vs. Acacia) on methane and nearly all individual milk fatty acids indicates that treating low emitting cows with such supplements will not prevent an efficient mitigation. There were milk fatty acids related to methane emission which were suitable to discriminate between emission categories and confirm methane suppression by dietary tannins. However, these fatty acids varied between regression equations. Further studies are needed to refine this approach with more advanced and complex models.

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**Fig. 1.** **A)** Development of methane emission (extrapolated to g/day) during the day on control diet (Day 0, filled circles), and the three days on the acacia diets (Day 1, empty squares; Day 2, crosses; Day 3, empty diamonds). **B)** Effect of the acacia diet on methane emission (g/day) measured in 10 min intervals in the first 120 min after feeding (Day 1) in relation to the methane emission (g/day) measured at the same time of the day in the same cows fed on the control diet (Day 0; set to 100%). Data are displayed as arithmetic means ± standard deviation (Fig. 1B). The asterisks indicate differences at *P <* 0.05 between measurements made at the same time on Day 0 and Day 1 (Fig. 1B)*.*

**Table 1**

Composition of the individual experimental feeds and amounts consumed (arithmetic means ± standard deviation).

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Diet component | Mixed ration | | | |  | Supplements | | |
| Feed item | Haya | Grass silagea | Corn silagea | Protein concentratebc |  | Energy concentratebd | Grass pelletsb | Tannin pelletsb |
| Composition (g/kg dry matter) |  |  |  |  |  |  |  |  |
| Dry matter (g/kg wet weight) | 886±15 | 352±40 | 370±28 | 931±0 |  | 888±0 | 903±4 | 895±1 |
| Organic matter | 910±11 | 880±19 | 968±4 | 910±0 |  | 937±0 | 874±4 | 898±1 |
| Crude protein | 98±23 | 139±16 | 67±9 | 317±3 |  | 228±3 | 173±6 | 240±3 |
| Starch | 0±0 | 3±3 | 350±24 | 114±0 |  | 312±1 | 19±1 | 244±3 |
| Neutral detergent fiber (aNDFom) | 493±46 | 399±36 | 452±40 | 145±15 |  | 271±12 | 402±25 | 143±9 |
| Acid detergent fiber (ADFom) | 358±38 | 319±31 | 278±34 | 110±1 |  | 108±1 | 263±30 | 69.5±15.2 |
| Lignin(sa) | 48.0±9.4 | 45.5±15.2 | 34.9±9.5 | 57.8±0.4 |  | 36.5±0.3 | 45.0±3.5 | 31.3±11.7 |
| Ether extract | 16.8±0.3 | 28.8±4.2 | 34.1±2.5 | 20.3±0.1 |  | 74.1±1.6 | 31.5±4.6 | 59.2±1.0 |
| Total extractable phenols | 12.8±1.9 | 13.8±1.7 | 8.8±0.1 | 4.6±2 |  | 5.4±1.0 | 12.3±2.2 | 83.3±15.1 |
| Non-tannin phenols | 9.9±0.5 | 11.5±0.1 | 7.3.±0.6 | 4.2±0.1 |  | 4.48±0.1 | 9.95±0.21 | 4.74±0.1 |
| Total tannins | 2.9±0.21 | 2.2±0.0 | 1.5±0.0 | 0.4±0.0 |  | 0.9±0.0 | 2.7±0.1 | 78.6±1.5 |
| Condensed tannins | 0.4±0.06 | 0.3±0.03 | 0.1±0.0 | 0.0±0.0 |  | 0.0±0.0 | 0.4±0.2 | 26.6±3.3 |
| Hydrolyzable tannins | 2.6±0.1 | 1.9±0.1 | 1.4±0.1 | 0.4±0.0 |  | 0.9±0.0 | 2.4±0.1 | 52.0±1.9 |
| Dry matter amounts consumed (kg/day) | |  |  |  |  |  |  |  |
| Day 0 | 0.63±0.11 | 4.91±0.26 | 7.24±0.67 | 1.75±0.20 |  | 1.11±0.36 | 0.65±0.23 | ‒ |
| Days 1 to 3 | 0.66±0.05 | 4.44±0.42 | 6.50±0.82 | 1.64±0.10 |  | ‒ | ‒ | 2.72±0.66 |

a n=19.

b n=3.

c UFA-250, UFA, Sursee, Switzerland.

d UFA 243, UFA, Sursee, Switzerland.

**Table 2**

Compositiona of the control diet (fed on Day 0) and the diet supplemented with *Acacia mearnsii* tannin extract (fed on Days 1 to 3) (means ± standard deviation).

|  |  |  |
| --- | --- | --- |
| Diet | Control | Acacia |
| Composition (g/kg dry matter) |  |  |
| Organic matter | 903±1 | 899±1 |
| Crude protein | 140±8 | 155±8 |
| Starch | 187±6 | 195±5 |
| Neutral detergent fiber (aNDFom) | 411±11 | 370±17 |
| Acid detergent fiber (ADFom) | 277±12 | 263±9 |
| Lignin(sa) | 46.4±4.3 | 44.3±3.9 |
| Ether extract | 34.1±1.7 | 37.4±2.0 |
| Total extractable phenols | 9.5±1.3 | 22.5±3.6 |
| Non-tannin phenols | 7.8±0.6 | 7.4±0.6 |
| Total tannins | 1.6±0.0 | 14.7±0.3 |
| Condensed tannins | 1.0±0.1 | 5.4±0.7 |
| Hydrolysable tannins | 1.5±0.4 | 10.1±0.7 |
| Fatty acids (g/100 g total fatty acids) |  |  |
| C12:0 | 0.160±0.007 | 0.179±0.007 |
| C14:0 | 0.274±0.008 | 0.377±0.007 |
| C16:0 | 15.5±0.1 | 19.7±0.1 |
| C18:0 | 2.605±0.079 | 10.08±0.074 |
| C20:0 | 0.591±0.017 | 0.591±0.160 |
| C22:0 | 0.275±0.008 | 0.218±0.007 |
| C24:0 | 0.649±0.027 | 0.574±0.025 |
| C16:1 | 0.247±0.005 | 0.206±0.004 |
| C18:1 n-9 | 19.7±0.6 | 16.5±0.6 |
| C20:1 n-7 | 0.204±0.077 | 0.183±0.710 |
| C20:1 n-9 | 0.189±0.069 | 0.149±0.063 |
| C24:1 n-9 | 0.533±0.031 | 0.491±0.029 |
| C18:2 n-6 | 32.6±0.5 | 28.0±0.5 |
| C20:2 | 0.057±0.007 | 0.051±0.006 |
| C22:2 | 0.166±0.005 | 0.144±0.004 |
| C18:3 n-6 | 0.118±0.004 | 0.109±0.003 |
| C18:3 n-3 | 23.5±0.2 | 19.7±0.3 |
| C20:5 n-3 | 0.117±0.068 | 0.112±0.064 |
| Saturated fatty acids | 20.9±3.6 | 32.4±7.9 |
| Monounsaturated fatty acids | 22.4±4.9 | 18.7±5.9 |
| Polyunsaturated fatty acids | 56.6±9.7 | 48.1±14 |

a Calculated from intake and analyzed composition of the different feed items.

**Table 3**

Least square means of intake, milk yield and composition of the 20 cows during the 4 days (Day) in the respiration chambers fed the control diet on Day 0 and the Acacia diet from Day 1 to Day 3. Cows were categorized (Category) into ten low and ten high emitters according to their methane yield (g methane/kg dry matter intake).

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Category | |  | Day | | | |  | *P*-values | | |
|  | Low | High |  | Day 0 | Day 1 | Day 2 | Day 3 | SEMa | Category | Day | Category × day |
| Dry matter intake (kg/day) | 16.0 | 15.6 |  | 16.1 | 15.8 | 15.8 | 15.6 | 0.41 | 0.13 | 0.39 | 0.44 |
| Milk yieldb (g/day) | 19.9 | 22.6 |  | 21.2 | 21.4 | 21.1 | 21.3 | 2.05 | 0.78 | 0.98 | 0.97 |
| Milk composition (g/kg) |  |  |  |  |  |  |  |  |  |  |  |
| Fat | 43.7 | 42.8 |  | 43.8 | 42.9 | 43.3 | 43.0 | 1.22 | 0.94 | 0.94 | 0.95 |
| Protein | 37.0 | 35.2 |  | 36.2 | 35.8 | 36.4 | 35.9 | 0.81 | 0.26 | 0.79 | 0.16 |
| Lactose | 48.1 | 47.2 |  | 47.3 | 47.8 | 47.8 | 47.6 | 0.22 | 0.06 | 0.84 | 0.91 |

a Standard error of the mean.

b Energy corrected milk yield.

**Table 4**

Least square means of fatty acid proportions in milk fat (g/100 total fatty acids) of the 20 cows during the 4 days (Day) in the respiration chambers fed the control diet on Day 0 and the Acacia diet from Day 1 to Day 3. Cows were categorized (Category) into ten low and ten high emitters according to their methane yield (g methane/kg dry matter intake.

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Category | |  | Day | | | |  | *P*-values | | | |
| Fatty acid (FA) | Low | High |  | Day 0 | Day 1 | Day 2 | Day 3 | SEM1 | Category | Day | Category × day | Contrasts2 |
| C4:0 | 1.78 | 1.79 |  | 1.81 | 1.79 | 1.75 | 1.79 | 0.524 | 0.84 | 0.61 | 0.92 |  |
| C6:0 | 1.88 | 1.83 |  | 1.88 | 1.90 | 1.81 | 1.83 | 0.563 | 0.88 | 0.06 | 0.77 |  |
| C8:0 | 0.033 | 0.031 |  | 0.032 | 0.032 | 0.032 | 0.033 | 0.0023 | 0.41 | 0.25 | 0.67 |  |
| C10:0 | 3.26 | 3.11 |  | 3.20 | 3.28 | 3.16 | 3.10 | 0.092 | 0.92 | 0.11 | 0.75 |  |
| C12:0 | 3.89 | 3.65 |  | 3.76 | 3.90 | 3.76 | 3.66 | 0.150 | 0.84 | 0.12 | 0.22 |  |
| C12:0-*iso* | 0.065 | 0.067 |  | 0.071ab | 0.092a | 0.055bc | 0.047c | 0.0061 | 0.84 | <0.001 | 0.54 | L, C |
| C13:0 | 0.117 | 0.117 |  | 0.117 | 0.121 | 0.117 | 0.114 | 0.0064 | 0.45 | 0.56 | 0.79 |  |
| C13:0-*iso* | 0.122 | 0.164 |  | 0.142 | 0.147 | 0.135 | 0.130 | 0.1035 | 0.004 | 0.12 | 0.24 |  |
| C14:0 | 13.3 | 13.1 |  | 13.2b | 13.6a | 13.1b | 12.9b | 0.27 | 0.26 | <0.001 | 0.77 | L |
| C14:0-*iso* | 0.219 | 0.252 |  | 0.236ab | 0.248a | 0.235ab | 0.223c | 0.0083 | <0.001 | 0.25 | 0.37 | L |
| C14:0-*anteiso* | 0.45 | 0.47 |  | 0.47 | 0.47 | 0.45 | 0.45 | 0.012 | 0.21 | 0.28 | 0.30 |  |
| C15:0 | 1.17 | 1.19 |  | 1.20 | 1.21 | 1.17 | 1.14 | 0.032 | 0.51 | 0.21 | 0.49 |  |
| C16:0 | 32.5 | 32.8 |  | 32.6 | 32.5 | 32.8 | 32.7 | 0.94 | 0.97 | 0.86 | 0.83 |  |
| C16:0-*iso* | 0.147 | 0.133 |  | 0.165ab | 0.193a | 0.104bc | 0.092c | 0.0217 | 0.15 | <0.001 | 0.42 | L, C |
| C16:0-*anteiso* | 0.283 | 0.291 |  | 0.293 | 0.281 | 0.292 | 0.229 | 0.0074 | 0.03 | 0.68 | 0.44 |  |
| C17:0 | 0.526 | 0.532 |  | 0.528 | 0.527 | 0.535 | 0.525 | 0.0123 | 0.57 | 0.85 | 0.67 |  |
| C17:0-*iso* | 0.073 | 0.070 |  | 0.077 | 0.069 | 0.070 | 0.070 | 0.0024 | 0.35 | 0.18 | 0.28 |  |
| C17:0-*anteiso* | 0.052 | 0.059 |  | 0.054 | 0.055 | 0.057 | 0.056 | 0.0431 | 0.56 | 0.97 | 0.89 |  |
| C18:0 | 9.60 | 9.39 |  | 9.62 | 9.26 | 9.45 | 9.65 | 0.432 | 0.72 | 0.88 | 0.92 |  |
| C20:0 | 0.122 | 0.119 |  | 0.116 | 0.123 | 0.122 | 0.122 | 0.0062 | 0.71 | 0.65 | 0.55 |  |
| C22:0 | 0.038 | 0.040 |  | 0.040 | 0.041 | 0.037 | 0.037 | 0.0344 | 0.23 | 0.32 | 0.79 |  |
| C10:1 | 0.341 | 0.310 |  | 0.33 | 0.33 | 0.32 | 0.32 | 0.192 | 0.29 | 0.96 | 0.86 |  |
| C12:1 | 0.060 | 0.057 |  | 0.059b | 0.094a | 0.047b | 0.035b | 0.0071 | 0.72 | <0.001 | 0.11 | L, C |
| C14:1 | 1.20 | 1.09 |  | 1.11 | 1.18 | 1.16 | 1.13 | 0.062 | 0.07 | 0.92 | 0.80 |  |
| C15:1 | 0.34 | 0.44 |  | 0.39 | 0.41 | 0.39 | 0.37 | 0.321 | <0.001 | 0.20 | 0.23 |  |
| C16:1 n-7 | 1.25 | 1.31 |  | 1.22 | 1.27 | 1.31 | 1.32 | 0.071 | 0.46 | 0.49 | 0.69 |  |
| C17:1 | 0.212 | 0.214 |  | 0.208 | 0.206 | 0.220 | 0.216 | 0.0143 | 0.86 | 0.10 | 0.92 |  |
| C18:1 n-9 | 19.2 | 19.5 |  | 19.3 | 18.6 | 19.4 | 20.0 | 0.52 | 0.92 | 0.12 | 0.99 |  |
| C18:1 c10 | 0.301 | 0.297 |  | 0.311a | 0.311a | 0.287b | 0.287b | 0.00912 | 0.83 | 0.02 | 0.57 | L |
| C18:1 c11 | 0.430 | 0.447 |  | 0.432 | 0.431 | 0.444 | 0.446 | 0.0244 | 0.63 | 0.82 | 0.99 |  |
| C18:1 c12 | 0.308 | 0.319 |  | 0.325 | 0.323 | 0.302 | 0.303 | 0.0123 | 0.39 | 0.15 | 0.45 |  |
| C18:1 c13 | 0.096 | 0.097 |  | 0.098 | 0.098 | 0.097 | 0.096 | 0.0373 | 0.35 | 0.95 | 0.77 |  |
| C18:1 c14 & t16 | 0.123 | 0.103 |  | 0.144 | 0.10 | 0.114 | 0.093 | 0.0414 | 0.18 | 0.59 | 0.003 |  |
| C18:1 t8 | 0.414 | 0.408 |  | 0.417 | 0.438 | 0.393 | 0.396 | 0.0213 | 0.78 | 0.60 | 0.73 |  |
| C18:1 t9 | 0.242 | 0.251 |  | 0.253 | 0.251 | 0.241 | 0.240 | 0.0081 | 0.23 | 0.49 | 0.94 |  |
| C18:1 t10 | 0.452 | 0.471 |  | 0.523 | 0.481 | 0.430 | 0.411 | 0.0322 | 0.65 | 0.17 | 0.78 |  |
| C18:1 t11 | 1.05 | 1.18 |  | 1.11 | 1.16 | 1.12 | 1.08 | 0.084 | 0.092 | 0.60 | 0.20 |  |
| C18:1 t12 | 0.371 | 0.363 |  | 0.388ab | 0.398a | 0.350ab | 0.325b | 0.0243 | 0.36 | 0.04 | 0.012 | L, C |
| C20:1 c11 | 0.124 | 0.123 |  | 0.121 | 0.122 | 0.123 | 0.123 | 0.0041 | 0.73 | 0.86 | 0.39 |  |
| C20:1 n-9 | 0.035 | 0.036 |  | 0.037a | 0.038a | 0.035ab | 0.034b | 0.0012 | 0.19 | 0.048 | 0.96 | L, C |
| C18:2 n-6 | 1.40 | 1.37 |  | 1.37 | 1.34 | 1.38 | 1.46 | 0.063 | 0.28 | 0.15 | 0.96 |  |
| C18:2 c9, c15 | 0.31 | 0.35 |  | 0.32 | 0.32 | 0.36 | 0.34 | 0.314 | 0.20 | 0.29 | 0.98 |  |
| C18:2 c9, t11 | 0.594 | 0.642 |  | 0.613 | 0.643 | 0.622 | 0.594 | 0.0931 | 0.69 | 0.92 | 0.86 |  |
| C18:2 c9, t12 | 0.084 | 0.08 |  | 0.079 | 0.088 | 0.080 | 0.083 | 0.0053 | 0.59 | 0.67 | 0.75 |  |
| C18:2 c9, t13 & t8, c12 | 0.210 | 0.216 |  | 0.223 | 0.216 | 0.208 | 0.206 | 0.0083 | 0.25 | 0.16 | 0.72 |  |
| C18:2 t9, t12 | 0.094 | 0.091 |  | 0.095 | 0.094 | 0.091 | 0.093 | 0.0031 | 0.39 | 0.06 | 0.31 |  |
| C18:3 n-3 | 0.502 | 0.514 |  | 0.514 | 0.501 | 0.493 | 0.503 | 0.0121 | 0.51 | 0.63 | 0.93 |  |
| C18:3 n-6 | 0.054 | 0.048 |  | 0.045 | 0.052 | 0.049 | 0.049 | 0.0024 | 0.16 | 0.69 | <0.001 |  |
| C20:3 n-6 | 0.046 | 0.042 |  | 0.059a | 0.050a | 0.033b | 0.035b | 0.0052 | 0.21 | <0.001 | 0.13 | L |
| C20:4 n-6 | 0.068 | 0.067 |  | 0.071 | 0.066 | 0.064 | 0.068 | 0.0042 | 0.32 | 0.46 | 0.52 |  |
| C20:5 n-3 | 0.050 | 0.045 |  | 0.047 | 0.046 | 0.049 | 0.049 | 0.0034 | 0.45 | 0.30 | 0.67 |  |
| C22:5 n-3 | 0.0.86 | 0.101 |  | 0.092 | 0.091 | 0.094 | 0.096 | 0.0818 | <0.001 | 0.35 | 0.65 |  |
| C22:6 n-3 | 0.049 | 0.031 |  | 0.051ab | 0.071a | 0.024bc | 0.015c | 0.0084 | 0.006 | <0.001 | 0.007 | L, Q, C |
| Total C18:1 trans | 2.56 | 2.63 |  | 2.65 ab | 2.79 a | 2.51 ab | 2.42b | 0.0138 | 0.46 | 0.011 | 0.92 | L, Q |
| Saturated FA | 68.2 | 67.7 |  | 67.9 | 68.3 | 68.1 | 67.6 | 0.06 | 0.79 | 0.63 | 0.96 |  |
| Monounsaturated FA | 26.6 | 27.0 |  | 26.9 | 26.3 | 26.8 | 27.4 | 0.55 | 0.86 | 0.36 | 0.95 |  |
| Polyunsaturated FA | 3.69 | 3.72 |  | 3.69 | 3.78 | 3.65 | 3.70 | 0.090 | 0.98 | 0.78 | 0.93 |  |

Day means carrying no common superscript are different at *P* < 0.05.

1 Standard error of the mean.

2 L, linear, Q, quadratic and C, cubic contrasts of Day effect at *P* <0.05.

**Table 5**

Least-square means of measured and calculated methane production (Pm; g/d), yield (Ym; g/kg dry matter intake (DMI)) and emission intensity (Im; g/kg energy-corrected milk (ECM)) of the 20 cows during the 4 days (Day) in the respiration chambers fed the control diet on Day 0 and the Acacia diet from Day 1 to Day 3. Cows were categorized (Category) into ten low and ten high emitters according to their methane yield (g methane/kg DMI).

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Category | | |  | Day | | | |  | *P*-values | | | |  |  |
| Methane | Low | High | |  | Day 0 | Day 1 | Day 2 | Day 3 | SEM1 | Category | Day | Category × day | Contrast2 | Equation3 | Reference |
| Measured by respiration chambers | | | | | | |  |  |  |  |  |  |  |  |  |
| g/day4 | 371 | | 387.1 |  | 424a | 387b | 359c | 347c | 8.75 | 0.030 | <0.001 | 0.54 | L |  |  |
| g/kg DMI | 22.5 | | 25.8 |  | 26.9a | 25.35ab | 23.7bc | 22.6c | 1.59 | 0.041 | 0.005 | 0.94 | L |  |  |
| g/kg ECM | 18.7 | | 19.9 |  | 19.8 | 19.7 | 19.0 | 18.6 | 3.89 | 0.57 | 0.77 | 0.88 |  |  |  |
| Calculated from milk fatty acid proportions via regression | | | | | | | |  |  |  |  |  |  |  |  |
| g/day4 | 377 | | 388 |  | 390ab | 395a | 376ab | 370b | 6.83 | 0.045 | 0.001 | 0.08 | L | 1 | Current study |
| g/day4,5 | 380 | | 385 |  | 390ab | 393a | 377ab | 370b | 6.91 | 0.029 | <0.001 | 0.09 | L | 2 | Current study |
| g/day | 305 | | 291 |  | 299 | 297 | 296 | 299 | 9.39 | 0.17 | 0.95 | 0.47 |  | 6 | van Gastelen et al. (2017) |
| g/day | 382 | | 380 |  | 377 | 384 | 382 | 382 | 5.02 | 0.51 | 0.79 | 0.99 |  | 11 | Engelke et al. (2019)7 |
| g/day (∑ MUFA) | 385 | | 385 |  | 384 | 391 | 385 | 382 | 11.1 | 0.87 | 0.30 | 0.96 |  | 12 | Engelke et al. (2019) |
| g/day5 | 365 | | 362 |  | 399 | 353 | 350 | 351 | 14.2 | 0.13 | 0.33 | 0.91 |  | 13 | Engelke et al. (2019) |
| g/day (∑ MUFA)5 | 352 | | 348 |  | 385 | 343 | 336 | 336 | 15.3 | 0.18 | 0.08 | 0.97 |  | 14 | Engelke et al. (2019) |
| g/day6 | 331 | | 323 |  | 332 | 317 | 332 | 327 | 22.4 | 0.60 | 0.88 | 0.80 |  | 15 | Engelke et al. (2019) |
| g/day (∑ MUFA)6 | 317 | | 313 |  | 322 | 309 | 317 | 312 | 23.8 | 0.73 | 0.90 | 0.80 |  | 16 | Engelke et al. (2019) |
| g/kg DMI | 22.2 | | 24.8 |  | 23.9 | 23.7 | 23.5 | 22.9 | 0.88 | 0.041 | 0.20 | 0.49 |  | 3 | Current study |
| g/kg DMI | 23.1 | | 22.7 |  | 23.0 | 22.7 | 22.9 | 23.1 | 0.21 | 0.090 | 0.69 | 0.92 |  | 7 | van Gastelen et al. (2017) |
| g/kg DMI | 18.3 | | 18.0 |  | 18.2 | 18.1 | 18.2 | 18.3 | 0.26 | 0.37 | 0.96 | 0.96 |  | 5 | Dijkstra et al. (2011) |
| g/kg DMI | 22.9 | | 22.7 |  | 23.1ab | 23.2a | 22.5bc | 22.4c | 0.16 | 0.093 | <0.001 | 0.32 | L, C | 9 | van Lingen et al. (2014) |
| g/kg ECM | 17.8 | | 19.4 |  | 19.04 | 18.5 | 18.8 | 18.1 | 0.76 | 0.11 | 0.83 | 0.55 |  | 4 | Current study |
| g/kg ECM4 | 10.9 | | 11.0 |  | 11.0 | 11.2 | 11.0 | 10.8 | 0.24 | 0.20 | 0.31 | 0.76 |  | 8 | van Gastelen et al. (2017) |
| g/kg ECM | 16.8 | | 16.6 |  | 17.1a | 17.2a | 16.4b | 16.2b | 0.192 | 0.42 | <0.001 | 0.53 | L, C | 10 | van Lingen et al. (2014) |

Means carrying no common superscript are different at P < 0.05. MUFA, mono-unsaturated fatty acids.

1 Standard error of the mean.

2 L, linear and C, cubic contrasts of Day effect at *P* < 0.05.

3 For full equations see Materials and Methods as well as Table 6 for equations derived from the current study.

4 Content of neutral detergent fiber (aNDFom) was used as a covariate when significant at *P* < 0.05.

5 Equation includes DMI.

6 Equation includes ECM yield.

7 Methane in g derived from methane in L/1.3962.

**Table 6**

Best regression equations (Eq) developeda for methane production, yield and emission intensity based on individual fatty acids in milk (g/100 g total fatty acids) as analyzed with gas chromatography as well as coefficients of determination (R2), concordance correlation coefficients (CCC) and root mean squared prediction errors (RMSPE).

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Milk fatty acids | Estimate | Standard error | *P*-value | Adjusted R2 | *P*-value | | rbf | | Cbcf | | CCCbf | RMSPEef |
| Methane production (Pm; g/day; Eq 1) | | | | | 0.591 | <0.001 | | 0.84 | | 0.99 | | 0.83 | 19.4 |
|  | Intercept | 586.4 | 35.83 | <0.001 |  |  | |  | |  | |  |  |
|  | C17:1 | -677.5 | 134.76 | <0.001 |  |  | |  | |  | |  |  |
|  | C18:2 c9, t13 & t8, c12 | 315.4 | 109.81 | 0.002 |  |  | |  | |  | |  |  |
|  | C18:2 c9, c15 | 144.4 | 34.37 | <0.001 |  |  | |  | |  | |  |  |
|  | C18:3 n-6 | -1815.1 | 208.99 | <0.001 |  |  | |  | |  | |  |  |
|  | C20:5 n-3 | -1793.4 | 411.86 | <0.001 |  |  | |  | |  | |  |  |
| Methane production (Pm; g/day) with dry matter intake; Eq 2) | | | | | 0.608 | <0.001 | | 0.85 | | 0.99 | | 0.84 | 18.5 |
|  | Intercept | 492.9 | 50.88 | <0.001 |  |  | |  | |  | |  |  |
|  | C17:1 | -635.2 | 130.76 | <0.001 |  |  | |  | |  | |  |  |
|  | C18:2 c9, t13 & t8, c12 | 204.4 | 114.53 | 0.063 |  |  | |  | |  | |  |  |
|  | C18:2 c9, c15 | 153 | 33.22 | <0.001 |  |  | |  | |  | |  |  |
|  | C18:3 n-6 | -1724.9 | 204.14 | <0.001 |  |  | |  | |  | |  |  |
|  | C20:5 n-3 | -1504.4 | 412.50 | 0.003 |  |  | |  | |  | |  |  |
|  | Dry matter intake | 5.54 | 2.22 | 0.050 |  |  | |  | |  | |  |  |
| Methane yield (Ym; g/kg dry matter intake; Eq 3) | | | | | 0.453 | <0.001 | | 0.69 | | 0.94 | | 0.65 | 3.19 |
|  | Intercept | 28.28 | 3.16 | <0.001 |  |  | |  | |  | |  |  |
|  | C17:0 anteiso | 170.94 | 36.32 | <0.001 |  |  | |  | |  | |  |  |
|  | C20:0 | -50.42 | 15.71 | <0.001 |  |  | |  | |  | |  |  |
|  | C17:1 | -54.37 | 13.41 | <0.001 |  |  | |  | |  | |  |  |
|  | C20:4 n-6 | -35.60 | 14.31 | <0.001 |  |  | |  | |  | |  |  |
| Methane emission intensity (Im; g/kg energy-corrected milk; Eq 4) | | | | | 0.431 | <0.001 | 0.68 | | 0.94 | | 0.64 | | 2.09 |
|  | Intercept | 19.14 | 15.3 | <0.001 |  |  | |  | |  | |  |  |
|  | C17:0 anteiso | 292.02 | 182.8 | <0.001 |  |  | |  | |  | |  |  |
|  | C20:0 | -368.9 | 77.28 | <0.001 |  |  | |  | |  | |  |  |
|  | C17:1 | -19.86 | 66.32 | 0.002 |  |  | |  | |  | |  |  |
|  | C18:2 c9, t12 | -61.27 | 6.01 | 0.002 |  |  | |  | |  | |  |  |
|  | C20:4 n-6 | -79.8 | 19.14 | <0.001 |  |  | |  | |  | |  |  |

a Developed based on Akaike’s information criterion, where data of all 4 days were used (n = 80).

b Person correlation coefficient.

e Bias correction factor.

d r × Cb.

e Expressed as g/day, g/kg dry matter intake and g/kg energy-corrected milk for methane production, methane yield and methane emission intensity, respectively.

f Comparison of calculated CH4 emission traits (based on displayed equation) with actual CH4 measurements.