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Increasing the proportion of hazel leaves in the diet of dairy cows reduced methane yield and excretion of nitrogen in volatile form, but not milk yield

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ABSTRACT

Various feeds for ruminants have been identified that help to mitigate the greenhouse gas methane. However, even when there has been success in suppressing absolute methane emissions, intake, digestibility, and performance often decline in parallel. Ideal dietary levels of effective feeds would reduce methane production without affecting performance-related variables. Such favorable associative effects have been demonstrated *in vitro* by combining a high-quality forage with plants rich in phenols. In the present study, the tannin-rich leaves of hazel (*Corylus avellana*) gradually replaced (from 0 to 820 g/kg) a high-quality forage (dried alfalfa) in 20 types of experimental pellets fed to 20 mid-to-late lactating cows. Additionally, the cows were fed a mixed basal ration and some concentrate. The proportion of hazel in the 20 complete diets ranged from 0 to 400 g/kg dry matter. After 14 days of adaptation, 8 days were used for intensive sampling of feces (including markers for determining digesta retention time), urine, and milk. In addition, cows stayed for 2 days in open-circuit respiration chambers. Hazel leaves reduced the feed intake only slightly. Digestibility declined and mean digesta retention time was prolonged with increasing hazel proportion, likely due to the lower feeding value of the hazel leaves compared to the alfalfa. As aimed for, there were no significant effects on energy-corrected milk yield, body energy, and body N retention with increasing hazel intake, even though methane emission clearly declined in absolute term and per unit of digestible organic matter and tended to decrease per unit of energy corrected milk. In addition, increasing hazel proportions strongly shifted N excretion from urinary N (which declined from about 300 to 100 g/kg N intake) to fecal N. This could also be anticipated from the sharp decline in milk urea concentration (from about 35 to 10

Abbreviations: ADFom, acid detergent fiber corrected for ash concentration; aNDFom, neutral detergent fiber assayed with α-amylase and corrected for ash concentration; BW, body weight; CH_4, methane; CP, crude protein; CT, condensed tannins; DM, dry matter; DMI, dry matter intake; ECM, energy-corrected milk; GE, gross energy; GIT, gastrointestinal tract; HT, hydrolysable tannins; lignin(sa), lignin determined by solubilization of cellulose with sulfuric acid; MBR, mixed basal ration; ME, metabolizable energy; MRT, mean retention time; NTP, non-tannin phenols; OM, organic matter; PSM, plant secondary metabolites; RR, reticulorumen; TEP, total extractable phenols; TT, total tannins; VFA, volatile fatty acids.

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mg/dL). In conclusion, hazel leaves as a feed supplement for dairy cows showed a high palatability within 3 weeks of feeding in dairy cows and great potential to mitigate emissions of methane and nitrogen in volatile form at maintained production levels. No favorable associative dosage effects seem to exist when combining tannin-rich hazel leaves with the high-quality forage alfalfa in a different proportions to a mixed basal ration. However, the present study is one of the few, where it was possible to mitigate noxious emissions of dairy cows by feeding a tannin rich feed supplement without concomitant negative impact on the animal's performance.

1. Introduction

Various attempts have been made to mitigate the emission of the greenhouse gas methane (CH_4) from ruminant husbandry in response to its large share of total greenhouse gas emissions (Gerber et al., 2013). Several feeding strategies have a high abatement potential (Hristov et al., 2013). This especially includes strategies based on feed supplements, some of which are ready to be applied in the field (Martin et al., 2010). Particularly promising is the strategic use of plants rich in secondary metabolites (PSM), including phenols and, within phenols, tannins (Hristov et al., 2013). Furthermore, as a result of the increasing food-feed competition for arable land, alternative feeds are becoming increasingly important (Makkar, 2018). In this respect, shrub and tree leaves gain interest as they are often rich in PSM (Frutos et al., 2004) and thus may mitigate the formation of CH_4 and, when tannins are prevalent, of ammonia in the rumen (Bodas et al., 2008; Jayanegara et al., 2011). When integrated into existing agricultural systems, shrubs may have further environmental benefits. Drawbacks of feeding leaves from woody plants may be their low palatability and adverse effects on nutrient digestibility (Moss et al., 2000; Tiemann et al., 2008). The ruminal and total tract retention time of the feed could also be affected by its phenol concentration (Baker and Hobbs, 1987; Melaku et al., 2005). One promising woody plant source is the leaves of the shrub hazel (*Corylus avellana*). They are rich in phenols, especially the flavanols myricetin 3-rhamnoside and quercetin 3-rhamnoside from the group of condensed tannins (CT) (Amaral et al., 2005). In previous *in vitro* studies, hazel leaves were found to be effective in reducing ruminal CH_4 and ammonia emissions without affecting digestibility (Terranova et al., 2018 and 2020). They proved to be highly palatable to dairy cows (Terranova et al., 2020) and to sheep, and they effectively suppressed CH_4 formation in sheep (Wang et al., 2018).

When describing effects of feeds on CH_4 or ammonia emissions often only the influence on absolute mitigation is reported. However, in several studies where mitigation with tannin-rich feed supplements was achieved, performance of the animals was concomitantly impaired, this often because of low palatability and adverse effects of tannins on nutritional value and digestibility (Tiemann et al., 2008; Grainger et al., 2009; Adejoro et al., 2020). However, mitigation is only sustainable when it concerns emission yield (per unit of intake of dry matter (DM) or digestible nutrients) or emission intensity (per unit of food produced; *i.e.* in relation to performance). This would be the case if diet digestibility and the animal's performance is not or not severely impaired by supplementing woody plants. In addition, CH_4 and ammonia mitigation would favorably occur already at dosages where there are no adverse effects on intake and digestibility, resulting in non-linear relationships of digestibility or performance and CH_4 formation with an increasing dosage of PSM would be required. Such non-linear effects, the so-called associative effects, are rarely investigated on a target variable with increasing dosage of the feed additive, as typically linear relationships are assumed. The concept of the associative effects was first described for a situation in which one dietary item affected the digestion of another item either positively or negatively (Van Soest, 1994; Niderkorn and Baumont, 2009). In general, dietary associative effects are assumed to be very common and cause over- or underestimations of the nutritional value of supplements (Van Soest, 1994). In the present context, combinations of high-quality feeds with phenol-rich plants that suppress ruminal CH_4 and ammonia formation while retaining a high total diet digestibility are of particular interest. The presence of this phenomenon was indeed previously demonstrated *in vitro* when incubating high-quality papaya leaves with several PSM-rich plants (Jayanegara et al., 2013). However, this specific phenomenon has only rarely been studied *in vivo*.

Therefore, the aim of the present study was to investigate the effect of replacing a high-quality forage (alfalfa) with a PSM-rich forage (hazel leaves) in increasing proportions in the diet of dairy cows. The hypotheses tested were that increasing the proportion of hazel leaves in the diet would result in (1) a decreasing milk yield, (2) a decreasing enteric CH_4 yield, and (3) a gradually increasing shift of nitrogen (N) in the excreta from urine to faeces. In addition, (4) the presence of favorable associative effects even at low hazel doses was hypothesized. Hazel leaves were chosen as a model for PSM-rich plants because there were indications of a dose-dependency of the effects of the hazel leaves *in vitro* (Terranova et al., 2018) and *in vivo* in sheep (Wang et al., 2018). In order to be able to determine the slope of the changes, a multiple regression analysis approach was applied in the present experiment using 20 experimental pellets with gradually increasing concentrations of hazel.

2. Materials and methods

2.1. Animals, diets, and experimental design

The experiment, which took place in late 2017 at AgroVet-Strickhof (Lindau, Switzerland), was approved by the Cantonal Veterinary Office of Zurich (license no. ZH271/16). Twelve Brown Swiss and eight Holstein cows, being in second to seventh lactation, 151–310 days in milk, yielding 23.4 ± 4.7 kg milk/day and weighing 711 ± 50 kg, were selected.

The experimental diets consisted of a mixed basal ration (MBR) and experimental pellets provided in a ratio of 0.4:0.6 as well as additional energy and protein supplements (Table 1). The MBR was composed (in g/kg DM) of corn silage, 550; grass silage, 380; hay, 20; dairy concentrate (UFA-250, UFA, Sursee, Switzerland), 50. Grass silage and hay were from ryegrass-dominated swards. Twenty different types of experimental pellets were produced with increasing hazel leaf proportions (hazel proportion; 0–800 g/kg pellet DM) and decreasing levels of dried alfalfa (970 to 170 g/kg pellet DM), all with 30 g molasses/kg DM (Table 2). Dried hazel leaves were provided by Alfred Galke GmbH (Bad Grund, Germany) in a particle size of 4–6 mm. The leaves were harvested in 2015 and 2016 in Albania. The alfalfa, purchased from Landi (Sense-Düdingen, Switzerland), was harvested in 2016 in France. The alfalfa was ground to a 3-mm particle length with a Sigma 5.2 hammer mill (Kuhn AG, Bottighofen, Switzerland). The hazel leaves, alfalfa, and molasses were mixed with a batch mixer (Speedmix DFML-1000, Bühler AG, Uzwil, Switzerland) and afterward pelleted to a 4.5-mm diameter (Kahl 40 PS, Amandus Kahl GmbH & Co, Reinbeck, Germany) using steam of max. 60 °C (Bühler AG, Uzwil, Switzerland).

Every cow received one out of the 20 experimental pellet types beside the MBR. The pellets of different type were randomly allocated to the animals, independent of breed, age, and performance. The experiment had to be staggered as only two respiration chambers were available. Concerning hazel level, the sequence with time, required to account for the limitation given by the two respiration chambers, was randomized as well. To meet the individual animals' requirements for protein and energy, pellet composition and milk yield were considered, and the supplementation level of a mixture of soybean meal and wheat flakes was adapted to this information. The average daily supplementation levels were 0.9 kg for soybean meal and 0.9 kg for wheat flakes. All cows received 80 g/day of a mineral-vitamin mix (details given in footnote 2 to Table 1).

Each animal performed 22 days of experiment. At first, animals were allowed to adapt to tie-stall housing and diets for 14 days. In that time, they also performed a first visit to the respiration chambers for 4 h. During this visit, the animals' heart rates were monitored with an electrode belt Polar Team2 (Polar Electro Oy, Kempele, Finland). In all cows, the heart rate fell below 80 beats per min after a 10–110 min stay in the chamber. After the adaptation, 8 days of sampling followed, of which on days 7 and 8, two cows each stayed in the two respiration chambers, with visual contact to each other through the glass walls.

2.2. Data recording and sampling

The individual tie-stalls were equipped with individual weighing plates (custom-made model, Mettler-Toledo, Greifensee, Switzerland) recording the daily feed intake. Cows were milked at 05:30 h and 16:30 h. The milk of the individual cows was collected in buckets and weighed on a scale (ID2 Multirange, Mettler-Toledo, Greifensee, Switzerland). During each milking event in the sampling period, milk samples were collected and preserved with Bronopol. Feed intake was recorded daily during the sampling period. Soybean meal, wheat flakes, and mineral supplements were offered together with the MBR on the weighing plates in the tie-stall or in the respiratory chamber troughs. Leftovers were recorded and removed before the morning feeding. The test pellets were fed in separate troughs, and leftovers were manually weighed before the morning feeding. Feeding was performed at the times of milking and, additionally, at 13:00 h (minerals were only given in the evening). The animals had unrestricted access to fresh water. Grass silage and corn silage were sampled weekly, resulting in eight samples each. Hay samples were collected three times, soybean meal and wheat flakes were sampled twice. Samples of each test pellet type were collected on days 1, 15, and 22 of the corresponding individual animal's experimental period. The cows were weighed with a truck load scale (Waagen Döhrn, model Terra ET, Wesel, Germany) on

Table 1
Diet composition and analyzed chemical composition of diet ingredients.

	Mixed basal ration (MBR)				Compensatory feeds[1,2]		Pellet ingredients	
Item	Grass silage	Corn silage	Hay	Concentrate	Soybean meal	Wheat flakes	Hazel leaves	Alfalfa
g/kg total dietary DM	380	550	20	50	217 ± 83 g/kg MBR			
Composition, g/kg DM								
Dry matter, g/kg	355	354	874	970	886	889	915	919
Organic matter	878	967	887	810	927	982	923	893
Crude protein	171	73	124	322	547	130	122	178
Ether extract	27.9	34.3	16.5	20.6	13.2	18.5	16.6	17.8
aNDFom	603	488	439	147	137	537	457	489
ADFom	387	299	344	111	93	129	408	447
Lignin(sa)	73.9	50.0	64.5	58.7	17.2	62.5	237.5	146.5
Total extractable phenols	13.8	8.8	12.8	4.6	3.6	1.2	103.2	9.9
Non-tannin phenols	11.5	7.3	9.9	4.2	3.0	0.8	21.8	8.6
Total tannins	2.23	1.50	2.89	0.35	0.50	0.37	81.41	1.27
Condensed tannins	0.31	0.08	0.31	0.00	0.00	0.00	79.29	0.009
Hydrolysable tannins	1.91	1.41	2.58	0.35	0.50	0.37	2.12	1.18
GE, MJ/kg DM	18.0	18.4	14.7	14.7	19.7	18.3	19.1	18.2

aNDFom, neutral detergent fiber assayed with α-amylase and corrected for ash concentration; ADFom, acid detergent fiber corrected for ash concentration; DM, dry matter; GE, gross energy; lignin(sa), lignin determined by solubilization of cellulose with sulfuric acid.

[1] Offered in a ratio of 1:1 and used to balance the energy and protein concentration of the different pellets and to account for different milk yields.

[2] Each cow received 80 g/day of a mineral-vitamin mix containing, per kg, 160 g calcium, 100 g magnesium, 80 g phosphorus, 32 g sodium, 10 g sulfur, 8 g zinc, 4 g manganese, 1 g copper, 100 mg iodine, 30 mg selenium, 30 mg cobalt, 1'200'000 IU vitamin A, 200'000 IU vitamin D_3, 3 g vitamin E, 150 mg biotin, and, in addition, 40 g/d of sodium chloride.

Table 2
Ingredient composition and analyzed chemical composition of the individual experimental pellets.

Pellet ingredient composition, g/kg DM																				
Hazel, g/kg DMI[1]	0	51	97	152	193	249	248	279	269	306	313	330	345	330	341	386	391	333	414	413
Pellet ingredients, g/kg DM																				
Hazel	0	88	175	263	351	438	464	490	516	542	567	593	619	645	671	697	722	748	774	800
Alfalfa	970	882	795	707	619	532	506	480	454	428	403	377	351	325	299	273	248	222	196	170
Molasses	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30
Pellet chemical composition, g/kg DM																				
Organic matter	884	892	893	896	901	903	906	906	905	910	912	914	914	913	909	916	917	896	918	920
Crude protein	190	171	169	163	163	151	151	148	151	145	144	143	143	136	134	136	133	135	132	128
Ether extract	22.4	17.8	18.3	16.8	19.5	18.3	19.1	18.4	17.8	18.4	19.4	20.9	21.5	20.1	19.7	22.1	21.6	19.9	21.0	19.4
aNDFom	407	486	481	466	483	488	483	475	523	512	522	486	512	505	498	500	511	492	486	481
ADFom	383	428	398	400	417	425	474	419	413	397	491	398	472	405	371	391	499	416	423	411
Lignin(sa)	91	148	138	166	229	168	175	171	183	175	227	173	194	172	180	181	224	199	194	191
Total phenols	5.2	18.3	23.6	33.2	48.5	46.4	49.5	57.2	62.9	66.2	67.0	68.1	69.4	73.6	77.2	83.4	83.8	81.6	85.7	86.2
Non-tannin phenols	4.5	10.4	11.1	13.1	16.7	14.2	14.4	14.9	16.1	17.1	18.0	16.9	16.8	17.5	18.6	19.9	18.9	18.5	19.2	19.5
Total tannins	0.8	8.0	12.5	20.0	31.8	32.2	35.1	42.2	45.1	49.2	45.6	48.8	52.7	56.1	58.7	63.5	64.9	63.1	66.5	64.9
Condensed tannins	0.3	4.0	7.6	14.1	24.2	24.9	27.1	32.9	37.2	35.0	34.4	37.3	41.5	47.6	49.4	46.6	48.9	49.1	49.2	49.3
Hydrolyz. tannins	0.5	4.0	4.9	5.9	7.6	7.3	8.1	9.3	7.0	6.6	11.2	9.5	11.2	5.5	9.2	6.3	16.1	14.1	19.4	15.5
GE (MJ/kg DM)	17.9	17.6	17.7	18.0	18.1	18.2	18.4	17.9	18.0	17.7	18.7	17.9	18.4	18.2	18.2	18.4	18.4	18.3	18.5	18.5

aNDFom, neutral detergent fiber assayed with α-amylase and corrected for ash concentration; ADFom, acid detergent fiber corrected for ash concentration; DM, dry matter; DMI, dry matter intake; GE, gross energy; Hazel, hazel leaves; lignin(sa), lignin determined by solubilization of cellulose with sulfuric acid.

[1] There was a certain variation in dietary pellet proportion due to cow-specific amounts of compensatory feeds.

day 1 of adaptation and on the first and last day of the sampling period.

During the sampling period, all feces were collected in steel trays located below a grid at the end of the tie-stall. Urine was collected separately from feces with urinals attached around the vulva of the cows. The urine was drained into a container, and a small subsample was diverted into a canister containing 30 g of 5 *M* sulfuric acid to prevent gaseous N losses. Feces and urine were weighed and sampled once per day. A proportion of 0.05 of the total feces and 50 mL each of acidified and non-acidified urine were frozen at −20 °C. Feces and non-acidified urine were later pooled to one sample per cow. To determine the retention time, Co-EDTA was used as a solute marker and fiber from hay cut to 2, 5, and 8 mm lengths and mordanted with Cr, La, and Ce, respectively, was used as particle markers. Details on the preparation of the markers are described in Grandl et al. (2018). On day 1 of the sampling period, the animals received, per kg of body weight (BW), a dosage of about 0.1 g of each particle marker and of 0.01 g of Co-EDTA. The frozen boli were administered orally with a commercial bolus applicator for cows. Fecal samples of about 250 g each were collected on the day before the marker application (three samples) and 4, 8, 12, 18, 22, 26, 30, 36, 42, 46, 52, 58, 66, 74, 82, 90, 98, 106, 114, 126, 138, and 150 h after the application. These samples were dried at 100 °C to constant weight and ground through a 1-mm screen with a centrifugal mill (Model ZM1, Retsch GmbH, Haan, Germany). The same procedure was applied for the fecal samples pooled across the entire sampling period, except that these were dried at 60 °C.

2.3. Respiration chamber measurements

Two open-circuit respiration chambers with a volume of 22.4 m^3 (*cf.* Grandl et al., 2016) were used to measure the gaseous exchange of the animals. The chambers were entered through an airlock for milking and feeding. For cleaning purposes, the measurements were interrupted for 0.5 h on day 2, 4.5 h after the morning feeding, and the missing data were interpolated from adjacent values. The chambers were set to an ambient temperature of 17 °C, a relative humidity of 60 %, and an air pressure of −60 Pa. Airflow was set to 800 L/min (Promethion FG-1000 flow generators, Sable Systems, Las Vegas, NV). Concentrations of CH_4, CO_2, and O_2 were measured in each chamber every 3 min for 1 min with a gas analyzer (Promethion GA-4, Sable Systems). Before starting the measurement, the gas analyzer was calibrated with pure N_2 (999.99 mL/L) and a mixed gas (198 mL O_2, 10 mL CO_2, 1 mL CH_4 per L, in N_2 as carrier). Recovery was tested three times in the experiment by burning propane gas. The mean recovery rate was 0.940 of the total. The gaseous exchange from 48 h was used, averaging the individual CH_4, CO_2, and O_2 data to daily amounts.

2.4. Laboratory analyses

Feed items and excreta were analyzed in duplicate or triplicate in the dried and fresh samples, respectively, according to standard procedures (AOAC International, 1997). Dry matter and total ash were analyzed with a TGA-701 furnace (Leco Corporation, St. Joseph, Michigan, USA; AOAC Official Method 942.05). Organic matter (OM) was calculated as DM minus total ash. Nitrogen concentration in feed items, non-dried feces, and acidified urine were quantified with a C/N analyzer (TruMac CN, Leco Corporation; AOAC Official Method 968.06). Crude protein (CP) was calculated as 6.25 × N. The carbon concentration of the non-acidified urine was determined with the same device. The concentration of the ether extract were determined with a Soxhlet extractor (Extraction System B-811, Büchi, Flawil, Switzerland; AOAC Official Method 963.15). Detergent fiber fractions were assessed in a Fibertherm system FT 12 (Gerhardt GmbH & Co. KG, Koenigswinter, Germany). Heat-stable α-amylase (Sigma-Aldrich, St. Louis, Missouri, USA) was used for neutral detergent fiber (aNDFom) analysis by method 6.5.1 from VDLUFA (2012), and both aNDFom and acid detergent fiber (ADFom) were corrected for ash concentration. Lignin(sa) was determined sequentially after the ADF analysis by incubation in sulfuric acid (720 mL/L) for 3 h. Gross energy (GE) concentration was measured in feed items and feces with a bomb calorimeter (C7000, IKA-Werke GmbH & Co. KG, Staufen, Germany). Analysis of total phenols and phenol fractions was performed according to Makkar (2003) using a double beam spectrophotometer (UV-6300PC, VWR, Leuven, Belgium). The feed samples were treated twice with a 700 mL acetone/L solution for the preparation of extracts. Concentrations of total phenols (TEP) and non-tannin phenols (NTP) were expressed as gallic acid equivalents. Total tannins (TT) were calculated as the difference of TEP and NTP. The CT were given as leucocyanidin equivalents. The difference between TT and CT was considered to be the hydrolysable tannins (HT).

The marker concentrations in the mordanted hay particles and in the feces were analyzed after wet ashing using inductively coupled plasma optical emission spectrometry (Optima 8000, Pekin Elmer, Rodgau, Germany). The mordanted particles of 2, 5 and 8 mm size and the EDTA contained, per kg DM, 32.8 g Cr, 49.5 g La, 41.5 g Ce and 151 g Co, respectively. The baseline concentrations measured in samples before the marker application were used to correct for fecal background levels in each individual cow.

The Bronopol-preserved milk was analyzed for fat, protein, lactose, and urea concentrations with a MilkoScan FT6000 (Foss, Hillerød, Denmark) at SuisseLab (Zollikofen, Switzerland).

2.5. Calculations and statistical analysis

Energy-corrected milk (ECM) was calculated as ECM [kg] = milk [kg] × (0.038 × fat [g/kg] + 0.024 × protein [g/kg] + 0.017 × lactose [g/kg])/3.14 (Agroscope, 2020).

Mean retention time (MRT) in the gastrointestinal tract (GIT) was computed for each marker according to Thielemans et al. (1978) as MRT GIT = (Σ C_i × t_i × dt_i) / (Σ C_i × dt_i), where t_i = mean time (h) after application of markers of two subsequent samplings i-1 and i, calculated as t_{i-1} + (t_i-t_{i-1})/2; C_i = marker concentration in the fecal sample voided in the interval represented by time t_i and t_{i-1}; and dt_i = sampling interval (h) of the respective sample, calculated as ([t_{i-1} –t_i] +[t_i –t_{i-1}])/2. The MRT of the solute marker Co-EDTA in the reticulorumen (RR) was calculated according to Grovum and Williams (1973). The MRT of the particles in the RR was calculated

according to Huhtanen and Kukkonen (1995) as MRT RR particles = MRT GIT particles – (MRT GIT solute – MRT RR solute).

Digestibility and energy-balance related variables were calculated as follows:

(1) Apparent digestibility = (intake [g or MJ/day] – fecal loss [g or MJ/day]) / intake [g or MJ/day];
(2) CH_4 energy (MJ/day) = CH_4 (L/day) × 0.03957 (Brouwer, 1965);

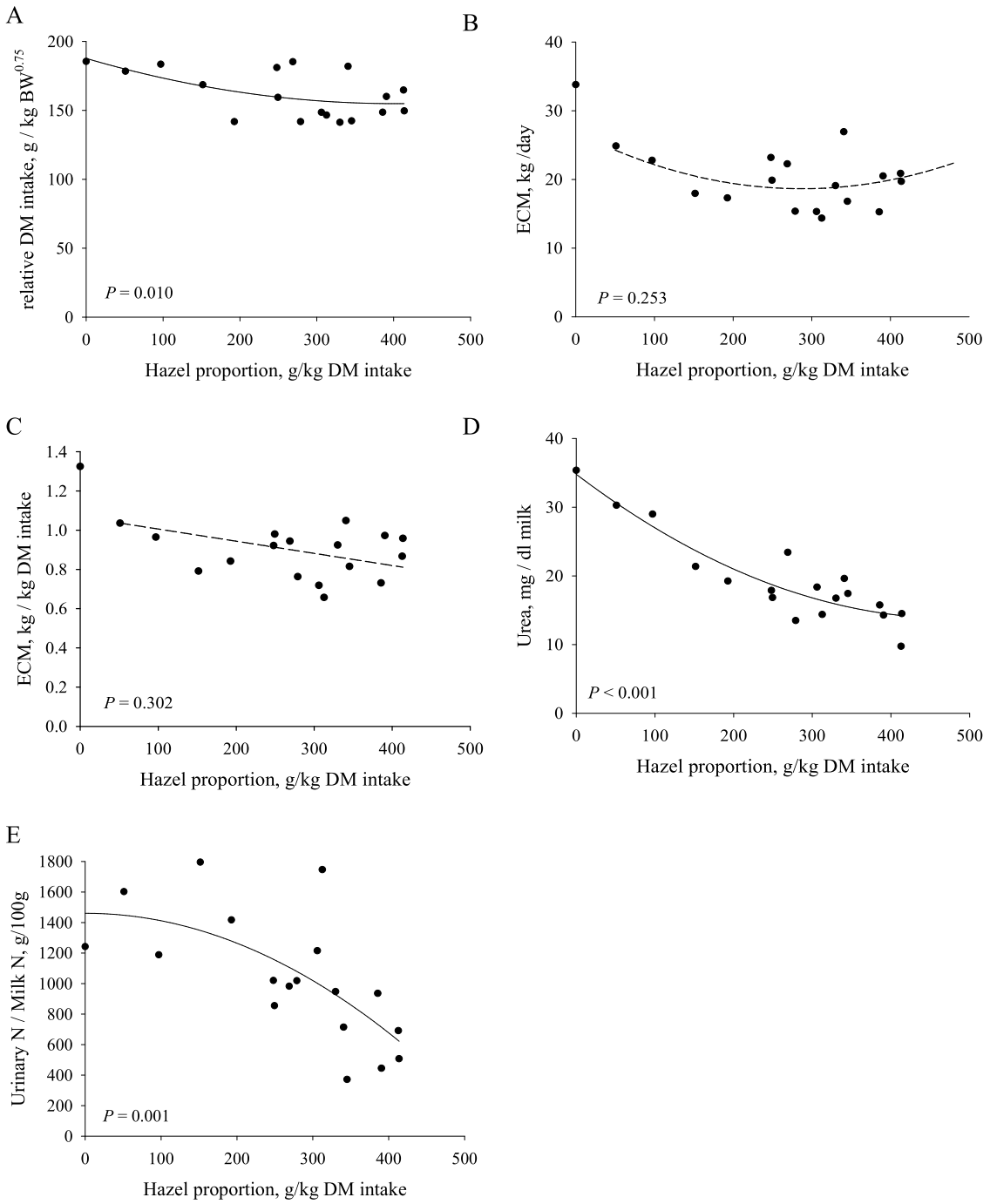

Fig. 1. Dry matter (DM) intake relative to metabolic body weight (BW) (A) as allocated considering individual milk yield, and changes in energy-corrected milk (ECM; B), feed conversion efficiency (C), milk urea concentration (D) and urinary-N to milk N ratio (E) occurring with increasing dietary hazel leaf proportion (for regression equations and coefficients see Supplementary Table S1 and S2). When the regression model was not significant a dashed regression line was drawn. Equations of the ECM related variables (B, C) were calculated without the value of the cow receiving no hazel leaves. *P*-values of the equations are presented in the legend.

(3) Urine energy (MJ/day) = 0.0331 × urine C (g/day) + 0.0092 × urine N (g/day) (Hoffmann and Klein, 1980);
(4) Heat energy (MJ/day) = 0.01618 × O_2 (L/day) + 0.00502 × (CO_2 [L/day] - 3 × CH_4 [L/day]) – 0.00217 × CH_4 (L/day) – 0.00599 × urine N (g/day) (Chwalibog et al., 1996);
(5) Body energy retention (MJ/day) = GE intake (MJ/day) – fecal energy (MJ/day) – CH_4 energy (MJ/day) – urine energy (MJ/day) – heat energy (MJ/day) – milk energy (MJ/day);
(6) Metabolizability = metabolizable energy (ME) intake (MJ/day) / GE intake (MJ/day).
(7) Metabolic BW was calculated as $BW^{0.75}$.

Data analysis was performed by multiple regression analysis with the procedure REG of SAS (version 9.4, SAS Institute, Carry NC, USA). The model applied was as follows:

$$Y_{ijk} = \mu + \beta_i H + \beta j H^2 + \beta_k \text{ days in milk} + \varepsilon_{ijk}$$

where Y_{ijk} is the individual observation of the respective variable, μ is the overall mean, β_{ijk} are the regression coefficients of the fixed linear (H) and quadratic (H^2) effects of hazel proportion, as well as of days in milk, and ε_{ijk} is the random residual. Including hazel proportion in both a linear and a quadratic term allowed the identification of linear and non-linear (associative) relationships of the variables with hazel proportion. To ensure that the effects of hazel proportion were corrected for individual variation in performance among animals, days in milk was included as a covariate. A first data evaluation by Student's *t* test showed that there were no significant differences between the cows' breed in any of the target variables. Therefore the breed was not included into the statistical model as covariate. For MRT, lactation number was used as a covariate instead, as it is known that animal age can influence MRT (Grandl et al., 2018) and because a preliminary correlation analysis showed no relationship between the days in milk and MRT. The model with the lowest Akaike's information criterion (Akaike, 1974) were used for model selection, which comprised either H, H^2, or both. The parameter estimates are given with the unadjusted R^2, SE, CV, and significance levels for the entire model as well as for individual parameters. The plots were drawn with SigmaPlot 13. The regression analysis was applied to 18 of the 20 experimental

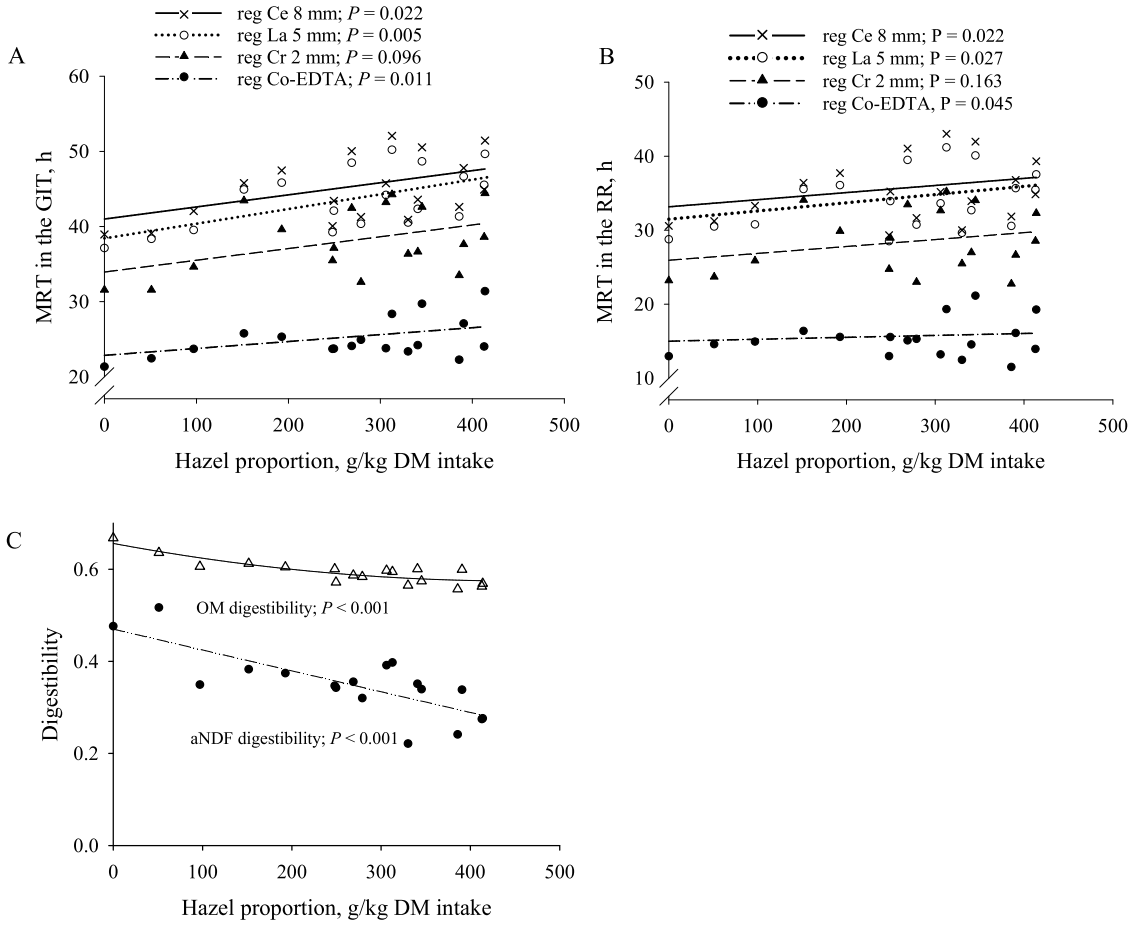

Fig. 2. Mean retention time (MRT; GIT, gastrointestinal tract (A); RR, reticulorumen (B)) and digestibility of organic matter (OM) and aNDFom (C) occurring with increasing dietary hazel leaf proportion (for regression equations and coefficients see Supplementary Tables S1 and S3). *P*-values of the equations are presented in the legend.

animals. One animal was excluded because of diarrhea during the sampling period, and the second, because it consumed hazel leaf pellets at only 0.60 of the total amount offered for most of the time and had widely varying amounts of total refusals across the sampling period. The figures show the measured individual data points and regression lines through the estimates where the covariates were held constant at their median (246.5 for days in milk and 3.00 for lactation number). Regression equations and coefficients are given in the supplementary material. The ECM-related variables were evaluated without the data of the cow receiving no hazel leaves. The apparent decline in ECM yield was 9 kg/day from this cow to the cow receiving the lowest hazel leaf level, an unlikely drastic depression, especially as no further clear decline in ECM was found with higher hazel proportions.

3. Results

Phenol concentrations were low in all feed items except for the hazel leaves (Table 1). Most of the hazel leaf phenols were CT.

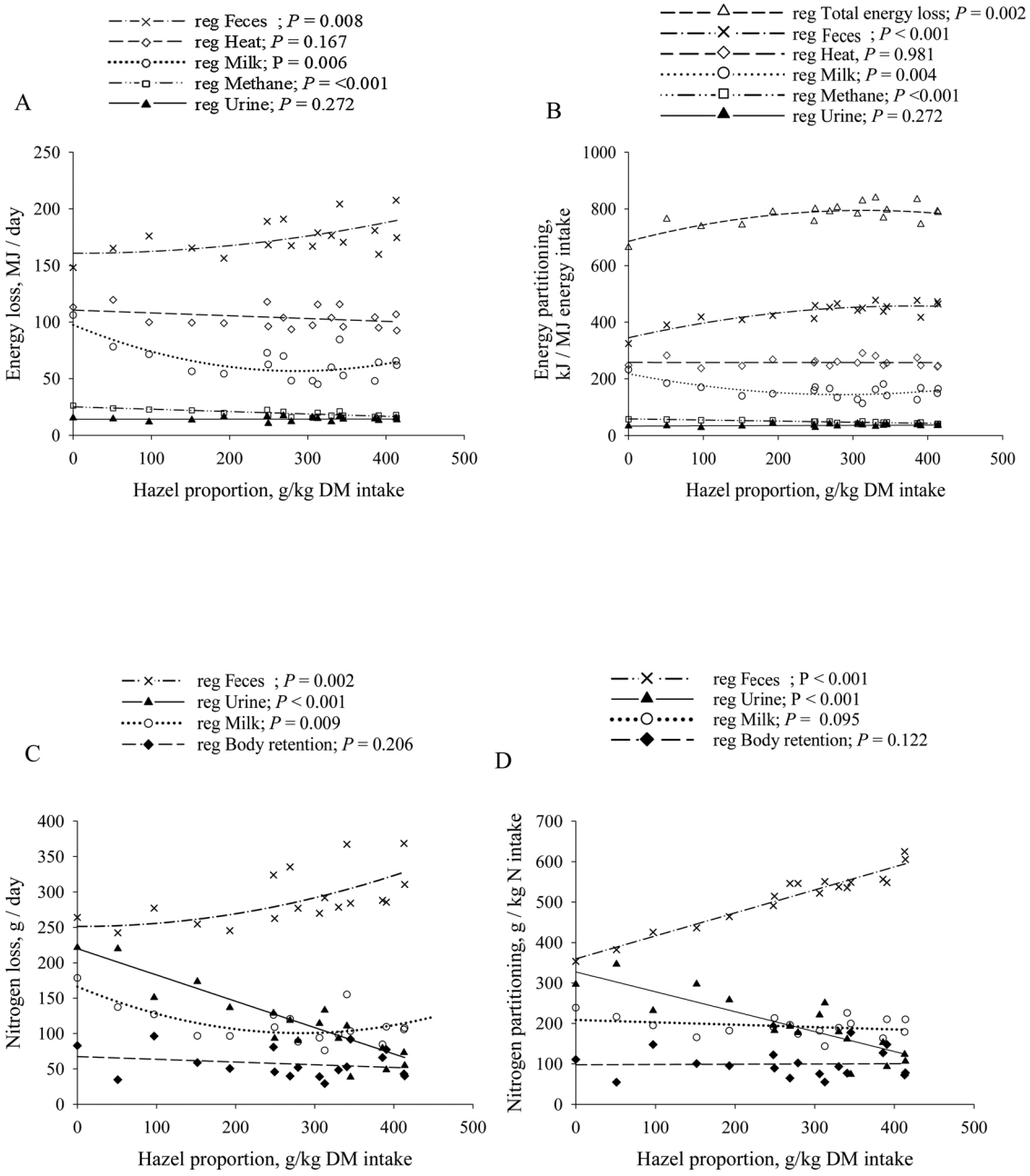

Fig. 3. Energy losses (A) and partitioning (B) as well as nitrogen losses (C) and partitioning (D) occurring with increasing dietary hazel leaf proportion (for regression equations and coefficients see Supplementary Table S2). *P*-values of the equations are presented in the legend.

Alfalfa contained 56 g/kg more CP than the hazel leaves, slightly more aNDFom and ADFom, and clearly less lignin(sa). The ratio between hazel leaves and alfalfa in the experimental pellets varied in the 20 pellet types in a gradient from 0:1 to 0.82:0.18 (Table 2). The dietary hazel proportion ranged from 0 to 414 g/kg. Along with this, the concentrations of TEP, NTP, TT, CT, and HT in the pellets increased from very low levels to 86, 20, 67, 49, and 19 g/kg dietary DM, respectively. The corresponding maximal concentrations were 50 g TEP, 14 g NTP, 36 g TT, 27 g CT, and 11 g HT/kg diet (data not shown in table). With increasing hazel proportion, CP concentration declined, and lignin(sa) concentration increased.

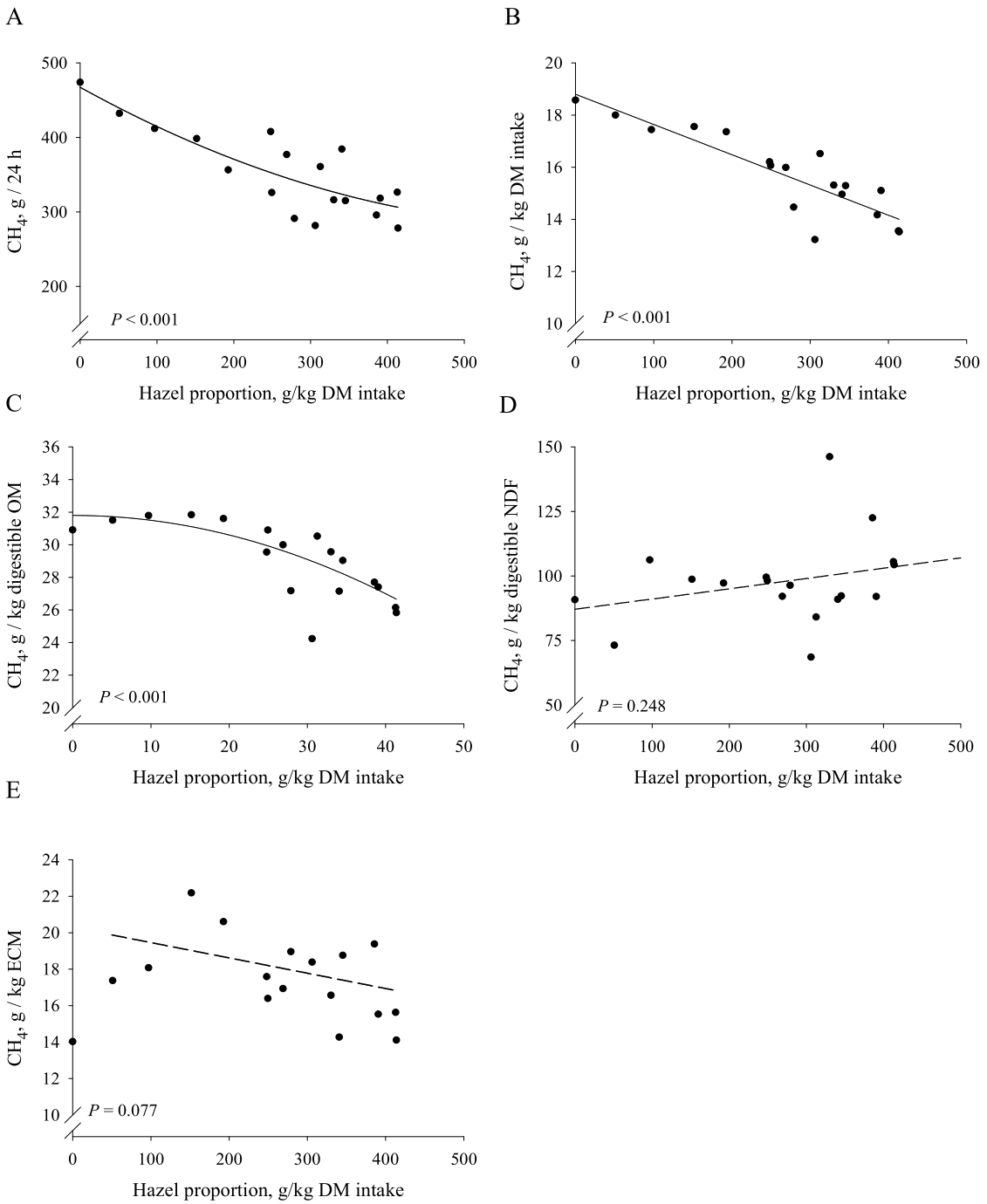

Fig. 4. Methane (CH_4) emission (A: absolute; B: per dry matter (DM) intake C: per digestible organic matter (OM); D per digestible aNDFom; E: per energy-corrected milk (ECM)) occurring with increasing dietary hazel leaf proportion (for regression equations and coefficients see Supplementary Table S4). When the regression model was not significant a dashed regression line was drawn. Methane emission per unit of ECM was calculated without the value of the cow receiving no hazel leaves.

The relative DM intake (DMI) was only slightly affected by hazel proportion ($P = 0.01$, Fig. 1A). When excluding the zero hazel cow, ECM yield and ECM per DMI (Fig. 1B and C) were not significantly affected by increasing hazel proportion (Supplementary Table S1). Milk fat, lactose, and protein concentrations were also not significantly affected by hazel proportion (data not shown). Milk urea ($P < 0.001$) declined clearly in a curvilinear manner with increasing hazel proportion from 35 mg/dL to almost 10 mg/dL, with a steep initial decline and a less pronounced decline at higher hazel proportion (Fig. 1D). By contrast, in the urinary-N to milk N ratio (N emission intensity), the decline became more prominent at higher hazel proportions (Fig. 1E; Supplementary Table S2).

Only weak effects of hazel proportion on MRT of particles and solute in the RR and the GIT were found, all pointing toward an increase with increasing hazel proportion (Fig. 2; Supplementary Table S3). Especially the passage of the solutes and that of the medium-sized as well as large particles (all $P < 0.05$) in the GIT and RR showed this effect, whereas there was a trend for small particles ($P < 0.1$) only in the GIT. The OM digestibility declined in a curvilinear manner ($P < 0.001$). The aNDFom digestibility declined in a linear manner ($P < 0.001$) with increasing hazel proportion. The decline was more pronounced than that of OM digestibility.

Fecal energy loss increased with increasing hazel proportion both in absolute amounts ($P = 0.008$) and as a proportion of GE intake ($P < 0.001$) (Fig. 3A and B; Supplementary Table S2). This resulted in a quadratic decline of energy digestibility ($P < 0.001$). Energy loss *via* CH_4 declined linearly. Overall, this decline was not sufficient to fully compensate for the fecal losses, causing a curvilinear decline in metabolizable energy supply as well ($P < 0.001$). Heat production was not affected by hazel proportion, whereas milk energy ($P = 0.006$) and body energy retention declined ($P = 0.003$) in absolute terms, and milk energy also declined in proportionate terms ($P = 0.004$).

With increasing hazel proportion, fecal N increased in a quadratic manner ($P = 0.002$) and urinary N decreased linearly ($P < 0.001$) in absolute levels g/day (Fig. 3C; Supplementary Table S2). When expressed per unit of N intake, both variables were changed linearly with increasing hazel proportion ($P < 0.001$) (Fig. 3D). Absolute milk N excretion changed in a curvilinear manner ($P = 0.009$), with the decline becoming less pronounced at higher hazel proportions. The N efficiency (milk N relative to N intake) was not affected by hazel proportion in the diet. The same was true for body N retention both in absolute terms and per unit of N intake.

Generally, CH_4 emissions declined with increasing dietary hazel proportion except when related to intake of digestible aNDFom (Fig. 4; Supplementary Table S4). The CH_4 emissions, in absolute terms, per BW, and per metabolic BW (presented in Supplementary Table S4 only) declined significantly with increasing hazel proportions ($P < 0.001$) as well as in a trend for ECM ($P = 0.077$). The regression for CH_4 per unit of digestible OM decreased significantly in quadratic hazel proportion terms, with the decline becoming more pronounced at higher hazel proportion ($P < 0.001$). The CH_4 per DMI and per GE intake (Y_m) declined linearly ($P < 0.001$) and substantially with increasing hazel proportion.

4. Discussion

The variation in the pellets achieved by replacing the high-quality forage alfalfa with the PSM-rich forage hazel leaves was large. The design thus allowed quite an even and large gradient of hazel proportion to be realized in the diets consumed. The main nutritional changes in the experimental pellets included a decrease in CP concentration by up to 0.33 compared to the pellets without hazel leaves, and an increase in phenol and lignin(sa) concentration by up to 16.5- and 2.5-times, respectively. Especially the latter was likely associated with a substantial decline in net energy concentration. As the pellets made up 580 g/kg of total diet on average, the individual diets also varied extensively in nutrient composition. Phenol concentrations and their composition may vary in forage due to cultivar and environmental conditions (Wam et al., 2017). The TEP level of the hazel leaf batch used in the present study was 103 g/kg DM, which was similar to that used earlier *in vitro* by Terranova et al. (2018 and 2020), but it was higher than found in the batch used by Wang et al. (2018). In the latter study, equal proportions of CT and HT in the TT were found, whereas in the present experiment and in the *in vitro* studies (Terranova et al., 2018 and 2020), the TT mostly consisted of CT.

4.1. Absence of adverse effects on milk yield and feed intake

Contrary to the first hypothesis, the milk yield of the animals was not negatively affected when supplemented with increasing proportions of hazel leaves. This was not even the case at high hazel supplementation level. This is contrary to a number of studies where tannin-rich feed additives were tested for their CH_4 mitigation potential. In the study of Grainger et al. (2009), for example, the CT extract from *A. mearnsii* was found to be effective in CH_4 mitigation but at the same time a significant decrease in milk production occurred. The same extract was found to severely reduce body weight gain of lambs and had no effect on CH_4 emission (Adejoro et al., 2020). In general, it seems that small amounts of tannins may even improve performance of ruminants, including milk yield as shown by Barry and McNabb (1999) in grazing sheep and by Dschaak et al. (2011) in dairy cows. However, high dosages of tannins, as provided in the present study with the higher proportions of hazel leaves in the diet, may impair performance. Accordingly, 57–100 g of CT/kg diet from *Lotus pedunculatus* were enough to impair rates of body and wool growth in the study of Barry and McNabb (1999). Highly tanniferous leaves from tropical leguminous shrubs in the diet even led to a body weight loss of growing lambs when included at high proportions (Tiemann et al., 2008). Only one study (Alves et al., 2017) is known to the authors that reported that mitigating greenhouse gas emissions from dairy cows could be accomplished by using tannins without negative impacts on milk production. A low level of supplementation of tanniferous feeds also remained without effect on milk yield or even increased yield in the study of Maasdorp et al. (1999), but this was likely owed to the improvement of the protein supply of the low-quality diet taking place with the supplements.

Despite the lower feeding value and the high concentrations of phenols and lignin(sa), the acceptance of the pellets was high even when containing only a small proportion of alfalfa and caused refusals in only one cow, which received pellets composed of 750 g hazel

leaves/kg. Apart from that, the tanniferous hazel leaves had no substantial effect on the relative DMI. This was astonishing as highly tanniferous feeds are often of low palatability in domestic ruminants (Frutos et al., 2004; Waghorn, 2008), as well as those high in lignin(sa) concentration, which is known for long. In a recent preference study (Terranova et al., 2020), hazel leaves were shown to be the most palatable out of six tannin-rich plants when processed with alfalfa to pellets. Hazel leaves pelleted with alfalfa (in a 0.6:0.4 ratio) were also consumed well by adult sheep (Wang et al., 2018). Further, Vandermeulen et al. (2016) showed that heifers, given the opportunity to browse shrubs on pasture, chose hazel as one of the most preferred among 11 shrub species.

4.2. Gradual mitigation of methane

In the present experiment, the CH_4 emissions clearly and substantially declined with increasing hazel proportion, confirming the second hypothesis. The level of decline was similar to that reported by Wang et al. (2018) with similar dietary hazel proportion. The major phenolic compounds of hazel leaves are the flavanols myricetin 3-rhamnoside and quercetin 3-rhamnoside, two CT (Amaral et al., 2005). The CT in several plants have been shown to have the potential to mitigate enteric CH_4 (reviewed by Beauchemin et al., 2008; Martin et al., 2009). In a meta-analysis, Jayanegara et al. (2012) observed a general linear relationship between dietary tannin concentration and methanogenesis, similar to that found in the present study. The decline found in the present study of 0.16 of the total CH_4 amount per g/kg of dietary TT was nearly the same as that Jayanegara et al. (2012) calculated. It is well-known that reduced fiber digestion reduces H_2 availability in the rumen, and this lack of substrate will decelerate the activity of the methanogens (Moss et al., 2000). In the present study, the reduction in fiber digestibility might indeed have been the cause of the CH_4 mitigating effect. Accordingly, the CH_4 emission in relation to the intake of digestible aNDFom did not significantly respond to hazel proportion. It therefore remains unclear whether the CH_4 mitigating effect of the hazel leaves was caused by the tannins or the lignified fiber, or both.

The mitigation of enteric CH_4 is only useful when related to a constant or less than proportionate decline in intake, digestibility, and milk yield. Methane yield per unit of DMI or Y_m declined by a similar magnitude to absolute CH_4 because relative intake was unchanged with hazel proportion. However, when related to intake of digestible OM, the efficiency of CH_4 reduction was lower due to the adverse effects on digestion, illustrating that purely intake-related CH_4 yields (per DMI, Y_m) may miss part of the information when digestibility is varying at the same time. In addition, the relationship was no longer linear, indicating that there were associative effects. When animals were fed with a mixture of forages with or without chicory, Niderkorn and Baumont (2009) and 2019) found favorable associative effects mediated by digestive interactions among plant components. Concerning the present context, Jayanegara et al. (2013) showed *in vitro* that the combination of phenol-rich plants with a high-quality forage reduced CH_4 not only in absolute terms but also per unit of short-chain fatty acids compared to single-plant incubations. In the present study, the CH_4 emission intensity (*i.e.*, in relation to ECM yield) seemed to decline in a linear manner with increasing hazel proportion, though not significantly. However, there was a curvilinear slope in CH_4 yield per unit of digestible OM, indicating unfavorable associative effects. According to Jayanegara et al. (2012), a tannin level of > 20 g/kg is needed to reduce CH_4 per unit of digestible OM effectively and substantially, which was the case only at > 250 g hazel leaves/kg diet DM in the present study. However, this was when the decrease in CH_4 per unit of digestible OM was actually weakening, thus yielding no explanation for the type of associative effects found.

Across the authors' *in vitro* (Terranova et al., 2018 and 2020) and *in vivo* studies (Wang et al., 2018; present study), hazel leaves showed a quite constant and repeatable CH_4 mitigation effect. The decrease in CH_4 yield per unit of OM digested was linear *in vitro* and non-linear *in vivo* (Fig. 5); *i.e.*, associative effects were observed only *in vivo*. The CH_4 mitigation effect in sheep and cows was very similar even though the basal diets were different, consisting of hay in the sheep and a mixed basal ration in the cows. These observations are noteworthy, as plant additives, promising when tested *in vitro*, are often less efficient when tested *in vivo* (only $R^2 = 0.26$

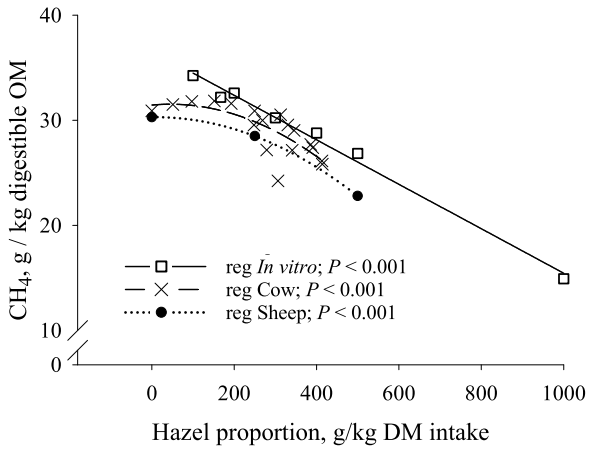

Fig. 5. Methane (CH_4) production per unit of digestible organic matter (OM) with increasing dietary hazel leaf proportion in *in vitro* (Terranova et al., 2018 and 2020) and *in vivo* experiments (sheep, Wang et al., 2018; cows, present study). For cow CH_4/digestible OM see Supplementary Table S4; Sheep CH_4/digestible OM $= 30.3 + 6.0 \times 10^{-4}$ hazel $- 3.1 \times 10^{-5}$ hazel^2; *in vitro* CH_4/digestible OM $= 36.6 - 0.021$ hazel. *P*-values of the equations are presented in the legend.

according to Flachowsky and Lebzien, 2009).

4.3. Gradual shift of nitrogen from a volatile form to faecal nitrogen

Our third hypothesis on a gradual increase of the shift of nitrogen in the excreta from easily volatile urinary nitrogen to less degradable faecal nitrogen was verified. In addition to CH_4 emissions, N emissions from manure are an environmental problem in animal production. Tannins, especially CT, have the capacity to bind to forage proteins through hydrogen bonds. These tannin-protein complexes are stable in a pH range from 3.5–8 and, therefore, under ruminal conditions (Frutos et al., 2004). In the present experiment, there was no clear statistical response to hazel proportion in apparent N digestibility, despite the increased fecal N excretion while N intake expressed per kg ECM (see Supplementary Table S2) did not changed significantly through the diets. This could indicate that the dietary N was bound by the tannins to pass the rumen and could not be released in the lower gut and the animal was therefore not able to metabolize it. This is supported by the sharp decline in milk urea levels. The protein availability in the rumen, became so low with high hazel proportion and low alfalfa proportion that notably low milk urea concentrations were found. However, the lack of effect on N digestibility could indicate that not all dietary N was bound by the tannins.

Livestock systems account for 65 % and 64 % of all anthropogenic N_2O and NH_3 emissions worldwide, respectively (Gerber et al., 2013). The N excretion with urine is a good marker for the potential N emissions from manure, as it is easily volatile (Dijkstra et al., 2013). Therefore, the substantial shift from urinary N to fecal N associated with increasing hazel proportion could be considered highly favorable from an environmental point of view. About 60–90% of all urinary N in cattle is excreted as urea that can be hydrolyzed rapidly after excretion to ammonia (NH_3). The transformation of urine N to N_2O, happening especially in highly N-dense urine patches on pasture (Giltrap et al., 2020) even let urinary N indicate potential nitrous oxides, further greenhouse gases (Dijkstra et al., 2013). The N emissions can be reduced through a number manure management methods, but reducing the N output by urine *via* dietary measures is the most immediate counteracting measure effective before most technical measures get active (Hristov et al., 2011). Diet effects on urine N losses were recovered as concomitant changes in NH_3 and, less clear, N_2O emissions from manure in different manure storage systems (Külling et al., 2003). The hazel leaves were highly efficient in reducing urine N losses and its proportion of total excreta N. In the present study, a number of indicators of improvements in N efficiency with gradually increasing hazel proportion were evaluated. These included absolute urine N excretion, urine N excretion relative to intake ("urine N yield"), urine N excretion relative to milk N formation ("urine N emission intensity"), and N losses with urea in milk and with urine relative to ECM yield. Also, in urinary N-related traits, associative (non-linear) effects of exchanging alfalfa by hazel leaves could be expected. Indeed, concerning urine N emission intensity (Fig. 1E), a curve with a similar shape to that of CH_4 emission intensity (related to digestible OM) was found, indicating greater efficiency of greater hazel proportion than smaller hazel proportion in mitigating urine N per kg of ECM. It remains unexplained why milk urea concentration showed the opposite type of slope, *i.e.*, large decline at low hazel levels.

4.4. Absence of favorable associate effects at low hazel doses

The fourth hypothesis concerning the presence of favorable associative effects even at low hazel doses was rejected. There was a clear decline in digestibility with increasing hazel proportion. The digestibility of energy and OM, especially of fiber, declined with increasing hazel proportion, as also found in sheep by Wang et al. (2018) with amounts of 50 % DM hazel in the diet. In the case of OM digestibility, the decline was slightly more pronounced between 0 and 200 g hazel leaves/kg, and hazel leaf proportion was less adverse above that level, thus indicating a slightly unfavorable associative effect. In contrast, Van Soest (1994) found favorable associative effects in digestibility when integrating a lower-quality feed into a high-quality diet. The decline from 0.48 to 0.28 in aNDFom digestibility when increasing hazel leaves from 0 to 414 g/kg was very pronounced. This was similar to the decline of 0.37 of the total found by Wang et al. (2018) in adult, non-lactating sheep receiving 0 or 500 g hazel leaves/kg DM. The lignin(sa) fraction in the diet has the property of decelerating physical and microbial feed degradation in the rumen because of its association with hemicellulose and cellulose (Waghorn and McNabb, 2003). A direct effect of the tannins is not very likely, but the fiber degrading microbes were also impaired by the decreasing dietary concentration of rumen-degradable protein because of the declining CP concentration and the CP-binding activity of the tannins. Compared to fiber digestibility, the effect of the hazel leaves on the OM digestibility was weaker. The fiber digestibility showed a highly significant linear hazel proportion effect, indicating that there were no associative effects. A reduced rate of fiber digestion could also delay RR clearance and hence increase MRT, especially of particles in the RR, but also in the entire GIT. In contrast, Melaku et al. (2005) found a faster passage of the particulate matter when multipurpose trees were added to tef straw compared to adding wheat bran in sheep.

In the present study, the ruminal digesta washing (difference in MRTs between particles and fluids) was not affected by hazel leaf proportion. Therefore, the observed differences in digestion cannot be explained by different levels of digesta washing. Our results are in line with previous findings indicating that the difference between particle and fluid outflow from the rumen is kept constant across a range of diet types (Clauss et al., 2014; Grandl et al., 2018).

Different from body N retention, body energy retention declined but in a curvilinear way with increasing hazel proportion. This indicates that the lower feeding value of the hazel leaves, compared to the alfalfa, had adverse effects on digestibility but were lower than anticipated.

The digestion of shrub leaves rich in PSC, like phenols and tannins may require a detoxification that costs the animal additional energy (White and Lawler, 2002). As a consequence, the changes in heat production should show the extent of this cost when comparing tannin-rich diets to low-tannin diets. However, the heat energy losses did not vary along the hazel proportion in the present study, which was consistent with findings of Wang et al. (2018). This could be explained by the high proportion of the indigestible CT

in the total hazel leaf phenols of the present study, as in that case only few digestible phenols would have needed metabolic detoxification. This is confirmed by the absence of an increase in total phenol concentration in the blood plasma of sheep with high dietary hazel proportions studied by Wang et al. (2019).

5. Conclusions

In the present experiment, feeding hazel leaves turned out to provide a promising way to mitigate greenhouse gas emission from dairy husbandry. It was shown to be a well palatable feed that is highly effective in mitigating CH_4 and, especially, urine N losses in dairy cows and especially without severely impairing milk yield when replacing alfalfa. This was astonishing for a tannin-rich plant also when considering the clearly lower digestibility compared to alfalfa. However, the associative effects concerning the relation to digestion and performance of both emission sources, CH_4 and easily volatile urine N, were not in a favorable direction by low hazel proportions, if any. Further studies have to confirm the absence of such beneficial associative effects in livestock, especially at low hazel doses. A wider spread implementation of the hazel shrub in animal nutrition would require an extension of cultivation. This could be a limitation, but also could open dairy husbandry to further positive environmental effects, such as a diversification of existing agricultural systems through the introduction of hazel shrubs. Their integration into pasture systems, *i.e.* a switch to a silvopastoral system, would be another possibility.

CRediT authorship contribution statement

M. Terranova: Data curation, Formal analysis, Validation, Visualization, Writing - original draft, Writing - review & editing. **L. Eggerschwiler:** Methodology, Writing - review & editing. **S. Ortmann:** Methodology, Writing - review & editing. **M. Clauss:** Methodology, Writing - review & editing. **M. Kreuzer:** Conceptualization, Project administration, Supervision, Writing - review & editing. **A. Schwarm:** Funding acquisition, Supervision, Conceptualization, Methodology, Writing - review & editing.

Declaration of Competing Interest

The authors report no competing interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.anifeedsci.2020.114790.

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