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Original article

Effect of coconut oil and defaunation treatment on methanogenesis in sheep

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Abstract — The present study was conducted to evaluate in vivo the role of rumen ciliate protozoa with respect to the methane-suppressing effect of coconut oil. Three sheep were subjected to a 2 × 2 factorial design comprising two types of dietary lipids (50 $g \cdot kg^{-1}$ coconut oil vs. 50 $g \cdot kg^{-1}$ rumen-protected fat) and defaunation treatment (with vs. without). Due to the defaunation treatment, which reduced the rumen ciliate protozoa population by 94% on average, total tract fibre degradation was reduced but not the methane production. Feeding coconut oil significantly reduced daily methane release without negatively affecting the total tract nutrient digestion. Compared with the rumen-protected fat diet, coconut oil did not alter the energy retention of the animals. There was no interaction between coconut oil feeding and defaunation treatment in methane production. An interaction occurred in the concentration of methanogens in the rumen fluid, with the significantly highest values occurring when the animals received the coconut oil diet and were subjected to the defaunation treatment. Possible explanations for the apparent inconsistency between the amount of methane produced and the concentration of methane-producing microbes are discussed. Generally, the present data illustrate that a depression of the concentration of ciliate protozoa or methanogens in rumen fluid cannot be used as a reliable indicator for the success of a strategy to mitigate methane emission in vivo. The methane-suppressing effect of coconut oil seems to be mediated through a changed metabolic activity and/or composition of the rumen methanogenic population.

lipid / nonyl phenol ethoxylate / Archaea / ciliate protozoa / ruminant

1. INTRODUCTION

Blaxter and Czerkawski [1] demonstrated a high potential of medium-chain fatty acids to suppress total digestive tract methane release in ruminants for the first time. Coconut oil as a source of medium-chain fatty acids also proved to significantly reduce methane release in vivo [2]. Concurrent to methanogenesis, the rumen

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ciliate protozoa population was reduced indicating that one reason for the methane-suppressing effect could be the inhibition of this group of hydrogen producers. Ciliate protozoa are known to have a specific role in interspecies hydrogen transfer since methanogens are associated ecto– and endosymbiotically with them [3, 4]. Stumm et al. [5] estimated that 10 to 20% of total methanogens could be attached to ciliate protozoa. From in vitro data, Newbold et al. [6] calculated that the methanogens associated with rumen ciliate protozoa are responsible for 9 to 25% of methanogenesis in the rumen fluid.

In an in vitro study, which was carried out to study the role of rumen ciliate protozoa regarding methane suppression by coconut oil [7], coconut oil reduced methane production in both faunated and defaunated rumen fluid and this seemed to have been the result of a direct inhibition of the rumen methanogens. The objective of the present study was to verify these in vitro results in sheep. This was considered necessary because ciliate protozoa are less well maintained in vitro, compared with in vivo conditions and the methanogen population could be different in composition [8].

2. MATERIALS AND METHODS

2.1. Experimental procedure

A 2 × 2 factorial design was applied varying the type of dietary lipids (coconut oil vs. rumen-protected fat) and defaunation treatment (with vs. without). Three castrated male sheep of the Swiss White Hill breed with an average live weight of 76 ± 8 kg were used. The whole experiment comprised six consecutive experimental periods of 28 days, feeding the two experimental diets alternately to each animal. The animals were subjected to the defaunation treatment in the third and fourth experimental period. With the observations from the first and second (faunated status) and the fifth and sixth experimental period (refaunated status) replicated observations for each diet in each individual animal were obtained. Prior to statistical evaluation, these data were combined to one average value each. With the exception of the fourth experimental period, on day 1 of each experimental period the sheep were inoculated with 250 mL fresh rumen fluid derived from a rumen-fistulated donor cow. Additionally, for the first 7 days of each experimental period the animals were fed the diet containing the rumen-protected fat in order to achieve a similar initial rumen microbial population. Thereafter, the animals were adapted to the respective experimental diets for 11 days, followed by 7 days of complete and separate collection of faeces and urine, and 3 days of quantitative measurement of gaseous exchange in open-circuit respiratory chambers. On day 28 of each experimental period, rumen fluid samples were taken from each sheep. These samples were always obtained 5 h after the morning feeding with a flexible stomach tube inserted through the oesophagus.

Defaunation treatment was carried out on day 4 of the third and fourth experimental period. For this purpose nonyl phenol ethoxylate (Synperonic NP9®, ICI Surfactants, Cleveland, UK) was administered into the rumen of the animals with a tube at an amount of 0.3 $g \cdot kg^{-1}$ live weight after dilution with 5 mL $water \cdot g^{-1}$ Synperonic NP9® according to Kreuzer and Kirchgessner [9]. Before administration of the defaunation agent, the animals were kept off feed and water for 24 h. First access to feed and water was allowed 8 h after the defaunation treatment. The success of the defaunation treatment was controlled in the rumen fluid samples on days 3 and 7 after treatment. Repetition of the defaunation treatment was carried out when ciliate protozoa were found which was the case for one animal in the third experimental period.

The two experimental diets were based on maize silage, hay and a concentrate containing either 50 $g \cdot kg^{-1}$ DM of a rumen-protected fat (Golden Flake-Prills®, Nutrition Trading International Ltd, Studley, UK) or coconut oil and were supplemented with a commercially available mineral-vitamin premix (Tab. I). It has been demonstrated in vitro [10] that the fat prills used are without any effect on rumen fermentation and ruminal methanogenesis. The metabolisable energy, calculated from tabulated values [11], was supplied to all sheep at a level of 1.3-fold of maintenance requirements, equivalent to 494 $kJ \cdot kg^{-0.75}$ live weight [11], in order to ensure a slightly positive energy balance. During the second week of each experimental period, the feed amount of the experimental diets was adjusted to the live weight and was kept constant afterwards. The diets were offered in two equal portions at 8.00 h and 16.00 h and were consumed without refusals. During the whole experiment, the animals had free access to fresh water. The animals were kept in individual pens (1.3 m × 1.9 m) fitted with automatic drinking bowls except in the 10-day measurement periods, when they were housed in metabolism crates (0.6 m × 1.1 m). In the crates, water troughs were installed and were filled twice a day with fresh tap water. Daily water consumption was measured during the time spent in the crates. The experiment was carried out in accordance with the Swiss guidelines for animal welfare.

Samples of maize silage, hay and concentrates were collected during each experimental period. For subsequent chemical analysis, the samples were milled through a 0.5 mm screen. Maize silage was lyophilised for 48 h before milling. For 7 days complete faeces and urine were collected separately twice a day and stored at –20 °C immediately after collection. The

Table I. Composition of the diets.

Lipid treatment	Protected fat	Coconut oil
Ingredients ($g \cdot kg^{-1}$ DM)		
Maize silage	522	522
Hay	52	52
Concentrate	416	416
Barley	96	96
Soybean meal	225	225
Potato protein	45	45
Rumen-protected fat	50	–
Coconut oil	–	50
Mineral-vitamin premix[1]	10	10
Analysed nutrient composition (per kg DM)		
Organic matter (g)	953	953
Crude protein (g)	203	196
Ether extract (g)	64	54
Cell wall constituents		
Neutral detergent fibre (g)	310	319
Acid detergent fibre (g)	188	186
Non-NDF carbohydrates (g)	376	385
Gross energy (MJ)	19.9	19.7

[1]Contained (per kg) 140 g Ca; 70 g P; 80 g Na; 30 g Mg; 15 mg Se; 500 000 IU vitamin A; 120 000 IU vitamin D_3; 2 500 mg vitamin E.

urine was immediately acidified in 3 M sulphuric acid to avoid N losses. For subsequent analysis of the carbon content, twice a day, a sample of non-acidified urine was obtained. At the end of the collection periods proportional samples of faeces and urine were taken and stored at –20 °C until chemical analysis. Part of the faeces were lyophilised for 48 h and milled through a 0.5 mm screen.

2.2. Analytical procedures

The contents of dry matter (DM), total ash, neutral detergent fibre (NDF), acid detergent fibre (ADF) and, subsequent to hydrolysis with 4M hydrochloric acid, ether extract in feed and lyophilised faeces were determined according to standard methods [12]. For DM and total ash, an automatic analyser was used (Thermogravimetric determinator, TGA-500, Leco Corporation, St. Joseph, MI, USA). Values of NDF and ADF were corrected for ash content, and α-amylase was used for NDF determination as recommended by Van Soest et al. [13]. Nitrogen was determined in the feed, non-lyophilised faeces and acidified urine with an automatic C/N analyser (Leco-Analysator Typ FP-2000, Leco Instrumente GmbH, Kirchheim, Germany) by the Dumas method. With the same analyser, the carbon content of non-acidified urine was measured. Crude protein content was calculated as 6.25 × N content. Non-NDF carbohydrates were defined as the organic matter (OM) content not incorporated into the crude protein, ether extract and NDF. Gross energy content of feed and lyophilised faeces was assessed through an anisothermic calorimeter (C 700 T System, IKA-Analysentechnik GmbH, Heitersheim, Germany). Urine energy was calculated according to the equation of Hoffmann and Klein [14] based on the contents of carbon and nitrogen in urine.

Immediately after collection, ciliate protozoa and bacteria were microscopically enumerated in rumen fluid samples using 0.1 mm and 0.02 mm depth Bürker counting chambers (Blau Brand®, Wertheim, Germany), respectively. Rumen fluid pH was determined by a pH meter (model 713, Metrohm, Herisau, Switzerland) equipped with the respective electrode. Frozen samples (–20 °C) of rumen fluid were used for the analysis of volatile fatty acids (VFA) by gas chromatography (GC Star 3400 CX, Varian, Sugarland, TX, USA) according to Tangerman and Nagengast [15]. For the enumeration of methanogens, samples of rumen fluid were frozen in liquid nitrogen and stored at –70 °C. The fluorescence in situ hybridisation technique (FISH) was applied as outlined by Stahl et al. [16] and modified by Soliva et al. [17]. To characterise the methanogens of the rumen, according to Lin et al. [18] five fluorescein-labelled (Cy3) probes were used, one domain-specific probe targeting 16S rRNA of total rumen Archaea, i.e. all methanogens (S-D-Arch-0915-a-A-20), and four order-specific probes for the Methanobacteriales (S-F-Mbac-0310-a-A-22), the Methanomicrobiales (S-O-Mmic-1200-a-A-21), the Methanococcales (S-F-Mcoc-1109-a-A-20) and the Methanosarcinales (S-O-Msar-0860-a-A-21). Labelled samples were examined with a microscope (BX-60, Olympus Optical AG, Volketswil, Switzerland) equipped for epifluorescence measurements. Individual fluorescence signals were automatically counted using a 3CCD color video camera (DXC-950P, Sony Corporation, Tokyo, Japan) and a software for image analysis (analySIS, version 3.1, Soft Imagine System GmbH, Uster, Switzerland). The repeated freezing of the rumen fluid samples along with the series of measurements was found to cause destruction of the methanogen cells. After defrosting for the second time, the complete 16S rRNA was thus liberated from the cells. Therefore, whole cell enumeration was possible only for the domain-specific probe which was used first. When using the four order-specific probes the liberated individual

16S rRNA copies and not the methanogen cells themselves were detected.

Oxygen consumption, carbon dioxide and methane release were measured with a dual chamber as elements of an open-circuit indirect respiration calorimetry system. The chambers were air conditioned (ambient temperature 17.3 ±0.6 °C (mean ± SD), relative humidity 67.9 ± 6.3, air flow 8.3 ±0.1 $m^3 \cdot h^{-1}$, atmospheric pressure 958 ±5 hPa). The air volume leaving the chambers was continuously recorded with in-line electronic flow meters (Type 8GD-LRM, Fluid Inventor AB, Stockholm, Sweden). Daily gaseous exchange data were calculated from three consecutive days (three measurement periods of 22.5 h each). The detectors used were an Oxymat 6 for O_2 (Siemens AG, Karlsruhe, Germany) and a Binos 1001 for CO_2 and CH_4 (Fisher-Rosemount, Baar-Walterswil, Switzerland). Prior to each measurement period, the detectors were manually calibrated. During the measurement periods, within an interval of 90 min, one automatic calibration, four measurements of the gaseous concentrations in the air flowing into the chambers (each 5.44 m^3) and 24 measurements of the gas concentrations in the outgoing air from each chamber were performed. Equations of Brouwer [19] were used for the calculation of methane energy loss and energy expenditure. Energy retention was calculated as metabolisable energy (ME) minus the energy expenditure (heat energy). The efficiency of utilisation of ME for maintenance (k_m) was estimated using the AFRC [20] equation. For the calculation of the efficiency of utilisation of ME for growth (k_f), the individual energy requirements for maintenance were determined considering fasting metabolism and activity assumptions for housed sheep [20].

2.3. Statistical analysis

The data were analysed by the general linear model (GLM) procedure (SAS, version 6.12, SAS Institute Inc., Cary, NC, USA). Analysis of variance was carried out regarding the lipid treatment, defaunation treatment, lipid × defaunation treatment and animal. The counts of bacteria, ciliate protozoa and FISH data were logarithmically transformed prior to statistical analysis. Multiple comparisons among means, regarding the interaction of lipid and defaunation treatment, were performed with the Tukey studentised range test considering $P < 0.05$ to be significant. The tables give the means, the standard errors of means (SEM) and the P-values for the treatment effects and interactions.

3. RESULTS

The analysed nutrient composition of the two experimental diets was similar since the rumen-protected fat was replaced by an equal amount of coconut oil (Tab. I). The overall realised DM intake of the animals was 1.1 $kg \cdot d^{-1}$ (data not shown). The ad libitum intake of tap water varied widely among the treatments (data not shown). Feeding coconut oil reduced ($P < 0.05$) tap water intake by up to 12% whereas defaunation treatment increased ($P < 0.01$) the intake by up to 23%. However, coconut oil only reduced water intake significantly when the animals were subjected to the defaunation treatment (treatment interaction, $P < 0.05$).

Exchanging rumen-protected fat by coconut oil neither affected pH nor total VFA concentration in rumen fluid (Tab. II). With coconut oil, the molar proportions of propionate and isovalerate were reduced ($P < 0.01$) and the molar proportion of butyrate ($P < 0.001$) and the acetate-to-propionate ratio were increased ($P < 0.05$). Defaunation treatment depressed pH ($P < 0.05$) but did not alter the total VFA concentration. The molar proportion of acetate was increased ($P < 0.01$) and the molar proportion of propionate was reduced ($P < 0.01$) with the defaunation treatment,

Table II. Treatment effects on rumen fluid properties, microbial cell counts and detected copies of rRNA as an indicator of the metabolic activity of the methanogens (n = 3 per treatment).

Lipid treatment (L)	Protected fat		Coconut oil		SEM	P-value[2]		
Defaunation treatment (D)	No	Yes	No	Yes		L	D	L × D
Rumen fluid properties								
pH	6.39	6.16	6.56	6.14	0.106	0.533	*	0.405
Volatile fatty acids ($mmol \cdot L^{-1}$)	124.6	122.2	93.9	111.5	13.74	0.183	0.599	0.497
Acetate (mol %)	65.0	70.1	66.0	69.5	1.07	0.865	**	0.466
Propionate (mol %)	24.9[a]	20.4[b]	20.6[b]	17.9[b]	0.87	**	**	0.345
Butyrate (mol %)	7.3[b]	6.3[b]	10.2[a]	10.1[a]	0.55	***	0.390	0.441
Isobutyrate (mol %)	0.3	0.3	0.6	0.3	0.23	0.484	0.444	0.653
Valerate (mol %)	1.4	1.5	1.8	1.7	0.15	0.103	0.966	0.472
Isovalerate (mol %)	1.2[ab]	1.4[a]	0.9[ab]	0.5[b]	0.13	**	0.692	0.071
Acetate-to-propionate ratio	2.6[b]	3.5[ab]	3.3[ab]	3.9[a]	0.18	*	**	0.569
Microbial cell counts (day 28)[1]								
Bacteria ($10^{10} \cdot mL^{-1}$)	2.34[ab]	3.29[a]	1.71[b]	2.80[a]	0.210	*	**	0.382
Ciliate protozoa ($10^{5} \cdot mL^{-1}$)	4.21[a]	0.33[ab]	1.44[a]	0.01[b]	1.614	*	**	0.078
Methanogens ($10^{8} \cdot mL^{-1}$)	2.31[b]	4.04[b]	2.24[b]	11.28[a]	0.848	*	**	*
Detected copies of rRNA ($10^{8} \cdot mL^{-1}$; day 28)[1]								
Methanobacteriales	3.84	6.67	4.56	3.37	0.593	0.187	0.415	*
Methanomicrobiales	2.27	3.26	2.65	2.54	0.381	0.839	0.296	0.222
Methanococcales	39.17[ab]	59.70[a]	43.81[ab]	24.18[b]	5.742	*	0.687	*
Methanosarcinales	0.03	0.04	0.01	0.04	0.014	0.297	0.177	0.409
Total rRNA copies	45.31[ab]	69.67[a]	51.04[ab]	30.13[b]	5.973	*	0.838	**

[1] Logarithmically transformed prior to analysis of variance.
[2] *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; P-value is shown when not significant.
[ab] Treatment means with different superscripts within the rows differ significantly ($P < 0.05$).

which also resulted in a reduced ($P < 0.01$) acetate-to-propionate ratio. At the second date of controlling the success of the defaunation treatment, i.e. day 11 of the third and fourth experimental periods, no ciliate protozoa were found in the rumen fluid samples of the animals but on day 28 two out of the three sheep had ciliate protozoa again. Coconut oil reduced ($P < 0.05$) the average cell concentration of bacteria and ciliate protozoa in the rumen. The defaunation treatment enhanced ($P < 0.01$) the cell concentration of bacteria and methanogens and reduced ($P < 0.01$) the cell concentration of ciliate protozoa by 96% on average. Treatment interaction ($P < 0.05$) occurred in methanogenic cell counts since coconut oil increased the cell concentration of methanogens only when the animals underwent the defaunation treatment. Table II also gives the results on the detected fluorescence signals of labelled 16S rRNA copies in rumen fluid when using the methanogen order-specific oligonucleotide probes. Independent from any experimental treatment, fluorescence signals of rRNA were the most frequent for Methanococcales. Fluorescence signal densities of rRNA of Methanosarcinales were the lowest and those of rRNA of Methanobacteriales and Methanomicrobiales were intermediate. The coconut oil diet reduced ($P < 0.05$) the density of rRNA copies of Methanococcales and an interaction ($P < 0.05$) was found for rRNA fluorescence signals of Methanobacteriales and Methanococcales. A significant reduction in the signal density of these methanogen orders was found only when the defaunation treatment was conducted and the coconut oil diet was fed. This was also true for the total signals of labelled rRNA copies detected ($P < 0.01$). The defaunation treatment alone did not significantly affect the rRNA fluorescence signal density of the individual rumen methanogenic orders.

Concerning the total tract nutrient digestibility, no significant effects of lipid treatment were recorded (Tab. III) as was also true for the nitrogen balance (data not shown). The defaunation treatment depressed ($P < 0.05$) ADF digestibility, but had no significant effects on the digestion of other nutrients and on N balance. Changing the lipid source from rumen-protected fat to coconut oil increased ($P < 0.05$) the energy expenditure. Other sources of energy loss and energy retention were not significantly affected by the type of lipid used except for the loss of energy due to the methane formation which was reduced by 15% on average ($P < 0.1$). There were no significant treatment effects on the calculated efficiencies of the utilisation of metabolisable energy.

Feeding the coconut oil diet increased CO_2 release ($P < 0.01$) and O_2 consumption ($P < 0.1$) of the animals, and the respiratory quotient was not altered (Tab. IV). In contrast, the defaunation treatment increased ($P < 0.01$) the respiratory quotient. Compared with the rumen-protected diet, the coconut oil diet reduced daily methane release when related to live weight, digested OM and digested NDF ($P < 0.01$), and consumed gross energy ($P < 0.1$). The methane emission of the animals was not affected by the defaunation treatment.

4. DISCUSSION

4.1. Defaunation treatment

For defaunation, nonyl phenol ethoxylate was administered into the rumen of the animals according to the procedure described by Kreuzer and Kirchgessner [9] as well as Veira and Ivan [21]. Compared with other defaunation agents, this compound does not appear to be harmful to the animals [22]. In the present study, no adverse effects of the treatment, even when repeated, were observed. However, the success in defaunation of the sheep was only partial. At the end of the experimental periods 3 and 4 (day 28), some of the sheep had ciliate protozoa again. Nevertheless, the average number of

Table III. Treatment effects on the apparent digestibilities of the nutrients, balance and utilisation of energy ($n = 3$ per treatment).

Lipid treatment (L)	Protected fat		Coconut oil		SEM	P-value[1]		
Defaunation treatment (D)	No	Yes	No	Yes		L	D	L × D
Total tract nutrient digestibility rate								
Organic matter	0.749	0.727	0.762	0.752	0.0139	0.221	0.296	0.705
Crude protein	0.804	0.796	0.802	0.801	0.0131	0.942	0.756	0.816
Ether extract	0.722	0.677	0.789	0.729	0.0488	0.267	0.325	0.876
Cell wall constituents								
Neutral detergent fibre	0.504	0.465	0.525	0.531	0.0236	0.115	0.507	0.379
Acid detergent fibre	0.505	0.460	0.526	0.457	0.0126	0.702	*	0.599
Non-NDF carbohydrates	0.925	0.913	0.929	0.920	0.0053	0.360	0.102	0.808
Energy balance ($MJ \cdot d^{-1}$)								
Energy intake (GE)	21.62	22.19	21.38	21.86	0.367			
Energy loss								
Faeces	5.87	6.53	5.49	5.89	0.426	0.275	0.258	0.775
Urine	1.04	1.29	1.17	1.09	0.160	0.855	0.624	0.332
Methane	0.91	0.99	0.80	0.82	0.060	0.055	0.434	0.586
Energy expenditure	10.84	11.02	11.43	11.55	0.226	*	0.519	0.894
Energy retention	2.97	2.35	2.49	2.51	0.460	0.747	0.545	0.517
Efficiency of ME utilisation								
Total ($RE \cdot ME^{-1}$)	0.213	0.173	0.179	0.179	0.0289	0.659	0.516	0.513
Maintenance (k_m)	0.727	0.713	0.731	0.729	0.0071	0.208	0.319	0.458
Growth (k_f)	0.526	0.465	0.445	0.435	0.0524	0.326	0.526	0.644

[1] *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; P-value is shown when not significant.
GE: gross energy; ME: metabolisable energy; RE: energy retention.

Table IV. Treatment effects on the gaseous exchange (*n* = 3 per treatment).

Lipid treatment (L)	Protected fat		Coconut oil		SEM	*P*-value[1]		
Defaunation treatment (D)	No	Yes	No	Yes		L	D	L × D
Exchange of O_2 and CO_2								
O_2 intake ($L \cdot d^{-1}$)	534	540	561	565	11.7	0.067	0.707	0.943
CO_2 production ($L \cdot d^{-1}$)	474[b]	492[ab]	498[ab]	511[a]	7.6	**	0.080	0.720
Respiratory quotient (CO_2/O_2)	0.887	0.914	0.889	0.906	0.0077	0.702	**	0.543
Daily methane release								
$mL \cdot kg^{-1}$ live weight	307	336	264	269	20.6	**	0.435	0.582
$L \cdot kg^{-1}$ digested OM	29.9	32.7	25.8	26.1	2.04	**	0.473	0.554
$L \cdot kg^{-1}$ digested NDF	137.6	159.6	114.0	108.9	12.23	**	0.515	0.311
$kJ \cdot MJ^{-1}$ GE intake	42.6	45.0	37.8	37.4	3.07	0.090	0.769	0.660

[1] *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; *P*-value is shown when not significant.
[ab] Treatment means with different superscripts within the rows differ significantly ($P < 0.05$).
OM: organic matter; GE: gross energy.

ciliate protozoa counted was only 4% compared with the number found in the faunated and refaunated status of the animals. Furthermore, data from Veira and Ivan [21] showed that a partial defaunation has almost the same effect as a complete defaunation.

In the present study, the defaunation treatment resulted in a reduced rumen pH, an increase in bacteria concentration in the rumen fluid and a depression in total tract degradation of ADF. This is consistent with the results of other defaunation experiments as reviewed by Jouany et al. [22]. As in the defaunation experiment of Itabashi et al. [23], no significant effect in the overall balance of nitrogen or energy occurred. The observed shift in the molar proportion of VFA, with increased acetate and reduced propionate proportions, was inconsistent with the bibliographic data [22]. However, Jouany et al. [22] commented that modifications by defaunation are not always systematic and, therefore, the results will vary between experiments. Concerning the propionate proportion, the sampling day could be a decisive factor as was shown by Demeyer et al. [24].

In reviews, defaunation is always mentioned as a method to reduce methane emission from ruminants with quoted ranges from 20 to 50% of depression [e.g., 25–27]. However, there is only little in vivo data supporting this assumption [28]. In the literature, six experiments were found, giving data on the methane release of faunated and defaunated animals measured with respiratory chambers [23, 29–33]. These experiments demonstrated that defaunation not necessarily results in a decreased methane emission. Whitelaw et al. [33], feeding a pelleted concentrate mixture, observed a significant reduction in total tract methane release by 50%. In the study of Itabashi et al. [23] a significant reduction (by 22%) was found, but only when using a hay-concentrate diet and not when feeding hay exclusively. Chandramoni et al. [29] showed that the supplementation of a diet (concentrate: roughage ratio of 70:30) with molasses could prevent any methane-suppressing effect of defaunation which otherwise was found to account for 30%. In other studies, using diets with a concentrate proportion below 35% [30, 32] or employing semi-synthetic diets [31], no significant

methane-suppressing effect of defaunation was observed. Therefore, diet composition seems to have a great influence on the expression of an effect of defaunation on methane production.

Ushida et al. [34] quoted an in vitro experiment using a hay and a hay-concentrate diet where defaunation non-significantly increased methane production by 6% with the hay diet and significantly decreased methane production by 30% with the hay-concentrate diet. With the hay diet the methanogen number was the same in the faunated and defaunated status. Only when the hay-concentrate diet was used the number of methanogens was depressed by defaunation. It is suggested [35] that the contribution of the ciliate protozoa to ruminal methanogenesis is only important – i.e. defaunation will suppress methane production – when the population of ciliate protozoa in the faunated status is sizeable and this will be determined by the diet type. According to Itabashi et al. [23] and Ushida et al. [35] comparing different types of diets, the experimental diets used in the present study supported a relatively small ciliate protozoa population.

Stumm et al. [5] concluded from their data that the rate of the ectosymbiotic association of methanogens with rumen ciliate protozoa is correlated with the relative contribution of the rumen ciliate protozoa to overall hydrogen production in the rumen. Therefore, a reduced rate of association may reflect that the overall ruminal hydrogen production is high and/or that hydrogen-producing bacteria become more important as the hydrogen source for the rumen methanogens. Accordingly, Morvan et al. [36] described a high correlation between the number of methanogens and the number of cellulolytic bacteria. In the present study, where methanogen density even increased with the defaunation treatment, it was assumed that the interspecies hydrogen transfer between ciliate protozoa and methanogens did not play an important role in ruminal methanogenesis.

Different microbe-microbe interactions may also explain the contradictory observations in the in vitro study of Dohme et al. [7] using almost the same diet composition and defaunation treatment as in the present study. Dohme et al. [7] observed a significant depression in methane production by 61% when defaunating rumen fluid. However, on the contrary to the present study, in the in vitro study the defaunation treatment did not result in a compensatory increase of bacteria number but even significantly depressed bacteria number and the calculated daily hydrogen production.

4.2. Coconut oil treatment

The methane-suppressing effect of coconut oil found in the present study verified the in vitro and in vivo observations achieved with relatively similar diet types [7, 37]. Nevertheless, the extent of the difference between the respective coconut oil and protected fat diets varied between the studies. In vitro [7], the replacement of 54 $g \cdot kg^{-1}$ DM of a rumen-protected fat with coconut oil resulted in a depression of 48% in methane production. With growing lambs [37], this replacement depressed total tract methane release per kg live weight by 23% and in the present experiment the average extent of depression was 14%. One reason for these differences might be the fact that the in vitro study reflected only the effects on ruminal methanogenesis whereas in vivo the effect on total tract methanogenesis was measured. Hindgut methanogenesis amounts to 11% of total tract methane production on average [38]. Additionally, on the contrary to the in vivo diets, the in vitro diets were not supplemented with a mineral-vitamin premix. The importance of mineral supply, especially of calcium, for reducing the methane-suppressing effect of lauric acid (predominant fatty acid in coconut oil) is known [39].

Overall, in the present experiment, the coconut oil treatment depressed rumen bacteria and ciliate protozoa which was consistent with the in vitro experiment of Dohme et al. [7]. However, differently to the in vitro study, the methane-suppressing effect of coconut oil occurring in the present experiment could not clearly be attributed to a direct depression of the methanogens. It is not quite clear whether the different methods used to count the methanogens, culture-based enumeration vs. FISH, had an influence on the results. Culture-based enumeration has limitations [40] because not all rumen microbes can be cultured. In the present study, the reduced methane production with coconut oil at unchanged methanogenic cell concentrations in the rumen fluid (animals in faunated and refaunated status) suggested an effect on the metabolism of the methanogens meaning a lower methane production rate per single methanogenic cell. However, this statement is based on methanogen concentrations within the rumen rather than on total tract methanogen numbers, and the concentration of methanogens could vary widely in vivo depending on the postprandial sampling time and sampling site within the rumen [41].

Generally, regarding all data on rumen fermentation in the present study, especially those on rumen microbial counts, part of the methane-suppressing effect of the coconut oil can probably be attributed to a depressed rumen fermentation, i.e. a shortage in available hydrogen. This was not obvious from total tract nutrient digestibility, since a certain shift of nutrient degradation to the lower gut is known to take place when feeding coconut oil [42].

Although energy expenditure was higher with the coconut oil diet, there were no significant differences in energy utilisation and energy retention between the two diet types because of a compensation by the reduced energy loss via methane. The difference in the energy expenditure was presumably caused by the different kinds of lipids used, since a triglyceride was supplied with coconut oil and fat non-esterified hydrogenated palm oil fatty acids were provided with the rumen-protected.

4.3. Interactions between defaunation and coconut oil treatment

In the present study, few significant interactions occurred showing that the effects of coconut oil feeding are not predominantly mediated by the simultaneously observed inhibition of the rumen ciliate protozoa. This is particularly true for methane since coconut oil reduced total tract methane release in animals with normal and suppressed fauna. These results verified the in vitro data obtained at the level of rumen fermentation [7] and was also shown for the methane-suppressing effect of polyunsaturated fatty acids [43].

Concerning the methane-producing microbes, the present data revealed an unexpected interaction. Although the overall total tract methane release of the animals was reduced with coconut oil independent of the defaunation treatment, the animals had the highest densities of methanogen cells in the rumen fluid when fed coconut oil and subjected to the defaunation treatment. With this treatment, the methanogen concentration was four times the average value of the other treatment groups. An analytical error concerning the methanogens symbiotically associated with ciliate protozoa could be ruled out since it was verified that the enumeration technique by FISH destroyed all ciliate protozoa. As stated earlier, in vivo, it is of limited accuracy to conclude from rumen microbial concentration on total rumen microbial population since the rumen fluid volume could vary. Depending on diet type, the postprandial evolution of the rumen methanogen concentration could vary widely [41] thus reflecting the different concentrations of dissolved hydrogen in the

rumen fluid at various stages of feed degradation. However, in the present study, data on water intake of the sheep suggest that the total rumen methanogen population was even more increased by the defaunation treatment as already obvious from counts per mL rumen fluid. For sheep, Broudiscou et al. [44] found that defaunation could significantly increase the rumen fluid volume by 26%.

For the present experiment and different from feeding coconut oil to the faunated and refaunated animals, it is hypothesised that coconut oil in combination with the defaunation treatment significantly affected the rate of methane production per methanogenic cell. This shift could either have been caused by a depressed metabolism of every methanogenic microbe, independent of species, or by an altered composition of the methanogenic community, or both. The data of Dohme et al. [10] and Ushida et al. [34] suggest that the methane production per methanogenic cell could be affected by coconut oil treatment and defaunation. However, in these in vitro experiments the metabolism of the single methanogenic cells seemed to have been enhanced with both treatments whereas in the present in vivo study the opposite took place.

Evidence for a depressed rate of methane production per methanogenic cell with coconut oil is given by the results on the rRNA copies detected when using the order-specific oligonucleotide probes. Although whole cell counting was not possible, the number of rRNA copies gave insight into the metabolic activity of the different groups of methanogens as discussed by Binelli et al. [45]. In contrast to the enumeration of total methanogenic cells showing a significant increase with coconut oil in combination with the defaunation treatment, the coconut oil treatment seems to have significantly inhibited the metabolic activity of Methanobacteriales and Methanococcales at the same time. Therefore, the reduced metabolic activity of these two methanogenic orders might explain the methane-suppressing effect of coconut oil in the defaunated animals.

The occurrence of an alteration of the composition of the rumen methanogenic community due to coconut oil and defaunation treatment is supported by several observations described in the literature. The species of the four methanogenic orders in the rumen vary widely in their cell wall structure and chemistry [46]. The cell wall determines the sensitivity of microbes to certain agents, including the fatty acids. Medium-chain fatty acids have been shown to inhibit the growth of *Methanobrevibacter ruminantium*, a species belonging to the Methanobacteriales [47], but up to now there are no bibliographic references regarding its effects on other methanogenic species.

There is evidence that the different phylogenetically defined groups of rumen methanogens are not equivalently associated with ciliate protozoa [8]. Sharp et al. [8] showed that, in the bovine rumen, representatives of the Methanobacteriaceae family are the most abundant methanogens associated with ciliate protozoa. In the absence of protozoa, their proportion of the total rumen methanogenic population will decrease for the benefit of Methanomicrobiales which seems to be an essentially free-living methanogenic group. From this increase in the proportion of Methanomicrobiales it can be supposed that perhaps especially methanogens of this order are among the microbes engulfed by ciliate protozoa since the ingestion of microbes by ciliate protozoa is non-random [48]. Tokura et al. [49] presumed that the free-living and ciliate-associated methanogens are different in their physiological characteristics and that, among the ciliate-associated methanogens, the endosymbiotic strains may also have different characteristics than those that adhere externally. A quick reaction in attaching and detaching to the cell surface of

ciliate protozoa, which was shown by Tokura et al. [49], probably requires a certain mobility of the methanogens involved and it is known that the mobility differs among the methanogenic species [50]. Since in the present study the coconut oil treatment appears to have inhibited the metabolic activity of Methanobacteriales and Methanococcales only in combination with the defaunation treatment it is assumed that in the ovine rumen, representatives of these orders are perhaps those preferentially endosymbiotically associated with ciliate protozoa.

5. CONCLUSIONS AND IMPLICATIONS

The present study once more confirmed the methane-suppressing effect of coconut oil, independent of the protozoal status. As a general conclusion, the present data illustrate that a depression of the concentration of ciliate protozoa or methanogens in rumen fluid cannot be used as a reliable indicator for the success of a methane-suppressing method in vivo. It seems that the methane-suppressing effect of coconut oil will change the metabolic activity and/or the composition of the rumen methanogenic population. Further research on the metabolism, growth rate and efficiency of methane production of the rumen methanogens under different treatments and with different ruminant species is required.

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