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The Trade-Off between Enteric and Manure Methane Emissions and Their Bacterial Ecology in Lactating Cows Fed Diets Varying in Forage-to-Concentrate Ratio and Rapeseed Oil

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Abstract: An experiment was conducted to examine how dietary interventions reducing enteric methane (CH_4) emissions influence manure CH_4 emissions in biogas production (as biochemical methane potential (BMP)) or under static conditions mimicking natural manure storage conditions. Experimental treatments consisted of a factorial arrangement of high (HF: 0.65) or low (LF: 0.35) levels of forage and 0 or 50 g of rapeseed oil per kg of diet dry matter. Oil supplementation reduced daily enteric CH_4 emissions, especially in the HF diet, by 20%. Greater dietary concentrate proportion reduced CH_4 yield and intensity (6 and 12%, respectively) and decreased pH, increased total volatile fatty acids, and molar proportions of butyrate and valerate in feces incubated under static conditions. Oil supplementation increased daily BMP and BMP calculated per unit of organic matter (OM) (17 and 15%, respectively). Increased dietary concentrate had no impact on daily BMP and BMP per unit of OM, whereas it reduced daily CH_4 production by 89% and CH_4 per unit of OM by 91% under static conditions. Dietary oil supplementation tended to decrease fecal CH_4 production per unit of digestible OM (23%) under static conditions. Diets had no impact on the alpha diversity of ruminal prokaryotes. After incubation, the fecal prokaryote community was significantly less diverse. Diets had no effect on alpha diversity in the BMP experiment, but static trial fecal samples originating from the HF diet showed significantly lower diversity compared with the LF diet. Overall, the tested dietary interventions reduced enteric CH_4 emissions and reduced or tended to reduce manure CH_4 emissions under static conditions, indicating a lack of trade-off between enteric and manure CH_4 emissions. The potential for increasing CH_4 yields in biogas industries due to dietary interventions could lead to a sustainable synergy between farms and industry.

Keywords: enteric methane; manure methane; trade-off; microbial community; dietary intervention

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1. Introduction

Ruminants contribute to anthropogenic greenhouse gas emissions, including methane (CH_4), which originates from enteric or, to a lesser extent, manure fermentation, with concerns over CH_4 emissions from the latter source growing over the past few years [1,2]. Alterations in ruminant diets aimed at mitigating enteric CH_4 emissions prompt shifts in nutrient digestibility, ultimately resulting in the modification of manure chemical composition. This, in turn, precipitates a change in manure CH_4 emissions [3,4].

Of the nutritional strategies used to reduce CH_4 emissions, lipid supplementation is a well-known method examined in many studies. Beauchemin et al. [5] indicated that

lipid supplementation in ruminant diets could potentially result in a reduction in daily CH_4 emissions by 1 to 5% for each 1% increase in dietary lipid supplementation. Such a change in CH_4 emission not only depends on the source of lipids or the profile of fatty acids included in the diet [6] but also may be influenced by the feed composition or even by the forage-to-concentrate (FC) ratio.

In Finland, cattle produce 9.75 million tons of manure annually, which is equal to approximately 75% of all manure produced in the country's livestock sector [7]. Manure management creates emissions of 0.7 million tons of CO_2 equivalent in 2021, equal to 12% of agricultural greenhouse gas emissions [8]. A cattle slurry-based system is the dominant method of manure management (40%), followed by solid storage (11%), and pasture (7%) [8]. Manure emissions have increased by 19% in 2021 compared with 1990 due to an increase in the number of animals kept in slurry-based systems.

Cattle manure is widely employed for the start-up of agricultural biogas plants or as a co-substrate in the anaerobic digestion of lignocellulosic feedstock [9], where gas production is assessed through biochemical methane potential (BMP) assays. The theoretical biogas yields from carbohydrates, proteins, and fats are 790–800, 700, and 1200–1250 m^3/t total solids, with theoretical CH_4 amounts of 50%, 70–71%, and 67–68%, respectively [10]. Although ruminal microbiota are efficient plant fiber digesters, the prokaryotes found in manure may function differently. Understanding the complex interactions between diet, manure prokaryotes, and CH_4 production in systems fed with manure holds enormous promise for the biogas industry. The composition of the microbial community in an anaerobic digester is dependent on the inoculum, operational conditions, and feedstock. Although inoculum plays an important role, it has been suggested that the inoculum's effect could fade in continuous long-term digestion as the operational conditions and feedstocks would modulate the microbial consortium; in cases where the organic loading rate was low, there was a small effect of the feedstock on the reactor populations [11]. Genera such as *Clostridium sensu stricto*, *Romboutsia*, and *Turicibacter* play crucial roles, as they can degrade a wide range of lignocellulosic biomasses; they are commonly found in animal manure [12] and have been reported to be producers of volatile fatty acids (VFA) and H_2 [13]. Methanogenic Archaea convert all the carbon present in the biomass into CH_4 and CO_2, and in cattle manure, *Methanosarcina* stands out [14]. The environmental conditions of feces, manure storage, and anaerobic digesters naturally differ, which will dictate which microorganisms are more abundant in each situation.

Studies on the simultaneous evaluation of enteric and manure CH_4 emissions, especially under different dietary conditions, are limited. Some experiments on dairy cows have shown that adding lauric acid [15] or rapeseed [3] to grass silage and corn silage-based diets reduces enteric CH_4 emissions with compensatory increases in manure CH_4 emissions. Hindrichsen et al. [16] demonstrated a 5 to 22% variation in manure CH_4 as a proportion of total CH_4 emissions in cows fed diets with contrasting compositions (lignified fiber, sugar, or starch). In addition, in a meta-analysis by Huhtanen et al. [4], mitigation of enteric CH_4 was associated with increased manure emissions, measured through BMP assays. It should be noted that these manure emissions were measured using BMP assays; however, those conditions may differ from practical farming scenarios, where manure storage conditions are often subject to fluctuations and may not align with the ideal conditions established in BMP assays (pH, temperature, and inoculum). Hence, while a single nutritional strategy or the synergy of two strategies could effectively reduce enteric CH_4 emissions, additional investigation is warranted to unveil their influence on manure CH_4 emissions and their possible trade-offs under diverse storage conditions—underscoring the novel dimension of this study.

We hypothesized that mitigation of CH_4 emissions by dietary interventions known to reduce enteric CH_4 emissions would not cause an increase in manure CH_4 emissions when measured under conditions similar to manure storage conditions, whereas it might increase CH_4 production in anaerobic digesters. Therefore, the present study aimed to examine the effects of FC ratio with or without rapeseed oil supplementation on enteric and

manure CH_4 emissions when measured under normal manure storage conditions (hereafter called static conditions) or used as substrate in an anaerobic digester. By elucidating these processes, we aim to shed light on diverse strategies to reduce the environmental impact of the dairy sector by capitalizing on the potential benefits that arise from harnessing the synergy between oil-rich substrates and anaerobic digestion in the context of cow manure.

2. Materials and Methods

Animal experimentation was conducted with regional State Administrative Agency approval (ESAVI/24435/2018, Hämeenlinna, Finland) in accordance with the guidelines established by the European Community Council Directive 2010/63/EU (EU 2010) and complied with the ARRIVE guidelines [17].

2.1. Animals, Experimental Design, and Treatments

A 4 × 4 Latin square design with a 2 × 2 factorial arrangement of treatments was applied to 4 multiparous Nordic Red dairy cows in mid-lactation (mean ± standard deviation, 101 ± 16 days in milk), producing 38.8 ± 1.9 kg milk/d with 21 d experimental periods. Sample size analysis was performed based on the assumption of achieving a 20% reduction in daily CH_4 production as grams per day when the power $(1 - \beta)$ of the study is 0.80 and α is 0.05 in a one-sided test, which resulted in 4 replicates. The cows were selected based on their similarities in parity, dry matter (DM) intake, milk yield, and body weight. The diets were randomly allocated to the cows in the first period, and thereafter, every diet was provided to the next cow in the next period. Each period consisted of dietary adaptation for 14 days and 7 days of sampling. Treatments comprised total mixed rations (TMR) based on grass silage containing either a high (65:35) or low (35:65) forage-to-concentrate (FC) ratio supplemented with 0 (HF and LF, respectively) or 5% rapeseed oil (RO) in diet DM (HFO and LFO, respectively; for details, refer to Razzaghi et al. [18]). In addition, the cows received 2 × 300 g of concentrate daily from the milking parlor. To ensure ad libitum feed intake, at least 5% of refusals were targeted daily based on the previous day's feed intake for every cow, and the diets were fed in 4 equal amounts at 0600, 0900, 1600, and 1900 h. Rapeseed oil (Avena Kantvik Ltd., Kirkkonummi, Finland) was stored at 4 °C until incorporated into the low or high FC ratio TMR, and the RO replaced concentrate pellets. The forage was restrictively fermented grass silage prepared from the primary growth of mixed timothy (*Phleum pratense*) and meadow fescue (*Festuca pratensis*) swards grown at Jokioinen (60°49′ N, 23°28′ E) treated with a formic-acid-based ensiling additive (5 L/tone, AIV 2 Plus, Valio Ltd., Helsinki, Finland). The cows were kept as a group in free stalls during the adaptation period and in respiratory chambers during the sampling period, with free access to water and salt blocks. The cows were milked in a 2 × 6 auto tandem milking parlor during the adaptation period and in situ in the chambers during the sampling period at 0700 and 1645 h.

2.2. Measurements and Chemical Analysis

To maintain the predetermined FC ratio and to formulate the experimental diets accurately, the DM content of grass silage was analyzed twice a week during the experiment at 105 °C for 20 h in a forced-air oven. Daily feed intake was measured by subtracting the refusals (measured daily at 1200 h before offering fresh feed) from the offered feed throughout the study, but intakes during d 17 to 21 of each experimental period were used for statistical analysis. Representative samples of silage and supplemental concentrates collected during the sample collection period were used for chemical analysis. The samples were pooled within each period before chemical analysis. Fresh silage samples were prepared for measurement of pH and analysis of VFA, lactic acid, formic acid, ethanol, water-soluble carbohydrate, soluble N, and ammonia N concentrations as described by Ahvenjärvi et al. [19]. In addition, the method proposed by Huida et al. [20] was used to correct silage DM content for the loss of volatiles. Concentrate pellets and leftovers were dried in a forced-air oven at 55 °C for 48 h, ground through a 1 mm screen (Sakomylly KT-

120, Koneteollisuus Oy, Klaukkala, Finland), and analyzed for DM, neutral detergent fiber (NDF), ash, ether extract (EE), and crude protein (CP) as described in detail by Ahvenjärvi et al. [19]. Indigestible neutral detergent fiber (iNDF) of silage, concentrates, and feces was determined by 12 d of ruminal incubation using nylon bags (60 × 120 mm, pore size 0.017 mm) followed by NDF analysis. Chemical analysis of silage, concentrates, and oils, plus their proportion in each diet, was used to calculate the chemical composition of each experimental TMR. Bomb calorimetry (1108 Oxygen bomb, Parr Instrument Co., Moline, IL, USA) was used to determine the gross energy (GE) of silage, concentrates, oil supplements, and excreta with benzoic acid (CAS 65-85-0, cat. no. 3415, Parr Instrument Co.) as the standard.

Milk yield was recorded throughout the experiment, but only measurements made between d 17 and 21 were used for statistical analysis. Milk samples were taken for 3 consecutive days (d 17, 18, and 19) in each experimental period during morning and evening milking, preserved with bronopol tablets (Valio Ltd., Finland), and stored at 4 °C until infrared analysis for fat, CP, lactose, urea, and somatic cells (MilkoScan FT+, Foss Electric, Hillerød, Denmark). Milk composition was calculated as the geometric average of morning and evening milk yields.

Total fecal and urine samples were collected from the cows for 3 consecutive days, starting on d 18 at 1000 h. The quantity of feces was weighed and sampled for in vitro tests (about 1 L), and a subsample (5% wt/wt) was taken for chemical and microbial composition analysis. The samples were stored at −20 °C, and at the end of the experiment, they were thawed (1–2 d at room temperature) and mixed thoroughly, and a subsample was obtained for every animal per period for chemical analysis. Feces were dried at 60 °C, and the sample was ground through a 1 mm sieve, and DM, ash, N, NDF, EE (after hydrolysis with 3 M HCl), and GE were determined as described earlier for feed samples. The urine was separated from the feces using a lightweight harness and flexible tubing attached to the vulva.

Rumen liquid (500 mL) samples for rumen fermentation and microbial community analysis were collected on d 21 of each experimental period, at 1000 h after respiratory chamber measurements, by stomach tubing using a Ruminator device (Profs Products, Wittibreut, Germany). Rumen pH was measured immediately after collection using a portable pH meter (pH110, VWR International, Radnor, PA, USA). Rumen sample processing for VFA and ammonia analyses was performed as described by Bayat et al. [21]. For bacterial community analysis, rumen liquid samples were immediately aliquoted into 2 mL tubes, snap-frozen in dry ice, and stored at −80 °C until DNA extraction.

Four open-circuit respiratory chambers (21.5 m^3) were used to measure the gas exchanges (oxygen, carbon dioxide, methane, and hydrogen) of the cows individually over 4 d (d 17 to 21), with the first day serving as acclimatization. The measuring system is described in detail elsewhere [21], but briefly, concentrations of the gases in the inlet and exhaust airflow were measured using a computer-controlled system using dedicated analyzers (Oxymax, Columbus Instruments, Columbus, OH, USA). Air outflow for each chamber was measured with an HFM-200 mass flow meter with a laminar flow element capable of measuring up to 3000 L/min (Teledyne Hastings Instruments, Hampton, VA, USA). Absolute gas exchanges were calculated by multiplying air flow and gas concentration differences.

2.3. Anaerobic Incubation of Manure

Biochemical CH_4 potential (BMP) was measured under mesophilic (37 °C) conditions using automated testing equipment (Bioprocess Control Ltd., Lund, Sweden) over 30 days. The bottles were mechanically mixed (84 rpm) for 1 min/h. Tests were conducted in 500 mL bottles with a 400 mL liquid volume in duplicate test bottles. Deionized water was added to achieve uniform gas space in every bottle. A sample-to-inoculum-organic-matter (OM) ratio of 1:1 was used. The inoculum was obtained from a farm-scale biogas plant treating cattle slurry (Luke Maaninka, Kuopio, Finland). All bottles were buffered with $NaHCO_3$ (3 g/L) and flushed with N_2 to obtain anaerobic conditions. The volume

of the produced gas was determined via water displacement. The gas was collected in gas bags and analyzed for CH_4 and CO_2 from five to seven times during the experiment using a gas chromatograph (Perkin Elmer Arnel Clarus 500) [22]. The daily CH_4 content between analyses was estimated by dividing the change in CH_4 content between the measurement days.

For static in vitro CH_4 measurements (75 days), the same equipment was used as in the BMP assay under a temperature of 25 °C without using inoculum, mixing, or $NaHCO_3$. Deionized water was added to each fecal sample with the ratio of excreted urine and feces from each cow. From the biogas, CO_2 was fixed with a 3 M sodium hydroxide solution, and the volume of CH_4 was determined via water displacement. Fecal samples for microbial community analysis were collected at the end of in vitro BMP and static CH_4 measurement trials. Samples were placed into 5 mL tubes, snap-frozen in dry ice, and kept at −80 °C until RNA extraction.

2.4. Microbial Analysis

Total DNA was extracted from 0.5 mL of rumen liquid, as described by Rius et al. [23]. RNA was extracted from ca. 65 mg of frozen feces using the NucleoSpin RNA Stool kit (Macherey-Nagel, Düren, Germany) and following the manufacturer's recommendations. The RNA was reverse-transcribed into cDNA using random primers and by following the protocol provided with the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Universal primers 515F and 806R [24] targeting the 16S ribosomal RNA gene V4 region were used for bacterial amplicon sequencing. Libraries were prepared as described by Huuki et al. [25] and sequenced in the Finnish Functional Genomics Centre (Turku, Finland) on the Illumina MiSeq platform using 2 × 300 bp chemistry. Demultiplexing of sequences, adapter removal, and sorting sequences with barcode were performed by the sequencing center. Sequencing data were processed using Qiime 2 [26]. Briefly, quality control, filtering of chimeric reads, and clustering of bacterial sequences into ASV were performed using DADA2 [27]. Bacterial ASV taxonomy was assigned using the Silva 138 database [28].

2.5. Calculations

Total-tract apparent digestibility coefficients were calculated based on the difference between the intake of a nutrient and its fecal output divided by the corresponding intake. Potentially digestible NDF was calculated as the difference between NDF and iNDF. Energy losses as CH_4 were calculated using the factor 55.24 kJ/g [29]. Energy-corrected milk (ECM) was calculated using the equation suggested by Sjaunja et al. [30] based on milk fat, protein, and lactose yields, and energy secretion (MJ/d) in milk was calculated as 3.14 × ECM yield (kg/d).

2.6. Statistical Analysis

Before statistical analysis, all data were tested for normality of distribution using Proc Mixed (version 9.4, SAS Institute Inc., Cary, NC, USA) using box plots and scatter plots of residuals and the generated fitted values. Experimental data were analyzed using ANOVA for a 4 × 4 Latin square with a 2 × 2 factorial arrangement of treatments through the mixed procedure with a model that included fixed effects of period, FC ratio, oil level, FC ratio via oil interaction, and random effects of cow. The data averages for cows within the period were calculated before statistical analysis. For the microbial data, only taxa observed at above 0.01% abundance in at least 50% of samples were included in the analysis. Before the test, the number of reads was log-base transformed [log2(x + 1)] and standardized by data centering. The values reported are least-squares means ± standard error of the mean (SEM). The significance level $p \leq 0.05$ was used to determine the significant effects of FC ratio, oil, and their interaction. In addition, probabilities at $0.05 < p < 0.10$ were considered as a trend.

The alpha diversity was calculated using observed ASV, Shannon, and Simpson diversity indexes, and the beta diversity was calculated as Bray–Curtis dissimilarities as described by Rinne et al. [31]. To identify if dietary treatment, concentrate proportion, or oil supplementation can explain rumen or fecal microbial community composition, a distance-based permutational multivariate analysis of variance (adonis) was performed using a *vegan* R package [32], and significance was declared at the $p < 0.05$ level after 999 permutations. To determine which fecal bacterial taxa were significantly different between BMP and static manure incubation trials, a linear discriminant analysis was performed as implemented in the *MicrobiotaProcess* R package [33]. To explore associations between CH_4 production and fecal microbial taxa in both manure incubation experiments separately, Spearman correlations were applied, and comparisons were declared significant at $p < 0.05$.

3. Results

3.1. Apparent Total-Tract Digestibility and Rumen Fermentation

No differences ($p \geq 0.13$) were observed in the digestibility of DM, OM, potentially digestible neutral detergent fiber (pdNDF), and starch with changing forage-to-concentrate ratios in dairy cow diets, while the LF diet had greater ($p \leq 0.04$) CP and GE digestibility and lower ($p = 0.04$) neutral detergent fiber (NDF) digestibility compared with the HF treatment (Table 1). Oil supplementation in the experimental diets had no impact ($p \geq 0.14$) on the digestibility of DM, OM, CP, NDF, starch, and GE, while the digestibility of ether extract (EE) and pdNDF tended ($p \leq 0.08$) to decrease.

Table 1. Effects of dietary forage-to-concentrate ratio and rapeseed oil supplement on apparent total tract nutrient digestibility in lactating dairy cows.

	Treatment [2]					*p*-Value [3]		
Item [1]	**HF**	**HFO**	**LF**	**LFO**	**SEM**	**FC**	**RO**	**FC × RO**
				Digestibility (%)				
DM	66.3	64.6	66.8	66.4	0.80	0.19	0.22	0.48
OM	67.8	65.9	68.6	68.0	0.80	0.13	0.17	0.48
CP	63.6	64.2	65.5	65.8	0.70	0.040	0.52	0.92
EE	58.8	53.5	61.4	58.5	2.00	0.073	0.059	0.52
NDF	60.3	57.1	55.6	54.5	1.44	0.040	0.18	0.51
pdNDF	70.5	66.3	67.2	65.4	1.75	0.20	0.082	0.43
Starch	97.8	98.0	98.3	97.8	0.28	0.62	0.57	0.26
GE	64.6	62.6	66.5	65.6	0.85	0.026	0.14	0.55

[1] DM, dry matter; OM, organic matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; pdNDF, potentially digestible NDF; and GE, gross energy. [2] Refers to diets based on high (0.65) or low (0.35) forage ratio supplemented with 0 (HF and LF, respectively) or 5.0% (HFO and LFO, respectively) of rapeseed oil on DM basis. Values are LS means and pooled SEM for $n = 4$. [3] FC, effect of forage-to-concentrate ratio in the diet; RO, effect of rapeseed oil supplement; and FC × RO, interaction of FC and RO.

The experimental diets had no impact ($p \geq 0.16$) on rumen pH, total VFA concentration, or ruminal ammonia-N (Table 2). Compared with the cows receiving an HF diet, cows fed LF diets had lower ($p \leq 0.04$) molar proportions of acetate and isobutyrate and greater ($p \leq 0.04$) molar proportions of propionate, butyrate, and valerate. Rapeseed oil supplementation in the HF and LF diets increased ($p = 0.04$) the molar proportion of isobutyrate while tending to decrease ($p = 0.09$) and increase ($p = 0.07$) the molar proportions of acetate and propionate, respectively. Acetate-to-propionate and acetate + butyrate to propionate ratios were lower ($p < 0.01$) for LF compared with HF diets, and dietary rapeseed oil (RO) inclusion decreased acetate-to-propionate and acetate + butyrate ratios ($p \leq 0.04$).

Table 2. Effects of dietary forage-to-concentrate ratio and rapeseed oil supplement on rumen fermentation characteristics in lactating dairy cows.

	Treatment [1]					*p*-Value [2]		
Item	**HF**	**HFO**	**LF**	**LFO**	**SEM**	**FC**	**RO**	**FC × RO**
pH	6.67	6.66	6.5	6.53	0.096	0.16	0.90	0.84
Ammonia-N (mmol/L)	2.22	2.66	3.12	2.63	0.461	0.18	0.94	0.16
Total VFA (mmol/L)	104	95	110	104	4.9	0.18	0.18	0.74
Molar proportion (mol/100 mol)								
Acetate	69.0	67.0	63.9	63.6	0.59	<0.01	0.093	0.20
Propionate	15.6	17.4	19.5	20.0	0.63	<0.01	0.068	0.26
Butyrate	12.0	11.5	13.0	12.7	0.62	0.043	0.43	0.90
Isobutyrate	0.64	0.72	0.50	0.63	0.044	0.035	0.044	0.55
Valerate	1.28	1.37	1.58	1.47	0.063	0.015	0.96	0.14
Isovalerate	0.68	1.19	0.72	0.94	0.097	0.28	<0.01	0.15
Caproate	0.80	0.74	0.77	0.65	0.072	0.30	0.13	0.66
Molar ratio								
Acetate/Propionate	4.43	3.86	3.29	3.20	0.139	<0.01	0.031	0.086
Acetate + Butyrate: Propionate	5.20	4.53	3.96	3.84	0.183	<0.01	0.036	0.11

[1] Refers to diets based on high (0.65) or low (0.35) forage ratio supplemented with 0 (HF and LF, respectively) or 5.0% (HFO and LFO, respectively) of rapeseed oil on DM basis. Values are LS means and pooled SEM for $n = 4$. [2] FC, effect of forage-to-concentrate ratio in the diet; RO, effect of rapeseed oil supplement; and FC × RO, interaction of FC and RO.

3.2. Enteric CH_4 and CO_2 Emissions

Oil supplementation reduced ($p < 0.01$) daily CH_4 emissions by an average of 14.6%; however, oil was more effective in reducing CH_4 emissions when added to the HF diet ($p = 0.015$ for FC ratio × RO interaction; Table 3). Methane yield (g/kg OMI or digested OM (DOM)) and intensity (g/kg milk or ECM) in the cows receiving oil supplements were lower ($p < 0.01$) than their control counterparts. Methane intensity and yield decreased ($p < 0.01$) with increasing concentrate levels. Daily CO_2 emissions and CO_2 per unit of milk produced decreased ($p < 0.01$) as a result of increased concentrate content in diet and oil supplementation.

Table 3. Effects of dietary forage-to-concentrate ratio and rapeseed oil supplement on enteric methane and carbon dioxide emissions in lactating cows.

	Treatment [2]					*p*-Value [3]		
Item [1]	**HF**	**HFO**	**LF**	**LFO**	**SEM**	**FC**	**RO**	**FC × RO**
Enteric CH_4								
g/d	535	428	516	470	27.7	0.25	<0.01	0.015
g/kg OMI	23.1	20.5	21.4	19.5	0.87	<0.01	<0.01	0.32
g/kg DOM	34.2	31.1	31.2	28.7	1.23	<0.01	<0.01	0.68
g/kg milk	17.2	14.0	14.3	11.6	0.64	<0.01	<0.01	0.56
g/kg ECM	16.0	13.0	13.2	12.0	0.64	<0.01	<0.01	0.087
% of GEI	6.66	5.64	6.05	5.26	0.244	<0.01	<0.01	0.26
Total CO_2								
g/d	14,374	13,133	15,628	15,203	710.5	<0.01	<0.01	0.11
g/kg OMI	622	631	648	631	19.2	0.22	0.70	0.23
g/kg milk	461	429	432	376	13.3	<0.01	<0.01	0.27
g/kg ECM	430	399	399	388	15.6	0.15	0.15	0.45

[1] OMI, organic matter intake; DOM, digested organic matter; ECM, energy-corrected milk; and GEI, gross energy intake. [2] Refers to diets based on high (0.65) or low (0.35) forage ratio supplemented with 0 (HF and LF, respectively) or 5.0% (HFO and LFO, respectively) of rapeseed oil on DM basis. Values are LS means and pooled SEM for $n = 4$. [3] FC, effect of forage-to-concentrate ratio in the diet; RO, effect of rapeseed oil supplement; and FC × RO, interaction of FC and RO.

3.3. Composition of Feces

The increased dietary concentrate proportion reduced ($p \leq 0.014$) OM, GE, NDF, and pdNDF concentrations and increased ($p < 0.01$) CP and starch concentrations in feces. Oil supplementation increased ($p < 0.01$) OM, EE, and GE concentrations and decreased ($p < 0.01$) CP and iNDF concentrations (Table 4).

Table 4. Effects of dietary forage-to-concentrate ratio and rapeseed oil supplement on fecal composition.

	Treatment [2]					*p*-Value [3]		
Item [1] (% DM Unless Stated)	**HF**	**HFO**	**LF**	**LFO**	**SEM**	**FC**	**RO**	**FC × RO**
DM (% fresh)	14.2	15.1	14.4	16.0	0.30	0.086	<0.01	0.23
OM	89.0	90.0	88.5	89.4	0.19	0.014	<0.01	0.62
CP	16.4	14.5	17.9	16.5	0.15	<0.01	<0.01	0.096
EE	4.17	9.19	4.16	8.99	0.247	0.64	<0.01	0.66
NDF	53.5	53.3	50.4	49.3	0.59	<0.01	0.20	0.38
iNDF	21.8	19.9	21.7	20.4	0.61	0.69	0.011	0.48
pdNDF	31.7	33.5	28.7	28.9	1.00	<0.01	0.24	0.32
Starch	0.541	0.428	0.774	0.922	0.0982	<0.01	0.86	0.22
GE (Mcal/kg DM)	4.54	4.80	4.45	4.73	0.014	<0.01	<0.01	0.40

[1] DM, dry matter; OM, organic matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; iNDF, indigestible NDF; pdNDF, potentially digestible NDF; and GE, gross energy. [2] Refers to diets based on high (0.65) or low (0.35) forage ratio supplemented with 0 (HF and LF, respectively) or 5.0% (HFO and LFO, respectively) of rapeseed oil on DM basis. Values are LS means and pooled SEM for $n = 4$. [3] FC, effect of forage-to-concentrate ratio in the diet; RO, effect of rapeseed oil supplement; and FC × RO, interaction of FC and RO.

3.4. Fermentation Characteristics in Incubated Feces

The increased dietary concentrate proportion decreased ($p = 0.048$) pH and increased ($p < 0.01$) total VFA and molar proportions of butyrate and valerate in feces under static conditions (Table 5). Also, feeding more dietary concentrate tended ($p \leq 0.067$) to increase the molar proportions of isobutyrate and caproate, whereas it tended ($p = 0.07$) to decrease the molar proportions of propionate. Dietary oil supplementation did not change ($p > 0.05$) total VFA and molar proportions of individual VFA in the incubated feces; however, it tended ($p \leq 0.09$) to decrease pH and increase the acetate-to-propionate ratio.

Table 5. Effects of dietary forage-to-concentrate ratio and rapeseed oil supplement on fermentation in incubated feces under static condition [1].

	Treatment [2]					*p*-Value [3]		
	HF	**HFO**	**LF**	**LFO**	**SEM**	**FC**	**RO**	**FC × RO**
pH	6.65	5.85	5.73	5.60	0.225	0.048	0.090	0.20
Total VFA (mmol/L)	62.4	90.4	152	152	15.0	<0.01	0.35	0.33
Molar proportion (mol/100 mol)								
Acetate	50.0	65.3	66.0	69.3	7.15	0.21	0.24	0.43
Propionate	37.6	19.3	7.4	6.0	9.89	0.070	0.36	0.43
Butyrate	5.26	7.14	15.9	14.5	2.01	0.004	0.89	0.45
Isobutyrate	1.05	1.54	2.53	2.25	0.490	0.067	0.83	0.47
Valerate	0.91	1.13	1.94	1.92	0.351	0.041	0.78	0.74
Isovalerate	4.35	3.65	3.56	3.44	0.997	0.63	0.70	0.78
Caproate	0.80	1.92	2.66	2.59	0.554	0.063	0.38	0.32
Acetate: propionate	4.95	14.0	9.46	12.0	2.68	0.64	0.06	0.24

[1] Static condition refers to in vitro incubation for 75 days without using inoculum, mixing (1 min every hour) $NaHCO_3$ buffer under temperature of 25 °C. [2] Refers to diets based on high (0.65) or low (0.35) forage ratio supplemented with 0 (HF and LF, respectively) or 5.0% (HFO and LFO, respectively) of rapeseed oil on DM basis. Values are LS means and pooled SEM for $n = 8$. [3] FC, effect of forage-to-concentrate ratio in the diet; RO, effect of rapeseed oil supplement; and FC × RO, interaction of FC and RO.

3.5. Methane Emissions from Feces and Biochemical Methane Potential from Manure

Oil supplementation in the HF and LF diets increased ($p \leq 0.029$) daily BMP and BMP calculated per unit of OM (Table 6). Increased dietary concentrate proportion had no impact ($p \geq 0.12$) on daily BMP and BMP per unit of OM. Daily fecal CH_4 production and CH_4 production per unit of OM, NDF, and DOM under static conditions, mimicking the manure storage conditions, decreased ($p \leq 0.032$) as a result of increasing dietary concentrate proportion. Dietary oil supplementation showed no significant effect ($p \geq 0.16$) on fecal CH_4 production per unit of OM and NDF, while it tended ($p = 0.089$) to decrease daily fecal CH_4 production per unit of DOM under static conditions.

Table 6. Effects of forage-to-concentrate ratio and dietary rapeseed oil supplement on methane emissions from feces.

	Treatment [2]					*p*-Value [3]		
Item	**HF**	**HFO**	**LF**	**LFO**	**SEM**	**FC**	**RO**	**FC × RO**
Biochemical CH_4 potential								
mL/g OM	219	267	238	266	9.2	0.36	<0.01	0.30
g/d	1164	1359	1293	1467	75.3	0.12	0.029	0.87
Fecal static CH_4 production [1]								
mL/g OM	26.4	10.8	3.2	0.3	5.83	0.028	0.16	0.32
g/d	143	48	19	1.6	28.4	0.022	0.089	0.21
mL/g DOM	99.4	40.1	16.3	0.8	19.2	0.016	0.089	0.28
mL/g NDF	145	66.0	16.0	1.5	34.71	0.032	0.23	0.39

[1] Static condition refers to in vitro incubation for 75 days without using inoculum, mixing $NaHCO_3$ buffer under temperature of 25 °C. [2] Refers to diets based on high (0.65) or low (0.35) forage ratio supplemented with 0 (HF and LF, respectively) or 5.0% (HFO and LFO, respectively) of rapeseed oil on DM basis. Values are LS means and pooled SEM for $n = 8$. [3] FC, effect of forage-to-concentrate ratio in the diet; RO, effect of rapeseed oil supplement; and FC × RO, interaction of FC and RO.

There was a major difference between CH_4 production from fecal samples in static and BMP experiments, as expected due to the inoculum added in the BMP experiment. In addition, there were two main discrete phases of gas production in the static assay affected by both the dietary concentrate ratio and the oil supplement (Figures 1 and 2). The first phase of gas production occurred during d 1 to 11, when high-concentrate diets had a faster CH_4 production rate. The second phase of gas production occurred between d 30 and 75 as well as between 56 and 75 for low- and high-concentrate diets, respectively. Diets supplemented with oil had a 10-day delay in starting the second phase of CH_4 production and had a lower rate of CH_4 production compared with un-supplemented diets. In the BMP experiment, all samples started to produce biogas on the first day and continued the production steadily, with total CH_4 production being higher with diets supplemented with oil (Figures 3 and 4).

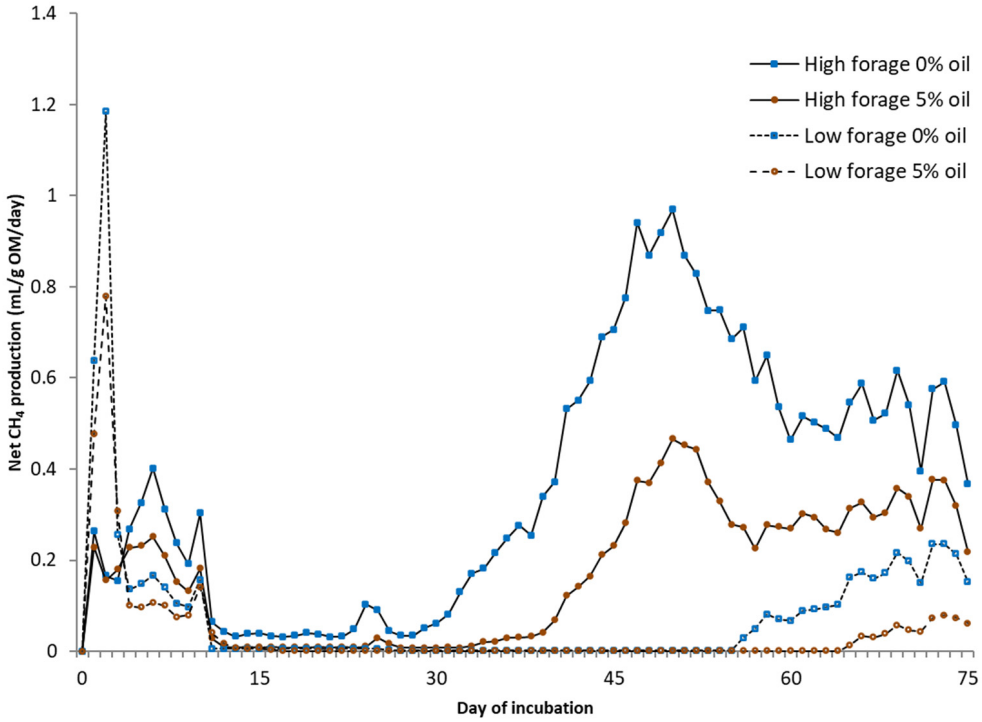

Figure 1. Methane production of incubated fecal samples of dairy cows fed different diets under static conditions.

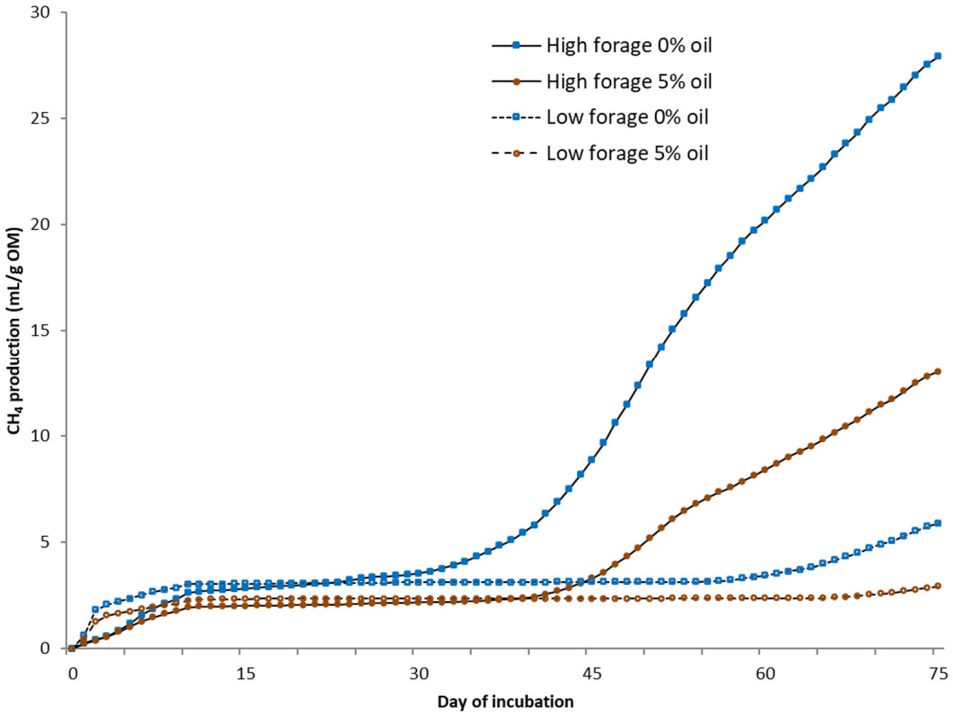

Figure 2. Cumulative methane production of incubated fecal samples of dairy cows fed different diets under static conditions.

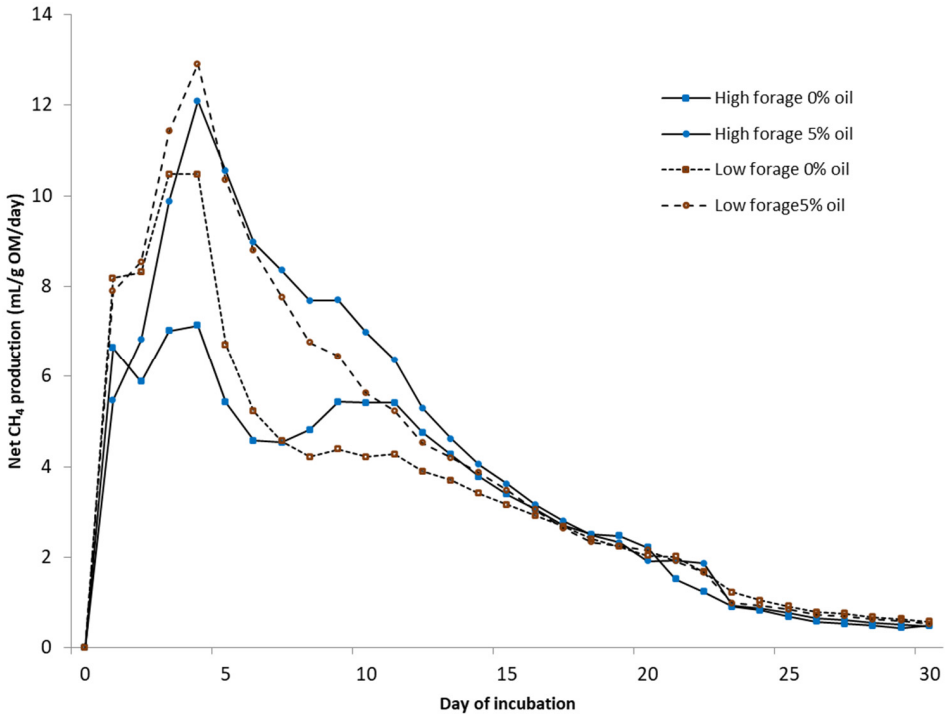

Figure 3. Methane production of incubated fecal samples of dairy cows fed different diets under BMP conditions.

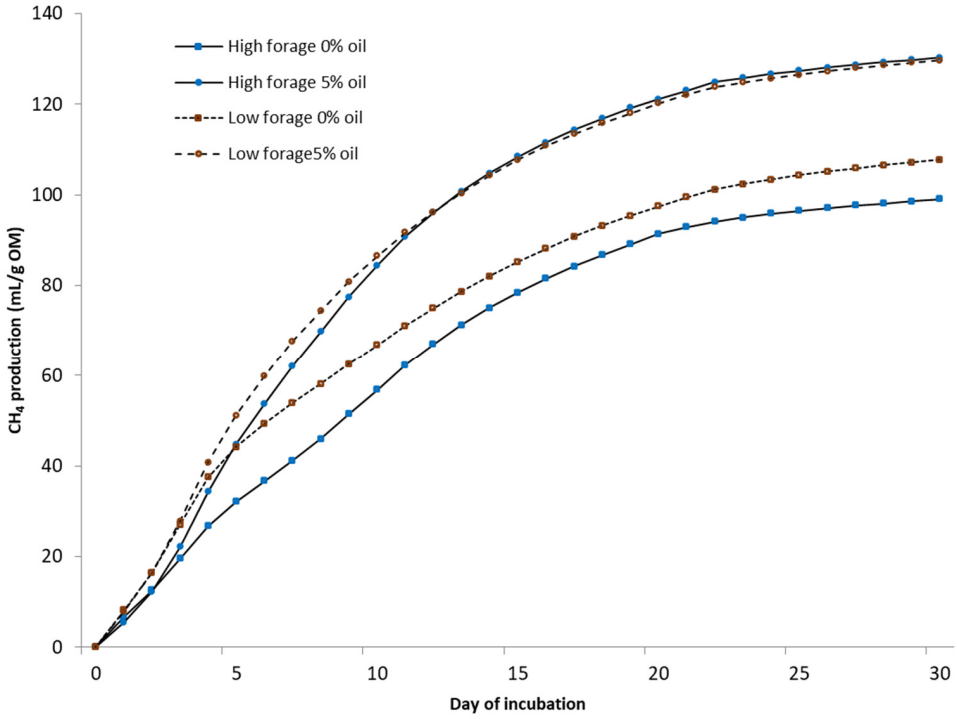

Figure 4. Cumulative methane production of incubated fecal samples of dairy cows fed different diets under BMP conditions.

3.6. Microbial Ecology

Sequencing resulted in 5260–28,189 good-quality reads per sample for rumen DNA and 13,957–44,371 reads per sample for fecal prokaryota cDNA. For alpha diversity estimation, sequencing data were subsampled to an even depth of 5200 reads for rumen and 13,900 for fecal prokaryota, respectively.

The experimental diets had no impact ($p > 0.01$) on the alpha diversity of ruminal prokaryota, estimated as observed amplicon sequence variants (ASVs), Shannon, or Simpson indexes (Supplementary Table S1). Beta diversity, evaluated as Bray–Curtis dissimilarities and visualized using a PCoA plot, indicated significant bacterial community differences based on experimental treatments (adonis $p < 0.01$), on dietary concentrate proportion ($p < 0.01$), but not on oil supplementation alone ($p = 0.16$) (Supplementary Figure S1). Dietary treatment with a higher forage proportion (HF) had a significantly higher abundance of *Christensenellaceae R-7* group, *Kiritimatiellae* WCHB1-41, or *Candidatus Saccharimonas*, while an increase in dietary concentrate proportion (LF) was linked with significantly more abundant *Acetitomaculum*, *Succiniclasticum*, *[Ruminococcus] gauvreauii* group, or *Ruminococcaceae* spp. (Supplementary Table S2). Rapeseed oil supplementation in the HF or LF diets significantly reduced *Candidatus Saccharimonas* and *Clostridia UCG-014* but increased the abundance of *Bacteroidales F082*.

To elucidate the rumen prokaryota association with enteric CH_4 production, Spearman correlations were calculated using genera-level taxonomical data. A decrease in CH_4 intensity (g/kg milk or ECM) was associated with a decrease in the abundance of *Bacteroidales RF16* group, *Rikenellaceae RC9* gut group, *Saccharofermentans*, and *Candidatus Saccharimonas* but an increase in *Succiniclasticum* or *Succinivibrionaceae UCG-002* genera (Supplementary Table S3).

Alpha diversity estimates indicated that before incubation trials, the fecal prokaryota community was significantly ($p < 0.05$) more diverse. After the anaerobic incubation of manure, richness was numerically higher in BMP as compared with the static trial (Supplementary Figure S2). Beta diversity analysis confirmed significantly different prokaryotic community structures in fecal samples collected before and after anaerobic incubation experiments (adonis $p < 0.01$) (Supplementary Figure S3).

The experimental diets had no significant impact on alpha diversity in the BMP experiment, but in the static trial, fecal samples collected from cows that received an HF diet showed lower Shannon ($p = 0.053$) and Simpson ($p = 0.035$) diversity indexes as compared with the LF diet (Supplementary Table S1). Beta diversity did not indicate significant bacterial community differences between dietary treatments in BMP manure incubation, but a significant diet effect (adonis $p = 0.001$) was observed in the static experiment. Also, fecal samples before manure incubation indicated significant prokaryotic community differences in response to diet (adonis $p < 0.01$) (Supplementary Figure S4).

Prokaryota taxonomical comparison in feces before and after both incubation experiments showed that BMP samples had a higher abundance of archaea from the *Methanomicrobiaceae*, *Methanosarcinaceae*, *Methanofastidiosaceae*, and *Methanomethylophilaceae* families and bacteria belonging to 20 phyla and 71 genera as compared with fecal samples before the trial. Fecal samples after the static incubation experiment were significantly enriched in archaea from the *Methanobacteriaceae* and *Methanosarcinaceae* families and bacteria from Firmicutes, Proteobacteria, and Actinobacteriota phyla and 44 genera, as compared with samples before the incubation (Figures 5 and 6).

Because beta diversity indicated a significant diet effect on the prokaryota community structure in fecal samples before the manure incubation trial and in the static experiment, we identified bacterial genera influencing this separation. Fecal samples from cows fed the HF diet were significantly enriched in *Bacteroides*, *Bacteroidales* spp., *Prevotellaceae UCG-004*, and the *Rikenellaceae dgA-11* gut group, while feces from the LF diet had a significantly higher abundance of the *Bacteroidales RF16* group, the *Rikenellaceae RC9* gut group, and *Ruminococcus*, among others (Table 7). Oil supplementation in the HF and LF diets resulted in increased abundances of *Clostridioides*, *Paeniclostridium*, and *Izemoplasmatales*. In fecal samples after static manure incubation experiments, *Methanosarcina*, *Oscillospirales UCG-010*, *Izemoplasmatales*, and the *Christensenellaceae R-7* group were detected at a significantly higher abundance in HF samples, while the *Alistipes*, *Rikenellaceae RC9*, and *Rikenellaceae dgA-11* gut groups, as well as *Clostridium sensu stricto* 1 and *Treponema*, were enriched in LF samples. Oil supplementation to both HF and LF diets resulted in increased abun-

dances of *Caproiciproducens*, *Peptostreptococcaceae* spp., *Clostridioides*, *Muribaculaceae*, and *Ethanoligenenaceae Incertae Sedis* (Table 7).

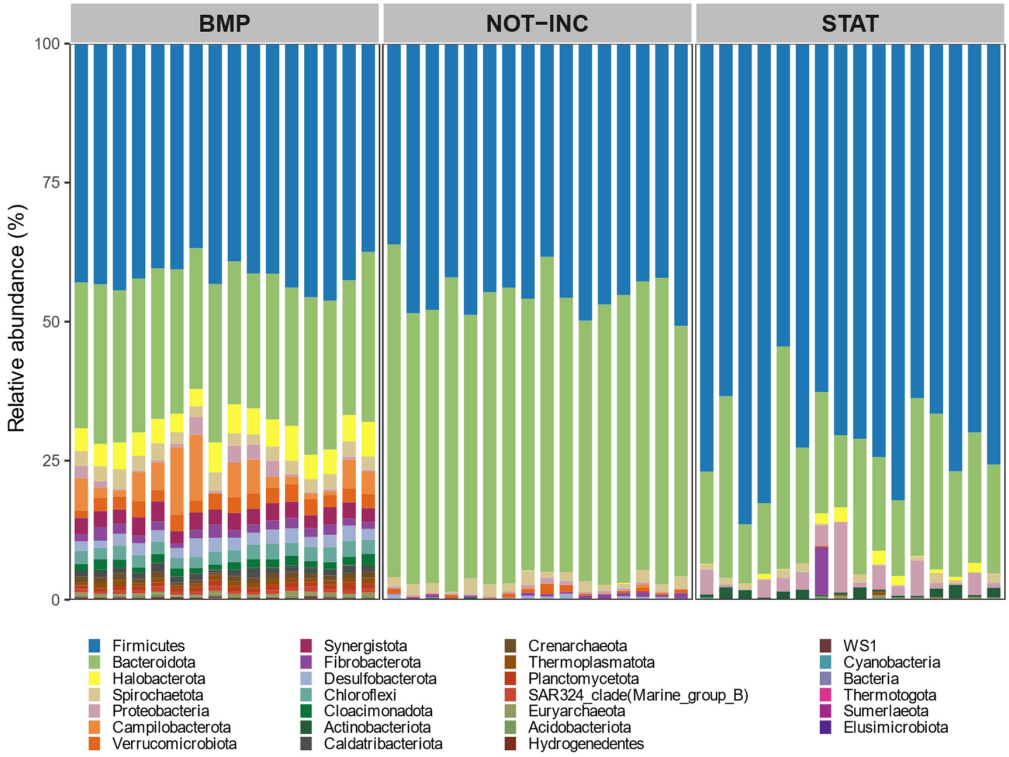

Figure 5. Fecal bacteria taxonomical composition at phylum level for samples before incubation experiment (NOT-INC), after manure BMP (BMP), and static (STAT) incubation trials.

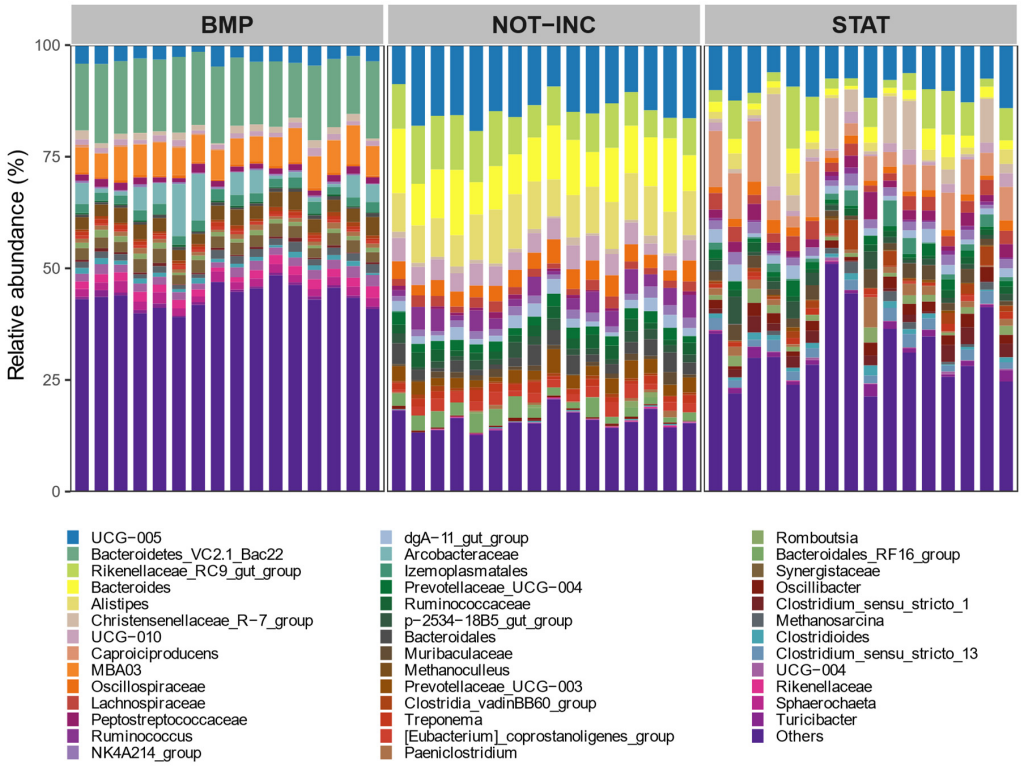

Figure 6. Fecal bacteria taxonomical composition at genus level for samples before incubation experiment (NOT-INC), after manure BMP (BMP), and static (STAT) incubation trials.

Table 7. Effects of forage-to-concentrate ratio and dietary rapeseed oil supplement on abundance (%) of fecal bacteria before incubation, as well as after BMP and after static manure incubation experiments.

	Treatment [1]					*p*-Value [2]		
Bacterial Taxa	**HF**	**HFO**	**LF**	**LFO**	**SEM**	**FC**	**RO**	**FC × RO**
Feces [3]								
Bacteroides	11.6	13.1	8.1	10.6	1.1	<0.01	0.096	0.57
Bacteroidales RF16 group	2.5	1.9	3.5	2.6	0.5	0.022	0.019	0.35
Prevotellaceae UCG-004	2.3	2.5	1.8	2.2	0.1	0.042	0.20	0.59
Rikenellaceae RC9 gut group	8.7	6.9	10.0	10.4	1.1	<0.01	0.055	0.17
Rikenellaceae dgA-11 gut group	3.0	2.3	1.3	1.2	0.2	<0.01	0.16	0.77
Bacteroidales spp.	1.7	2.5	1.0	0.89	0.2	<0.01	0.82	0.15
Clostridia vadinBB60 group	0.54	0.31	0.88	0.72	0.1	0.025	0.070	0.31
Lachnospiraceae spp.	1.4	1.6	2.3	2.2	0.2	<0.01	0.90	0.24
Ruminococcus	1.5	3.0	4.8	3.9	0.6	<0.01	0.44	0.040
[Eubacterium] coprostanoligenes group	1.6	1.6	2.2	3.0	0.2	0.017	0.81	0.34
Clostridioides	0.13	0.26	0.04	0.17	0.07	0.052	0.040	0.62
Paeniclostridium	0.15	0.61	0.07	0.41	0.20	0.12	<0.01	0.20
Izemoplasmatales	0.01	0.13	0.05	0.09	0.03	0.39	<0.01	0.11
Monoglobus	1.7	0.96	1.1	1.4	0.2	0.70	0.093	0.013
Feces, BMP								
Oscillospiraceae UCG-005	3.4	2.6	4.2	3.2	0.4	0.040	0.020	0.83
Fibrobacterales BBMC-4	1.6	1.3	2.1	1.5	0.2	0.16	0.047	0.49
Clostridium sensu stricto 1	0.56	0.31	0.63	0.39	0.06	0.20	<0.01	0.95
Feces, static								
Methanosarcina	1.6	1.0	0.16	0.15	0.32	0.014	0.24	0.22
Oscillospirales UCG-010	2.1	2.3	1.5	0.68	0.31	<0.01	0.32	0.12
Alistipes	0.87	1.1	3.6	1.6	0.33	0.011	0.17	0.26
Rikenellaceae RC9 gut group	1.8	4.1	9.9	6.0	1.2	<0.01	0.97	<0.01
Rikenellaceae dgA-11 gut group	1.5	2.3	2.6	1.0	0.2	0.015	0.014	<0.01
Acholeplasma	0.30	0.07	0.78	0.73	0.12	0.028	0.62	0.11
Izemoplasmatales	3.0	2.1	0.65	0.79	0.51	<0.01	0.42	0.24
Christensenellaceae R-7 group	13.2	4.4	2.4	1.0	1.4	<0.01	0.012	0.18
Fonticella	1.8	1.4	1.2	1.3	0.2	0.027	0.86	0.34
Clostridium sensu stricto 1	0.61	0.90	2.7	3.1	0.30	<0.01	0.20	0.31
Lachnospiraceae spp.	2.2	2.7	2.2	1.9	0.2	0.031	0.96	0.22
Treponema	0.18	0.45	1.4	1.1	0.15	0.001	0.46	0.17
Incertae Sedis	0.60	2.6	1.4	2.2	0.64	0.084	0.048	0.18
Caproiciproducens	3.6	6.6	7.1	9.7	1.4	0.074	0.032	0.70
Peptostreptococcaceae spp.	1.4	2.2	1.9	3.6	0.5	0.58	0.044	0.52
Clostridioides	0.89	1.2	0.77	1.4	0.24	0.27	0.023	0.47
Muribaculaceae	1.3	2.4	2.1	3.3	0.3	0.30	0.014	0.88
Oscillibacter	2.3	1.7	1.5	2.9	0.3	0.13	0.17	<0.01

[1] Refers to diets based on high (0.65) or low (0.35) forage ratios supplemented with 0 (HF and LF, respectively) or 5.0% (HFO and LFO, respectively) of rapeseed oil on DM basis. Values are LS means and pooled SEM for $n = 4$. [2] FC, effect of forage-to-concentrate ratio in the diet; RO, effect of rapeseed oil supplement; FC × RO, interaction of FC and RO. [3] Feces refer to feces collected from animals. BMP, biochemical methane potential; feces, static, in vitro incubation for 75 days without using inoculum, mixing, and $NaHCO_3$ buffer under temperature of 25 °C.

Spearman correlations, calculated to explore the fecal prokaryota association with the CH_4 output, demonstrated that in a static manure incubation trial, a decrease in CH_4 was significantly associated with a decrease in abundances of *Methanosarcina*, *Izemoplasmatales*, or the *Clostridia vadinBB60* group but increased abundances of *Muribaculaceae*, *Prevotellaceae UCG-004*, *Alistipes*, the *Rikenellaceae RC9* gut group, *Oscillospiraceae UCG-005*, *Romboutsia*, or *Treponema* (Supplementary Table S4).

4. Discussion

4.1. Dry Matter Intake and Nutrient Digestibility

As mentioned earlier, the effect of diets on intake, milk production, energy and N metabolism, and milk fatty acid composition are presented and discussed by Razzaghi et al. [18], but briefly, rapeseed oil supplementation at 5% diet DM tended to decrease DM intake, leading to lower intake of other nutrients (CP, NDF, pdNDF, water-soluble carbohydrate, and starch) without affecting GE intake, which is consistent with the previous studies on oil [34] or oilseeds [35]. On the other hand, cows receiving a diet lower in forage level (65 vs. 35% DM) increased DM intake by about 9%, leading to increased intake of OM, CP, EE, water-soluble carbohydrates, starch, and GE and reduced intake of NDF and pdNDF. The increased DM intake observed aligns with findings from other studies [34,36].

The lack of oil supplementation effect on the digestibility of DM, OM, NDF, CP, starch, and GE is in line with Bayat et al. [34] using a grass silage-based diet supplemented with rapeseed oil (at 5% DM). Similarly, Brask et al. [37] reported that OM, NDF, starch, and fatty acid digestibility in dairy cows did not significantly change compared with the control group as a result of rapeseed supplementation (at 2–3% DM) for diets based on grass silage. Previous studies have well established that oilseeds or oils have inconsistent effects on nutrient digestibility depending on the oil content, oil source, and composition of the basal diet [38–40].

Reduced NDF digestibility with lower dietary forage content has been well indicated by other studies [34,36,41] and can be attributed to increased DM intake and shorter ruminal retention time [36,41,42]. In addition, more available non-structural carbohydrates decrease the rate of NDF digestion, and higher levels of iNDF in high-concentrate diets contribute to lower NDF digestibility compared with high-forage diets [36]. However, despite lower NDF digestibility, OM, and therefore GE digestibility, was greater with lower dietary forage content.

4.2. Enteric Methane Emission and Ruminal Fermentation

The lower enteric CH_4 emissions and intensities are in line with studies that supplemented dairy cow diets with linseed or rapeseed oil [43,44] or oilseeds [39,40]. Fats impact CH_4 emissions through various mechanisms, including reduced ruminal OM fermentation, negative effects of C14:0 and C12:0 on protozoa, methanogen inhibition by 18-carbon unsaturated fatty acids [39], and changes in hydrogen utilization for biohydrogenation of unsaturated fatty acids. In this experiment, the ruminal molar ratio of acetate to propionate decreased, which is expected to reduce enteric CH_4 emissions [45,46]. These results suggested that the inclusion of oil in the experimental diets resulted in an 8.5% drop both in the acetate-to-propionate ratio and in enteric CH_4 emissions calculated as grams per kilogram DOM.

A well-known strategy for reducing enteric CH_4 emissions is to increase the level of concentrate, which shifts the ruminal fermentation pattern from acetate to propionate with the development of starch-fermenting bacteria [47]. This results in lower enteric CH_4 emissions due to the competition between propionate and methanogenesis in using metabolic hydrogen. In this experiment, a considerable change was observed in the ruminal acetate-to-propionate ratio with a high concentration level in the diet. However, no significant difference was observed in daily enteric CH_4 emission, which was due to the greater feed intake associated with the high-concentrate diet. Therefore, a lower CH_4 yield with high concentrate diets was consistent with a lower ruminal acetate-to-propionate ratio. The results of our experiment showed that at high concentration levels, enteric CH_4 emissions per unit of milk or energy-corrected milk decreased by 17 and 13%, respectively. Similar reductions in CH_4 emissions due to reduced dietary forage content were reported by Bayat et al. [34] and Aguerre et al. [48].

4.3. Chemical Composition of Feces

Oil supplementation considerably increased EE in feces (9.1 vs. 4.2% DM). Previous studies [3,49] have indicated a direct relationship between diet EE and fecal EE. Increased GE in the feces of cows fed oil-supplemented diets is consistent with the higher EE content. Oil supplementation increased fecal DM content by 8.7%, which is consistent with Møller et al. [50] and Hellwing et al. [3].

The higher starch and lower NDF and pdNDF concentrations in fecal samples of cows receiving a high-concentrate diet followed their concentrations in feed regardless of having similar or different digestibility. In contrast, Aguerre et al. [48] reported that decreased forage content (based on alfalfa and corn silage) from 68 to 47% DM did not change NDF or starch concentration in feces. In addition, Uddin et al. [51] found no change in NDF concentration but reported increased starch in feces as a result of decreasing forage content (based on alfalfa and corn silage) from 68 to 54% DM.

4.4. Fermentation Characteristics of Incubated Feces

Dietary oil supplementation tended to decrease fecal pH under static incubation, consistent with rapeseed oil in grass and corn silage diets [44]. However, Hassanat and Benchaar [49] found no pH change when linseed oil was added to red clover and corn silage diets over 17 weeks. In the current study, at the end of the incubation period, concentrations of total total VFA and molar proportion of individual VFA in feces were not influenced by dietary oil supplementation, except for a tendency for an increased acetate-to-propionate ratio. In the same vein, Hassanat and Benchaar [49] reported that oil supplementation had no impact on total VFA and acetate concentrations over the incubation period, while Ramin et al. [44] reported unchanged concentrations of total VFA and propionate and reduced acetate and butyrate in feces during the incubation period for oil-supplemented diets compared with the control group, with no change in CH_4 emission (g/d) in feces. Such a relationship was not observed in this experiment, as static CH_4 production (g/d) tended to decrease with increased propionate concentration in feces.

In this experiment, fecal pH dropped with high-concentrate content in the experimental diets, which was in line with higher concentrations of total VFA and short-chain fatty acids, except for propionate, in incubated fecal samples. This increased concentration of total VFA and acetate might be due to the activities of acetogenic bacteria to convert OM to VFA.

4.5. Methane Emissions and Biochemical Methane Potential from Manure

Oil supplementation in the experimental diets increased fecal BMP by 15 and 16.5% for daily production and calculated per unit of OM, respectively. This indicates that dietary oil supplementation reduces enteric CH_4 emissions and simultaneously increases the biogas production from manure, which can be an advantage in a production system that exploits biogas. Møller et al. [50] documented an elevation in BMP (30-day incubation) following dietary supplementation of dairy cow feed with whole rapeseed and rapeseed oil in comparison to the control group. In fact, oil supplementation in diets increases crude fat and OM in the diet, indicating higher levels of BMP. Fats tend to generate more CH_4 compared to carbohydrates and proteins in BMP conditions [52], as supported by a linear correlation between feces and dietary fat content in BMP conditions [50]. Moreover, there could be an inverse association between fecal iNDF and BMP [4]. In this study, introducing fat into experimental diets led to a 16% decrease in fecal iNDF and a 15% rise in BMP (g/d) compared to controls. Also, an inverse relationship was found between BMP and enteric CH_4 emission as oil supplementation reduced enteric CH_4 and increased BMP, which is consistent with Møller et al. [50] and Huhtanen et al. [4]. In contrast, in our study, dietary oil supplementation resulted in a 62% reduction in CH_4 production under static conditions designed to mimic natural manure storage conditions. Under the same condition, CH_4 production per unit of DOM tended to decrease. These significant and numerical reductions in static CH_4 production were in line with reduced enteric

CH_4, indicating no trade-off between enteric and manure CH_4 emissions under conditions mimicking the natural storage of manure. The first phase of gas production under static conditions probably reflects the fermentation of soluble fractions, whereas the second phase may reflect the fermentation of slowly degradable fractions or the secondary fermentation of microbial mass after depletion of DOM. Moreover, our results show different behavior of BMP and static conditions on manure CH_4 production, indicating that BMP conditions are not representative of natural conditions of manure storage, and instead, static conditions should be used to study manure CH_4 production and the possible trade-off between enteric and manure CH_4 emissions. However, BMP conditions are useful to study the maximum potential of manure CH_4 production, which is suitable for biogas production.

In this experiment, fecal BMP was not influenced by the FC ratio, while, under static conditions, daily CH_4 production decreased by about 89% with an increased concentrate content of the diet. This downward trend was also observed for static CH_4 calculated per unit of OM, DOM, and NDF. Aguerre et al. [53] measured CH_4 content in samples containing mixtures of feces, urine, bedding straw, and diluting water and reported that CH_4 emission was not influenced by forage level in diet (47 and 68% of diet DM), which is consistent with the chemical composition of feces since changes in forage level did not alter starch and fiber contents.

This outcome could have a substantial environmental impact by mitigating CH_4 emissions from dairy farming. Simultaneously, it raises a favorable economic dimension for the farm, specifically in terms of achieving an increased CH_4 yield per unit of mass. The circularity of manure management involves a sustainable approach where the OM from manure is efficiently processed to reduce ruminant greenhouse gas emissions while maximizing biogas production, thus contributing to both environmental and energy sustainability. The study findings align with this concept, highlighting the potential of anaerobic digestion systems to convert manure into valuable biogas, mitigate greenhouse gas emissions, and promote a closed-loop nutrient cycle in agriculture.

4.6. Prokaryota in Feces

Our results show that both BMP and static fecal incubation trials reduced prokaryota diversity when compared with freshly collected fecal samples. Due to changed conditions during the incubation trials, we anticipated having a fraction of dead prokaryota originating from fresh fecal samples that were not able to function in the new environment. As we were interested in only active prokaryota contributing to fermentation, VFA synthesis, and gas production in fecal incubation trials, the microbiota was studied at the RNA level.

We demonstrate that oil supplementation in cows' diets induced differences in prokaryota community composition in rumen as well as fresh fecal samples. We also show that diet-related microbial differences are visible in manure samples after static incubation but could not be distinguished in manure after BMP, as BMP is affected by the microbial community present in the inoculum and the influence of the manure as feeding material in a batch experiment is limited [54]. For instance, the prokaryota taxonomical community composition showed that BMP samples had 6.6% sequencing reads affiliated with archaea as opposed to 1% detected after static manure incubation and 0.05% detected in fresh fecal samples. Also, the Firmicutes-to-Bacteroidota (F/B) ratio changed in both incubation trials. While in fresh fecal samples the F/B ratio was similar (0.9:1), in BMP samples the F/B ratio was 1.6:1, with additional phyla replacing the diminished Bacteroidota. In contrast, after static manure incubation, which lasted for 75 days, the F/B ratio increased to 3.4:1. It is well known that certain genera of the phyla Firmicutes and Bacteroidota are effective lignocellulose degraders, and bioaugmentation of anaerobic digesters with a hemicellulolytic consortium of *Clostridium* and *Bacteroides* has proven to enhance biogas production [55,56]. However, Bacteroidota usually accumulate and proliferate in the initial hydrolysis and acidogenesis stages, whereas at the later stages, when the growth substrate becomes limited, the numbers and abundance of these bacteria decrease (reviewed by Xu et al. [57]). An increase in the abundance of Firmicutes is related to CH_4 reduction,

which is due to their role in the metabolism of VFA. Accumulation of VFA is detrimental to sensitive methanogens [58], which was visible in the static manure incubation trial where samples collected from cows that received LF or LFO diets produced more total VFA but less CH_4. Our results demonstrate that different conditions during the BMP and static incubation trials stimulated the proliferation of different prokaryota communities that were responsible for differences in VFA production and CH_4 emissions, as well as BMP from manure.

5. Conclusions

A high-concentrate diet reduced enteric CH_4 yield and intensity. Oil supplementation reduced daily enteric CH_4 emissions; however, it was more effective when added to a high-forage diet. Oil supplementation increased daily BMP and BMP per unit of OM but tended to decrease daily manure CH_4 production and CH_4 per unit of digested OM under static conditions. The experimental diets had no significant effect on prokaryota alpha diversity in the BMP experiment, but in the static trial, fecal samples collected from cows receiving a high forage diet showed significantly lower diversity indexes. Overall, the reduction of manure CH_4 emissions under static conditions indicated a lack of trade-off between enteric and manure CH_4 emissions when animals received a diet with an oil supplement. At the same time, dietary oil supplements increase manure BMP production, which can be exploited for more efficient biogas production, and suggest diverse manure utilization options aimed at reducing the environmental impact of the dairy livestock sector. In addition, the different behavior of manure CH_4 production under static and BMP conditions implies considering manure storage conditions in calculating greenhouse gas inventories.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/methane3010002/s1, Table S1: Effects of forage-to-concentrate ratio and dietary rapeseed oil supplement on alpha diversity of ruminal and fecal bacteria; Table S2: Effects of forage-to-concentrate ratio and dietary rapeseed oil supplement on abundance (%) of ruminal bacteria; Table S3: Association between relative abundance of rumen bacteria at genus level and enteric methane production calculated as methane intensity (g/kg milk or ECM); Table S4: Association between relative abundance of fecal bacteria at genus level and enteric methane production from static manure incubation experiment. Methane output was calculated as g/d; Figure S1: Effects of forage-to-concentrate ratio and dietary rapeseed oil supplement on beta diversity of ruminal bacteria. Treatments refer to diets based on high (0.65) or low (0.35) forage ratio supplemented with 0 (HF and LF, respectively) or 5.0% (HFO and LFO, respectively) of rapeseed oil on DM basis; Figure S2: Alpha diversity estimates for fecal samples before incubation experiment (NOT-INC) and after manure BMP (BMP) and static (STAT) incubation trials; Figure S3: Beta diversity for fecal samples before incubation experiment (NOT-INC) and after manure BMP (BMP) and static (STAT) incubation trials. Figure S4: Effects of forage to concentrate ratio and dietary rapeseed oil supplement on beta diversity of fecal bacteria before incubation (A), after BMP (B) and static manure incubation experiments (C). Treatments refer to diets based on high (0.65) or low (0.35) forage ratio supplemented with 0 (HF and LF, respectively) or 5.0% (HFO and LFO, respectively) of rapeseed oil on DM basis.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request. Raw sequences have been deposited in the NCBI SRA repository under the BioProject accession number PRJNA831716.

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