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Changes in the Rumen Microbiota of Cows in Response to Dietary Supplementation with Nitrate, Linseed, and Saponin Alone or in Combination

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ABSTRACT Dietary supplementation with linseed, saponins, and nitrate is a promising methane mitigation strategy in ruminant production. Here, we aimed to assess the effects of these additives on the rumen microbiota in order to understand underlying microbial mechanisms of methane abatement. Two 2-by-2 factorial design studies were conducted simultaneously, which also allowed us to make a broad-based assessment of microbial responses. Eight nonlactating cows were fed diets supplemented with linseed or saponin in order to decrease hydrogen production and nitrate to affect hydrogen consumption; also, combinations of linseed plus nitrate or saponin plus nitrate were used to explore the interaction between dietary treatments. Previous work assessed effects on methane and fermentation patterns. Rumen microbes were studied by sequencing 18S and 16S rRNA genes and ITS1 amplicons. Methanogen activity was monitored by following changes in *mcrA* transcript abundance. Nitrate fed alone or in combination in both studies dramatically affected the composition and structure of rumen microbiota, although impacts were more evident in one of the studies. Linseed moderately modified only bacterial community structure. Indicator operational taxonomic unit (OTU) analysis revealed that both linseed and nitrate reduced the relative abundance of hydrogen-producing *Ruminococcaceae*. Linseed increased the proportion of bacteria known to reduce succinate to propionate, whereas nitrate supplementation increased nitrate-reducing bacteria and decreased the metabolic activity of rumen methanogens. Saponins had no effect on the microbiota. Inconsistency found between the two studies with nitrate supplementation could be explained by changes in microbial ecosystem functioning rather than changes in microbial community structure.

IMPORTANCE This study aimed at identifying the microbial mechanisms of enteric methane mitigation when linseed, nitrate, and saponins were fed to nonlactating cows alone or in a combination. Hydrogen is a limiting factor in rumen methanogenesis. We hypothesized that linseed and saponins would affect hydrogen producers and nitrate would affect hydrogen consumption, leading to reduced methane production in the rumen. Contrary to what was predicted, both linseed and nitrate had a deleterious effect on hydrogen producers; linseed also redirected hydrogen consumption toward propionate production, whereas nitrate stimulated the growth of nitrate-reducing and, hence, hydrogen-consuming bacterial taxa. This novel knowledge of microbial mechanisms involved in rumen methanogenesis provides insights for the development and optimization of methane mitigation strategies.

KEYWORDS linseed, methane, microbiota, nitrate, rumen, saponin

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Methane emissions associated with ruminant livestock production are an important contributor to global greenhouse gas emissions (1). Rumen methanogenesis is a naturally occurring process that involves methanogenic archaea consuming hydrogen to reduce carbon dioxide. Hydrogen and carbon dioxide production occurs during feed fermentation by bacteria, protozoa, and fungi; hydrogen availability is a limiting factor for methane production. In addition, there is a significant linear relationship between protozoan concentration in the rumen and methane emissions (2). Among the measures that have been undertaken to reduce methane production by ruminants, diet composition and inclusion of feed additives have received the most attention (3). Among them, nitrate added to ruminants' diets consistently and persistently lowers methane emissions (4). Linseed oil, which is rich in linoleic acid, has proven to be one of the most efficient lipid sources used in methane mitigation strategies (4). Saponins are natural phytogenic feed additives used to improve animal feeding and production characteristics (5). Theoretically, these three additives lead to decreased methane production via different modes of action. Nitrate is an alternative electron acceptor, as its reduction competes with methane production for hydrogen (6). Additionally, nitrate or its reduced forms might be toxic to rumen methanogens and protozoa (7), but this effect was not systematically reported (8, 9). Lipids from linseed (and fats in general) added to diets replace a proportion of dietary carbohydrates and, as rumen microbes do not ferment them, less hydrogen is produced. Protozoal numbers have been reported to decrease with supplementary linseed oil (8, 10), although this effect was not always observed (11). Saponins can reduce methanogenesis by a toxic effect on rumen protozoa (5), but *in vivo* results indicate otherwise, as rumen microbes can deglycosylate and, thus, inactivate saponins (12).

Based on available information, we hypothesized that linseed oil and saponins would mainly affect hydrogen production (by a toxic effect on protozoa or by providing alternative substrates for rumen fermentation) and nitrate would mainly modulate hydrogen consumption pathways (by providing an alternative hydrogen sink). We performed amplicon-type sequencing analysis of rumen contents, sampled during two previous studies (8, 13); the first one reported the effect of linseed, nitrate, and linseed plus nitrate supplementation on enteric methane production; tea saponin replaced linseed in the second one. The primary aim of the current study was to search for changes in rumen microbiota structure and methanogenic activity that could explain observed reductions in methane emissions.

Minor but significant changes induced by treatment can be masked by spurious between-group differences unrelated to the treatment but rather to the host animal, the diet, or sample management. Moreover, it is not unusual to find reports on nitrate and fatty acid supplementation where methane decreased in a similar way, but effects on rumen microbiota were contrasting (14–17). On the other hand, it was recently shown that combination of microbial data from multiple sets of hosts with supposed similar microbiota should increase specificity and allow identification of causal microbes (18). Therefore, we took advantage of the data available from two independent studies, analyzed it separately but by following the same procedures, and made an integrated interpretation. Our secondary objective was to try to find clues to explain inconsistency in results from published studies.

(This article was submitted to an online preprint archive [19].)

RESULTS

Eight nonlactating dairy cows were randomly allocated to two 2-by-2 factorial designs. In study 1, dietary treatments consisted of control (CTL) diet, supplemented alternatively with linseed oil (LIN), nitrate (NIT), and linseed plus nitrate (LIN+NIT); in study 2, tea saponin (TEA) replaced linseed oil. In order to achieve adequate statistical power, the statistical model for both studies included cow as random effect, and fixed effects were experimental period and the following: (i) in study 1, linseed (CTL and NIT versus LIN and LIN+NIT), nitrate (CTL and LIN versus NIT and LIN+NIT), and their interaction, termed linseed×nitrate (or lin×nit), and (ii) in study 2, saponin (CTL and NIT

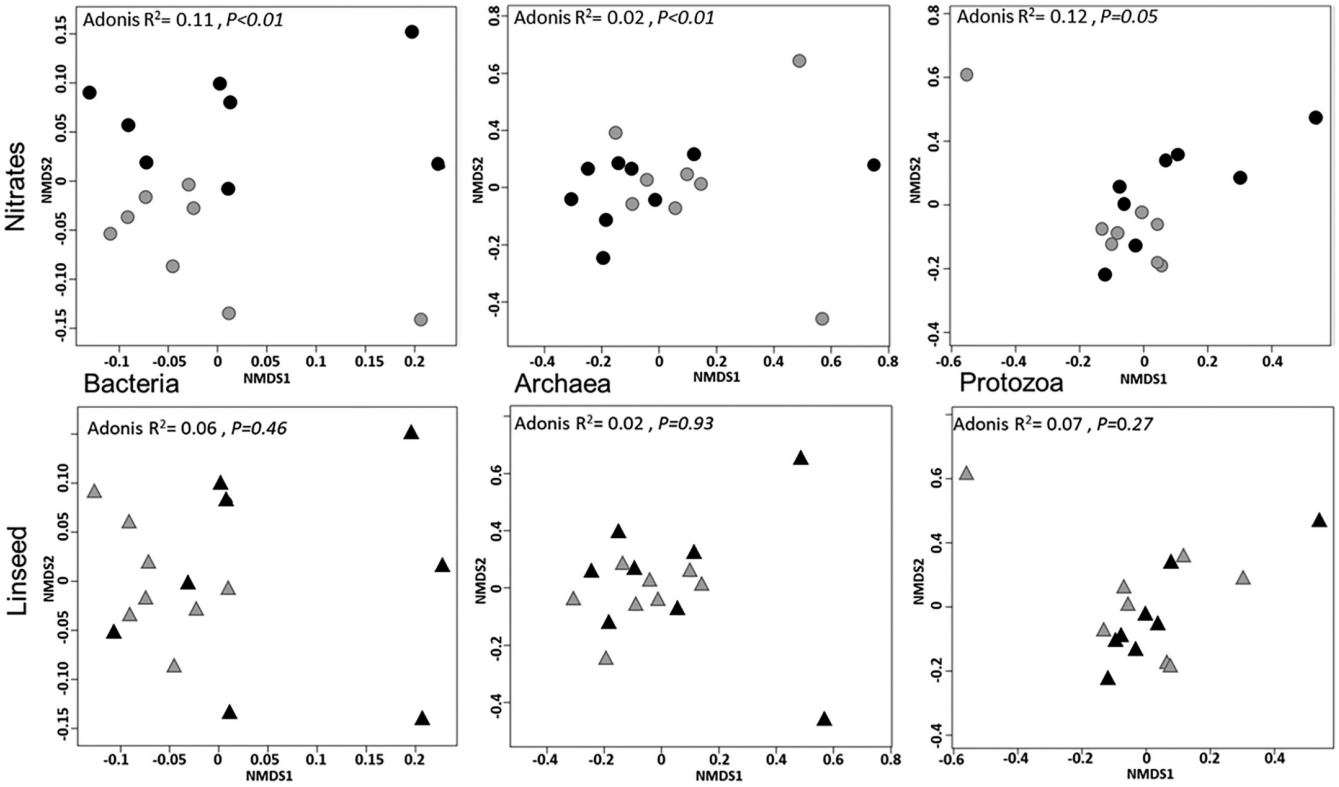

FIG 1 Structure and composition of bacterial, archaeal, and protozoal communities in study 1, related to nitrate or linseed treatments (black symbols) and respective controls (gray symbols), were examined by multivariate analysis. NMDS plots derived from Bray-Curtis dissimilarities between cows are shown. Each symbol is representative of a single cow. Samples are plotted along the first two-component axes. Microbial composition was compared using Adonis.

versus TEA and TEA+NIT), nitrate (CTL and TEA versus NIT and TEA+NIT), and their interaction, saponin×nitrate (sap×nit). Throughout the text, linseed, nitrate, and saponin will refer to diet contrasts detailed above.

In study 1, compared to CTL, dietary treatments LIN, NIT, and LIN+NIT decreased methane production (g/day) by 22%, 29%, and 33%, respectively, and methane yield (g/kg of dry matter intake [DMI]) by 25%, 29%, and 32% (8). In study 2, NIT and TEA+NIT decreased methane production by 42% and 34% and methane yield by 36% and 29%, respectively, compared to CTL (13). TEA alone had no effect on methane production or on volatile fatty acid (VFA) profiles.

In both studies, *Bacteroidales* and *Clostridiales* were the dominant bacterial orders and accounted for more than 88% of the classified reads, regardless of the dietary treatment (see Fig. S1 in the supplemental material). Sequences affiliated with the *Methanobrevibacter* genus accounted for 80% of all archaeal sequences in both studies, followed by *Methanosphaera,* unclassified methanogens, and three *Methanomassiliicoccaceae* genera (Fig. S1). In both studies, *Piromyces* represented more than 60% of rumen fungi, followed by *Orpinomyces* and *Caecomyces*. Dietary treatments did not affect fungal community composition or its structure (Fig. S2), and we are not going to discuss it further.

Linseed moderately affected bacterial community composition with no effect on rumen methanogens and protozoa. Nonmetric multidimensional scaling (NMDS) did not reveal any distinct clustering of bacterial communities (Fig. 1), and total bacterial numbers were similar (Table 1) in cows receiving or not receiving linseed-supplemented diets. Accordingly, CowPI predictions showed no changes in metabolic profiles (Table S1). However, the richness index was reduced by the linseed treatment (Table S2), and linseed increased ($P < 0.05$) relative abundance of *Selenomonadales, Synergistales, Elusimicrobiales,* and *Micrococcales* (Table 2). Moreover, indicator species

TABLE 1 Abundance of total bacteria and abundance and activity of methanogenic archaea in the rumen of nonlactating cows fed methane-reducing additives

	Abundance and activity															
	Study 1[a]					Effect			Study 2[b]					Effect		
Parameter	CTL	LIN	NIT	LIN+NIT	SEM	Linseed	Nitrate	lin×nit	CTL	TEA	NIT	TEA+NIT	SEM	Saponin	Nitrate	sap×nit
Total bacterial concn ($\log_{10}$ *rrs* copies/ng DNA)	5.09	4.94	4.95	4.98	0.051	0.50	0.54	0.33	5.04	4.96	5.02	5.15	0.057	0.64	0.12	0.05
Methanogen concn ($\log_{10}$ *mcrA* copies/ng DNA)	2.95	2.68	2.72	2.76	0.051	0.36	0.15	<0.1	2.97	2.84	2.97	3.07	0.081	0.84	0.25	0.24
Methanogen activity[c] ($2^{-\Delta CT} \times 10^6$)	23.91	21.54	10.49	8.19	3.384	0.51	<0.01	0.99	18.67	16.08	7.40	8.28	4.463	0.76	<0.01	0.53

[a]In study 1, cows were fed a control (CTL) diet and CTL diet supplemented with linseed (LIN), nitrate (NIT), and linseed plus nitrate (LIN+NIT). Tested effects were linseed (CTL and NIT versus LIN and LIN+NIT) and nitrate (CTL and LIN versus NIT and LIN+NIT) and their interaction, lin×nit.
[b]In study 2, cows were fed a control (CTL) diet and control diet supplemented with tea saponin (TEA), nitrate (NIT), and tea saponin plus nitrate (TEA+NIT). Tested effects were saponin (CTL and NIT versus TEA and TEA+NIT) and nitrate (CTL and TEA versus NIT and TEA+NIT) and their interaction, sap×nit.
[c]Methanogen activity is measured as *mcrA* expression levels.

analysis showed that *Ruminococcaceae*-related operational taxonomic units (OTUs) characterized the bacterial community of cows not receiving linseed supplementation (Fig. 2 and Table S3).

Regarding methanogen concentration, *mcrA* copy numbers per nanogram of extracted DNA were not affected by linseed supplementation (Table 1), and neither was overall community structure (Fig. 1 and Table 3).

Feeding linseed did not modify protozoan community structure and composition compared to the respective control treatment (Fig. 1 and Table 4). There were 3 indicator OTUs identified, 2 associated with CTL diet and 1 with LIN diet, but they all represented less than 0.01% of the rarefied data set (13,809 reads per individual).

Tea saponins had only minor effects on rumen microbial population. Adding tea saponin to diets only affected the low-abundance order of unclassified *Deltaproteobacteria* (Table 2). No changes in diversity indices were noticed (Table S2). NMDS (Fig. 3) and permutational multivariate analysis of variance (PERMANOVA) analysis did not reveal significant changes in bacterial community, although *Lachnospiraceae* were highly abundant in cows supplemented with saponin (Fig. 2). Similarly, concentration and taxonomic composition of the archaeal community were not influenced by tea saponin (Fig. 3 and Table 3), and neither was the protozoan community structure (Fig. 3 and Table 4).

TABLE 2 Bacterial orders significantly affected by at least one dietary treatment in the rumen of nonlactating cows fed methane-reducing additives[c]

	Relative abundance (%)															
	Study 1[a]					Effect			Study 2[b]					Effect		
Bacterial order	CTL	LIN	NIT	LIN+NIT	SEM	Linseed	Nitrate	lin×nit	CTL	TEA	NIT	TEA+NIT	SEM	Saponin	Nitrate	sap×nit
Bacteroidales	46.5	44.0	46.5	44.0	0.010	0.88	0.54	0.35	39.98	37.55	45.92	42.18	0.012	0.12	0.01	0.76
Selenomonadales	1.54	2.27	1.77	2.76	0.002	0.03	0.42	0.82	2.14	1.95	2.11	2.66	0.001	0.40	0.14	0.11
Coriobacteriales	0.29	0.20	0.39	0.36	0.000	0.16	0.01	0.39	0.22	0.19	0.32	0.37	0.000	0.73	0.00	0.18
Gastranaerophilales	0.18	0.17	0.07	0.07	0.000	0.99	0.09	0.83	0.26	0.21	0.11	0.10	0.000	0.34	0.00	0.57
Unclassified	0.14	0.20	0.14	0.05	0.000	0.30	0.02	0.03	0.17	0.18	0.14	0.11	0.000	0.59	0.10	0.47
Synergistales	0.05	0.06	0.03	0.06	0.000	0.04	0.26	0.26	0.05	0.03	0.05	0.03	0.000	0.07	0.96	0.60
Elusimicrobiales	0.02	0.10	0.06	0.08	0.000	0.04	0.56	0.22	0.03	0.04	0.07	0.04	0.000	0.71	0.87	0.46
Burkholderiales	0.01	0.01	0.06	0.06	0.000	0.82	0.00	0.62	0.01	0.01	0.11	0.04	0.000	0.29	0.01	0.44
Unclassified *Deltaproteobacteria*	7.08	1.42	2.03	2.66	0.000	0.09	0.26	0.15	4.36	0.31	2.97	1.00	0.000	0.03	0.93	0.43
Victivallales ($\times 10^{-3}$)	6.44	4.65	1.09	6.17	0.000	0.28	0.27	0.04	1.38	3.44	1.54	4.18	0.000	0.12	0.97	0.92
Xanthomonadales ($\times 10^{-3}$)	4.68	2.37	3.64	8.64	0.000	0.98	0.45	0.48	7.74	1.28	2.77	9.45	0.000	0.99	0.37	0.00
Micrococcales ($\times 10^{-3}$)	4.26	13.5	5.16	10.2	0.000	0.01	0.59	0.65	8.22	6.51	15.9	9.85	0.000	0.19	0.13	0.62
Opitutae vadin HA64 ($\times 10^{-3}$)	0.36	7.24	1.53	3.94	0.000	0.06	0.78	0.31	0.68	1.16	3.14	-	0.000	0.40	0.97	0.14

[a]In study 1, cows were fed a control (CTL) diet and CTL diet supplemented with linseed (LIN), nitrate (NIT) and linseed plus nitrate (LIN+NIT). Tested effects were linseed (CTL and NIT versus LIN and LIN+NIT) and nitrate (CTL and LIN versus NIT and LIN+NIT) and their interaction, lin×nit.
[b]In study 2, cows were fed a control (CTL) diet and control diet supplemented with tea saponin (TEA), nitrate (NIT) and tea saponin plus nitrate (TEA+NIT). Tested effects were saponin (CTL and NIT versus TEA and TEA+NIT) and nitrate (CTL and TEA versus NIT and TEA+NIT) and their interaction, sap×nit.
[c]Values are the means from four observations, and analysis was performed on square root-transformed taxonomic tables using the aov function in R.

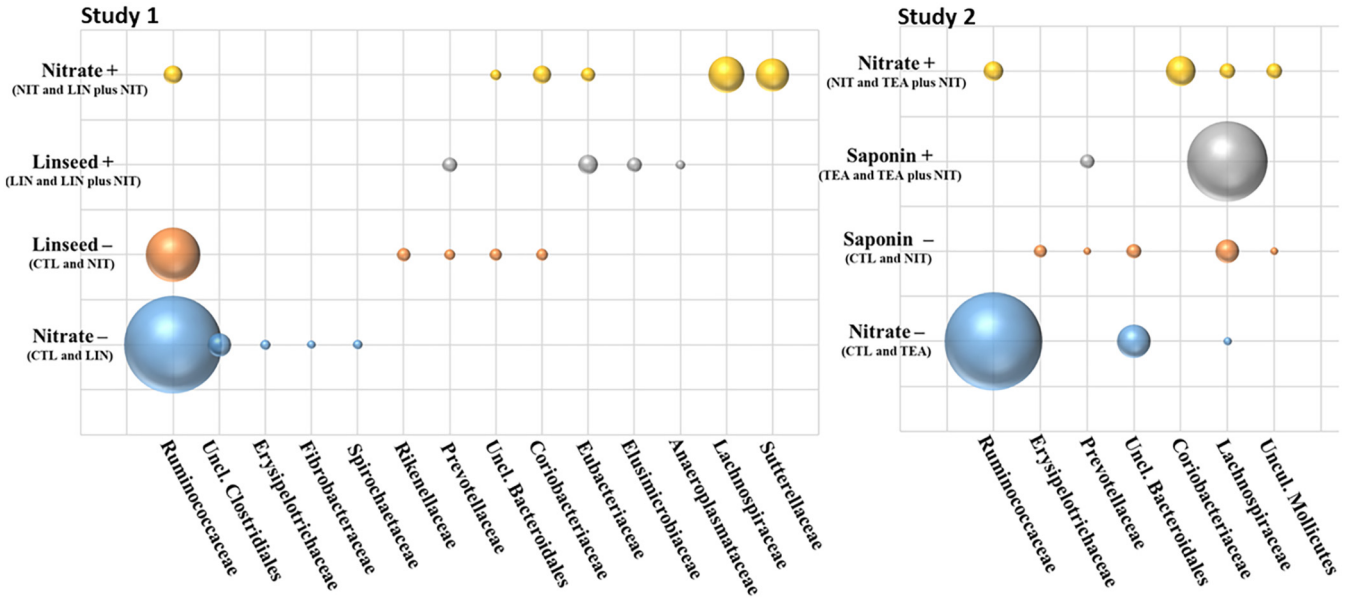

FIG 2 Bubble charts showing indicator OTU distribution by dietary treatment in the rumen of nonlactating cows fed methane-reducing additives. Bubble size reflects the count number in the rarefied data set.

Nitrate remodels bacterial and archaeal communities. In order to have an integrated discussion on the effects of nitrate on microbes from both studies, we needed to compare like to like. This is why we compared microbial communities of cows fed CTL diet in each study. Bacterial communities of these cows were similar (Adonis R^2 of 0.16 and P value of 0.26). A small numerical difference was noted in the *Bacteroidales*/*Clostridiales* ratios, which were 1.02 and 0.81 in study 1 and study 2,

TABLE 3 Archaeal species detected in the rumen of nonlactating cows fed methane-reducing additives[c]

	Relative abundance (%)															
	Study 1[a]					Effect			Study 2[b]					Effect		
Archaeal species	CTL	LIN	NIT	LIN+NIT	SEM	Linseed	Nitrate	lin×nit	CTL	TEA	NIT	TEA+NIT	SEM	Saponin	Nitrate	sap×nit
Methanobacterium alkaliphilum	0.04	0.71	0.03	0.01	0.002	0.166	0.126	0.138	0.01	0.56	0.05	0.00	0.001	0.184	0.173	0.120
Methanobrevibacter acididurans	0.01	0.00	0.01	0.01	0.000	0.966	0.506	0.649	0.00	0.00	0.00	0.01	0.000	0.448	0.876	0.623
Methanobrevibacter boviskoreani clade	2.72	1.44	2.72	1.86	0.018	0.502	0.891	0.887	3.89	0.50	0.40	2.46	0.018	0.776	0.740	0.234
Methanobrevibacter gottschalkii clade	38.08	45.63	21.10	19.28	0.051	0.584	<0.001	0.371	39.39	38.46	29.32	27.01	0.051	0.862	0.203	0.824
Methanobrevibacter oralis	0.47	0.34	0.32	0.25	0.001	0.158	0.115	0.641	0.27	0.46	0.30	0.27	0.001	0.271	0.250	0.137
Methanobrevibacter ruminantium clade	36.28	33.06	54.18	62.10	0.043	0.742	<0.01	0.365	33.80	37.75	47.87	47.66	0.043	0.766	0.107	0.735
Methanobrevibacter sp. strain RT	0.02	0.03	0.01	0.00	0.000	0.999	0.059	0.102	0.04	0.02	0.00	0.01	0.000	0.673	0.078	0.168
Other *Methanobrevibacter*	2.54	1.64	1.70	1.13	0.002	0.109	0.136	0.712	1.56	2.05	1.52	1.50	0.002	0.331	0.220	0.296
Methanosphaera cuniculi	0.22	0.04	0.25	0.23	0.001	0.575	0.561	0.668	0.05	0.09	0.08	0.10	0.001	0.641	0.760	0.774
Methanosphaera sp. strain A4	0.08	0.03	0.05	0.04	0.000	0.193	0.746	0.456	0.05	0.03	0.02	0.03	0.000	0.912	0.089	0.066
Methanosphaera sp. strain ISO3-F5	7.97	6.41	8.60	8.36	0.011	0.691	0.581	0.777	9.43	8.59	6.26	11.61	0.011	0.143	0.937	0.053
Other *Methanosphaera*	0.83	0.35	0.88	0.34	0.001	0.081	0.934	0.913	0.45	0.57	0.25	0.50	0.001	0.211	0.375	0.639
Other *Methanobacteriaceae*	0.07	0.05	0.08	0.05	0.000	0.255	0.976	0.806	0.04	0.05	0.04	0.06	0.000	0.418	0.841	0.565
Other *Methanococcales*	0.01	0.00	0.00	0.00	0.000	0.337	0.337	0.337	0.00	0.01	0.00	0.00	0.000	0.337	0.337	0.337
Methanomicrobium mobile	0.24	0.08	0.01	0.00	0.003	0.320	0.081	0.376	0.51	0.46	0.62	0.04	0.003	0.399	0.679	0.477
Other	0.09	0.07	0.00	0.01	0.001	0.918	0.156	0.849	0.00	0.23	0.00	0.01	0.001	0.257	0.306	0.311
Group 10 species	0.60	0.00	0.16	0.00	0.003	0.166	0.405	0.405	0.99	0.84	0.61	0.56	0.005	0.820	0.435	0.908
Candidatus "Methanomethylophilus alvus"	0.02	0.01	0.02	0.01	0.000	0.360	0.642	0.689	0.00	0.02	0.01	0.01	0.000	0.243	0.845	0.110
Group 12 species	1.99	2.21	2.50	2.05	0.006	0.902	0.878	0.745	1.51	2.41	3.27	2.36	0.006	0.999	0.244	0.218
Group 8 species	0.10	0.09	0.03	0.00	0.000	0.797	0.234	0.876	0.02	0.00	0.00	0.00	0.000	0.214	0.156	0.297
Group 9 species	0.03	0.02	0.09	0.16	0.001	0.677	0.244	0.648	0.04	0.21	0.03	0.02	0.001	0.052	0.025	0.039
Other *Methanomassiliicoccaceae*	2.96	3.12	3.87	1.58	0.000	0.842	0.687	0.226	2.74	1.51	3.44	1.67	0.000	0.987	0.859	0.883
Other	4.59	4.66	3.37	2.52	0.001	0.918	0.156	0.849	5.18	5.15	5.92	4.11	0.001	0.257	0.306	0.311

[a]In study 1, cows were fed a control (CTL) diet or CTL diet supplemented with linseed (LIN), nitrate (NIT), or linseed plus nitrate (LIN+NIT). Tested effects were linseed (CTL and NIT versus LIN and LIN+NIT) and nitrate (CTL and LIN versus NIT and LIN+NIT) and their interaction, lin×nit.

[b]In study 2, cows were fed a control (CTL) diet or CTL diet supplemented with tea saponin (TEA), nitrate (NIT), or tea saponin plus nitrate (TEA+NIT). Tested effects were saponin (CTL and NIT versus TEA and TEA+NIT) and nitrate (CTL and TEA versus NIT and TEA+NIT) and their interaction, sap×nit.

[c]Values are the means from four observations, and analysis was performed on square root-transformed taxonomic tables using the aov function in R.

TABLE 4 *Protozoa* genera detected in the rumen of nonlactating cows fed methane-reducing additives[c]

	Relative abundance (%)															
	Study 1[a]					Effect			Study 2[b]					Effect		
Protozoa genus	CTL	LIN	NIT	LIN+NIT	SEM	Linseed	Nitrate	lin×nit	CTL	TEA	NIT	TEA+NIT	SEM	Saponin	Nitrate	sap×nit
Entodinium	59.84	49.66	46.52	33.94	0.036	0.67	0.05	0.10	46.21	51.05	47.55	50.55	0.040	0.68	0.90	0.88
Polyplastron	11.57	8.50	17.51	10.43	0.021	0.75	0.26	0.27	7.94	9.73	10.39	7.19	0.016	0.62	0.92	0.63
Unclassified *Trichostomatia*	9.93	11.59	15.26	16.70	0.023	0.97	0.36	0.75	22.28	9.34	14.59	14.16	0.032	0.23	0.72	0.47
Isotricha	1.52	3.92	6.00	4.34	0.009	0.15	0.06	0.68	5.43	5.27	6.72	6.43	0.008	0.56	0.65	0.76
Dasytricha	1.31	0.80	1.86	4.01	0.006	0.65	0.10	0.76	0.90	1.92	1.67	1.77	0.004	0.19	0.34	0.13
Unclassified	0.17	0.17	0.06	0.19	0.000	0.08	0.15	0.06	0.14	0.18	0.17	0.14	0.000	0.85	0.82	0.57
Ophryoscolex	0.16	0.00	0.46	1.33	0.002	0.06	0.00	0.67	3.26	3.08	0.41	1.47	0.010	0.64	0.52	0.78
Unclassified *Ciliophora*	0.04	0.09	0.04	0.06	0.000	0.59	0.50	0.27	0.03	0.07	0.06	0.05	0.000	0.09	0.81	0.09
Unclassified SAR[d]	0.04	0.02	0.01	0.02	0.000	0.09	0.38	0.66	0.04	0.04	0.01	0.02	0.000	0.15	0.00	0.57
Trichostomatia	0.04	0.28	0.02	0.02	0.000	0.04	0.01	0.06	0.10	0.08	0.24	0.03	0.000	0.41	0.94	0.34
Pseudoplatyophyra	0.00	0.05	0.02	0.00	0.000	0.00	0.18	0.12		0.03	0.00	0.00	0.000	0.21	0.50	0.17

[a]In study 1, cows were fed a control (CTL) diet and CTL diet supplemented with linseed (LIN), nitrate (NIT) and linseed plus nitrate (LIN+NIT). Tested effects were linseed (CTL and NIT versus LIN and LIN+NIT) and nitrate (CTL and LIN versus NIT and LIN+NIT) and their interaction, lin×nit.

[b]In study 2, cows were fed a control (CTL) diet and control diet supplemented with tea saponin (TEA), nitrate (NIT) and tea saponin plus nitrate (TEA+NIT). Tested effects were saponin (CTL and NIT versus TEA and TEA+NIT) and nitrate (CTL and TEA versus NIT and TEA+NIT) and their interaction, sap×nit.

[c]Values are the means from four observations, and analysis was performed on square root-transformed taxonomic tables using the aov function in R.

[d]SAR, Stramenopiles-Alveolata-Rhizaria cluster.

respectively. Similar to bacteria, methanogenic communities in animals fed CTL diets were similar between studies (Adonis R^2 of 0.028 and P value of 0.942). Regarding protozoa, some differences were revealed by NMDS and PERMANOVA analyses between the two control groups. NMDS graphs (Fig. S3) showed only a small overlap between the protozoan population fed CTL in each study, which was confirmed by an Adonis test ($P < 0.1$). Also, *Entodinium*-related sequences accounted for 60% of total classified sequences in study 1, whereas they represented 46% of sequences in study

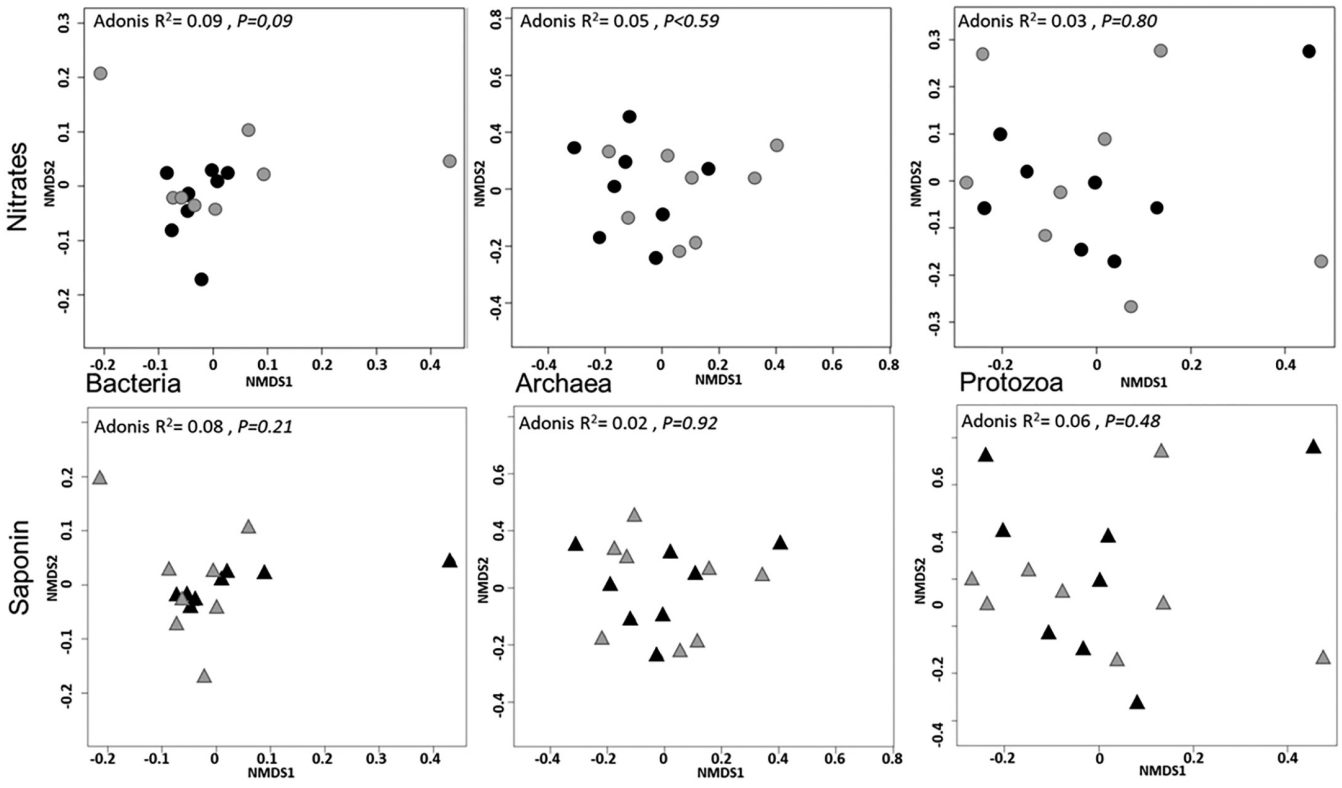

FIG 3 Structure and composition of bacterial, archaeal, and protozoal communities in study 2, related to nitrate or saponin treatments (black symbols) and respective controls (gray symbols), were examined by multivariate analysis. NDMS plots derived from Bray-Curtis dissimilarities between cows are shown. Each symbol is representative of a single cow. Samples are plotted along the first two-component axes. Microbial composition was compared using Adonis.

2 (Table 4). Although this difference was not statistically significant, it was accompanied by significantly higher numbers of *Trichostomatia*- and *Isotricha*-related sequences in study 1 than study 2 (Table 4).

Feeding nitrate, in both studies, increased *Coriobacteriales* and *Burkholderiales* relative abundance and decreased (study 2), or tended to decrease (study 1), abundance of *Gastranaerophilales* (Table 2). In addition, in study 2, nitrate supplementation increased the relative abundance of *Bacteroidales* (Table 2). Diversity indices were not influenced by dietary treatment (Table S2) in any study. NMDS analysis (Fig. 1) revealed that while in study 1 nitrate supplementation was the major driver of phylogenetic dissimilarity among bacterial communities (Adonis R^2 of 0.11, *P* value of <0.01), in study 2 nitrate only moderately affected community structure (Adonis R^2 of 0.09, *P* value of 0.09). Indicator species analysis revealed that 10 OTUs in study 1 and 21 in study 2 were differentially abundant between cows fed and not fed nitrate (*P* value of <0.05 and indicator value of >0.7; Table S3 and Table S4). *Lachnospiraceae* and *Sutterellaceae* characterized nitrate-supplemented diets (Fig. 2) in study 1, and *Coriobacteriaceae* and the uncultured *Mollicutes* family were identified as indicator OTUs for nitrate-supplemented diets in study 2. More interestingly, in both studies *Ruminococcaceae*-related OTUs characterized the bacterial community of control cows (Fig. 2).

CowPI predictive analysis suggested that nitrogen metabolism was increased in both studies when nitrate was fed to cows (Table S1). Nitrate supplementation induced numerous changes in metabolic profiles. Regarding carbohydrate metabolism, nitrate supplementation would affect most of the described pathways, but observed changes were different in each study. Predictions regarding lipid metabolism were more consistent between studies and suggested that dietary supplementation with nitrates would decrease biosynthesis of fatty acids.

In both studies, feeding nitrate had no effect on methanogen concentration in the rumen (*mcrA* copy numbers) but reduced methanogen activity (*mcrA* expression levels) (Table 1). When cows were fed nitrate, Shannon and Simpson diversity indices decreased or tended to decrease (Table S2), although the overall taxonomic composition was not affected (Table 3). NMDS and PERMANOVA analyses showed that feeding nitrates deeply modified archaeal community structure in study 1 but had no effect on community structure in study 2 (Fig. 1 and 3).

In study 1, *Entodinium* relative abundance tended to decrease and *Isotricha* tended to increase in animals receiving nitrate-supplemented diets (Table 4). Diversity indices remained similar between diets and contrasts (Table S2). However, there was some evidence (Adonis R^2 of 0.12, *P* value of 0.05) that nitrate modulated the rumen protozoan population in cows (Fig. 1). In contrast, in study 2, nitrate had no effect on protozoan community in the rumen of nonlactating dairy cows (Fig. 3).

Correlation patterns of microbial population. We analyzed the correlation between bacterial families and genera of methanogens and protozoa (Fig. 4 and 5). Values for methane production (g/day), yield (g/kg DMI), hydrogen production (only for study 1), and (acetate + butyrate)/propionate ratio from the data sets of Guyader et al. (8, 13) were also included in the analysis. Only significant correlations are discussed.

In study 1 (Fig. 4), methane production (g/day) and yield (g/kg DMI) were positively correlated ($R^2 = 0.83$ and $R^2 = 0.69$, respectively) with the (acetate + butyrate)/propionate ratio when cows were not fed nitrate; in these animals, methane yield correlated positively with *Rikenellaceae* ($R^2 = 0.56$). In the absence of nitrate, *Methanobrevibacter* negatively correlated with unclassified *Methanomassiliicoccaceae* ($R^2 = -0.64$), and *Ruminococcaceae* correlated positively with members of the protozoal *Polyplastron* genus ($R^2 = 0.97$). When diets were supplemented with nitrate, methane production and yield as well as (acetate + butyrate)/propionate ratio were strongly correlated with a group of unclassified *Methanomassiliicoccaceae* ($R^2 = 0.69$, $R^2 = 0.85$, and $R^2 = 0.59$, respectively). In addition, when diets were nitrate supplemented, a positive correlation was established between *Prevotellaceae* and *Dasytricha* ($R^2 = 0.70$ and $R^2 = 0.73$). There was a strong negative correlation between *Methanobrevibacter*

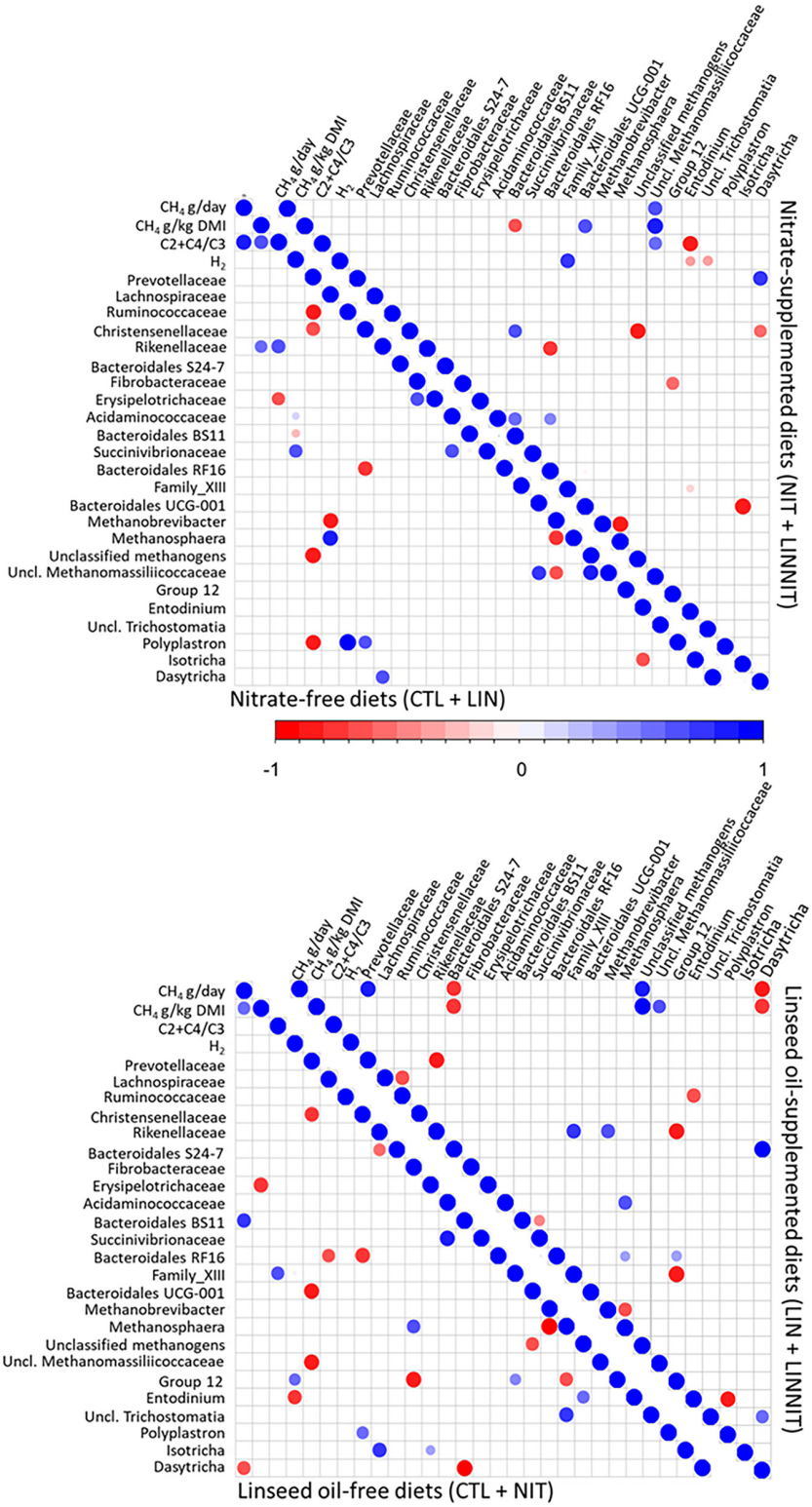

FIG 4 Spearman's rank correlation matrix of the dominant ruminal bacterial families, genera of archaea and protozoa, and fermentation parameters in study 1. Illustrated correlation patterns are for nitrate and linseed supplementations. Listed microbial populations were detected in at least 50% of the rumen samples analyzed and represent at least 1% of the bacterial, archaeal, protozoal, or fungal communities. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colors of the scale bar denote the nature of the correlation, with 1 indicating perfect positive correlation (dark blue) and −1 indicating perfect negative correlation (dark red) between two microbial populations.

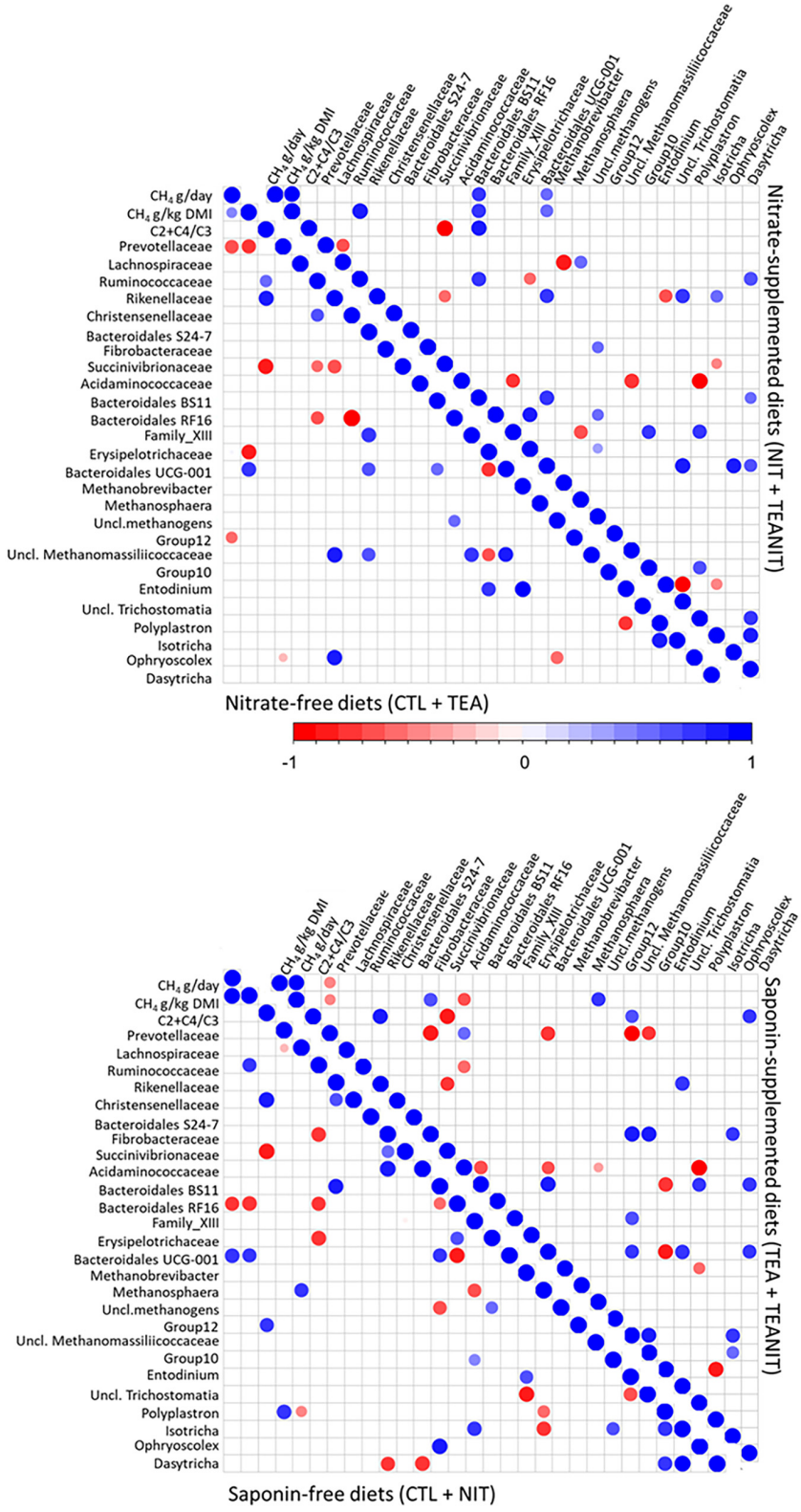

FIG 5 Spearman's rank correlation matrix of the dominant ruminal bacterial families, genera of archaea and protozoa, and fermentation patterns in study 2. Illustrated correlation patterns are for nitrate and tea saponin supplementations. Listed microbial populations were detected in at least 50% of the rumen samples analyzed and represent at least 1% of the bacterial, archaeal, protozoal, or fungal communities. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colors of the scale bar denote the nature of the correlation, with 1 indicating perfect positive correlation (dark blue) and −1 indicating perfect negative correlation (dark red) between two microbial populations.

and *Methanosphaera* independently of nitrate supplementation (R^2 of −0.76 in cows not fed nitrate and R^2 of −0.83 in cows fed nitrate).

Methane production and yield when linseed was fed to cows correlated negatively with the *Bacteroidales* S 24.7 group ($R^2 = -0.76$) and *Dasytricha* ($R^2 = -0.83$) populations and positively with an unclassified archaeal taxon ($R^2 = 0.83$) and unclassified *Methanomassiliicoccaceae* ($R^2 = 0.61$). Independently of linseed supplementation, a negative correlation between *Methanobrevibacter* and *Methanosphaera* was observed ($R^2 = -0.64$ and $R^2 = -0.95$).

In study 2 (Fig. 5), when diets were not supplemented with nitrate, methane production correlated negatively with *Prevotellaceae* ($R^2 = -0.69$) and methanogen group 12 ($R^2 = -0.50$). When diet was supplemented with tea saponin, *Prevotellaceae* correlated negatively with methane production ($R^2 = -0.47$) and yield ($R^2 = -0.43$) as well as with *Fibrobacteraceae* ($R^2 = -0.88$), *Bacteroidales* ($R^2 = -0.76$), and two families of *Methanomassiliicoccaceae* (R^2 of −0.90 for unclassified *Methanomassiliicoccaceae* and R^2 of −0.70 for group 10).

DISCUSSION

Guyader et al. (8) showed that combining dietary strategies acting theoretically on hydrogen production (lipids) and consumption (nitrate) can have an additive effect on methane reduction. In a second study, they confirmed the antimethanogenic potential of nitrate supplementation but observed no effect of tea saponin on methane production (13). These studies were conducted simultaneously; cows were selected at random from the same experimental herd and were randomly allocated to a study. Given the consistency of results for methane production and fermentation patterns reported in the two articles of Guyader et al. (8, 13), we decided to analyze the rumen microbiota from both studies at the same time (from DNA extraction up to statistical tests). Although linseed and nitrate have a medium to high potential methane-mitigating effect (the effect of saponins being less reproductive) (20), microbial data are scarce and inconsistent between studies. This could be explained by different methodologies for rumen sample collection, conservation, and nucleic acid extraction, as well as on how data were obtained and analyzed (21, 22). Thus, second, we compiled the microbial data in order to get insight into the mode of action of nitrate on the rumen microbial ecosystem.

To this aim, we first checked that the microbiota of the two groups of cows was comparable; hence, we performed a detailed analysis of microbial community structure and composition in rumen contents sampled during the period when CTL diet was fed to each animal. No major differences in bacterial communities were observed, except a nonsignificant shift in the *Bacteroidales*/*Clostridiales* ratio, which is known to vary widely across individual animals (23). However, we observed numerical differences in the relative abundance of *Entodinium* (60% in study 1 versus 46% in study 2), which is consistent with enumeration results reported previously (5.71 and 5.38 $\log_{10}$ cells/ml in study 1 and study 2, respectively [8, 13]), showing more abundant ciliate populations in cows from study 1.

In study 1, dietary supplementation with linseed increased the relative abundance of *Selenomonadales*. This is in accordance with our previous work exploring the effects of linseed plus nitrate on rumen microbiota (24) in bulls, where we reported increased numbers in sequences affiliated with three *Selenomonas* genera and one unclassified *Selenomonadales* genus. As these microbes are potential nitrate reducers (25), we hypothesized that their growth was supported by the higher nitrate availability, but the present study suggests that it is a linseed effect. Oleic acid (representing, on average, 20% of linseed oil fatty acids) stimulated the growth of *Selenomonas ruminantium* in pure cultures (26). However, for *in vivo* studies, results are contrasting: *Selenomonas* was among the genera explaining differences in bacterial community structure between lambs fed a linseed diet and those fed a control diet (27), but there was no change in *Selenomonas* abundance when cows were fed sunflower oil (30% oleic acid) (23). Members of the *Selenomonadales* order are also known to reduce succinate to propi-

onate, which is in agreement with a higher molar proportion of propionate in the rumen of cows fed linseed (8). Linseed supplementation also increased abundance of uncultured *Bacteroidetes*, and the *Bacteroidales* S27-7 family was negatively correlated with methane production and yield. On the other hand, linseed diets were characterized by decreased abundance of *Ruminococcaceae*, which is in agreement with previous findings that fatty acids are toxic to these cellulolytic microbes (23, 26, 28). We observed no effect on rumen protozoan numbers (8) and diversity, although *Dasytricha* correlated negatively with methane emissions and positively with the *Bacteroidales* S27-7 family. Linseed oil supplementation also had no effect on the abundance or diversity of the rumen methanogenic community. In accordance with previous results (9, 24), the antimethanogenic potential of linseed oil fatty acids was not related to archaeal numbers in the rumen but rather to a lower metabolic activity of these microbes, which could be explained by lower availability of hydrogen.

Adding tea saponins to the diet had no effect on microbial numbers or on diversity. This is consistent with the lack of changes in methane production or VFA profiles reported by Guyader et al. (13). The efficacy of saponins in suppressing methane production varies considerably depending on the chemical structure, source, dose, and diet (29). Saponins have been reported to inhibit rumen protozoa (5) and, thus, limit hydrogen production in the rumen. However, in our previous work (13) and the study of Ramírez-Restrepo et al. (30), adding tea saponins to ruminants' diets had the opposite effect on protozoan numbers. Saponins break down the membrane of protozoa by interacting with their sterols. However, rumen microbes can degrade the sugar moiety of saponins, rendering them inactive. To improve the antiprotozoal effect of saponins, changing their chemical structure and, thus, protecting them from microbial degradation, was recently proposed (12).

Nitrate supplementation induced changes in the relative abundance of CowPI-generated functional profiles of bacteria, although metabolic pathways were affected in a dissimilar way between studies. This was expected, as predictions are based on 16S rRNA gene data and multivariate analysis of OTU tables also show differences between studies. In study 1, multivariate analysis revealed that nitrate supplementation altered bacterial and archaeal communities. However, in study 2, NMDS and PERMANOVA results were less conclusive, although reductions of methane emissions and changes in fermentation parameters were comparable between experiments. Nevertheless, both studies pinpointed a limited number of taxa associated with decreased methane emissions in nitrate-fed cows. Nitrate supplementation increased the abundance of *Coriobacteriales* and *Burkholderiales* orders, which contain taxa with known nitrate-reducing activity (31–33). This coincides with predicted higher nitrogen metabolism functions and is in accordance with the numerically higher nitrite concentrations measured by Guyader et al. (8, 13) in nitrate-fed cows. Also, cows not fed nitrate presented an enhanced cellulolytic community, which is in accordance with our previous results showing a toxic effect on *Ruminococcaceae* in animals fed linseed plus nitrate diets (24). *Ruminococcus flavefaciens* and *Ruminococcus albus* populations decreased in the rumen of goats when nitrate was added to the diet (25). An *in vitro* study (34) showed that the growth of these two cellulolytic bacteria was inhibited by nitrite at a level of 3 mmol/liter, but measured nitrite levels in our studies rarely exceeded 0.08 mmol/liter (8, 13). Lower concentrations could still be toxic, as another study showed that the specific growth rate of *R. flavefaciens*, but not *R. albus*, was decreased by less than 0.03 mmol/liter of nitrate (35). Marais et al. (35) also argued that nitrite inhibits electron transport systems (*R. flavefaciens*), so bacteria not possessing an electron transport system (*R. albus*) are less affected. *R. flavefaciens* and *R. albus* are the only cultured *Ruminococcus* species able to degrade cellulose (36), making them an important part of a functional rumen ecosystem. *In vitro*, *R. albus* produces acetate, hydrogen, and carbon dioxide, and its metabolic activity is stimulated by the presence of methanogens (37). Thus, reducing *Ruminococcaceae* numbers by nitrate supplementation would decrease the amount of hydrogen produced, which could indirectly reduce methane production. This conclusion is also supported by the decreased

expression levels of the methanogenic *mcrA* gene, which has been shown to correlate with methane emissions (24, 38, 39). However, *Ruminococcaceae* are an important group of bacteria inhabiting the rumen and are able to degrade plant cell wall polysaccharides into metabolizable energy. This implies that inhibition of the rumen fibrolytic community decreases fiber degradation. In the present studies, nitrate supplementation did not affect total tract digestibility (8, 13), but linseed tended to reduce fiber digestibility (8).

We also observed a strong positive correlation between unclassified *Methanomassiliicoccaceae* and methane production when cows were fed nitrate-supplemented diets. Veneman et al. (9) also reported an increase in the abundance of *Methanomassiliicoccaceae*-related methanogens in the rumen of nitrate-fed animals. *Methanomassiliicoccaceae* are obligate hydrogen-dependent methylotrophic methanogens (40), whereas most of the other rumen methanogens perform methanogenesis from hydrogen and carbon dioxide. They are part of a unique methanogen order with a characteristic set of genes involved in the methanogenesis pathway (40). It is likely that their particular physiology confers on them a competitive advantage when the activity of other methanogens is affected in a nitrate/nitrite-enriched environment.

We conducted this study to understand how the rumen microbial ecosystem responds to dietary methane mitigation by linseed, saponin, and nitrate supplementation alone or in combination. We hypothesized that adding linseed or saponins to the diet reduces hydrogen production by a toxic effect on rumen protozoa and by replacing dietary carbohydrates with nonfermentable fatty acids; additionally, we were expecting that nitrate supplementation would redirect hydrogen consumption toward nitrate reduction rather than methanogenesis. Changes in the rumen microbial ecosystem were monitored using archaeon-, bacterium-, eukaryote-, and fungus-specific primers targeting either 16S or 18S rRNA genes and ITS1. Our sequencing strategy allowed us to accurately draw the parallel between changes in methane emissions and microbiota structure. Our study showed that linseed oil decreases methane emissions by reducing the number of hydrogen producers (cellulolytic *Ruminococcaceae*) and by stimulating propionate producers (*Selenomonas*), thereby diverting hydrogen from methanogenesis. Nitrate supplementation favored the development of nitrate-reducing bacteria (*Coriobacteriales* and *Burkholderiales*) and had a negative effect on cellulolytic *Ruminococcaceae*; as a consequence, nitrate supplementation also significantly affected methanogen community structure and activity. In contrast, we did not show any shifts in rumen microbiota structure and activity due to dietary supplementation with tea saponins.

In a secondary aim of our work, we capitalized on data available from two independent studies, expecting to draw relevant conclusions. It is common that studies exploring microbial mechanisms of the same methane abatement strategy come to dissimilar conclusions. Authors generally argue that these differences are due to differences in diet, animal species, physiologic stage, and different sample processing or bioinformatics pipelines. In the present work, we minimized the impact of study design on data interpretation, despite some inconsistent results being observed for nitrate-supplemented diets from study 1 and study 2. Nitrate reduced methanogen activity and stimulated nitrate-reducing bacterial populations in both studies. Similarly, *Ruminococcaceae*-related OTUs characterized nitrate-free diets in both studies. In contrast, multivariate analysis showed that nitrate altered bacterial and archaeal communities in study 1, whereas only a moderate effect on bacteria was observed in study 2. In both experiments, each experimental period lasted 5 weeks. It is possible that microbiota shifted as a result of imposed dietary treatments and did not completely migrate back to the initial state. In a massive rumen contents exchange study, Weimer et al. (41) found that cows almost completely reconstructed their microbiota in 3 weeks, with a complete return to its original host-specific state in 9 weeks. However, pH and VFA profiles returned to the original values much more quickly, within 1 day. We could argue that changes induced by nitrate supplementation were at the level of microbe function rather than species composition. This is supported by the fact that reductions

in methane emissions and shifts in VFA profiles were comparable between studies. A metatranscriptomic approach will be more fruitful to further explore microbial mechanisms of methane mitigation using linseed and/or nitrate.

MATERIALS AND METHODS

The experiments were conducted at the animal facilities of INRA Herbipôle Unit (Saint-Genès Champanelle, France). All procedures involving animals were conducted in accordance with the French Ministry of Agriculture guidelines for animal research and all applicable European guidelines and regulations on animal experimentation. The experiments were approved by the Auvergne Regional Ethics Committee for Animal Experimentation, approval number CE50-12.

Animals, experimental design, and feeding management. Animals and experimental design were described by Guyader et al. (8, 13). Briefly, eight nonlactating Holstein cows were separated into two groups conducted in parallel according to a two-by-two factorial design. Within each study, four cows were randomly assigned to four dietary treatments during 5-week experimental periods. In study 1, diets were on a dry matter (DM) basis: control diet (CTL; 50% natural grassland hay and 50% concentrate), control diet with 4% linseed oil (LIN; 2.6% added fat), control diet with 3% calcium nitrate (NIT; 2.3% nitrate), and control diet with 4% linseed oil plus 3% calcium nitrate (LIN+NIT; 2.6% added fat plus 2.3% nitrate) (8). In study 2, diets were on a DM basis: control diet (CTL; 50% natural grassland hay and 50% concentrate), control diet with 0.77% tea saponin (TEA; 0.5% saponin), control diet with 3% calcium nitrate (NIT; 2.3% nitrate), and control diet with 0.77% tea saponin plus 3% calcium nitrate (TEA+NIT; 0.5% saponin plus 2.3% nitrate) (13). The chemical compositions of the diets CTL and NIT were similar between the two studies. Methane emissions and fermentation parameters are those described in companion papers of Guyader et al. (8, 13).

Rumen content sampling for microbial analysis. At the end of each experimental period, whole rumen content samples (200 g) were taken, through cannula, from multiple sites within the rumen. Sampling was done 3 h after the morning feeding, when methane emission differences between diets measured in the same animal were maximal (42). A part of each sample (~30 g) was mixed with 30 ml ice-cold phosphate-buffered saline (PBS), pH 6.8, and homogenized using a Polytron grinding mill (Kinematica GmbH, Steinhofhalde, Switzerland) for three cycles of 1 min with intervals of 1 min on ice. Approximately 0.5 g was transferred to a 2.5-ml Eppendorf tube and mixed with 1 ml of RNA*later* stabilization solution (Applied Biosystems, Austin, TX, USA). Tubes were immediately stored at −80°C until further processing.

Total nucleic acid extraction and cDNA synthesis. Total nucleic acids (DNA and RNA) were coextracted from all samples by bead beating and phenol-chloroform extraction, followed by saline-alcohol precipitation (43). The yield and purity of extracted DNA and RNA were assessed using a NanoDrop lite spectrophotometer (Thermo Fisher Scientific, Wilmington, DE); RNA integrity was estimated with an Agilent RNA 6000 Nano kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. Following extraction and quality assessment, RNA was reverse transcribed using a reverse transcriptase kit with random primers (Promega, Madison, WI), according to the manufacturer's instructions, on a T-100 thermocycler (Bio-Rad, Hercules, CA).

Quantification and gene expression of microbial communities. Samples from each cow from the two sampling days of each experimental period were pooled by mixing an equal quantity of DNA or equal volumes of cDNA. Quantification of gene targets was performed on microbial DNA and cDNA by quantitative PCR (qPCR) using a StepOnePlus apparatus (Applied Biosystems, Villebon sur Yvette, France). Reactions were run in triplicate in 96-well plates, using 15.5 μl of 1× TaKaRa SYBR *Ex Taq* premix (Lonza, France), 0.25 μmol each forward and reverse primer, and 20 ng of DNA or 2 μl of cDNA in a final volume of 20 μl. Primer description, average amplification efficiency, slope, and R^2 values from qPCR are described in Table S4 in the supplemental material as required by MIQE guidelines for PCR (44). Negative controls without templates were run in each assay to assess overall specificity.

Abundances of total bacteria (based on 16S rRNA gene copies) and methanogens (based on *mcrA* DNA copies) were assessed using absolute quantification as previously described (39). The level of expression of the functional *mcrA* gene (based on *mcrA* cDNA copies) was assessed using the $2^{-\Delta CT}$ method (45) (C_T is threshold cycle) with 16S rRNA gene copies as an internal reference: $2^{-\Delta CT} = 2^{-(CT\ mcrA\ \times\ CT\ rrs)}$.

Technical triplicates were averaged while checking overlaying of amplification plots at the C_T value. Absolute quantification of total bacteria and methanogenic archaea was expressed as $\log_{10}$ 16S rRNA gene and *mcrA* copies/ng extracted DNA, respectively.

Sequencing strategy and data analysis. Approximately 3 μg of extracted DNA was sent to the Roy J. Carver Biotechnology Center (Urbana, IL, USA) for fluidigm amplification and sequencing of bacterial and archaeal 16S rRNA genes, eukaryotic 18S ribosomal DNA (rDNA) for protozoa, and internal transcribed region 1 (ITS1) for fungi (Table S5). The libraries were sequenced on a 250-paired-end MiSeq run and generated 8,249,698 raw reads for bacterial 16S rRNA genes, 1,778,521 for archaeal 16S rRNA genes, 836,803 for ITS1, and 2,245,531 for eukaryotic 18S rDNA (Table S6). Data were analyzed on an in-house Galaxy-based graphic user interface for QIIME (46), PIPITS (47), and IM Tornado (48) (Table S5). All pipelines included a quality control step, removing sequences with Phred scores of <33 and trimming based on expected amplicon lengths, as well as merging paired reads, chimera search, and removal and OTU picking (Table S6). Merging paired-end archaeal 16S rRNA gene reads was performed by mothur's (49) make.contigs command before input in the QIIME pipeline. Taxonomic classification for *Bacteria* and *Protozoa* was based on the SILVA v123 database (50), for *Archaea* on RIM-DB (51), and for fungi on the UNITE database (52). CowPI (53), the rumen microbiome-focused version of PICRUSt (Phylogenetic

Investigation of Communities by Reconstruction of Unobserved States) (54), was used to predict dietary treatment-induced changes in bacterial metabolic profiles.

Statistical analysis. Results from qPCR quantification, relative abundance (after square root transformation) of microbes at different taxonomic levels, diversity indices, and CowPI functional gene relative abundances were analyzed by analysis of variance (ANOVA) in R (version 3.4.0). The statistical model included the random effect of cow ($n = 4$); fixed effect of period ($n = 4$); contrasts for nitrate (CTL and LIN versus NIT and LIN+NIT in study 1, CTL and TEA versus NIT and TEA+NIT in study 2), linseed (CTL and NIT versus LIN and LIN+NIT in study 1), and tea saponin (CTL and NIT versus TEA and TEA+NIT in study 2); and the interaction linseed×nitrate or saponin×nitrate. Significance was considered at a P value of ≤ 0.05. Trends were discussed at $0.05 < P \leq 0.1$. Least-square means are reported throughout.

OTUs with fewer than 3 sequences were withdrawn from further analysis. OTU tables were imported in R and rarefied to minimize the variations created by different sample depths of subsampling. Further analysis was performed using the vegan R package (55). Alpha diversity values for all microbial communities were obtained using various diversity indices (Shannon and Simpson diversity indices, richness, and evenness) and analyzed by ANOVA for the effect of contrasts and the interactions described above. NMDS was used to ordinate microbial libraries (4 cows and 4 experimental periods per study and per microbial group). We used the betadisper function to check the homogeneity of group dispersions before performing a PERMANOVA analysis via the Adonis function of vegan. The multipatt function from R package indicspecies (56) was used to find indicator OTUs using a 5% significance level for selecting indicators in cows fed linseed, tea saponin, and nitrate. The species-site group association parameter was IndVal.g.

Correlation analyses between microbial populations and some fermentation parameters (methane, hydrogen, and VFA ratio) were performed in R. Only microbial groups that represented more than 1% (average of all samples) of the total community within each of the three microbial groups (bacteria, archaea, or protozoa) and that were detected in at least 50% of rumen samples were included in the analysis. Spearman's rank correlations and P values were calculated by the above-described contrasts and plotted using the packages hmisc (57) and corrplot (58).

Accession number(s). Raw sequence data are available in the Sequence Read Archive (SRA) under BioProject ID PRJNA415383.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02657-18.

SUPPLEMENTAL FILE 1, PDF file, 1.3 MB.

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