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Increasing linseed supply in dairy cow diets based on hay or corn silage: Effect on enteric methane emission, rumen microbial fermentation, and digestion

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ABSTRACT

We investigated the effects of increasing extruded linseed supply in diets based on hay (H; experiment 1) or corn silage (CS; experiment 2) on enteric methane (CH_4) emission, rumen microbial and fermentation parameters, and rumen and total-tract digestibility. In each experiment, 4 lactating Holstein cows fitted with cannulas at the rumen and proximal duodenum were used in a 4×4 Latin square design (28-d periods). Cows were fed ad libitum a diet [50:50 and 60:40 forage:concentrate on a dry matter (DM) basis for experiments 1 and 2, respectively] without supplementation (H0, CS0) or supplemented with extruded linseed at 5% (H5, CS5), 10% (H10, CS10), and 15% (H15, CS15) of dietary DM (i.e., 1.8, 3.6 and 5.4% total fatty acids added, respectively). All measurements were carried out during the last 8 d of each period. Linseed supply linearly decreased daily CH_4 emission in cows fed H diets (from 486 to 289 g/d for H0 to H15, on average) and CS diets (from 354 to 207 g/d for CS0 to CS15, on average). The average decrease in CH_4 per kilogram of DM intake was, respectively, -7 , -15 , and -38% for H5, H10, H15 compared with the H0 diet, and -4 , -8 , and -34% for CS5, CS10, and CS15 compared with the CS0 diet. The same dose-response effect was observed on CH_4 emission in percent of gross energy intake, per kilogram of nutrient digested, and per kilogram of 4% fat- and 3.3% protein-corrected milk (FPCM) in both experiments. Changes in the composition of rumen volatile fatty acids in response to increasing linseed supply resulted in a moderate or marked linear decrease in acetate:propionate ratio for H or CS diets, respectively. The depressive effect of linseed on total protozoa concentration was linear for H diets (-15 to -40% , on average, for H5 to H15 compared with H0)

and quadratic for CS diets (-17 to -83% , on average, for CS5 to CS15 compared with CS0). Concentration of methanogens was similar among H or CS diets. The energetic benefits from the decreased CH_4 emission with linseed supply in diets based on hay or corn silage did not improve digestibility or milk yield. Milk efficiency (kg of FPCM/kg of DM intake) was improved with linseed supply up to H10 in H diets and was unchanged in CS diets. Lower CH_4 enteric emission from dairy cows fed linseed helps limit the environmental footprint of ruminant livestock.

Key words: dairy cow, digestion, hay or corn silage, linseed, methane

INTRODUCTION

Methane (CH_4) released by ruminants is the main greenhouse gas at the farm level (Veyssset et al., 2010) and constitutes an energetic loss for the animal ranging from 2 to 12% of its gross energy (GE) intake (Johnson and Johnson, 1995). Decreasing enteric CH_4 emission from ruminants without altering animal production is desirable both as a strategy to reduce global greenhouse gas emissions, and thus the negative environmental impact of livestock, and as a means of improving feed conversion efficiency.

Several reviews have reported that dietary fatty acid (FA) supply (Grainger and Beauchemin, 2011; Hristov et al., 2013), particularly FA from linseed (Martin et al., 2010), is one of the most efficient dietary strategies to mitigate enteric CH_4 emission from dairy cows. In addition, linseed supply to dairy cow diets helps improve the nutritional value of milk through a slight increase in linolenic acid (Doreau et al., 2011). The extent of the linseed CH_4 -mitigating effect varies depending on many factors, including FA availability in the rumen and the dose included in the diet (Martin et al., 2010). Low supply of linseed oil ($<2\%$ added FA) did not modulate CH_4 emission in dairy cows (Livingstone et al., 2015), whereas higher supply (3.7–5.7% added FA) of differ-

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ent linseed forms (crude, crushed, or extruded whole grains, or oil) resulted in a lower CH₄ yield (g/kg of DM intake) but had deleterious effects on OM and fiber digestibility and, for the highest FA level, impaired intake and dairy cow performance (Martin et al., 2008; Beauchemin et al., 2009b). In addition, the extent of the effect of linseed supply on CH₄ emission may also depend on diet composition, especially the nature of forage and the percentage of concentrate (Martin et al., 2010). Only Chung et al. (2011) compared the effect of linseed with different forage-based diets fed to dry cows, and found a decrease in CH₄ emission with barley silage, but not with hay. To our knowledge, the dose-response effect of dietary linseed FA on CH₄ emission has not yet been studied. In addition, only a few *in vivo* studies have considered, in the same experiment, the effects of linseed FA supply on both CH₄ emission and the rumen fermentation and microbial processes involved.

To address these questions, we performed 2 experiments in dairy cows, the first with a hay-based diet and the second with a corn silage-based diet. These diets are representative of winter diets used frequently in Europe for dairy cows. Diets were supplemented with extruded linseed at 3 levels of FA supply (1.8, 3.6, and 5.4% of DM). The highest FA supply used here is not currently recommended at the farm level, but the wide variation in FA level was chosen to establish the dose-response relationship regarding enteric CH₄ emission and associated digestive processes such as rumen fermentation and microbial parameters and rumen and total-tract digestibility of diets. Data on milk production and composition have been published previously (Ferlay et al., 2013).

MATERIALS AND METHODS

Two experiments were performed during 2 successive years at the animal experimental facilities of the Herbivores Research Unit at Institut National de la Recherche Agronomique (INRA, Saint-Genès-Champagne, France). Procedures on animals were in accordance with the guidelines for animal research of the French Ministry of Agriculture and all other applicable national and European guidelines and regulations for experimentation with animals (see http://www2.vet-lyon.fr/ens/expa/acc_regl.html for details).

Animals, Experimental Design, and Diets

The main description of the experiments are given below. Additional details are found in Ferlay et al. (2013). In each experiment, 4 lactating multiparous Holstein

cows fitted with rumen and proximal duodenum cannulas were used after lactation peak (experiment 1: 117 ± 26 DIM; experiment 2: 96 ± 29 DIM) in 4×4 Latin square designs. Each experimental period lasted 28 d with the first 5 d as a transition period between treatments and the last 8 d in wk 4 (d 1 to 8) as a measurement period. Cows were housed in a tiestall barn during the whole experiment.

Linseeds were supplied as extruded linseed mixture (70% linseed, 30% wheat bran, Valorex, Combourtillé, France) at 0, 7, 14, or 21% of inclusion in the diet, which represented 0, 5, 10, or 15% of linseed in dietary DM. This mixture was included in the diets substituting for expeller linseed meal, wheat bran, and corn grain, and it provided a supply level of 1.8, 3.6, or 5.4% FA for 5, 10, and 15% of linseed, respectively. In experiment 1, the diet was composed of 50% hay and 50% concentrates on a DM basis; the experimental diets were called **H0**, **H5**, **H10**, and **H15**, according to the level of inclusion of linseeds. In experiment 2, the diet was composed of 60% forages (55.5% corn silage plus 4.5% hay) and 40% concentrates on a DM basis; experimental diets were called **CS0**, **CS5**, **CS10**, and **CS15** according to the level of inclusion of linseeds. Diets were given *ad libitum*. In both experiments, concentrates were offered at 60 and 40% of the daily amount at 0900 and 1630 h, respectively. In experiment 1, hay was offered in equal amounts at 0900, 1330, and 1630 h. In experiment 2, forages was offered in equal amounts at 0900 and 1630 h. The forage:concentrate ratio was maintained as close as possible to the target by adjusting the amount of feed offered daily. Ingredient and chemical composition of experimental diets as consumed are presented in Table 1. The diets were formulated to cover 105% of the INRA energy and protein requirements for maintenance and lactation of dairy cows (INRA, 2007).

Measurements

Intake, Milk Production, and BW. Intake and milk production were recorded daily by weighing throughout the experiment. Feeds (offered and refused) and milk were sampled in wk 4 until biochemical analyses as described in Ferlay et al. (2013). Animals were weighed at the beginning and the end of the experiment.

Total-Tract and Rumen Digestibility. Total-tract digestibility was measured by total feces collection from d2 to d7 of each experimental period. Duodenal flow was determined using ytterbium chloride (YbCl₃) as described by Fanchone et al. (2013). Briefly, a YbCl₃ solution was infused continuously into the rumen (1.2 g of Yb daily) via the rumen cannula using a peristaltic

Table 1. Composition of experimental diets as consumed based on hay (H; experiment 1) or corn silage (CS; experiment 2) without supplemented extruded linseed (H0, CS0) or supplemented with extruded linseed at 5% (H5, CS5), 10% (H10, CS10) or 15% (H15, CS15) of dietary DM

Item	Hay-based diet (experiment 1)				Corn silage-based diet (experiment 2)			
	H0	H5	H10	H15	CS0	CS5	CS10	CS15
Ingredient, % of DM								
Hay ¹	49.6	49.3	49.4	50.2	4.5	4.4	4.3	4.8
Corn silage ²	0	0	0	0	55.7	55.6	55.3	55.4
Dairy concentrate ³	27.7	27.9	27.7	27.3	12.1	11.7	12.1	11.6
Soybean meal	0	0	0	0	6.0	6.1	6.1	6.0
Corn grain	6.3	4.5	2.6	0.8	6.0	4.0	1.8	0
Wheat bran	6.3	4.2	2.1	0	6.0	4.1	1.8	0
Linseed meal	9.2	6.2	3.1	0	8.8	6.0	3.3	0
Extruded linseed:wheat bran (70:30)	0	7.0	14.1	20.8	0	7.1	14.2	21.2
Mineral-vitamin mix ⁴	0.8	0.9	0.9	0.9	1.0	1.0	1.0	1.1
Chemical composition, % of DM								
OM	92.1	92.1	92.1	93.1	92.6	92.9	93.2	93.7
NDF	46.8	47.1	47.6	48.5	40.5	41.5	42.4	43.6
ADF	20.6	20.9	21.3	21.9	19.3	20.3	21.3	22.3
Starch	11.9	11.4	10.6	10.1	27.1	25.6	24.1	22.9
Nitrogen	2.2	2.2	2.2	2.2	2.3	2.3	2.3	2.3
Fatty acids (FA)	2.3	4.1	6.0	7.6	2.8	4.6	6.3	7.9
Gross energy, MJ/kg of DM	18.2	18.6	19.0	19.6	20.5	20.7	20.9	21.1

¹Hay (% of DM): OM (92.5), NDF (61.9), ADF (31.4), nitrogen (1.3), FA (1.1) for experiment 1; OM (93.3), NDF (63.5), ADF (36.8), nitrogen (1.3), FA (1.2) for experiment 2.

²Corn silage (% of DM): OM (95.2), NDF (49.7), ADF (24.5), starch (31.0), nitrogen (1.2), FA (2.6) for experiment 2.

³Dairy concentrate (% of DM): OM (92.3), NDF (31.1), ADF (10.7), starch (28.1), nitrogen (2.9), FA (1.4) for experiment 1; OM (92.3), NDF (24.2), ADF (11.0), starch (26.5), nitrogen (2.9), FA (1.2) for experiment 2.

⁴Minerals (%), P (0.8), Ca (1), Mg (0.5), Na (0.5), Cu (0.015); trace elements (mg/kg): Cu (15); vitamins (IU/kg): vitamin A (6,000), vitamin D₃ (1,250), and vitamin E (10 mg/kg).

pump from 6 d before duodenal samplings to d 6 to ensure a steady state before sampling. Sixteen 250-mL duodenal samples were collected day and night from d 5 to 7 and then pooled, providing representative samples of duodenal contents, which were frozen at -20°C . After thawing, subsamples were used for nitrogen (N) and ammonia N (N-NH_3) determination, and the rest of the sample was lyophilized for OM, NDF, ADF, starch, and Yb determination. Feces were separated from urine by a device diverting urine in a bottle, removed once daily, and then weighed and mixed. A 1% feces aliquot was sampled for DM determination (60°C for 72 h) and then pooled for the whole period, and was kept for OM, NDF, ADF, starch, and Yb determinations. A 0.5% feces aliquot was sampled for N and Yb determination and frozen at -20°C until analyses.

Methane Emission. Methane emission was determined from d 1 to 4 according to the sulfur hexafluoride (SF_6) tracer technique described by Martin et al. (2008). Brass permeation tubes were filled with approximately 600 mg of SF_6 gas while tubes were kept in liquid N_2 and then calibrated for 10 wk. Permeation rates of tubes averaged 1.720 ± 0.318 mg/d for experiment 1 and 1.334 ± 0.319 mg/d for experiment 2. Tubes were introduced in the rumen 2 wk before the beginning of

measurements and remained in the rumen throughout the experiment. Representative breath samples of each animal were collected daily in evacuated collection devices by a capillary tubing fitted to a halter. Ambient air samples were collected once daily in the shed during the 4 d of sampling breath gas.

Rumen Fermentation Parameters and Microbial Ecosystem. On d 6 and 7, 300 g of rumen contents were collected by hand from different sites of the rumen just before and 2.5 h after the morning feeding. Samples were immediately strained through a 250- μm -pore nylon filter to obtain rumen liquid and solid phase fractions. The liquid phase was used for pH determination using a digital pH meter (CG837, Ag/AgCl electrode, Schott Geräte, Hofheim, Germany) and then sampled as follows. First, 0.5 mL of 5% H_3PO_4 was added to a 5-mL sample and then frozen before VFA analysis. Then, 4 mL of 20% NaCl were added to a 1-mL sample and frozen before N-NH_3 determination. Five milliliters of a methyl green-formalin-salt solution (0.92 mM methyl green, 0.14 M sodium chloride, 35 mL/L formaldehyde) were added to a 5-mL sample and shielded from light at room temperature until protozoa counting. In addition, before feeding, a 10-mL sample of liquid phase and a 30-g sample of solid phase were

stored without preservative in sterile tube at -20°C (Mosoni et al., 2007) until microbial analyses by quantitative PCR (qPCR).

Analyses

Biochemical Analyses. Organic matter was determined by ashing at 550°C for 6 h, N by the Kjeldahl procedure, and starch using a polarimetric method (AOAC International, 1997). Fiber (NDF and ADF) was determined by sequential procedure (Van Soest et al., 1991) after pretreatment with amylase and was expressed inclusively of residual ash. Gross energy was determined using an adiabatic bomb calorimeter (Gallenkamp Autobomb; Loughborough, UK).

Ytterbium was determined in feces and duodenal contents by atomic absorption spectrophotometry (model 2380 spectrophotometer, PerkinElmer, Waltham, MA) at a wavelength of 398.8 nm with an acetylene/ N_2O flame after extraction of the marker from lyophilized samples (Hart and Polan, 1984). Duodenal DM flow was calculated from fecal DM output and Yb concentrations in duodenum and feces.

Concentrations of SF_6 and CH_4 in breath and ambient air samples were determined by gas chromatography as described by Martin et al. (2008). A gas chromatograph (Varian-Chrompack, CP-9003, Les Ulis, France) fitted with an electron capture detector and a gas chromatograph (PerkinElmer; Autosystem XL, Courtaboeuf, France) fitted with a flame-ionization detector were used to determine SF_6 and CH_4 concentrations, respectively. The samples were run on chromatographs equipped either with a Molecular Sieve 0.5 nm column ($3\text{ m} \times 3.2\text{ mm}$ internal diameter; Interchim, Montluçon, France) maintained at 60°C for the SF_6 analysis or with a Porapak N 80-100 mesh column ($3\text{ m} \times 3.2\text{ mm}$ internal diameter; Alltech, Templemars, France) maintained at 40°C for the CH_4 analysis. Flow rate of the carrier gases was 30 mL/min of N_2 and 40 mL/min of helium for the SF_6 and the CH_4 analyses, respectively. Chromatographic analyses were performed after calibration with standard gases (Air Liquide, Mitry-Mory, France) for SF_6 (195 ppt) and CH_4 (99.9 ppm). Daily CH_4 emission by each animal was calculated using the permeation rate of SF_6 for each animal and the concentrations (above background) of SF_6 and CH_4 in the breath samples, as follows: $\text{CH}_4\text{ (g/d)} = \text{SF}_6\text{ permeation rate (g/d)} \times [\text{CH}_4]/[\text{SF}_6]$, where gas concentrations are expressed in micrograms per cubic meter.

Volatile fatty acids were analyzed in rumen fluid by gas chromatography (CG 8000 gas chromatography, Carlo Erba Instruments, Milan, Italy) using a fused-silica column (length 25 m, inner diameter 0.25 mm)

CP-wax 58 FFAP CB (Varian, Middelburg, the Netherlands) and 4-methylvaleric acid as internal standard (Jouany, 1982). Ammonia N was determined in rumen fluid and duodenal contents by the colorimetric method of Weatherburn (1967) using an autoanalyzer (System II, Technicon Instruments, Tarrytown, NY).

Microbial Analyses. Total DNA was extracted in duplicate from 1-mL liquid and 0.250-g solid phases of the rumen content using the method described by Yu and Morrison (2004). Major cellulolytic bacteria—*Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *Ruminococcus albus*—as well as methanogenic *Archaea* were quantified by targeting *rrs* and *mcrA* genes, respectively, using the qPCR conditions described by Mosoni et al. (2011). Data were expressed as copy number of target gene per microgram of DNA. Protozoal numbers in the rumen fluid (cells/mL) were counted by microscopy using a Dolfuss cell (Elvetec Services, Clermont-Ferrand, France) according to the procedure described in Jouany and Senaud (1982) and were categorized as either entodiniomorphs or holotrichs ciliates (Williams and Coleman, 1992).

Statistical Analyses

Before statistical analyses, data of each variable collected on several days in wk 4 were averaged and microbial data were \log_{10} transformed to achieve normal distribution of the residuals. For each experiment, data were analyzed as 4×4 Latin squares using the Mixed procedure of SAS (SAS Institute, 2003). The statistical models included the random effect of cow and fixed effects of period ($n = 4$), diet ($n = 4$) for some variables (intake, milk production, CH_4 emission, digestibility and microbial qPCR), and sampling time ($n = 2$) and the interaction diet \times sampling time for repeated variables (rumen fermentation and protozoa). Variance component was the covariance structure used in both models. Linear and quadratic contrast statements were used to test the effect of increasing amounts of extruded linseed. Linear or quadratic effects are denoted as P_L or P_Q , respectively. Least squares means are reported with the standard error of the mean derived from the model. Data were considered significant if $P \leq 0.05$ and trends were presented at $0.05 < P < 0.10$.

RESULTS

Experiment 1

Final BW of cows was similar to initial BW (655 ± 68.2 and 655 ± 55.0 kg, respectively) in experiment 1. Increasing supply of extruded linseed in the hay-based diets did not significantly modify intake of DM,

OM, NDF, N, or GE (Table 2), whereas starch intake decreased linearly with linseed supply ($P_L < 0.001$). Milk yield and 4% fat- and 3.3% protein-corrected milk (FPCM) were similar among diets based on hay, whereas a curvilinear decrease was reported for milk efficiency (kg of FPCM/kg of DMI; $P_Q = 0.04$), the lowest value being reported for H15 and the highest for H10 (Table 2).

Nutrient digestibilities in the rumen (DM, OM, NDF) and NAN duodenal flow were unaffected by linseed supply of H diets, except starch digestion, which increased ($P_L = 0.03$; Table 3). Total-tract digestibility of all nutrients was unaffected by linseed supply of H diets (Table 3). Increasing linseed supply decreased linearly daily CH_4 emission (g/d) in cows fed H diets from 486 to 289 g/d for H0 to H15 ($P_L < 0.01$; Table 4). Expressed in grams per kilogram of DMI, the extent of the decrease in CH_4 was, respectively, -7 , -15 , and -38% with H5, H10, and H15 compared with H0, the decrease being more important for diets supplied with 15% of extruded linseed. The same significant response to increasing supply of extruded linseed was observed for CH_4 emission expressed in proportion of GE intake ($P_L < 0.01$ and $P_Q = 0.03$), per kilogram of nutrient digested ($P_L < 0.01$), and per kilogram of milk or FPCM ($0.03 \leq P_L \leq 0.02$).

The effect of linseed supply on rumen fermentation and protozoa parameters was similar at both sampling times (sampling time \times diet interaction not significant). Rumen pH, total VFA, and N-NH_3 concentrations were similar among H diets (Table 5). Acetate (C2) proportions were similar with increasing linseed supply, whereas propionate (C3) and butyrate (C4) proportions were affected ($P_L = 0.02$), involving a significant decrease in C2/C3 and (C2 + C4)/C3 ratios ($P_L \leq 0.04$). For protozoa, increasing linseed supply linearly depressed their total numbers from -15 to -40% on average for H5 to H15 compared with H0 ($P_L < 0.01$; Table 5). The concentration of the 3 cellulolytic bacteria in the liquid and solid phases of the rumen content was unaffected before feeding by linseed supply of H diet; only *F. succinogenes* tended to decrease in the solid phase ($P_L = 0.06$; Table 6). The concentration of methanogens was unaffected by linseed supply in the liquid phase and tended to be lower for H10 compared with other diets in the solid phase ($P_Q = 0.06$; Table 6).

Experiment 2

Body weight of cows fed CS diets did not change during experiment 2 (initial and final BW: 668 ± 95.7 and 665 ± 108.9 kg). Increasing supply of extruded linseed in the corn silage-based diets did not significantly modify intake of DM, OM, NDF, and N (Table

Table 2. Intake and milk production in lactating cows receiving natural grassland hay diet (H; experiment 1) or corn silage diet (CS; experiment 2) without supplemented extruded linseed (H0, CS0) or supplemented with extruded linseed at 5% (H5, CS5), 10% (H10, CS10), or 15% (H15, CS15) of DM

Item	Hay-based diet (experiment 1)						Corn silage-based diet (experiment 2)						Contrast <i>P</i> -value	
	H0	H5	H10	H15	SEM	Contrast <i>P</i> -value ¹			SEM	CS15	CS10	CS5		
						L	Q							
Intake, kg/d														
DM	20.8	18.9	19.9	19.9	0.70	0.55	0.15	18.7	18.4	17.7	16.7	1.08	0.14	0.68
OM	19.1	17.5	18.3	18.4	0.65	0.62	0.14	17.3	17.1	16.5	15.7	1.02	0.18	0.68
NDF	9.71	8.89	9.46	9.60	0.334	0.86	0.13	7.63	7.65	7.52	7.29	0.570	0.50	0.75
Starch	2.35	2.02	1.99	1.86	0.066	<0.001	0.10	5.07	4.73	4.28	3.82	0.335	0.01	0.83
Nitrogen	0.46	0.41	0.43	0.42	0.015	0.18	0.17	0.43	0.42	0.41	0.38	0.023	0.10	0.56
Gross energy intake, MJ/d	378.4	350.9	378.3	385.6	13.35	0.35	0.16	383.3	381.7	371.1	353.4	22.78	0.26	0.67
Milk														
Milk yield, kg/d	26.1	25.8	28.3	26.4	1.68	0.58	0.59	25.3	22.6	23.6	25.1	2.62	0.96	0.43
FPCM, ² kg/d	24.5	23.7	25.8	22.6	1.66	0.49	0.30	23.0	18.7	20.9	22.3	2.78	0.25	0.49
Milk efficiency ³	1.18	1.26	1.29	1.13	0.071	0.63	0.03	1.23	1.02	1.21	1.34	0.156	0.13	0.34

¹L = linear; Q = quadratic.

²FPCM = milk converted to fat and protein with 4% fat and 3.3% protein according to the equation of Gerber et al. (2011).

³Milk efficiency = FPCM/DMI.

Table 3. Intake, rumen and total-tract digestibility in lactating cows receiving natural grassland hay diet (H; experiment 1) or corn silage diet (CS; experiment 2) without supplemented extruded linseed (H0, CS0) or supplemented with extruded linseed at 5% (H5, CS5), 10% (H10, CS10), or 15% (H15, CS15) of DM

Item	Hay-based diet (experiment 1)						Corn silage-based diet (experiment 2)					
	Contrast <i>P</i> -value ¹						Contrast <i>P</i> -value					
	H0	H5	H10	H15	SEM	Q	L	Q	SEM	CS15	CS10	CS5
DM												
Rumen digestibility, ² %	35.1	37.6	35.6	33.8	4.03	0.57	0.73	0.57	4.47	29.1	42.2	41.2
Total-tract digestibility, %	70.5	68.8	70.5	69.8	0.84	0.58	0.89	0.58	1.84	65.4	66.6	67.8
OM												
Rumen digestibility, %	43.7	47.0	44.4	43.6	3.43	0.53	0.84	0.53	4.38	36.3	49.1	48.0
Total-tract digestibility, %	72.1	70.5	72.1	71.4	0.84	0.61	0.92	0.61	1.84	67.3	68.2	69.3
NDF												
Rumen digestibility, %	53.3	59.9	61.7	57.0	4.76	0.28	0.57	0.28	6.41	44.4	50.4	56.1
Total-tract digestibility, %	61.4	60.8	63.5	61.9	1.29	0.66	0.45	0.66	2.76	49.3	50.1	55.5
Starch												
Rumen digestibility, %	65.9	77.2	78.3	84.9	4.51	0.62	0.03	0.62	3.22	72.6	85.8	83.1
Total-tract digestibility, %	97.5	96.9	97.5	97.7	0.96	0.29	0.45	0.29	0.61	93.5	93.5	92.8
Nitrogen												
NAN duodenal flow/N intake, %	1.22	1.16	1.18	1.17	0.088	0.73	0.64	0.73	0.065	0.99	0.84	0.97
Total-tract digestibility, %	67.3	67.1	66.0	67.4	0.70	0.31	0.78	0.31	1.78	67.5	67.3	65.7

¹L = linear; Q = quadratic.²Rumen digestibility = apparent rumen digestibility.**Table 4.** Methane emission in lactating cows receiving natural grassland hay diet (H; experiment 1) or corn silage diet (CS; experiment 2) without supplemented extruded linseed (H0, CS0) or supplemented with extruded linseed at 5% (H5, CS5), 10% (H10, CS10), or 15% (H15, CS15) of DM

Item	Hay-based diet (experiment 1)						Corn silage-based diet (experiment 2)					
	Contrast <i>P</i> -value ¹						Contrast <i>P</i> -value					
	H0	H5	H10	H15	SEM	Q	L	Q	SEM	CS15	CS10	CS5
CH ₄ , g/d	485.6	411.2	394.6	289.5	29.90	0.58	0.002	0.58	36.24	206.8	306.8	332.7
CH ₄ , % of gross energy intake	7.1	6.5	5.8	4.1	0.42	0.03	0.002	0.03	0.32	3.2	4.5	4.8
CH ₄ , g/kg of DM intake	23.4	21.8	19.9	14.6	1.45	0.25	0.005	0.25	1.26	12.3	17.3	18.0
CH ₄ , g/kg of OM intake	25.5	23.7	21.5	15.8	1.56	0.24	0.004	0.24	1.34	13.1	18.5	19.4
CH ₄ , g/kg of NDF intake	49.9	46.4	41.7	29.8	3.07	0.22	0.003	0.22	2.53	28.2	40.5	43.5
CH ₄ , g/kg of digested DM	33.3	31.7	28.2	20.9	2.56	0.23	0.006	0.23	2.08	18.9	26.1	27.2
CH ₄ , g/kg of digested OM	35.4	33.7	29.9	22.1	2.25	0.22	0.005	0.22	2.18	19.6	27.3	27.9
CH ₄ , g/kg of digested NDF	80.1	76.5	66.3	47.8	5.33	0.23	0.004	0.23	8.02	58.2	81.9	78.3
CH ₄ , g/kg of milk	18.6	16.9	14.2	11.7	1.60	0.81	0.02	0.81	1.65	9.1	13.6	14.1
CH ₄ , g/kg of FPCM ²	19.8	17.8	15.5	13.6	1.73	0.96	0.03	0.96	2.35	10.3	15.4	18.7

¹L = linear; Q = quadratic.²FPCM = milk converted to fat and protein with 4% fat and 3.3% protein according to the equation of Gerber et al. (2011).

Table 5. Rumen pH, fermentative parameters and protozoa concentration in lactating cows receiving natural grassland hay (H; experiment 1) or corn silage (CS; experiment 2) diets without supplemented extruded linseed (H0, CS0) or supplemented with extruded linseed at 5% (H5, CS5), 10% (H10, CS10), or 15% (H15, CS15) of DM

Item ¹	Hay-based diet (experiment 1)							Corn silage-based diet (experiment 2)						
	Contrast P -value ²							Contrast P -value						
	H0	H5	H10	H15	SEM	L	Q	CS0	CS5	CS10	CS15	SEM	L	Q
pH	6.42	6.44	6.46	6.46	0.083	0.71	0.96	6.60	6.58	6.77	6.85	0.09	0.03	0.71
Total VFA, mM	106.0	107.7	112.3	110.3	6.31	0.53	0.77	96.4	95.2	95.8	88.2	3.55	0.15	0.38
Acetate (C2), %	63.4	62.6	62.8	62.1	0.69	0.24	0.95	64.5	63.0	60.9	59.8	0.80	<0.001	0.82
Propionate (C3), %	20.2	21.2	21.3	22.6	0.79	0.02	0.82	20.0	21.2	22.3	25.5	0.64	<0.001	0.08
Butyrate (C4), %	12.7	12.4	12.2	11.4	0.51	0.02	0.48	11.8	11.9	11.7	9.3	0.63	0.004	0.03
C2/C3	3.19	3.01	2.99	2.82	0.133	0.04	0.98	3.36	3.05	2.89	2.44	0.102	<0.001	0.49
(C2+C4)/C3	3.83	3.60	3.58	3.34	0.166	0.02	0.96	3.94	3.61	3.41	2.80	0.113	<0.001	0.19
N-NH ₃ , mg/L	100.0	95.0	97.1	111.4	14.81	0.52	0.45	117.7	110.9	113.6	102.1	11.05	0.31	0.82
Protozoa, log ₁₀ cells/mL														
Total	5.61	5.55	5.55	5.38	0.045	0.002	0.24	5.68	5.59	5.44	4.73	0.103	<0.001	0.002
Entodiniomorphs	5.60	5.54	5.54	5.37	0.045	0.003	0.23	5.68	5.58	5.44	4.73	0.103	<0.001	0.003
Holotrichs	3.85	3.73	3.68	3.61	0.103	0.02	0.62	1.81	2.66	1.87	0.77	0.642	0.08	0.06

¹Average data for the 2 sampling times (0 and 2.5 h after feeding). No sampling time \times diet interactions were found for any of the variables listed except for total VFA in diets CS ($P_L < 0.01$ and $P_Q = 0.65$ at 0 h; $P_L = 0.13$ and $P_Q = 0.42$ at 2.5 h).

²L = linear; Q = quadratic.

Table 6. Rumen methanogens and cellulolytic bacteria concentrations¹ in lactating cows receiving natural grassland hay (H; experiment 1) or corn silage (CS; experiment 2) diets without supplemented extruded linseed (H0, CS0) or supplemented with extruded linseed at 5% (H5, CS5), 10% (H10, CS10), or 15% (H15, CS15) of DM

Item ¹	Hay-based diet (experiment 1)							Corn silage-based diet (experiment 2)						
	Contrast P -value ²							Contrast P -value						
	H0	H5	H10	H15	SEM	L	Q	CS0	CS5	CS10	CS15	SEM	L	Q
Methanogens, log ₁₀ <i>mcrA</i> copies/ μ g of DNA														
Liquid	7.32	7.29	7.25	7.30	0.048	0.41	0.69	8.42	8.44	8.20	7.95	0.074	0.66	0.17
Solid	7.17	7.16	7.08	7.19	0.032	0.87	0.06	8.23	8.25	8.17	8.29	0.084	0.78	0.58
Cellulolytic bacteria, log ₁₀ <i>rrs</i> copies/ μ g of DNA														
<i>Fibrobacter succinogenes</i>														
Liquid	7.15	7.14	7.21	7.28	0.089	0.14	0.55	7.08	6.82	6.42	6.94	0.286	0.15	0.67
Solid	7.00	6.86	6.65	6.77	0.094	0.06	0.18	7.37	7.02	6.70	6.70	0.424	0.26	0.68
<i>Ruminococcus flavefaciens</i>														
Liquid	5.82	5.96	5.99	6.02	0.103	0.10	0.47	5.84	6.27	5.96	6.19	0.226	0.67	0.17
Solid	6.25	6.36	6.55	6.43	0.182	0.37	0.50	7.78	7.98	7.86	7.97	0.163	0.53	0.80
<i>Ruminococcus albus</i>														
Liquid	5.71	5.79	5.78	5.90	0.56	0.88	0.59	6.12	6.27	6.40	6.30	0.202	0.67	0.17
Solid	5.36	5.44	5.48	5.39	0.158	0.77	0.46	5.93	6.62	6.43	6.27	0.161	0.29	0.04

¹Data from different fractions of the rumen content (liquid and solid phase) sampled before feeding (0 h).

²L = linear; Q = quadratic.

2), even though a numerical and linear decrease was observed for DM intake from 18.7 to 16.7 for CS0 to CS15. Intake of starch decreased linearly with linseed supply ($P_L = 0.01$). Milk yield and FPCM were similar in diets based on corn silage as well as milk efficiency (Table 2).

Rumen digestibility of DM, OM, and NDF, and NAN duodenal flow were similar among CS diets, whereas starch digestion increased in the rumen, particularly for CS5 and CS10 compared with C0 and CS15 ($P_Q = 0.01$; Tables 3). In the total tract, NDF digestion decreased with linseed supply (from 57.8 to 49.3% for CS0 to CS15; $P_L = 0.05$), which is related to the numerical decline in NDF digestion in the rumen. Total-tract starch digestion was higher for diets supplemented with linseed compared with CS0 ($P_L < 0.01$ and $P_Q = 0.09$; Table 3).

Increasing linseed supply decreased daily CH_4 emission (g/d) from 354 to 207 g/d for CS0 to CS15 ($P_L < 0.01$; Table 4). Compared with CS0, the extent of decrease in CH_4 expressed per kilogram of DMI was -4 and -8% with CS5 and CS10 and was more substantial (-34%) with CS15 ($P_L < 0.01$ and $P_Q = 0.08$). The same significant response to increasing supply of extruded linseed was observed for CH_4 output expressed in proportion of GE intake ($P_L < 0.01$ and $P_Q = 0.09$), per kilogram of nutrient digested ($0.02 \leq P_L \leq 0.07$), and per kilogram of milk or kilogram of FPCM ($P_L = 0.04$ and $0.04 \leq P_Q \leq 0.08$).

Rumen pH increased with linseed supply in CS diets ($P_L = 0.03$) (Table 5). The effect of extruded linseed supply on the total VFA concentration in the rumen differed between the 2 sampling times (sampling time \times diet interaction: $P < 0.001$; data not shown); VFA concentration decreased significantly before feeding with linseed supply (-26 mM between CS0 and CS15; $P_L < 0.01$) and was unchanged 2.5 h after feeding. The main changes in VFA profiles were a decrease in acetate ($P_L < 0.001$) and butyrate proportions ($P_L < 0.001$ and $P_Q = 0.03$) and an increase in propionate proportion ($P_L < 0.001$), which resulted in lower ratios of acetate:propionate and (acetate + butyrate):propionate ($P_L < 0.001$) with linseed supply (Table 5). No change in N-NH_3 concentration was observed among diets. Total protozoa and entodiniomorph concentrations in the rumen decreased with increasing linseed supply for CS diets ($P_L \leq 0.001$ and $P_Q \leq 0.01$; Table 6).

The concentration of the 3 cellulolytic bacteria in the liquid and solid phases of the rumen content was unaffected by linseed supply before feeding, except that of *R. albus*, which increased for CS5 and CS10 compared with CS0 and CS15 ($P_Q = 0.04$) in the solid phase. The concentration of methanogens was similar among CS diets in both phases of the rumen content.

DISCUSSION

This is the first report on the dose-response effect of extruded linseed supply in diets based on hay or corn silage on enteric CH_4 emissions in dairy cows. Quantitative digestion in the rumen and the total-tract as well as rumen fermentation and microbial mechanisms of methanogenesis observed in both experiments were also considered. The interaction between linseed FA and the type of forage was not assessed statistically in this work because the 2 forages were studied in 2 different experiments. However, this point is discussed in the light of the available literature.

Effect of Increasing Linseed Supply on Digestibility and Rumen Processes

The OM digestibility of H and CS diets was not significantly decreased by linseed supply, although it was numerically lower by 8.0 and 3.1 percentage points between CS0 and CS15 for rumen and total-tract digestibility, respectively. This decrease was accompanied by a numerical decrease in OM intake (-1.6 kg between CS0 and CS15), which resulted in a decrease in digestible OM intake, without negative consequences for milk production or animal BW. This trend might have become significant if more animals had been used in these experiments. Linseed supply (3–4% FA) in 50 to 55% silage-based diets had no effect on OM digestibility in dairy cows (Gonthier et al., 2004; Benchaar et al., 2012) or in dry cows fed 2.6% linseed oil in a 50% hay-based diet (Guyader et al., 2015). Others have also reported no change in OM digestibility with 4 to 5% linseed FA supply, although the numerical decrease was greater with corn silage-based than with red clover silage-based diets in dairy cows (4% linseed oil; Benchaar et al., 2015) and with barley silage-based than with hay-based diets in dry cows (5% added FA from crude linseed; Chung et al., 2011). Taken together, these results show that an increase of more than 4 to 5% of FA from linseed may present a risk of reduction of OM digestibility, at least with cereal silage-based diets, as previously shown (Martin et al., 2008).

In the present study, the absence of a significant decrease in OM digestibility for CS diets may be related to the opposite effects of linseed supplementation on NDF and starch digestibility in the total tract. Indeed, NDF digestibility linearly decreased with increasing linseed supply, whereas the opposite was observed for starch digestibility. With CS diets, the higher starch digestibility with linseed supply can be explained by a lower starch intake because starch is replaced by lipids. It has been shown that starch digestibility is decreased when the amount of corn ingested is excessive

(Nozière et al., 2010). In the rumen, the trend toward a decrease in NDF digestibility with increasing linseed supply of CS diets is consistent with the preprandial decrease in VFA concentration. With H diets, neither NDF digestibility nor VFA concentration in the rumen was modified by linseed supply. In agreement with our results, the extent of rumen fermentation as reflected by VFA concentration was not affected by linseed supply for diets based on hay (Chung et al., 2011) or red clover silage-based diets (Benchaa et al., 2015), but was reduced for barley silage-based diets (Chung et al., 2011; Benchaa et al., 2015). In these 2 last studies, the decrease in rumen VFA concentration may also have been related to the decrease in the digestibility of the fiber fraction of diets.

Neither ruminal ammonia concentration nor duodenal NAN flow varied with lipid supply in our experiments. This confirms the general effect observed with lipids whatever their source (Doreau and Ferlay, 1995) and results obtained with linseed supply (Ueda et al., 2003). A decrease in ammonia could have been expected because of the decrease in protozoa, which is often associated with a decrease in protein degradation (Morgavi et al., 2008). In this experiment, the absence of change in ammonia suggests that protein metabolism in the rumen was not modified. Chung et al. (2011) and Guyader et al. (2015) also observed the absence of variation of ruminal ammonia and urinary N despite a decrease in protozoa when linseed was supplied in the diet. The changes in protozoa population may have been too small to result in changes in rumen protein metabolism.

Polyunsaturated FA have been shown to have a toxic effect on some rumen bacteria species, especially cellulolytic bacteria (Maia et al., 2007; Mao et al., 2010). This toxic effect of linseed FA on the concentrations of the 3 main cellulolytic bacteria species (*F. succinogenes*, *R. albus*, *R. flavefaciens*) was not observed in our work with H or CS diets. Therefore, the lower NDF rumen digestion in CS diets in response to linseed supply cannot be explained by a decline in cellulolytic bacteria number but a decrease in their fibrolytic activity can be supposed.

Effect of Increasing Linseed Supply on Enteric Methane Emission and Rumen Processes

The CH₄ emission per kilogram of DM from published data (Beauchemin et al., 2009a) is close to the average value for the H0 diet and in the lower range of variation for the CS0 diet. The latter observation may be due to the high starch content of the CS diet. Daily CH₄ emission decreased with increasing FA supply from linseed, confirming that lipid supplementation is one

of the most effective dietary strategies in lowering enteric CH₄ emission by ruminants (Martin et al., 2010; Grainger and Beauchemin, 2011; Hristov et al., 2013). The CH₄-mitigating effect of extruded linseed (2–3% added FA) persisted for up to 1 yr in dairy cows fed diets based on grazed pasture (80%) or grass silage (60%; Martin et al., 2011). However, the CH₄-mitigating effect of linseed oil in dairy cows was not systematic, probably because of the low level of FA included in the diets in some studies (1–2%; van Zijderveld et al., 2011; Livingstone et al., 2015).

We reported a linear effect of extruded linseed supply on CH₄ yield (g/kg of DMI) with both types of forages. The decrease was greater with the highest FA supply than with the 2 other levels of supplementation (−3.1, −3.2, and −6.7% per 1% added FA on average for the 2 diets supplemented with 1.8, 3.6, and 5.4% of FA, respectively). To our knowledge, the dose-response effect of dietary FA on methanogenesis in ruminants has never been studied with linseed and with only a few with other FA sources. A linear effect of FA supply on CH₄ emission in cattle was reported with rapeseed oil (0, 5.4, 9.5% added FA; Jentsch et al., 1972), and coconut oil (0, 1.3, 2.7, 3.3% added FA in Hollmann et al., 2012; 0, 1.3, 2.7, 4.6% added FA in Jordan et al., 2006). A quadratic effect was reported with the highest supply of coconut oil in sheep (0, 3.5, 7% added FA; Machmüller and Kreuzer, 1999). In their quantitative review of the literature, Grainger and Beauchemin (2011) reported a linear reduction in CH₄ yield of dairy cattle with diets containing up to 80 g of total dietary FA/kg of DM. The relationship was not affected by the source of FA but differed between cattle and sheep.

The interaction effect between lipids and the nature of the forage in the diet on CH₄ emission has seldom been studied. Chung et al. (2011) observed an effect of 5% added FA from crude linseed with barley silage-based but not with grass hay-based diets fed to dry cows (50:50). Benchaa et al. (2015) reported an effect of 4% linseed oil that was greater with corn silage (60:40) than with red clover silage in dairy cows. All of these results suggest that the effect of dietary linseed supply on enteric CH₄ emission in cows appears to be modulated by the source of dietary forage source, and it appears to be greater with diets containing more starch and less fiber.

In our study, the dose-response curve of the effect of extruded linseed on methanogenesis was similar for CH₄ emission expressed per kilogram of nutrient intake, per kilogram of digested nutrient, and per kilogram of milk. This suggests that CH₄ emission variations with linseed FA cannot be explained by changes in intake, digestibility, or milk production. Milk efficiency (kg of FPCM/DMI) was highest for H5 and H10 among

the H diets, and did not vary among the CS diets, for unexplained reasons. The changes in net energy value due to FA addition was probably too limited to entail an increase in energy value of the diet.

A first explanation of the CH₄-mitigating effect of FA is that, whatever their nature, they decrease the amount of OM fermented in the rumen if they replace a proportion of dietary carbohydrates, as was the case in our experiments. The biohydrogenation process in the rumen of added PUFA from linseed may also help to decrease methanogenesis, but the hydrogen sink function of PUFA in the rumen was estimated to be negligible (i.e., 1–2%) in studies based on stoichiometric (Czerkawski, 1986) and modeling (Mills et al., 2001) approaches. The specific effect of linseed FA is generally related to changes in rumen microbes and associated fermentation patterns. Concerning rumen protozoa, their concentration was systematically reduced by linseed supply. This effect was linear in H diets and both linear and quadratic in CS diets, which may explain the greater decrease in methanogenesis with CS15 than with CS5 and CS10 compared with CS0. This adverse effect of FA from linseed on rumen protozoa is classical but not systematic. It was reported with similar levels of ground linseed in barley silage-based diets (–82%, Chung et al., 2011), linseed oil in hay-based diets (–52%, Guyader et al., 2015) fed to nonlactating cows, and linseed oil in corn silage-based diets fed to dairy cows (–78%, Benchaar et al., 2015). In contrast, linseed oil supply (up to 4%) in silage-based diets had no effect on the total number or the genera distribution of protozoa in dairy cows (Benchaar et al., 2012). Protozoa act as physical hosts for methanogens and during fermentation produce a high quantity of H₂, which is used by methanogens to reduce CO₂ to CH₄ (Morgavi et al., 2010). Consequently, the toxic effect of linseed FA on protozoa, associated with lower H₂ availability to methanogens, appears to be involved in CH₄ mitigation in ruminants. This confirms recent findings from a meta-analysis of the literature (Guyader et al., 2014). Abundance of rumen methanogens was not affected by linseed supply in the H and CS diets. In previous studies in which methanogenesis was reduced, rumen methanogens were unaffected by a supply of 3% FA from extruded linseed in dairy cows (Martin et al., 2011), whereas their number decreased in dry cows supplemented with 4% linseed oil (Guyader et al., 2015). All these results support the view that the number of rumen methanogens is not the key factor affecting CH₄ emission (Morgavi et al., 2010). A modulation of the activity and diversity of methanogens might be responsible for the CH₄ mitigating effect of linseed FA, as observed in bulls fed a starch-rich diet (Popova et al., 2011).

Reported effects of linseed supply on rumen fermentation parameters remain inconsistent in the literature. An increase in the proportion of propionate in response to 2 to 4% linseed FA supplementation was reported with diets based on hay in dry cows (Guyader et al., 2015) or on corn and grass silages in dairy cows (Gonthier et al., 2004). Others authors have reported no effect of different linseed forms (rolled, extruded, oil) on rumen VFA composition in dairy cows fed grass silage (Martin et al., 2011) or corn silage (Doreau et al., 2009). Direct comparisons in dry cows (Chung et al., 2011) or dairy cows (Benchaar et al., 2015) indicate that the effect of linseed FA supply on rumen VFA composition varied with the nature of the dietary forage. No changes in VFA composition were reported with diets based on hay or red clover silage, whereas the proportion of propionate increased with diets based on cereal silages. In the present work, increasing the linseed supply changed rumen VFA composition by increasing propionate at the expense of butyrate in H diets and at the expense of butyrate and acetate in CS diets. This shift of rumen fermentation patterns toward the H₂-consuming pathway (propionate) at the expense of the H₂-producing pathway (acetate and butyrate) may indicate an H₂ excess due to the diminished CH₄ production (Janssen, 2010) and may be related to the decline in protozoa observed with linseed supply in both diets. This is supported by Morgavi et al. (2008), who reported an increase in propionate production with a concomitant decrease in butyrate production in the rumen of sheep without protozoa compared with faunated sheep.

CONCLUSIONS

The effect of increasing extruded linseed FA supply on enteric CH₄ emission, digestibility, and rumen function was studied in dairy cows fed diets based on hay or corn silage in 2 separate experiments. Extruded linseed efficiently and linearly reduced CH₄ emission in both experiments without altering intake, digestibility, or milk production with the hay-based diets. With the corn silage-based diets, intake and fiber digestibility decreased numerically with the highest linseed FA supply. The decrease in rumen protozoa number and the shift of fermentation toward propionate production appear to be related to the CH₄ mitigating effect of linseed with the hay- and corn silage-based diets. The energetic benefits of decreased CH₄ emission improved the milk efficiency of cows fed hay-based diets supplemented with the lowest linseed FA levels but not that of cows fed corn silage-based diets. Lower CH₄ emission from dairy cows fed linseed helps to limit the negative environmental impact of ruminant livestock.

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