



Effects of ruminal protozoa on methane emissions in ruminants—A meta-analysis

X. Dai,¹ K. F. Kalscheur,² P. Huhtanen,³ and A. P. Faciola^{4*}

¹Department of Clinical Science and Services, Royal Veterinary College, University of London, Hatfield, AL97TA, United Kingdom

²US Dairy Forage Research Center, USDA-Agricultural Research Service, Madison, WI 53706

³Production Systems, Natural Resources Institute Finland (LUKE), 31600 Jokinen, Finland

⁴Department of Animal Sciences, University of Florida, Gainesville 32608

ABSTRACT

The effects of different ruminal protozoa (RP) on CH₄ emissions from ruminants were evaluated in a meta-analysis, using 64 publications reporting data from 79 *in vivo* experiments. Experiments included in the database reported CH₄ emissions (g/d) and total RP (TRP, log₁₀ cells/mL) from the same group of animals. The relationship between CH₄ emissions and RP (TRP, entodiniomorphids, and isotrichids), and TRP-, entodiniomorphid-, and isotrichid-based CH₄ emission prediction models, were evaluated as mixed models with experiment as a random effect and weighted by the reciprocal of the standard error of the mean and centered around one. Positive associations existed between TRP and isotrichids with CH₄ emissions but not between entodiniomorphids and CH₄ emissions. A reduction in CH₄ emissions was observed, averaging 7.96 and 4.25 g/d, per log unit reduction in TRP and isotrichid concentrations, respectively. Total RP and isotrichids were important variables in predicting CH₄ emissions from ruminants. Isotrichid CH₄ prediction model was more robust than the TRP, evidenced by lower predicted sigma hat study (%), and error (%), and with higher concordance correlation coefficient. Both TRP and isotrichid models can accurately predict CH₄ emissions across different ruminant types, as shown by the low square root of the mean square prediction error, with 6.59 and 4.08% of the mean of root of the mean square prediction error in the TRP and isotrichid models, respectively. Our results confirm that isotrichids are more important than entodiniomorphids in methanogenesis. Distinguishing these 2 populations yielded a more robust CH₄ prediction model than combining them as total protozoa.

Key words: greenhouse gas, modeling, ruminal fermentation

INTRODUCTION

Methane is a greenhouse gas, as well as an energy waste, for ruminants. Ruminal methanogens use dihydrogen and carbon dioxide produced during ruminal fermentation; therefore, methanogens have a symbiotic relationship involving interspecies hydrogen transfer with other ruminal microorganisms, including ruminal protozoa (RP; Balch et al., 1979). Ruminal protozoa are involved in methanogenesis, partially through butyrate and acetate production, which releases 2- and 4-pair mol of H-atoms, respectively, per mole of fermented glucose (Guyader et al., 2014).

In our previous work (Dai and Faciola, 2019), we observed that different strategies (defaunation, lipids, and phytochemicals supplementation) reduced total RP (TRP) concentration, in conjunction with a decrease in CH₄ emissions (g/kg DMI). Others reported a CH₄ reduction ranging from 11 to 35% associated with RP reductions (Hegarty, 1999; Morgavi et al., 2008; Morgavi et al., 2012; Newbold et al., 2015). In line with these observations, others have reported a linear correlation between CH₄ emissions and TRP concentration (Morgavi et al., 2010; Guyader et al., 2014; Dai and Faciola, 2019), which confirms that RP play a critical role in methanogenesis.

Ruminal protozoa are classified into 2 groups, namely entodiniomorphids and isotrichids. It has been demonstrated that isotrichids have different endosymbiotic methanogens than entodiniomorphids (Belanche et al., 2014) and have a more significant effect on ruminal methanogenesis (Belanche et al., 2015), because of greater O₂ consumption (Firkins et al., 2020). This illustrates that variations in RP composition could affect CH₄ emissions, and simply looking at total RP may not provide a complete picture of their specific effects on CH₄ emissions. In Belanche et al. (2015), isotrichid (holotrichs) inoculation increased both methanogens (+0.41 log₁₀) and methanogenesis (+54%). Currently, little information exists for evaluating the effects of different RP (entodiniomorphids and isotrichids) on CH₄

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*Corresponding author: afaciola@ufl.edu

emissions across different experimental conditions. In addition, there is a knowledge gap in how the relationship between CH₄ emissions and RP (TRP, entodiniomorphids, and isotrichids) can be quantitatively affected by other factors such as dietary components, ruminal fermentation, and nutrient digestibility. Therefore, we hypothesized that isotrichids are more likely to contribute to methanogenesis than entodiniomorphids, and thus distinguishing these 2 populations should yield a more robust model than combining them as a total RP. The objectives of this meta-analysis were to (1) evaluate the relationships between CH₄ emissions with TRP and different RP (entodiniomorphids and isotrichids) concentrations and (2) generate TRP, entodiniomorphid, and isotrichid CH₄ emissions prediction models.

MATERIALS AND METHODS

Because no human or animal subjects were used in this analysis, no Institutional Animal Care and Use Committee or Institutional Review Board approval was required.

Data Collection

The database search included publications reporting in vivo data from experiments published in English, in which total RP concentration and CH₄ emissions were measured from the same group of animals. To access publications, the editorial platforms of the US National Library of Medicine National Institutes of Health through PubMed (<http://www.ncbi.nlm.nih.gov/pubmed>), the ISI Web of Science (<http://apps.webofknowledge.com>), Agricola (<https://agricola.nal.usda.gov/vwebv/selectDatabase.do?dbCode=AGRI2DB>), Proquest (<http://www.proquest.com/>), and CSIRO (<http://www.publish.csiro.au/>) were searched with the following keywords: methane, protozoa, and ruminants. The data collection was conducted in April 2020. The search aimed to identify publications with experiments that were suitable for meta-analysis. Quantitative factors included TRP concentration, entodiniomorphids, isotrichids, CH₄ emissions, ruminal fermentation variables, total-tract digestibility, and DMI dietary chemical compositions were added to the database when available, with pooled standard error of the mean (SEM). Entodiniomorphids included the ones reported as entodiniomorphs, genera *Entodinium*, *Epidinium*, *Diplodinium*, or a sum of these genera, when any of them were recorded together in the same study, as well as the family *Ophryoscolecidae*. Isotrichids included those reported as holotrichs, genera *Isotricha* and *Dasytricha*, or the sum of the genera *Isotricha* and

Dasytricha, when reported together within the same study. Ruminal fermentation variables included pH, total VFA, molar proportion of acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, branched-chain volatile fatty acid, ratio of acetate, and propionate and ammonia N concentration. Total-tract digestibility included DM, OM, CP, and NDF digestibility. Dietary components included dietary DM, OM, CP, NDF, lipid, and starch. The names “entodiniomorphids” and “isotrichids” for RP followed recommendations according to Firkins et al. (2020). We also recorded CH₄ emissions and ruminal fluid collection methods.

Inclusion and Exclusion Criteria

Supplemental Figure S1 (https://osf.io/mdsu4/?view_only=baa648d766a048a68d8b7775f3f03f31) depicts a Prisma diagram (Moher et al., 2009) of the data collection flow for the meta-analysis. The initial criteria to include an experiment for the meta-analysis were that both CH₄ emissions and RP concentration were reported in vivo. Searched works in the literature were screened for duplicates. Then, suitability for inclusion was evaluated by reading the abstract to check that the experiment was conducted for mitigating CH₄ emissions and that both CH₄ emissions and RP concentration were reported. After reading the Materials and Methods section of each publication, experiments in which treatments were not implemented as previously described in the initial inclusion criteria of the abstract were excluded. After the initial search and screening, 83 publications, including those with multiple experiments, were further assessed for eligibility. When relevant, one publication could supply multiple experiments if controls were different or several CH₄ mitigation strategies were evaluated with the same basal diet. From those 83, 16 publications were excluded because of the following reasons: publications used 18S rRNA sequencing to determine RP concentration, or the RP concentration and CH₄ emissions were not measured from the same group of animals. Even though both microscopy and 18S rRNA sequencing allow for identifying dominant members of the ciliate communities and classifying the RP community, microscopy is considered a more accurate method for evaluating total numbers or relative abundance of different RP genera in a sample (Kittelman et al., 2015). In addition, 3 studies (Martin et al., 2011; Doreau et al., 2014; Moate et al., 2019) that used rumenocentesis to collect ruminal fluid were excluded, as rumenocentesis from the ventral rumen would bias against the larger entodiniomorphids that are abundant in the rumen mat. After that, the database contained 64 publications with 79 experiments and a total of 242 treatment means for the following data analysis.

In sum, the studies were conducted on dairy cows (49%), beef steers (19%), and small ruminants (32%). The experiments were classified into 5 groups according to the application of experimental treatments: (1) 38% of the experiments used phytochemicals (tannins, saponins, and essential oils); (2) 25% of the experiments used lipids (long-chain fatty acids and medium-chain fatty acids); (3) 11% of the experiments tested probiotics and prebiotics; (4) 8% of the experiments tested chemicals (iodopropane, nitrate, sulfate); (5) the remaining experiments tested dietary factors (forage, concentrate, and so on). Among all experiments included in the database, 65% of the studies reported a reduction in CH₄ emissions. Meanwhile, for this database, 57.9% of CH₄ emissions were measured in chambers, 38.7% were measured by sulfur hexafluoride (SF₆), and 3.4% were measured using the GreenFeed system (C-Lock Inc.). In the database, 56.7% of ruminal fluid was collected directly from the rumen, and 43.3% of ruminal fluid was collected through stomach tubing. The data structure and the percentage of observations that reported specific quantitative factors of interest in the selected experiments used for the meta-analysis included: ruminal VFA concentration and individual VFA molar percentage; ammonia N (NH₃-N) concentration; total-tract digestibility. Descriptive statistics of the data included in the meta-analysis are presented in Table 1. For this meta-analysis, total RP concentration, entodiniomorphids, and isotrichids were expressed as log₁₀ cells per milliliter to normalize the data. The CH₄ emissions were expressed as grams per day. Total VFA and NH₃-N concentrations were expressed as millimolars. The molar proportions of VFA were expressed as a percentage of total VFA concentration. Dry matter intake was presented as kilograms per day.

Statistical Analysis

Data were analyzed by mixed models with PROC MIXED in SAS (SAS Institute, 1999). All mixed models included the random intercept of experiment identification and repeated measurements grouped by type (REPEATED or GROUP) to calculate a different residual variance for different ruminant types. The unstructured covariance structure was always initially applied. If the model did not converge, variance components were used as covariance structures. The response variable (CH₄ g/d) was weighted with the inverse of pooled standard error of the mean (SEM) of CH₄ (St-Pierre, 2001; Roman-Garcia et al., 2016). To prevent overweighting of the studies with extremely low SEM, the SEM of CH₄ was trimmed to one-fourth of the mean of SEM (Firkins et al., 2001; Roman-Garcia et al.,

2016). The trimming process was done separately for mixed and fixed effects models that were used for statistical analysis in the studies. Then, calculated weights (reciprocals of trimmed SEM) were centered around 1 by whether a fixed or mixed model was used for statistical analysis in the studies (St-Pierre, 2001). Regression model diagnostics were checked for all the evaluated models. The influence points were evaluated by the INFLUENCE statement in PROC MIXED of SAS. Observation had a significant influence if Cook's distance exceeded $\frac{4}{(n-p-1)}$, where n is the number of observations and p is the number of predictor variables (Bruce and Bruce, 2017). Observations with studentized residuals greater than 3 in absolute value were considered outliers (Gareth et al., 2014). In addition, one experiment with 4 observations that tested defaunation was excluded for downstream analysis, due to a high leverage effect.

The quantitative relationship between CH₄ emissions with TRP, entodiniomorphids, and isotrichids was first evaluated, and the random slopes of TRP, entodiniomorphids, and isotrichids were included in the model. The class variables of the ruminal fluid collection method (MeR), the methane measurement method (MeS), and the treatment effect of whether the treatment mean is a control or treated were included in the analysis. All respective interactions of RP with class variable and squared terms of TRP, entodiniomorphids, and isotrichids were evaluated, and only significant variables were kept in the models.

The primary purpose of this study was to predict CH₄ emissions based on different RP. A backward elimination procedure was applied to select the best TRP, isotrichid, and entodiniomorphid CH₄-predicted models. Considering the significant positive association between CH₄ emissions and DMI (Supplemental Table S1; https://osf.io/mdsu4/?view_only=baa648d766a048a68d8b7775f3f03f31), the DMI was included in all initial models, as well as the class variables (MeR, MeS, and treatment effect). The dietary, ruminal fermentation, and total-tract digestibility variables were all included in the initial TRP, isotrichid, and entodiniomorphid CH₄ prediction models. All respective interactions and squared terms were assessed in all models. The RP, isotrichid, or entodiniomorphid variables were forced to stay until the end of each model during the model derivation procedure. If, in the end, the RP, isotrichids, or entodiniomorphids were the only ones that were not significant, then the predicted model was not generated. The variables with the highest nonsignificant ($P > 0.05$) P -values were iteratively removed from the model, and only significant variables

Table 1. Statistical description of diet and animal characteristics in the database used for the meta-analysis

Item	Control					Treated						
	N	Mean	SD	Median	Minimum	Maximum	N	Mean	SD	Median	Minimum	Maximum
CH ₄ , g/d	72	239	192	214	7.13	749	170	200	181	164	4.86	637
DMI, kg/d	72	12.1	9.56	9.80	0.42	27.4	170	10.9	9.32	7.94	0.43	27.6
Ruminal protozoa, log ₁₀ cell/mL												
Total	72	5.67	0.86	5.78	0	8.44	170	5.60	0.57	5.68	3.83	8.09
Entodiniomorphids	40	5.66	0.35	5.70	4.82	6.47	79	5.52	0.47	5.54	3.77	6.44
Isotrichids	39	3.94	0.63	4.08	1.81	5.37	74	3.69	0.86	3.87	0	4.92
Dietary component, g/kg												
DM	25	620	247	575	160	923	67	585	241	530	119	923
OM	59	922	22.9	927	828	948	136	923	29.6	931	800	960
CP	71	147	40.5	155	39.0	243	164	149	34.3	148	39.0	233
NDF	70	399	121	366	178	726	161	382	108	356	169	726
Ether extract	36	29.6	12.8	26.8	10	70.2	73	42.6	23.8	35.7	9.00	138
Starch	31	211	106	243	6.00	384	75	221	95.9	245	22.0	384
Ruminal fermentation												
pH	62	6.47	0.37	6.4	5.72	7.22	141	6.46	0.33	6.44	5.91	7.43
Total VFA, mM	67	99.2	28.6	103	35.2	162	154	97.9	28.7	101	30.1	164
Acetate, molar %	68	65.9	5.24	65.4	51.8	77.5	157	65.5	5.44	65.0	48.2	78.4
Propionate, molar %	68	20.0	3.93	20.2	11.3	33.4	157	20.4	4.52	20.6	9.50	36.0
Butyrate, molar %	68	10.4	2.17	10.7	5.4	16.5	157	10.5	2.35	10.9	4.20	16.3
Isobutyrate, molar %	39	0.84	0.42	0.85	0.07	1.76	92	0.9	0.51	0.90	0	2.23
Valerate, molar %	51	1.43	0.53	1.49	0.4	2.66	111	1.45	0.49	1.47	0.40	3.02
Isovalerate, molar %	42	1.33	0.75	1.40	0.10	4.03	96	1.38	0.83	1.39	0.09	4.13
Branched-chain VFA, mM	45	2.23	1.02	2.33	0.21	4.58	98	2.27	1.06	2.26	0.14	5.20
NH ₃ , mM	59	8.39	4.83	7.87	0.66	20.7	129	7.97	4.93	6.75	0.82	28.3
Total-tract digestibility, %												
DM	44	66.2	6.03	67.7	48.0	79.9	97	63.7	9.57	67.5	39.0	87.9
OM	47	69.4	4.98	70.6	55.1	77.5	109	67.0	8.78	69.3	39.9	83.3
CP	39	66.0	5.57	66.1	52.1	74.3	86	64.5	8.44	66.1	28.0	79.7
Ether extract	7	60.1	8.77	59.2	51.4	72.0	13	62.5	14.7	60.7	35.9	87.9
NDF	53	54.2	11.0	54.4	29.1	79.5	109	52.6	10.7	50.8	23.7	81.0

were kept in the final model. However, linear effects were kept in the model if the corresponding quadratic or interaction term was significant ($P < 0.05$). The variables' collinearity was measured by the variance inflation factor (**VIF**), and variables with $VIF > 100$ were removed during the model derivation procedure (Roman-Garcia et al., 2016). In all cases reported herein, variables were considered highly correlated when $VIF > 10$ (St-Pierre and Glamocic, 2000), except for the squared terms, which typically had $VIF > 10$, because they are correlated by calculation (Roman-Garcia et al., 2016).

Because of the congruency of the results from models in SAS and R (R Core Team, 2020), the models' performance evaluation was performed in R. The models' performance was evaluated by concordance correlation coefficient (**CCC**), sigma hat study, and sigma hat residual to evaluate the overall model prediction error and accuracy. The CCC was calculated based on the `epi.ccc` function from the package `epiR` in R (Stevenson et al., 2021), and a greater CCC indicates the better prediction of the observed values, according to Lin (1989). The predicted sigma for study (the estimated standard deviation, SD, of the study) and predicted sigma for residual (the estimated SD of the errors or residual) were calculated with `VarCorr` functions from the package `lme4` (Bates et al., 2015) in R. The residual SD indicates the accuracy of a model to predict external observations (Gelman et al., 2020). The σ can be considered a measure of the unexplained variation in the data or inference precision about the regression coefficients. The predicted sigma for residual was divided by the mean of the dependent variable and multiplied by 100 to calculate an equivalent to the traditional coefficient(s) of variation (CV); similarly, the predicted sigma error was divided by the dependent variable mean and multiplied by 100 to generate a CV equivalent for study (Boerman et al., 2015).

Random cross-validation was performed by randomly partitioned data in 10 groups of folds among study and 10 iterations, as recommended by Rodríguez et al. (2010). Ten-fold cross-validation was performed for the prediction models to calculate the performance parameters of models based on square root of the mean square prediction error (**RMSPE**), calculated as below:

$$\text{RMSPE} = \frac{\sqrt{\frac{1}{n} \times \sum_{i=1}^n (O_i - P_i)^2}}{\frac{1}{n} \sum_{i=1}^n O_i} \times 100,$$

where n is the number of observations, O_i is the i th observed value, and P_i is the i th predicted value.

The root of the mean square prediction error, expressed as a percentage of the observed mean, estimates the overall prediction error. The cross-validation fold was created by using the `creatFolds` function from the package `caret` in R (Kuhn, 2008). For each iteration, a model was developed as described above using 9 folds of the data set. The selected model was subsequently evaluated as described above with the remaining data. Cross-validation performance values were reported as the mean of the 10-fold cross-validation results with 95% confidence interval.

RESULTS AND DISCUSSION

Relationship Between CH_4 Emissions and RP Concentration

Methane emissions had a positive association with TRP ($P < 0.01$) and isotrichids ($P = 0.01$). Reductions in CH_4 emissions averaged 7.96 and 4.25 g/d per log unit reduction in TRP and isotrichid concentrations, respectively. A significant positive correlation between RP concentration and CH_4 emissions was also observed in the previous meta-analysis (Morgavi et al., 2010; Guyader et al., 2014; Newbold et al., 2015; Dai and Faciola, 2019), and the research evaluating defaunation reported a CH_4 decrease ranging from 11 to 35% (Hegarty, 1999; Morgavi et al., 2008, 2012; Newbold et al., 2015). This indicates the critical role of RP on methanogenesis. However, no significant association was found between CH_4 emissions and entodiniomorphids (Table 2).

The role of ruminal protozoa in methanogenesis could be based on their ability to produce H_2 in their hydrogenosomes and their ability to host epi- and endosymbiotic methanogens and protect them from oxygen toxicity (Fenchel and Finlay, 2006). Isotrichids have different endosymbiotic methanogens than entodiniomorphids (Belanche et al., 2014) and have a more significant effect on ruminal methanogenesis than entodiniomorphids (Belanche et al., 2015). It has been demonstrated that isotrichids have more active hydrogenosomes than entodiniomorphids (Paul et al., 1990) and greater O_2 consumption (Firkins et al., 2020). In addition, different associations with dietary components and ruminal fermentation variables between isotrichids and entodiniomorphids (Supplemental Table S1) also suggest their different metabolic activities in the rumen. All of these could explain a significant association of CH_4 emissions with isotrichids but not with entodiniomorphids. We analyzed the association of CH_4 emission with entodiniomorphid concentration separately within ruminant types. We found a significant positive association between CH_4 emissions and entodiniomorphids in

Table 2. Models that evaluated the relationship between CH₄ yield (g/d) with total ruminal protozoa (TRP), entodiniomorphids, and isotrichids as predictors and related model evaluation parameters¹

Predictor	Total rumen protozoa			Entodiniomorphids			Isotrichids		
	Estimate	SE	<i>P</i> -value	Estimate	SE	<i>P</i> -value	Estimate	SE	<i>P</i> -value
(Intercept)	90.9	30.3	<0.01	148	61.1	0.02	151	39.7	<0.01
TRP, log ₁₀ cells/mL	7.96	2.55	<0.01						
Entodiniomorphids, log ₁₀ cells/mL				4.44	3.58	0.22			
Isotrichids, log ₁₀ cells/mL							4.25	1.46	0.01
Model evaluation parameter									
CCC ²		0.24			0.19			0.23	
Predicted sigma hat study ³		166			183			183	
Predicted sigma hat study/mean, %		77.2			72.6			71.9	
Predicted sigma hat error		27.2			26.4			24.9	
Predicted sigma hat error/mean, %		12.6			10.4			9.81	
ExpN ⁴		78			41			41	
Observations		238			118			111	

¹Among both quantitative relationship of TRP and isotrichids, the rumen fluid collecting methods was significant ($P < 0.05$).

²CCC = concordance correlation coefficient.

³Sigma hat = a measure of the unexplained variation in the data or inference precision about the regression coefficients.

⁴ExpN = number of experiments.

dairy cows, but not in beef cattle, and small ruminants (data not shown). This may suggest that the effects of entodiniomorphids on CH₄ emissions could vary among ruminant types and requires further evaluation within the types.

Isotrichids could better explain the quantitative relationship between CH₄ emissions and RP concentration, compared with TRP as a predictor, indicated by lower predicted sigma hat error (%) with similar CCC (Table 2). Therefore, separating RP populations, especially isotrichids, would yield a more robust quantitative model between CH₄ emissions and RP concentrations than combining them as total RP. Meanwhile, the MeR ($P < 0.05$) was significant in both quantitative models of TRP and isotrichids, implying that the ruminal fluid sampling method could affect the quantitative relationship between CH₄ emissions and RP. The effect of the ruminal fluid sampling method on the quantitative relationship could be due to either its effect on RP counting or its effect on the measurements of ruminal fermentation variables, and thus on CH₄ emission. When the ruminal fermentation variables were included in the TRP and isotrichid models, the significant effect of MeR was removed (data not shown). A previous study also observed that total RP counts were not different between stomach tubing and rumen cannula collection but differed in absolute values for VFA concentrations in dairy cows (de Assis Lage et al., 2020). However, the low CCC observed in both quantitative models for TRP (0.24) and isotrichids (0.23) indicates a poor prediction of the observed CH₄ emissions, by only having TRP or isotrichids as the quantitative predictor, suggesting that the study of other quantitative factors is required to improve CH₄ prediction model performance.

Ruminal Protozoa CH₄ Prediction Model

The TRP and isotrichid CH₄ prediction models are presented in Table 3 and Figure 1. Even after controlling other important predictors of CH₄ emissions, such as DMI, NDF, and OM digestibility, both TRP and isotrichids still explained substantial proportions of the variation of CH₄ emissions ($P < 0.01$), confirming the unique and essential role of TRP and isotrichids in CH₄ emissions in ruminants. Ruminal protozoa had no interaction with other continuous variables kept in the final prediction models, suggesting that the effect of TRP and isotrichids on CH₄ emission may be independent of other variables. However, for entodiniomorphid CH₄ prediction models, entodiniomorphids were nonsignificant, and thus failed to generate any CH₄ prediction model. As discussed previously, this could be due to its less critical role in CH₄ emissions, supporting our hypothesis that isotrichids are more likely to promote methanogenesis than entodiniomorphids. The variation of CH₄ emissions explained by entodiniomorphids could be explained by DMI, dietary components, ruminal fermentation, or total-tract digestibility variables. Therefore, the role of TRP (including both entodiniomorphids and isotrichids) on CH₄ emissions is likely associated with isotrichids.

The RP (TRP, isotrichids), DMI, digestion (OM and NDF digestibility), and ruminal fermentation (valerate, pH) variables were the parameters kept in both final models to predict CH₄ emissions, indicating the crucial roles of the total amount of substrate and fermentation products on methanogenesis. Brask et al. (2015) concluded that the amount of feed fermented was the primary factor determining variations in CH₄ emissions.

Table 3. The CH₄ emissions (g/d) prediction equation developed from total ruminal protozoa (TRP) and isotrichids and related model evaluation parameters

Predictor ¹	TRP				Isotrichids			
	Estimate	SE	<i>P</i> -value	VIF	Estimate	SE	<i>P</i> -value	VIF
(Intercept)	54.2	26.3	0.05		-77.2	13.8	<0.01	
TRP	-33.8	9.97	<0.01	49.0				
TRP × TRP	3.37	0.93	<0.01	49.5				
Isotrichids					6.03	0.99	<0.01	1.20
DMI	32.7	1.78	<0.01	2.27	40.0	2.27	<0.01	7.07
OMD	0.27	0.05	<0.01	1.04				
VAL	6.13	1.34	<0.01	2.45	11.5	1.57	<0.01	1.72
DMI × VAL	-9.48	0.88	<0.01	2.21	-11.8	0.77	<0.01	4.52
NDFD					-0.062	0.08	0.45	1.90
DMI × NDFD					0.293	0.06	<0.01	4.14
pH					5.58	2.21	0.02	1.12
Model evaluation parameter								
CCC		0.94				0.96		
Predicted sigma hat study		63.8				62.4		
Predicted sigma hat study/mean, %		32.7				29.7		
Predicted sigma hat error		19.4				11.1		
Predicted sigma hat error/mean, %		10.0				5.28		
10-fold cross-validation								
RMSPE, % (95% CI)		6.59 (5.53–7.65)				4.08 (4.62–4.54)		
ExpN		36				23		
Observations		111				57		

¹Two-way interactions of other variables with RP were evaluated and were not significant ($P \geq 0.05$). RP = rumen protozoa; VAL = valerate, molar percentage; OMD = total-tract digestibility of OM, %; NDFD = total-tract digestibility of NDF, %; CCC = concordance correlation coefficient; sigma hat = a measure of the unexplained variation in the data or inference precision about the regression coefficients; RMSPE = square root of the mean square prediction error; VIF = variation inflation factor; ExpN = number of experiments.

Both CH₄ prediction models had a significant positive correlation with DMI, with the coefficient factor of 32.7 in the TRP model and 40.0 in the isotrichid model. This analysis confirmed that DMI is the most important variable to predict enteric CH₄ emissions in ruminants, which agrees with previous studies (Reynolds et al., 2011; Hristov et al., 2013; Niu et al., 2018). Arndt et al. (2021) also found that greater DMI increased CH₄ emissions in dairy cows. In addition, we also observed a significant positive relationship between CH₄ emissions with total-tract OM digestibility and a significant positive relationship between CH₄ emissions and the interaction of DMI and total-tract NDF digestibility in the TRP and isotrichid CH₄ prediction models, respectively, indicating an increased intake of digestible organic matter. These significant positive relationships suggest that greater CH₄ emissions may be due to the greater substrate availability for methanogenesis in the rumen. Furthermore, NDF digestibility, instead of OM digestibility, was included in the isotrichid CH₄ prediction model. This could be due to isotrichids mainly converting sugars and small starch granules into glycogen (Dehority, 2003). Including NDF digestibility accounts for the variations that could not be accounted for by isotrichids. We also found a significant positive correlation between CH₄ emissions and NDF digestibility ($P < 0.01$) and a tendency for a positive correlation between CH₄ emissions and OM digestibility ($P = 0.06$;

Supplemental Table S1). More structural carbohydrates generally favor acetate and butyrate production by di-hydrogen producers (Moe and Tyrrell, 1979; Bannink et al., 2008), and thus more CH₄ production. Defaunation generally decreases NDF digestibility in the ruminants (Newbold et al., 2015; Li et al., 2018); however, in the current database, defaunation studies were excluded. The isotrichid CH₄ prediction model can distinguish the role of NDF digestibility, and more accurately, evaluate the role of isotrichids on the methanogenesis after controlling for NDF digestibility.

The negative correlation between CH₄ emissions and the interaction between DMI and molar percentage of valerate was observed in both TRP and isotrichid CH₄ prediction models. This negative association suggests a greater valerate concentration with less CH₄ emissions. Production of valerate involves net uptake of electrons that arise from fermentation and reduce the total amount of H₂ formed (Czerkawski, 1986; Janssen, 2010), and therefore, decreases CH₄ production. Interestingly, we also observed a positive association between CH₄ emissions and pH in the isotrichid model. Lower ruminal pH generally reduces CH₄ production in the rumen by inhibiting methanogens and RP growth (Van Soest, 1994; Hegarty, 1999), because both producers (fibrolytic bacteria and RP) and consumers (methanogens) of hydrogen are more susceptible to low pH (Kessel and Russell, 1996). Furthermore, it

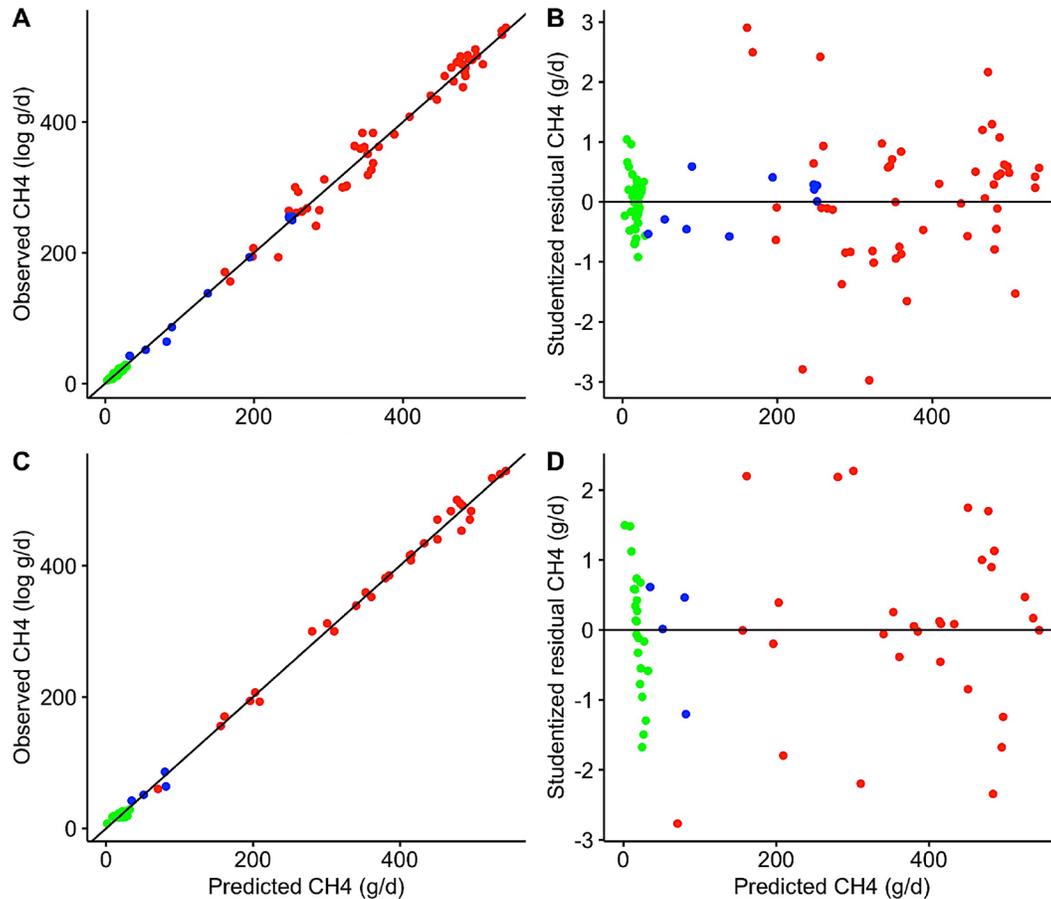


Figure 1. Observed and predicted methane (CH_4) emissions [A: total ruminal protozoa (TRP) model; C: isotrichid model]; studentized residual of CH_4 emissions (B: TRP model; D: isotrichid model) from the regression analysis. The slope of the residuals regressed on predicted values did not differ significantly from zero. Dots represent observations from the data set. Green = small ruminants; blue = beef cattle; red = dairy cows.

suggests that isotrichids are more susceptible to low pH. In addition, a significant negative correlation with pH was only observed with entodiniomorphids but not with isotrichids (Supplemental Table S1); this could be due to a stronger negative correlation between starch and pH ($P < 0.01$; data not shown), and entodiniomorphids' preference for ingesting larger starch granules (Williams and Coleman, 1992). Both TRP and isotrichid CH_4 prediction models did not include any dietary variables. This indicates a marginal effect of dietary variables in CH_4 emissions. The variation of dietary variables on CH_4 emissions could be explained by OM or NDF digestibility in the CH_4 prediction models. Interestingly, we found a negative correlation between dietary ether extract and isotrichids, but did not observe it in entodiniomorphids and TRP ($P = 0.04$; Supplemental Table S1), suggesting that isotrichids were more sensitive to dietary ether extract, compared with entodiniomorphids.

In addition, isotrichids are more important in methanogenesis than entodiniomorphids, as discussed previously. Therefore, the effectiveness of dietary lipids on suppressing CH_4 emissions, as shown in previous studies (Kobayashi, 2010; Grainger and Beauchemin, 2011; Patra, 2014), could be due to the negative effects of lipids on isotrichids.

As hypothesized, separating isotrichids and entodiniomorphids made it possible to produce a more robust model, rather than combining them as total protozoa. The isotrichid CH_4 prediction model had better model performance than that of TRP, evidenced by lower predicted sigma hat study (%) and error (%) and higher CCC (Table 3). According to the result of 10-fold cross-validation, the isotrichid model had a lower mean of RMSPE (4.08 vs. 6.59%), which also confirmed the better model performance of the isotrichid CH_4 prediction model. Furthermore, both models had low RM-SPE ($< 10\%$), suggesting that both RP CH_4 prediction

models can predict CH₄ emissions across different ruminant types. However, the predicted sigma study hat was much higher than the predicted sigma hat error in both CH₄ emission prediction models (Table 3), which could be due to variations of the sampling procedures or other potential unresolved random effects that were not uncovered but were still accounted for in the study effect. Therefore, CH₄ production might not be simply estimated by fitting total RP or isotrichid counts based on the generated CH₄ prediction models. The accuracy of the prediction models should be further tested against independent observations in future studies.

CONCLUSIONS

By quantifying the relationship between CH₄ emissions and different RP, we observed that CH₄ emissions had positive associations with TRP and isotrichids but not with entodiniomorphids. A reduction in CH₄ emissions averaged 7.96 and 4.25 g/d per log unit reduction in TRP and isotrichid concentrations, respectively. Total RP and isotrichids are important variables to predict CH₄ emissions in ruminants. Our TRP and isotrichid models can predict CH₄ emissions across different ruminant types. The isotrichid CH₄ prediction model had better model performance than the TRP CH₄ model. Our results confirm that isotrichids are more important in methanogenesis, compared with entodiniomorphids. Separating these 2 populations yielded a more robust CH₄ emissions prediction model than combining them as total protozoa.

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ORCID

- X. Dai  <https://orcid.org/0000-0003-2916-663X>
 K. F. Kalscheur  <https://orcid.org/0000-0002-5290-3602>
 P. Huhtanen  <https://orcid.org/0000-0001-7855-7448>
 A. P. Faciola  <https://orcid.org/0000-0003-0935-6233>