

Priority microbial targets for methane mitigation in ruminant production: a meta-analysis

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Abstract

Back ground: Methane emissions from livestock are one of the most significant contributing factors to global warming. Although it is now understood that methanogenic archaea in the rumen of ruminants utilize hydrogen, formate, and methyl compounds produced by bacteria, fungi, and ciliate protozoa to generate methane, their relative contributions remain unclear.

Results: This study aims to identify priority microbial targets for methane mitigation strategies in ruminant systems, thereby supporting more sustainable and low-carbon animal production. After retrieval and screening in the Web of Science database and Google Scholar database, 210 studies containing the required data were obtained. The obtained data were constrained using Z-score and then reanalyzed. The results showed that there were significant positive correlations between methane production and bacteria and fungi ($P < 0.05$). According to the correlation analysis between methane production and rumen microbiota, four regression models were constructed. Equation iv demonstrated a favorable fit (Adj. $R^2 = 0.74$, $P < 0.01$). Significant differences in the copy numbers of bacterial 16S rRNA, fungal 18S rRNA, and protozoal 18S rRNA were observed between high and low methane production conditions. When the dietary NDF content increased, the relative contribution of bacteria decreased, while those of fungi and protozoa increased relatively. However, overall, the contribution to methane production followed the order: bacteria > fungi > protozoa, and the driving factor for methane production contribution was primarily determined by microbial abundance.

Conclusion: These findings facilitate the prioritization of rumen bacteria as microbial targets for methane mitigation, thereby enhancing feed energy utilization efficiency and reducing agricultural greenhouse gas emissions.

Key words: Methane mitigation, Ruminant, Bacteria, Fungi, Protozoa

Background

Symbiotic microorganisms in the ruminant digestive tract break down recalcitrant carbohydrates into volatile fatty acids, carbon dioxide, and hydrogen, supplying essential energy for both the host and the symbionts, while also generating methane [1]. Methane generated by ruminants is not only a primary contributor to global warming but also results in the loss of dietary energy. After ruminants ingest feed, chewing and rumination promote microbial adhesion to the feed surface, and also hydrate and disrupt the protective cuticle of the plants [2]. Microorganisms adhering to the feed surface produce cell-bound or free enzymes that hydrolyze cellulose, as well as pectin and soluble plant cell contents [3]. The resulting hexoses undergo the EMP (Embden-Meyerhof-Parnas) pathway to generate NADH (reduced nicotinamide adenine dinucleotide) and pyruvate, whereas pentoses are metabolized via the pentose phosphate pathway or the phosphoketolase pathway, yielding products (such as glyceraldehyde-3-phosphate and fructose-6-phosphate) that can enter the EMP pathway for further degradation [4]. During the conversion of the intermediate product pyruvate into volatile fatty acids catalyzed by microbial enzymes, the process is accompanied by the regulation of NADH/NAD⁺ (oxidized nicotinamide adenine dinucleotide) redox balance and the transfer of hydrogen and carbon dioxide between hydrogen-producing microorganisms (such as bacteria, protozoa, and fungi) and hydrogen-consuming microorganisms (primarily methanogens). This process is key to maintaining the homeostasis of ruminal anaerobic metabolism [5]. Methane production in the rumen occurs primarily through the hydrogenotrophic pathway, the acetoclastic pathway, and the methylotrophic pathway. Among these, the hydrogenotrophic pathway is the dominant pathway; the methylotrophic pathway contributes relatively little to ruminal methane production; and the acetoclastic pathway represents another important route for monitoring and regulating methane emissions [1]. Although the process of methane emission in the rumen is highly complex, it is nonetheless the result of the collaborative action of ruminal microorganisms.

The rumen harbors a large number of microorganisms, with bacterial concentrations of approximately 10^{11} cells/mL, fungi around 10^6 cells/mL, and protozoa ranging from approximately 10^4 to 10^6 cells/mL [6, 7]. Bacteria are the most abundant microbial community in the rumen and play an important role in fiber degradation, as well as in the utilization of starch and protein [8]. Upon the entry of feed into the rumen, the abundant cellulolytic bacterial community (comprising primarily *Fibrobacter succinogenes*, *Ruminococcus albus*, and *Ruminococcus flavefaciens*) degrades cellulose

and other polysaccharides into cellodextrins, cellobiose, and glucose. During glycolysis and subsequent metabolic processes, these bacteria generate hydrogen, carbon dioxide, acetate, and other metabolites, thereby providing essential precursors for methanogens to synthesize methane [9]. More than 90% of fungi in the rumen remain unclassified, and all currently validated anaerobic fungi belong to the phylum Neocallimastigomycota [10]. They possess a complex life cycle. Upon release, zoospores typically attach to straw within 0.5 hours and generate mycelia that penetrate plant tissues. The cellulases secreted by these fungi assemble into cellulosome-like organelles dedicated to the degradation of plant fibers. The end products of this process include acetate, formate, lactate, succinate, hydrogen, and carbon dioxide [9]. Due to their ability to prey upon, engulf, and sequester bacteria, as well as to ferment starch and participate in fiber degradation, protozoa have received significant attention in past research [9, 11]. Achieving *in vitro* cultivation of protozoa is difficult; as a result, research on protozoa has largely focused on defaunation studies. After defaunation, ruminants exhibit improved feed conversion efficiency and a 10 - 13% reduction in methane emissions [12]. However, fiber degradation is also significantly decreased, which indicates that protozoa play a positive role in ruminal fiber degradation [11, 13].

Although bacteria, fungi, and protozoa play key roles in ruminal fiber degradation and methane production, the relative contributions of these groups remain controversial [14]. The objective of this study is to determine the relative contributions of three microbial communities within the ruminant rumen to methane production, and to identify the primary factors contributing to the discrepancies in their contributions.

Materials and Methods

Study eligibility

The systematic literature search in this study was conducted in strict accordance with the PRISM (Preferred Reporting Items for Systematic Reviews and Meta-Analyses). Studies were included based on the following criteria: (1) The selected studies must include quantitative data for bacteria (based on the 16S rRNA gene), fungi (based on 18S rRNA), and protozoa (based on 18S rRNA), with all units expressed as log₁₀ copies/mL, and must include methane production data, with the unit expressed as mM. (2) The microbial community must be from the rumen of ruminants. (3) The selected studies must be original research articles, not reviews. Actually, only considering the above inclusion criteria may introduce bias; thus, the following exclusion criteria are proposed. The presence of any of the following

criteria led to study exclusion: (1) The animals had physiological diseases. (2) The diet contained chemicals affecting the rumen microbiota. (3) The feeding trial had multiple stages, each with different feeding strategies. (4) The diet fed to the ruminants contained methane inhibitors.

Data sources

Relevant studies were retrieved from the Web of Science [15] and Google Scholar databases [16]. The search time-frame covered the period from database inception to January 17, 2026. In the MeSH module of the PubMed platform [17], subject headings and free-text words for ruminants, cattle, sheep, and fungi were searched and used as keywords for literature screening. The search strategy is presented in Supplementary File S1. Duplicate studies were removed from the screened literature, and the included articles were filtered for confounding factors according to the exclusion criteria.

Publication Bias Assessment

To avoid publication bias, the JBI (Joanna Briggs Institute) critical appraisal checklist for single-group studies was used to assess the included studies [18]. The two authors independently assessed the studies based on the evaluation criteria outlined in the checklist. If the two authors failed to reach agreement on a particular study, the study was referred to a third author. The authors discussed the disagreements that arose during the assessment process and eventually resolved all of them.

Data analysis

Outlier detection was performed on the collected data using Z-scores. Z-scores within the interval [-2, 2] were regarded as acceptable:

$$Z = \frac{X_i - \bar{X}}{S}$$

Where X_i is the value of the variable collected in the articleis, which is the average of multiple values from the author's original experimental results, $\bar{X}$ is the average value of X , and S is the standard deviation.

The standardized regression coefficient (β weight) was used as a tool to assess relative contribution. It eliminates the influence caused by differences in variability and objectively reflects the relative contribution of each microbial group to methane production:

$$\beta_i = b_i \times \frac{SD_{X_i}}{SD_Y}$$

Where β_i is the standardized regression coefficient of the i-th independent variable, b_i is the unstandardized regression coefficient of the i-th independent variable, SD_{X_i} represents the standard deviation of the i-th independent variable, and SD_Y is the standard deviation of the dependent variable Y. Normalized total contribution (NTC) is calculated as the product of the standardized regression coefficient (β) and the log10 - transformed mean abundance of each microbial group.

Pearson correlation analysis and regression fitting was performed using IBM SPSS Statistics (version 26.0; IBM Corp., Armonk, NY, USA). Prior to regression analysis, the data were checked for multicollinearity, normality, and outliers. A stepwise regression approach was used to build the regression model. Statistical significance was set at $\alpha = 0.05$, VIF (variance inflation factor) < 5 . The data collection site map was created using ArcGIS (version 10.8; Esri, Redlands, CA, USA). The flow diagram of the collection process was constructed using Review Manager (version 5.3; The Cochrane Collaboration, London, United Kingdom). The line box plots, heatmaps, fitting curves, and radar charts were all generated using Origin (version 2024; OriginLab Corporation, Northampton, MA, USA).

Results

Study screening process and results

The study screening process is illustrated in Figure 1A. Following the search strategy described in Supplementary File S1, 9,842 studies were identified from the Web of Science database and 5,874 from the Google Scholar database. After removing 4,271 duplicates and 470 review articles that lacked the required data, 10,975 articles remained. Upon screening, 9,791 articles were excluded for being irrelevant to this study. Of the remaining 1,184 articles, 974 were excluded because they did not contain the required statistical data on microbial counts. Ultimately, 210 articles met the inclusion criteria (Fig. 1A). The experimental sites of these studies are shown in Figure 1B. They are widely distributed between 50° N and 50° S latitude (Fig. 1B).

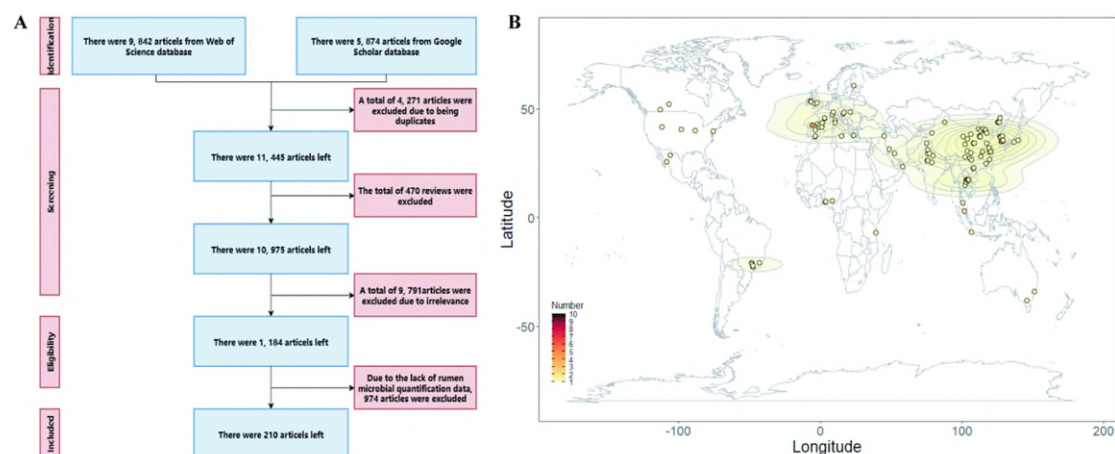


Figure 1. The literature search flowchart and geographic location map. A total of 15,716 studies were retrieved. After screening, 210 studies ultimately remained (Fig. 1A). The geographic locations of the selected studies are marked on the map (Fig. 1B, map lines delineate study areas and do not necessarily depict accepted national boundaries).

Relationships between microbial abundance and methane production

To elucidate the trends in methane production as a function of the abundance of individual microbial taxa, we performed descriptive statistics, contribution calculations, and fitted curves for the relationships between rumen microorganisms and methane production. The standardized regression coefficients (β) followed the order of fungi (0.12) > bacteria (0.10) > protozoa (0.03), whereas the normalized total contribution (NTC) followed the order of bacteria (51.9) > fungi (38.8) > protozoa (9.3) (Table 1). The results of linear regression analyses revealed that the abundances of bacteria, fungi, protozoa, and methanogenic archaea each exhibited highly significant positive correlations with methane production ($P < 0.01$) and the adjusted coefficients of determination (Adj. R^2) were 0.13, 0.44, 0.23, and 0.26, respectively (Figs. 2A-2D). Following stratification of methane production into high and low yield groups, the abundances of all microbial taxa in the high methane production group were found to be significantly greater than those in the low methane production group ($P < 0.05$) (Fig. 2E). Comparison of normalized standardized regression coefficients among different microbial groups revealed that fungi exhibited the highest coefficient (0.45), followed by bacteria (0.37) and protozoa (0.18). Regarding the normalized total contribution, the ranking was bacteria > fungi > protozoa, with corresponding values of 0.51, 0.48, and 0.12, respectively. After stratifying methane production into high-yield and low-yield groups, the standardized coefficient of bacteria decreased, while those of fungi and protozoa increased (Fig. 2F).

Table 1. Descriptive statistics and contribution indices of rumen microorganisms and methane production.

	Number of articles	Number of treatment	Mean	SD	CV	Slope	Standardized β	NTC
Bacteria	82	167	10.38	1.81	2.23	1.81	0.10	51.9
Fungi	58	96	6.40	1.63	2.58	4.73	0.12	38.8
Protozoa	44	82	6.13	1.15	1.7	4.51	0.03	9.30
Methane	41	109	22.21	7.51	3.53	-	-	
Methanogen	69	74	7.19	2.18	2.62	-	-	

Note: Mean = mean value (Microbial abundance is expressed as log10 copies/mL; methane is expressed as mM), SD = Standard deviation, CV = Coefficient of variation, Slope = slope of linear regression, VIF = Variance inflation factor, Standardized β = standardized regression coefficient (β), NTC = Normalized total contribution (%).

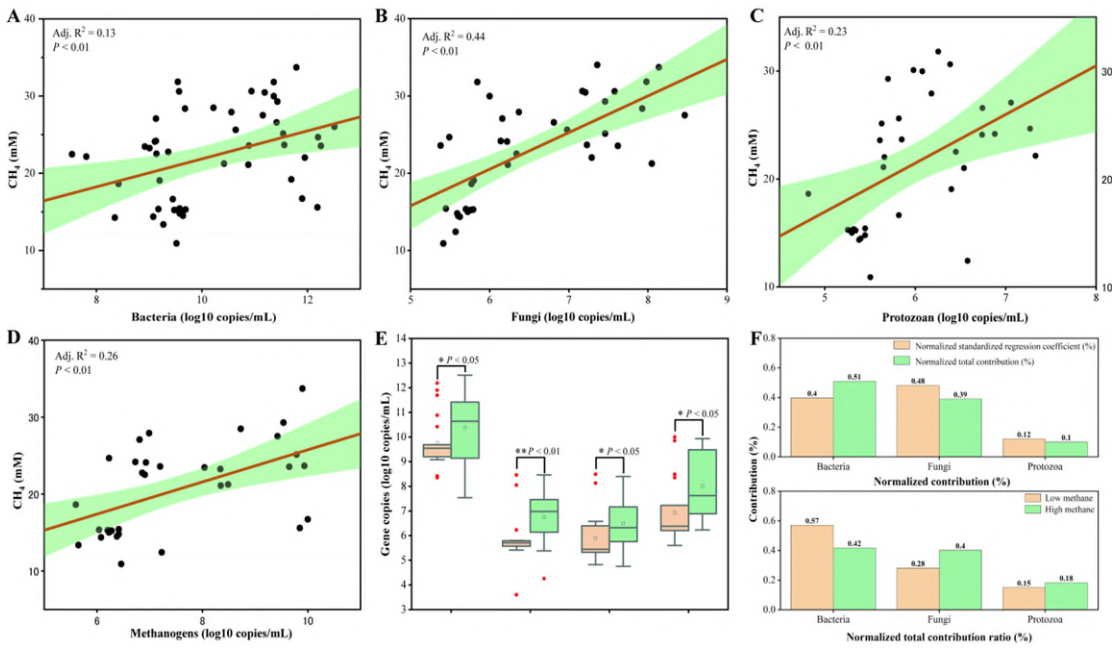


Figure 2. The relationship between the microbiota and methane emissions, and a comparison of the standardized coefficients. The relationship between bacteria, fungi, protozoa, methanogenic archaea, and methane emissions (A-D). The comparison of the abundance of a given microbiota under high versus low methane production conditions (E). Comparison of standardized coefficients among

microbiota (F).

Correlations between microbiota and methanogenic archaea

To investigate the causes underlying the variation in standardized coefficients among different microbiota with respect to methane production, linear regression curves for each microbiota against methanogenic archaea were generated. Bacteria, fungi, and protozoa all exhibited significant positive correlations with methanogenic archaea ($P < 0.05$), with Adj. R^2 of 0.52, 0.54, and 0.11, respectively (Fig. 3A-C). Pearson correlation results showed that bacteria, fungi, and protozoa all exhibited positive correlations with methane production, with correlation coefficients of 0.38, 0.52, and 0.24, respectively. Similarly, positive correlations were observed between bacteria, fungi, protozoa, and methanogenic archaea, with coefficients of 0.71, 0.74, and 0.36, respectively. Among the three microbial groups, the Pearson correlation between bacteria and protozoa was negative (-0.06), while the correlations between the other pairs were positive. (Fig. 3D). After stratifying methane production into high and low groups, the Pearson correlations between each microbial group and methane production, as well as between each microbial group and methanogenic archaea, were improved compared to the low methane production group, with the exception of the correlation between fungi and methanogenic archaea (Fig. 3E and 3F).

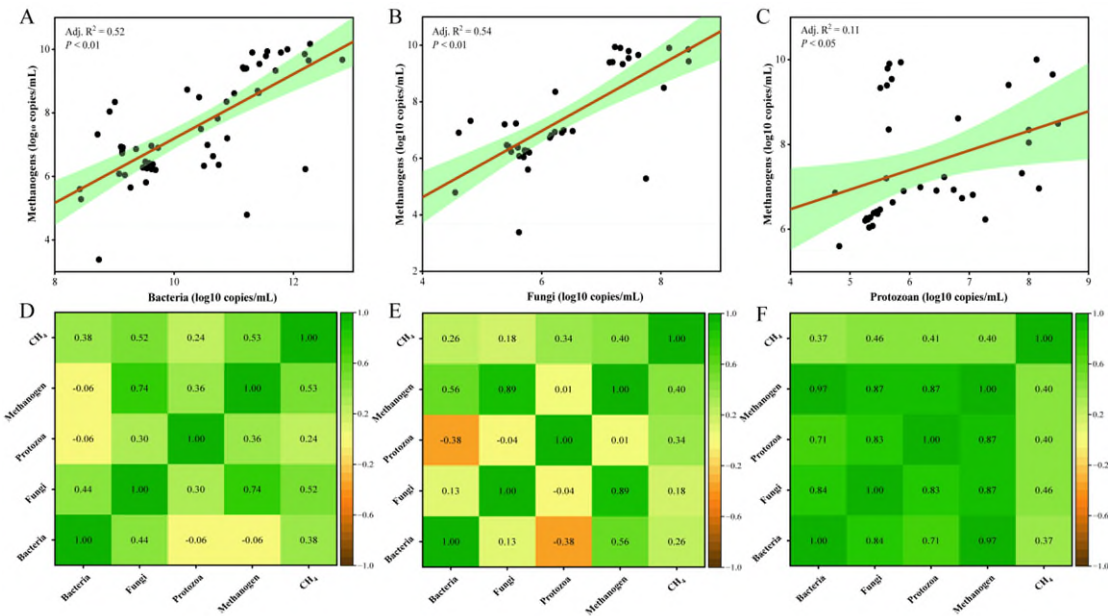


Figure 3. The linear regression fitting curves of each microbiota with methanogenic archaea, and the Pearson correlations between each microbiota, methanogenic archaea, and methane production.

Regression fitting curves between each microbiota and methanogenic archaea (A-C). Pearson correlations among microbial abundances and with methane production (D-F).

Correlation Analysis and Regression Fitting

Pearson correlation analysis among methane production, rumen microorganisms, and dietary components showed that methane production was significantly and positively correlated with bacterial 16S rRNA abundance and fungal 18S rRNA abundance ($P < 0.01$). Methane production also showed a positive correlation with protozoa, but the correlation was not significant ($P > 0.05$). With the exception of ether extract, the correlations between dietary components and methane production were not significant ($P > 0.05$), although NDF and ADF showed positive correlations with methane production (Table 2).

Table 2. Pearson correlation of methane production, rumen microbiota abundance, and dietary composition.

	Methane	Bacteria	Fungi	Protozoa	NDF	ADF	CP	EE	Ash
Methane	1								
Bacteria	0.38**	1							
Fungi	0.52**	0.44**	1						
Protozoa	0.24	-0.06	0.3	1					
NDF	0.15	-0.03	0.181	.468**	1				
ADF	0.127	-0.02	0.174	.564**	0.98**	1			
CP	-0.019	0.15	-0.109	-0.052	0.73**	0.67**	1		
EE	0.29*	0.15	0.212	.62**	0.77**	0.73**	0.67**	1	
Ash	-0.14	-0.95**	0.76**	-0.76*	0.82**	0.80**	0.77**	0.86**	1

Note: NDF = neutral detergent fiber, ADF = acid detergent fiber, CP = crude protein, EE = ether extract,

* indicates $P < 0.05$, ** indicates $P < 0.01$.

The Adj. R^2 for the multiple linear regression equations (Eq. i - iii) were 0.46, 0.20, and 0.26, respectively. The fit of Equation ii was not significant ($P = 0.23$). The multiple nonlinear regression equation (Eq. iv) had an adjusted R^2 of 0.74, indicating a statistically significant fit ($P < 0.01$) (Table 3).

Table 3. Linear and nonlinear fitting equations for methane production

Equation	Eq.	Adj. R^2	P-value
$CH_4 = -27.456_{(9.315)} + 2.576B_{(0.888)} + 1.807F_{(1.319)} + 1.943P_{(1.102)}$	i	0.46	< 0.01

$\text{CH}_4 = -5.527_{(15.927)} - 0.008\text{NDF}_{(0.008)} + 2.81\text{B}_{(1.438)} - 0.903\text{F}_{(2.036)} + 1.224\text{P}_{(1.868)}$	ii	0.20	0.23
$\text{CH}_4 = -6.507_{(8.702)} + 1.113\text{B}_{(0.909)} + 2.681\text{F}_{(1.038)}$	iii	0.26	< 0.01
$\text{CH}_4 = -314.85_{(11.459)} + 13.966\text{B}_{(12.439)} + 75.992\text{F}_{(11.812)} + 0.495\text{B}^2_{(0.645)} - 2.606\text{F}^2_{(0.794)} - 3.579\text{BF}_{(0.668)}$	iv	0.74	< 0.01

Note: B = bacteria, F = fungi, P = protozoa, NDF = neutral detergent fiber, Eq. = equation. The values in parentheses represent standard error, Adj. R^2 = adjusted R^2 , and $P < 0.01$ indicates extremely significant differences, $P < 0.05$ indicates significant differences.

Based on the predicted values and true values of methane yield, fitting curves were plotted for the three equations (Fig. 4). The scatter points in the fitting curves of all three equations were uniformly distributed around the $X = Y$ line. Among them, Equation 4 had an Adj. R^2 of 0.46, $P < 0.01$, and a Slope of 0.42, showing the smallest difference between the predicted and true values (Fig. 4C).

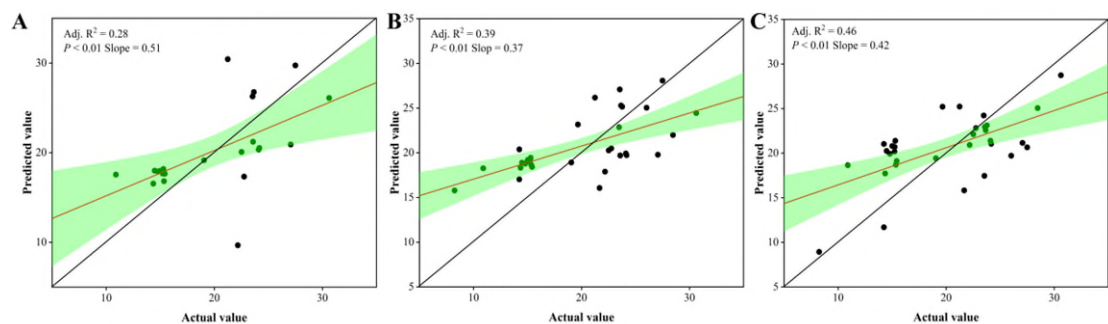


Figure 4. Fitting curves of the true values and predicted values of methane yield. Figures A - C show the fitting curves of the predicted and true values for Equation i, Equation ii, and Equation iv, respectively. The black line in each figure represents the equation $X = Y$.

Effects of dietary NDF on microbial contributions to methanogenesis

The results of Pearson correlation analysis demonstrated that bacteria, fungi, and protozoa were all positively correlated with acetate, propionate, and butyrate (Fig. 4A). The relative contribution of each microbiota based on volatile fatty acids ranked as bacteria > fungi > protozoa, with values of 0.45, 0.28, and 0.26, respectively (Fig. 4B). To explore the factors influencing the contribution, fitting curves of methane production as a function of dietary NDF content (Fig. 5C), as well as curves of the relative contribution of each microbial group in response to NDF content, were plotted (Fig. 5D-F). The results showed that methane production increased with increasing dietary NDF content ($P < 0.01$, Adj. R^2 = 0.13), and methane production in the high NDF group was significantly higher than that in the low NDF group ($P < 0.05$) (Fig. 4C). According to the fitting curves of NDF content against the relative

contributions of each microbial group, the relative contribution of bacteria showed a decreasing trend with increasing NDF content ($P = 0.18$, Adj. $R^2 = 0.02$) (Fig. 5D). In contrast, the relative contributions of fungi and protozoa increased with increasing NDF content ($P = 0.12$ and 0.17 , respectively; Adj. $R^2 = 0.04$ and 0.04 , respectively) (Fig. 4E and 4F).

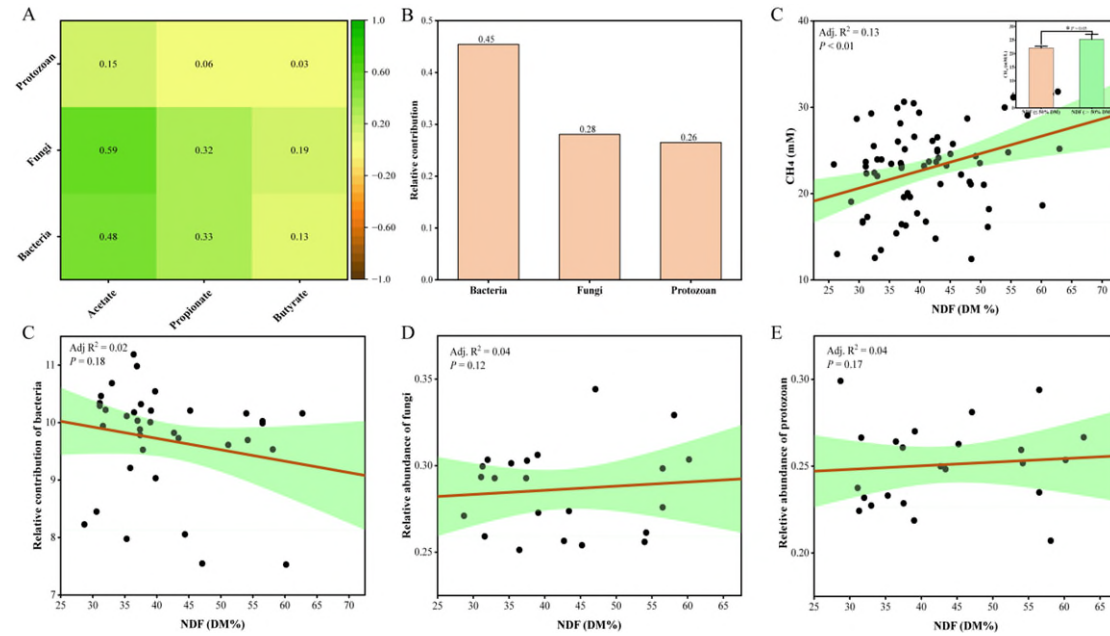


Figure 5. The relative contribution of each microbiota based on volatile fatty acids, and the fitting curves between feed NDF and each microbiota. Pearson correlations between volatile fatty acids and bacteria, fungi, and protozoa (Fig. 5A). Relative contributions of different microbiota based on volatile fatty acids (Fig. 5B). Linear fitted curve of feed NDF content versus methane production (Fig. 5C). Linear fitted curves of feed NDF content versus the relative contributions of bacteria, fungi, and protozoa (Fig. 5C-E).

Factors influencing methane emissions

The comparison of various factors potentially influencing methane emissions indicated that, across the range of animal species examined in this study, neither ruminant species nor sex exerted significant effects on methane emissions from ruminants ($P > 0.05$) (Fig. 6A, 6B). Methane production was significantly higher in *in vivo* experiments compared to *in vitro* experiments ($P < 0.05$) (Fig. 6C). Additionally, ruminants at altitudes of 500 - 1000 m exhibited significantly higher methane production than those at low (0 - 500 m) and high altitudes (> 1000 m) (Fig. 6D).

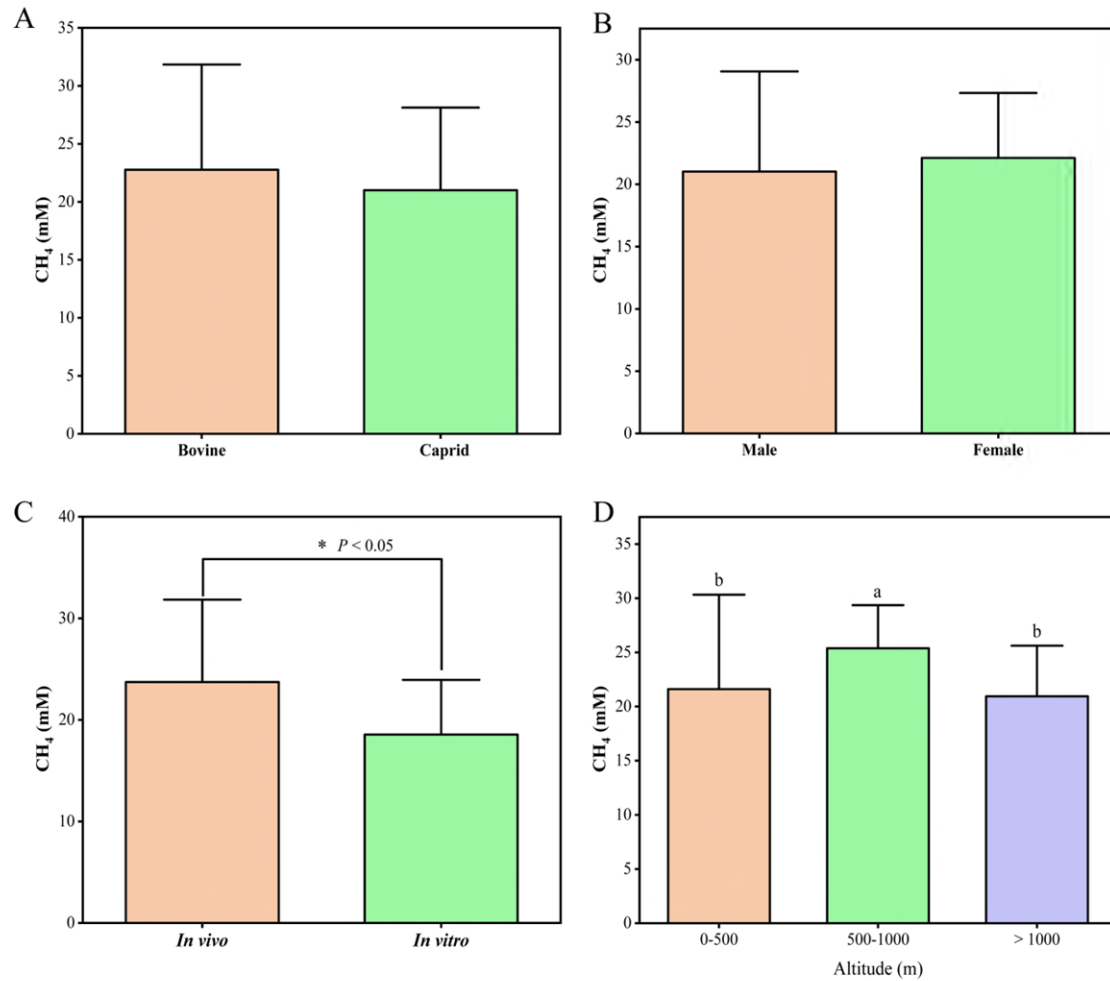


Figure 6. Factors affecting methane emissions from ruminants. Effect of ruminant species on methane emissions (Fig. 6A). Effect of sex on methane production (Fig. 5B). Effect of *in vivo* and *in vitro* experiments on methane emissions (Fig. 6C). Effect of altitude on methane production (Fig. 6D). Effects of experimental conditions and altitude on microbiota-methane correlations

Under *in vivo* conditions, the Pearson correlation of bacteria with methane production was higher than that of fungi and protozoa. Under *in vitro* conditions, the Pearson correlation of fungi was higher than that of bacteria and protozoa. Compared with *in vitro* conditions, the Pearson correlation of bacteria with methane production was higher under *in vivo* conditions, whereas that of fungi was lower (Fig. 7A). Compared with *in vitro* conditions, the abundances of bacteria and fungi were higher under *in vivo* conditions, with the difference being significant for bacteria ($P < 0.05$) (Fig 7B). At altitudes of 0 - 500 m and 500 - 1000 m, the Pearson correlation of bacteria with methane production was higher than that of fungi and protozoa. At altitudes > 1000 m, the Pearson correlations of both bacteria and

fungi were higher than that of protozoa (Fig. 7C). Compared with the altitude conditions of 0 - 500 m and > 1000 m, the abundances of bacteria and fungi were significantly higher at 500 - 1000 m ($P < 0.05$), whereas no significant difference was observed for protozoa (Fig. 7D).

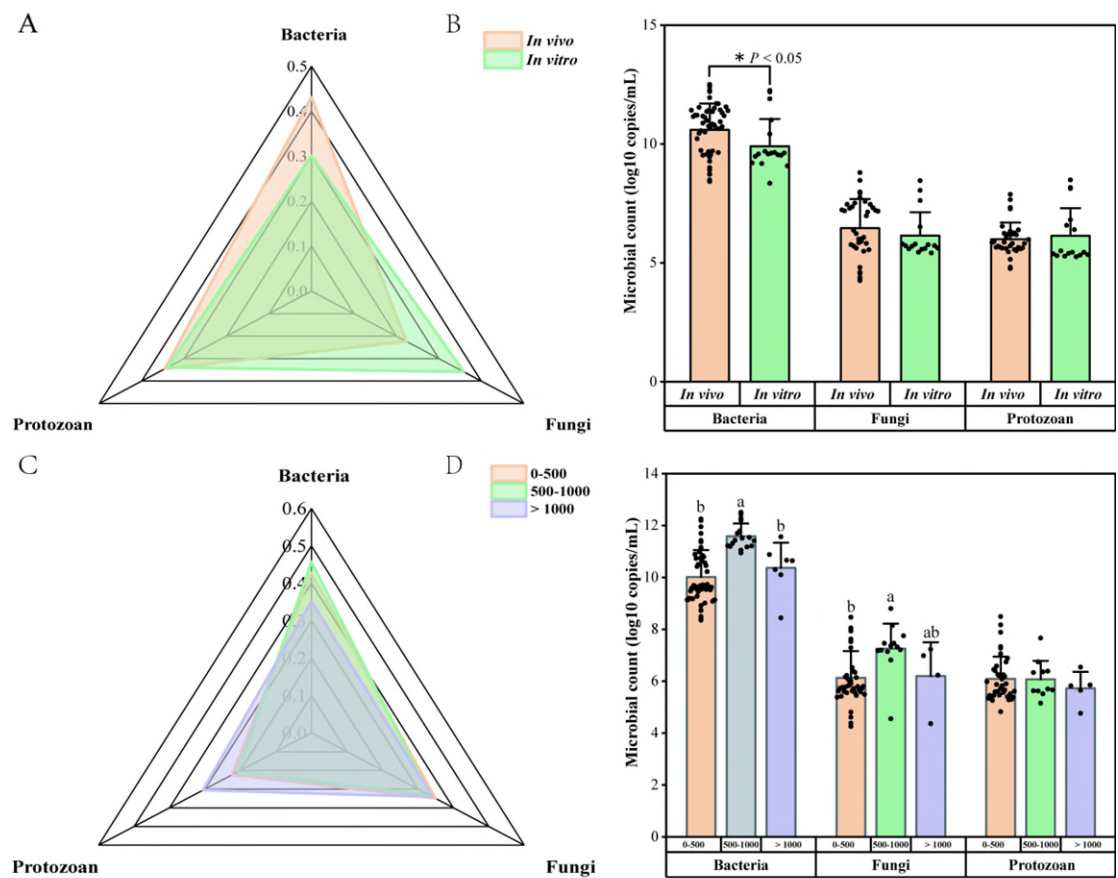


Figure 7. Microbial factors contributing to differences in methane emissions. Radar plot of Pearson correlations of bacteria, fungi, and protozoan with methane emissions under *in vivo* versus *in vitro* conditions (Fig. 7A). Comparison of bacterial, fungal, and protozoal abundances under *in vivo* and *in vitro* conditions (Fig. 7B). Radar plot of Pearson correlation coefficients for bacteria, fungi, and protozoa with methane emissions at different altitudes (Fig. 7C). Comparison of bacterial, fungal, and protozoan abundances at different altitudes (Fig. 7D).

Discussion

After screening 210 articles following the PRISMA guidelines, the selected studies were assessed using the JBI checklist. The JBI checklist is a standardized critical appraisal tool employed in systematic reviews to evaluate the methodological quality and assess the risk of bias of primary studies

[9, 19]. The checklist comprises 10 assessment items, each of which can be answered with 'Yes,' 'No,' or 'Not applicable' by the respondents. After screening 210 articles following the PRISMA guidelines, the selected studies were assessed using the JBI checklist. The quality scores were distributed as follows: 27 studies received a score of 5, 54 studies received a score of 6, 74 studies received a score of 7, 44 studies received a score of 8, and 11 studies received a score of 9. The JBI quality assessment scores for the 210 selected studies could be found in Supplementary File S2.

Bacteria are the most abundant microbial community in the rumen. The phyla Firmicutes, Fibrobacterota, and Bacteroidota are the primary cellulose- and hemicellulose-degrading lineages, comprising *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Ruminococcus albus*, *Clostridium lochheadii*, *Clostridium longisporum*, *Butyrivibrio fibrisolvens*, *Prevotella spp.*, and *Streptococcus spp.* Furthermore, *Fibrobacter succinogenes*, *Streptococcus bovis*, and *Prevotella ruminicola* (primary amylolytic bacteria); *Fibrobacter succinogenes*, *Ruminococcus albus*, *Butyrivibrio fibrisolvens*, and *Streptococcus bovis* (primary pectinolytic bacteria); as well as *Butyrivibrio fibrisolvens*, *Butyrivibrio proteoclasticus*, and *Streptococcus bovis* (primary proteolytic bacteria) play key roles in the degradation of their respective substrates [20]. The metabolites derived from their breakdown of feed provide essential substrates that fuel methane production. Previous studies have primarily focused on the positive correlation between the archaea-to-bacteria abundance ratio and methane production [21], and some studies have also reported a positive correlation trend between bacterial abundance and methane production [22, 23]. Methanogenic archaea assume a major role in this process, as they remove hydrogen from the rumen and reduce the ruminal hydrogen partial pressure, thereby creating optimal functional conditions for the rumen microorganisms involved in fermentation [24]. Anaerobic fungi (*Neocallimastigomycetes*) in the rumen are also important fiber decomposers [25]. Currently, four families are recognized within this phylum: *Caecomycetaceae* fam. nov., *Piromycetaceae* fam. nov., *Neocallimastigaceae*, and *Anaeromycetaceae*, encompassing a total of 22 genera [26]. The metabolic end products of anaerobic fungi also constitute an important source of methane production. In the study of López-García et al., long-read sequencing technology was used to analyze the composition and metagenome of rumen microorganisms, and it was found that methane emissions were positively correlated with fungal abundance [27]. Ciliate protozoa vary between different ruminant species and also among individuals of the same species [28, 29]. The genera *Entodinium* and *Epidinium* are the core groups that dominate in terms of abundance [30]. Due to the difficulty of cultivating protozoa in

vitro, most research on the impact of protozoa on methane production has focused on defaunation studies [11]. Multiple studies have shown that defaunation leads to a decrease in methane production [12, 13, 31]. The primary reasons are the symbiotic association between methanogenic archaea and protozoa [32], and the ability of most protozoa (e.g., holotrich and entodinomorph genera) to produce hydrogen via [FeFe] - hydrogenases, thereby fueling methanogenesis [30].

The composition of ruminant feed is one of the most important factors affecting ruminal methane production [33]. When the concentrate-to-forage ratio of the diet decreases, methane production increases correspondingly [34]. When the crude fiber content of the diet increases, rumen fermentation shifts toward an acetate-type pattern, leading to elevated levels of acetate and butyrate in the rumen, accompanied by the release of hydrogen, which provides ample substrate for hydrogenotrophic methanogens and subsequently promotes methane production [35]. In addition, the dietary concentrate-to-forage ratio also affects the composition of the rumen microbiota. When the crude fiber content in the diet increases, fibrolytic bacteria (such as *Butyrivibrio fibrisolvens*, *Ruminococcus flavefaciens*, *Ruminococcus albus*, etc.) and anaerobic fungi in the rumen increase as well [36]. In most studies, dietary crude fiber content does not significantly affect rumen bacterial abundance. In the study by Agustina et al., total bacterial count declined as increasing NDF content, but the decrease was not significant [37, 38]. While in the study by Wanapat et al., the bacterial abundance did not show a simple monotonic increase or decrease with increasing NDF content [39]. Consistent with their observations, the present study found that bacterial abundance decreased with increasing dietary crude fiber content; however, this reduction was not statistically significant ($P > 0.05$). In contrast, the abundances of fungi and protozoa increase with an elevation in the dietary fiber ratio. A higher NDF content provides a relatively higher pH and a greater diversity of cellulosic substrates, thereby facilitating the proliferation of these microorganisms [40]. The changes in microbial abundance induced by an increase in dietary fiber structure suggest that the relative contribution to methane production is closely correlated with microbial population size.

Methane emissions from ruminants are influenced by a variety of factors, including dietary composition, body size, animal species, growth rate, activity level, productive performance, physiological status, and environmental conditions [41-43]. Previous research has demonstrated that methane yield per unit varies among different ruminant species, and that cattle have significantly

higher methane production per unit than goats and sheep ($P < 0.05$) [44], whereas sex has no significant effect on methane yield per unit ($P > 0.05$) [45]. Altitude is a significant factor influencing methane emissions from ruminants. In the study by Han et al., the medium-altitude group (4,500 m) exhibited a lower acetate-to-propionate ratio and lower microbial richness and diversity compared to the low-altitude (3,800 m) and high-altitude (4,800 m) groups, which was mainly attributed to multidimensional differences in environment and forage nutrition across altitudes [46]. In the study by Zhang et al., there was no significant difference in the alpha diversity of rumen microorganisms between the low-altitude (700 m) and high-altitude (3,750 m) groups [47]. This indicates that the effect of altitude on the rumen microbial composition of ruminants is not a single, linear relationship. The results of this study indicate that rumen microbial abundance varies significantly across different altitudes, with a peak observed at medium altitude (500 - 1000 m), suggesting that this altitude range may represent an optimal balance between environmental stress and nutrient availability. When experimental conditions are restricted, *in vitro* assays can serve as an effective alternative to *in vivo* experiments. However, due to the exclusion of key physiological factors such as rumen volume and digesta passage rate, *in vitro* assays cannot serve as a complete substitute for *in vivo* experiments [48]. In the study by Fernández et al., some plant compounds that effectively inhibited methane production in *in vitro* assays showed significantly reduced inhibitory effects in *in vivo* experiments ($P < 0.05$), which may be attributed to the exposure of microorganisms to oxygen and the removal of most solid material during filtration, leading to decreased microbial density and alterations in microbial community structure [49]. In our study, similar results were observed, with higher methane production and higher microbial community abundance in *in vivo* experiments compared with *in vitro* experiments. The findings from *in vivo* and *in vitro* experiments, together with the effects of experimental altitude on

methane emissions and microbial community composition, demonstrated the important role of rumen microbial abundance in driving methane production. Furthermore, they established the order of relative contributions among bacteria, fungi, and protozoa.

Conclusion

This study investigated the relative contributions of rumen bacteria, fungi, and protozoa to methane emissions, as well as the primary factors influencing methane production. The results demonstrated that the relative contribution of methane emissions followed the order of bacteria > fungi > protozoa, and that the richness of each microbial group was the predominant factor affecting methane emissions. Consequently, future methane mitigation strategies should prioritize interventions targeting rumen bacteria rather than fungi or protozoa. Therefore, prioritizing bacterial-mediated methanogenesis pathways contributes to reducing methane emissions while maintaining production efficiency.

Abbreviations

ADF	Acid detergent fiber
Adj. R ²	Adjusted coefficients of determination
B	Bacteria
CP	Crude protein
CV	Coefficient of variation
EE	Ether extract
EMP	Embden-Meyerhof-Parnas
Eq.	Equation
Fig.	Figure
F	Fungi
JB	Joanna Briggs Institute
NAD ⁺	Oxidized nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NDF	Neutral detergent fiber
NTC	Normalized total contribution
P	Protozoa
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
SD	Standard deviation
VIF	Variance inflation factor

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

Yanfen Cheng is a Guest Editor of the Journal of Animal Science and Biotechnology and was not involved in the editorial review or the decision to publish this article. The other authors declare that they have no competing interests.

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Authors' contributions

Guanghai Hu: Writing – review and editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. Varijakshapanicker Padmakumar: Data curation, Project administration. Weiyun Zhu: Methodology, Data curation, Validation, Supervision. Yanfen Cheng: Writing – review and editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. All authors read and approved the final manuscript.

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