TEA SAPONIN MODULATES *IN-VITRO* RUMEN FERMENTATION PROFILE AND REDUCES METHANE PRODUCTION: A META-ANALYSIS

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ABSTRACT

Saponins are secondary plant metabolites that bind to hydrophobic non-sugar components which are capable of reducing rumen enteric CH_4 production. However, the effect of different levels of dietary tea saponin on rumen fermentation remains inconclusive. This study aimed to investigate the effect of tea saponin on *in-vitro* rumen fermentation and rumen enteric CH_4 production through a meta-analysis approach. A total of 6 articles were selected and included in the meta-analysis database, continued by statistical analysis using SAS software by performing mixed model formula. The results showed that tea saponin levels lowered *in-vitro* organic matter degradability (IVOMD) and *in-vitro* dried matter degradability (IVDMD; P = 0.01) by a linear response. Furthermore, increased tea saponin levels in the diet reduced enteric CH_4 emission expressed as CH_4/DM substrate and CH_4/IVOMD by a quadratic (P = 0.01) and a linear response (P = 0.04), respectively. Increased tea saponin levels also tended to decrease gas production linearly (P = 0.08). Tea saponins inclusion also decreased pH levels linearly (P = 0.04), but increased NH_3 concentration quadratically (P = 0.01). Moreover, the inclusion of tea saponin quadratically increased the total volatile fatty acids (VFA) concentration in the rumen and linearly increased acetate (C_2) and propionate (C_3) proportions ($P < 0.05$). As a result, the C_2/C_3 ratio linearly decreased (P = 0.01) by the increased tea saponin levels, where protozoa population also quadratically diminished (P = 0.01). In conclusion, tea saponin levels directly reduced enteric CH_4 production by diminishing protozoa populations.

Keywords: *in-vitro*, meta-analysis, methane, protozoa, rumen, tea saponin

INTRODUCTION

Ruminants are known to contribute 20% of enteric methane (CH_4) emissions of the world's total greenhouse gases (GHG) or around 47% of the overall GHG emissions in the livestock sector (Haque 2018). Besides being noticed as one of environmental pollutant, CH_4 production also results in the losses of total gross dietary energy by approximately 15% (Giger-Reverdin & Sauvant 2000; Yanza *et al.* 2021a). Currently, biologically active components (BACs) plants are considered as alternative antibiotics in decreasing CH_4

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production from ruminants due to the negative impact of antibiotics residue in products of animal origins (Cieslak *et al.* 2013; Yanza *et al.* 2021b). Previous studies showed positive evidence of BACs effects in reducing CH_4 production from ruminants by their supplementation in the diet (Jayanegara *et al.* 2012; Irawan *et al.* 2022). One prospective BACs often used to reduce CH_4 production is saponin (Cieslak *et al.* 2013; Jayanegara *et al.* 2014).

Saponins are BACs made up of sugar components that bind to hydrophobic non-sugar components (Patra 2012; Guyader *et al.* 2015). Due to their chemical structures, saponins have anti-bacterial and anti-methanogenic capabilities which directly interact with ruminal microbes, modulate rumen fermentation processes and reduce rumen enteric CH_4 production (Belanche *et al.* 2014; Jayanegara *et al.* 2019). Saponins are present in plants, such as alfalfa, soybeans, almonds, *Quillaja* sp., *Saponaria* sp., *Yucca schidigera*, and tea leaves (Wina 2012; Guyader *et al.* 2015; Jadhav *et al.* 2016). In addition, tea is considered a saponin-rich plant that has been frequently studied concerning its effect on rumen fermentation and CH_4 mitigation (Jayanegara *et al.* 2014).

Previous studies demonstrated the beneficial effects of saponins on bovine, such as ruminants (Wina 2012; Cieslak *et al.* 2014; Canul-Solis *et al.* 2020). However, a high level of tea-saponin extract supplemented in the ruminant diet can reduce enteric CH_4 production by up to 29%, followed by a reduced protozoa population of about 51%, depending on the saponin levels (Goel *et al.* 2008; Guyader *et al.* 2017). Previous studies' showed different effects due to different sources of saponin and different supplementation levels which resulted in varied CH_4 production (Cieslak *et al.* 2013; Tan *et al.* 2020). Hence, the effects of tea saponin on rumen fermentation and CH_4 production may remain inconclusive because the effect of different levels of tea saponin supplementation varies (Jayanegara *et al.* 2014). Therefore, this study aimed to determine the effects of dietary tea saponin on *in-vitro* rumen fermentation and CH_4 production through a meta-analysis approach.

MATERIALS AND METHODS

Database Development

The present meta-analysis followed the systematic reviews and meta-analyses (PRISMA) guidelines, a method in the preferred reporting items for the meta-analysis approach (Liberati *et al.* 2009). A database was created using existing studies concerning the effects of tea saponin levels on *in-vitro* rumen enteric CH_4 production. Google Scholar, Scopus, and Science Direct were used as search engines to find articles containing the keywords "saponin", "ruminant", and "methane" (Fig 1).

The screening and selection phases resulted in a total of 34 articles. The subsequent evaluation of abstract and substantial data resulted in only 6 selected articles with 10 studies (n = 62 total treatments) which matched with the meta-analysis criteria concerning the utilization of tea saponin on ruminants and the effect of tea saponin on rumen enteric CH_4 production, which were included in the database (Table 1).

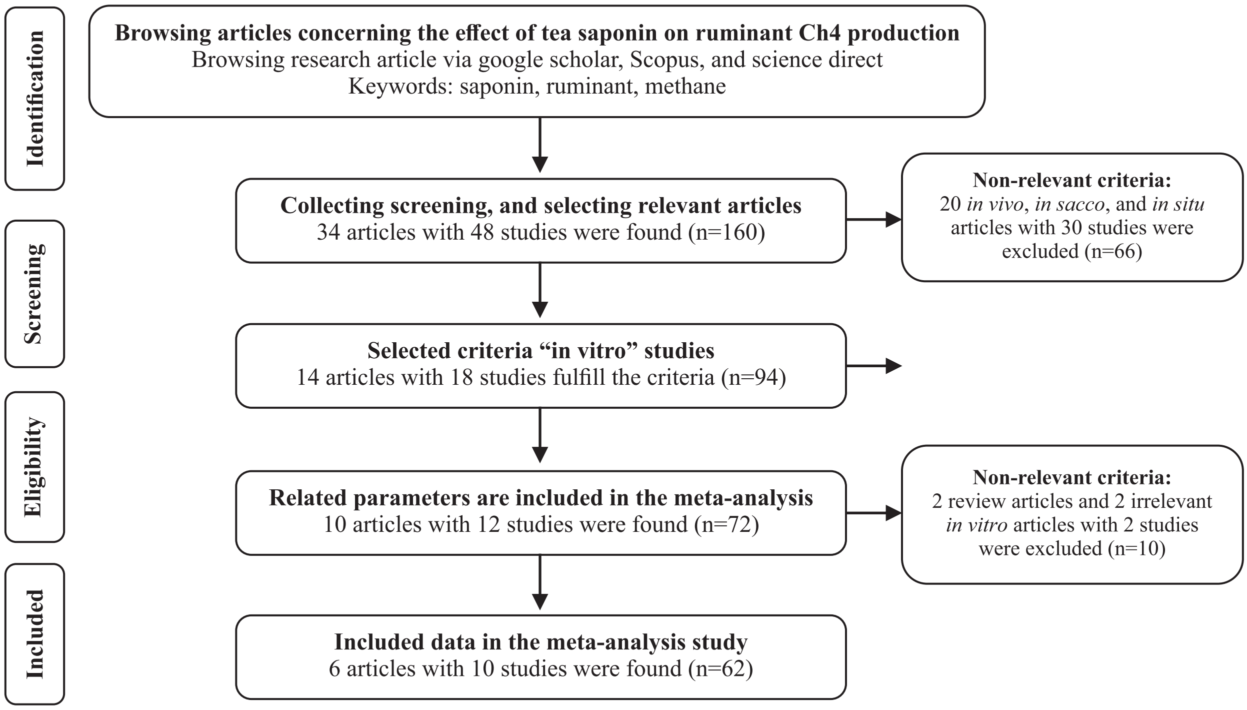


Figure 1 The PRISMA diagram for selecting articles and studies matched with designated criteria in the meta-analysis approach

Table 1 List of studies included in the meta-analysis of the effect of tea saponin on *in-vitro* rumen fermentation profile and enteric CH_4 production

No	Reference	Year	Σ Study	Animal donor	n Exp	Source of tea saponin	Level of tea saponin (g/kg DM substrate)
1	Hu *et al.*	2005a	1	Sheep	4	Commercial tea saponin containing more than 60% triterpenoid saponin	0 - 40
2	Hu *et al.*	2005b	2-3	Sheep	4	Commercial tea saponin containing more than 60% triterpenoid saponin	0 - 53
3	Hu *et al.*	2006	4	Sheep	4	Commercial tea saponin containing 60% triterpenoid saponin	0 - 107
4	Jadhav *et al.*	2016	5-8	Steer	6	Tea (*Camellia sinensis*) seed	0 - 10
5	Guyader *et al.*	2017	9	Cow	4	Commercial tea saponin	0 - 50
6	Zhang *et al.*	2021	10	Sheep	5	Tea seeds (*C. sinensis*)	0 - 20

Notes: n Exp = number of experiments; DM = dry matter.

All constructed data were only based on *in-vitro* experiments involving small ruminants, (sheep) and large ruminants (cows and steer). The tea saponin supplementation levels were measured in g/kg Dry Matter (DM) of feed. Data included in the database were: (1) *in-vitro* feed degradability, such as organic matter degradability (OMD) and dry matter degradability (DMD); (2) *in-vitro* gas production, such as total gas and rumen enteric CH_4 production; and (3) rumen fermentation profile, such as pH, ammonia (NH_3) concentration, volatile fatty acids (VFA) concentration, microbial protein synthesis (MPS), and protozoa population (Table 2). All data were inputted, verified, and evaluated carefully before being statistically analyzed. Data having different measurement units in a similar parameter were converted into a similar unit commonly used to allow direct analysis within a particular parameter.

Table 2 Descriptive statistics of analyzed variables on the effect of tea saponin toward respective parameters

Parameter	Unit	n	Mean	Min	Max	SEM
DMD	g/kg	36	766	732	783	1.90
OMD	g/kg	46	740	547	814	12.0
pH		26	6.95	6.41	7.94	0.09
NH_3 concentration	mmol/L	51	14.6	10.0	20.8	0.36
VFA	mmol/L	62	87.3	33.9	120	3.37
C_2	%	26	63.1	23.5	72.0	2.63
C_3	%	26	19.4	7.53	25.9	0.98
C_4	%	26	8.74	2.86	13.4	0.75
C_2/C_3		26	3.34	2.69	4.54	0.11
Gas production	mL	51	58.4	37.2	119	3.24
Gas production/DMs	mL/g	58	238	121	394	8.96
CH_4 production	mL	51	8.76	5.99	23.6	0.48
CH_4/DMs	mL/g	62	35.2	8.86	55.5	1.34
CH_4/OMD	mL/g	46	45.7	20.7	68.2	1.79
MPS	mg/mL	15	1.27	0.71	2.61	0.15
Protozoa	log	58	4.45	3.00	5.76	0.08

Notes: DMD = digested dry matter; OMD = organic matter degradability; NH_3 = ammonia concentration; VFA = volatile fatty acids; C_2 = acetate; C_3 = propionate; C_4 = butyrate; DMs = dry matter substrate; CH_4 = rumen enteric methane production; MPS = microbial protein synthesis; SEM = standard error of means.

Statistical Analysis

All parameters were analyzed using a mixed-model methodology in the present meta-analysis. SAS 9.4 software was used to perform the PROC MIXED model (SAS Institute Inc. 2021). The levels of tea saponin were treated as the fixed effects, while the studies were treated as random effects. Both continuous and discrete variables were subjected to statistical modeling (St-Pierre 2001; Jayanegara *et al.* 2014; Yanza *et al.* 2021a). Tea saponin levels were categorized as a continuous predictor variable. The statistical model used was (Equation 1):

$$Y_{ij} = B_0 + B_1X_{ij} + B_2X^2_{ij} + s_i + b_iX^{ij} + e_{ij} \text{ .... (1)}$$

where: Y_{ij} = dependent variable; B_0 = overall intercept across all studies (fixed effect); B_1 = linear regression coefficient of Y on X (fixed effect); B_2 = quadratic regression coefficient of Y on X (fixed effect); X_{ij} = value of the continuous predictor variable (saponin levels); s_i = value of random effect of study i; b_i = the random effect of study on the regression coefficient of Y on X in study i; and e_{ij} = the unexplained residual error. The CLASS statement was established using the tea saponin levels and the study variable, while the RANDOM statement was made based on a variety of studies (Yanza *et al.* 2021). According to Jayanegara *et al.* (2014), these models were performed by weighting the number of replicates in the experiments. The model was considered very significant at $P \leq 0.05$ and significant at $0.05 < P \leq 0.10$. When the respective quadratic regression models were not significant at $P < 0.05$, a linear regression model was applied.

RESULTS AND DISCUSSION

As a contributor to greenhouse gases (GHG) that affects global warming, one of the effective strategies to reduce CH_4 from ruminants is through dietary manipulation with natural additive supplementation (Cieslak *et al.* 2013; Jayanegara *et al.* 2014). Tea saponin is considered a source of biologically active components (BAC) that can suppress rumen enteric CH_4 production with a minimum negative effect (Guo *et al.* 2008; Guyader *et al.* 2017).

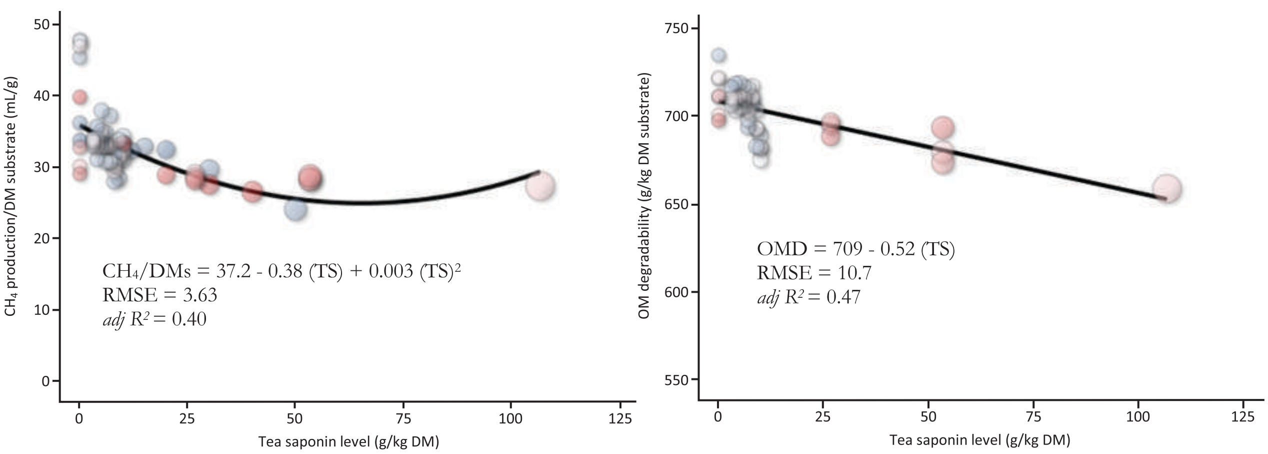
Table 3 The effect of tea saponin on *in-vitro* rumen feed digestibility, total gas production and enteric CH_4 production

Parameter	Unit	n	Response	Intercept	SE	Slope	SE	P value	RMSE	AIC	Trend
DMD	g/kg	36	L	776.50	4.30	-1.90	0.48	< 0.01	7.89	261.10	↓
OMD	g/kg	46	L	708.50	25.43	-0.52	0.12	< 0.01	10.68	397.60	↓
Gas production	mL	51	L	69.49	7.53	-0.06	0.04	0.08	3.26	325.80	↓
Gas/DMs	mL/g	58	L	221.69	23.57	-0.06	0.14	0.69	14.03	522.60	↓
CH_4 production	mL	51	Q	10.72	1.31	-0.08	0.02	< 0.01	0.86	194.90	↓
						0.0006	0.0002	0.01			
CH_4/DMs	mL/g	62	Q	35.89	2.78	-0.33	0.08	< 0.01	3.63	400.9	↓
						0.003	0.001	0.01			
CH_4/OMD	mL/g	46	L	44.06	3.70	-0.12	0.06	0.04	5.20	316.10	↓

Notes: DMD = digested dry matter; OMD = organic matter degradability; DMs = dry matter substrate; CH_4 = rumen enteric methane production; L= linear response; Q = quadratic response; SE = standard of error; RMSE = root mean square of error; AIC = Akaike information criterion. The model is considered significant at $0.05 < P \leq 0.10$ and very significant at $P \leq 0.05$.

In the present study, increased tea saponin levels tended to decrease gas production ($P = 0.08$). Increased tea saponin levels also reduced rumen enteric CH_4 production expressed as CH_4/DM substrate and CH_4/IVOMD, with a quadratic ($P = 0.01$) and a linear responses ($P = 0.04$), respectively. Apparently, there is a relationship between tea saponin levels and the reduction of rumen enteric CH_4 production. When methane is expressed as CH_4/DM substrate, rumen enteric CH_4 production is reduced following a curvilinear pattern (Fig. 2a) with the increased levels of tea saponin supplementation (Fig. 2a). The present study also showed that tea saponin became ineffective in reducing rumen enteric CH_4 production at a supplementation level of 100 g/kg DM.

Previous studies mostly connected the decreased rumen enteric CH_4 production with the rumen microbial population. The presence of saponin can inhibit the activity of rumen microbes, such as protozoa, cellulolytic bacteria and fungi (Hu *et al.* 2005; Guyader *et al.* 2017; Kozlowska *et al.* 2021). Our present study resulted in a diminished protozoa population by means of a quadratic response ($P < 0.01$). Furthermore, the study also showed that tea saponin could inhibit and/or diminish the activity of rumen microorganisms to release free hydrogen, which directly resulted in limited hydrogen supply for methanogens to produce CH_4 (Guo 2008; Jayanegara *et al.* 2014). Saponin mode of action is considered toxic for most rumen microorganisms, including bacteria or even methanogens. Hence, the methanogenesis process was directly inhibited by tea saponin supplementation (Guyader *et al.* 2017).



(a) (b)

Figure 2 Relationship between the level of tea saponin (TS; g/kg DM) and (a) enteric CH_4/DM substrate (mL/g); (b) OM degradability (g/kg DM substrate)

A study by Jadhav *et al.* (2016) showed that saponin supplementation affects rumen digestibility. Our present meta-analysis found that tea saponin supplementation linearly lowered both *in-vitro* organic matter degradability (IVOMD; $P < 0.01$) and *in-vitro* dried matter degradability (IVDMD; $P < 0.01$).

Although tea saponin is considered a natural and safe additive, its supplementation in ruminants feed negatively influences rumen degradability of nutrients (Hu *et al.* 2005b). Therefore, it is suggested that high levels of tea saponin supplementation indirectly interfere with rumen degradability rates due to the inhibited activity of rumen microbes (Hu *et al.* 2005b; Hu *et al.* 2006; Jadhav *et al.* 2016).

Cieslak *et al.* (2014) reported that supplementation of saponin extracted from *Saponaria officinalis* at 1 - 500 g/kg DM had no detrimental effects on *in-vitro* rumen DM degradability of insoluble nutrients, but effectively reduced rumen enteric CH_4 production up to 34%. Our present study proved the negative effect of saponin on rumen degradability rates.

Although there was no such data concerning soluble and insoluble nutrients, the relationship between tea saponin and OM degradability in the present study showed an obvious negative slope followed by a linear pattern (Fig. 2b). Hence, tea saponin has detrimental effects on digestibility, depending on the source of saponins and the level of supplementation.

The concentration of volatile fatty acids (VFA) in the rumen was quadratically affected ($P < 0.05$), by which the saponins increased propionate (C_3) and lowered butyrate (C_4) proportions, where both C_3 and C_4, in partial showed a linear response of VFAs ($P < 0.01$) toward tea saponin supplementation (Table 4).

On the contrary, there was no effect of increased supplementation levels of tea saponin on acetate (C_2) proportion, which caused the linear reduce of C_2/C_3 ratio ($P = 0.01$) (Fig. 3a).

Several genera of microorganisms may be tolerant to tea saponin during fermentation in the rumen, such as *Megasphaera*, *Mitsuokella*, *Schwartzia* and *Selenomonas,* due to saponin mode of action in directly inhibiting methanogens (Jayanegara *et al.* 2014; Belanche *et al.* 2016). Therefore, the VFA concentration increased by the curvilinear pattern at a certain level of tea saponin (Fig. 3c). Most saponins have anti-protozoal effects, including tea saponin (Kozlowska *et al.* 2021).

Saponin chemical structures are known to be bound to lipids, which then disrupt the sterol of the protozoa cell membrane, causing the leakage of cell content and finally kill the protozoa (Jadhav *et al.* 2016; Kozlowska *et al.* 2021). Besides, the increased level of saponin also linearly decreased pH levels ($P = 0.04$), in which rumen protozoa are sensitive to decreased pH value (Jadhav *et al.* 2016). Hence, the presence of tea saponin in rumen fermentation indirectly decreased the methanogens population due to the limited hydrogen supply from protozoa to form CH_4. On the other hand, most of the free hydrogen molecule produced by rumen microbial activity was shifted for the propionate (C_3) formation instead of CH_4 formation (Moss *et al.* 2000).

Moreover, decreased protozoa population positively impacted the utilization of N molecule from the feed. Hence, tea saponins inclusion also quadratically decreased NH_3 concentration ($P = 0.01$) (Fig. 3b).

Such evidences occurred due to protozoa activity involved in the intra-ruminal cycle of microbial N (Jouany 1996). Another study showed that saponins reduced ammonia-N due to the ability of saponins to reduce urease activity, as a result of saponins capability to bind ammonia (Hussain & Cheeke 1995). Our present study indicated that microbial protein synthesis results tended to linearly increased by the increased levels of tea saponins ($P = 0.08$).

Jadhav *et al.* (2016) stated that a lower concentration of ammonia-N indicated that adding tea saponin improved microbial protein synthesis in all substrates. Hence, the present study showed the efficiency of microbial protein in synthesizing the released ammonia.

Table 4 The effect of tea saponin on *in-vitro* rumen fermentation profile and protozoa population

Parameter	Unit	n	Response	Intercept	SE	Slope	SE	P-value	RMSE	AIC	Trend
pH		26	L	7.01	0.21	-0.001	0.0004	0.04	0.04	-30.60	↓
NH_3	mmol/L	51	Q	16.12	0.61	-0.17	0.04	< 0.01	1.70	240.50	↓
						0.0012	0.0004	0.01			
Total VFA	mmol/L	62	Q	73.14	8.15	0.31	0.13	0.02	6.03	470.60	↑
						-0.003	0.001	0.05			
C_2	%	26	L	63.71	5.83	-0.021	0.027	0.44	2.52	160.00	↓
C_3	%	26	L	17.88	1.95	0.06	0.01	< 0.01	1.22	121.40	↑
C_4	%	26	L	8.69	1.72	-0.02	0.01	< 0.01	0.51	86.80	↓
C_2/C_3		26	L	3.60	0.24	-0.011	0.002	< 0.01	0.21	34.60	↓
MPS	mg/mL	15	L	1.02	0.32	0.005	0.003	0.08	0.23	28.00	↑
Protozoa	log	58	Q	4.64	0.22	-0.015	0.002	< 0.01	0.11	-1.80	↓
						0.0001	0.00003	< 0.01			

Notes: NH_3 = ammonia; VFA = volatile fatty acids; C_2 = acetate; C_3 = propionate; C_4 = butyrate; MPS = microbial protein synthesis; L = linear response; Q = quadratic response; SE = standard error; RMSE = root mean square of error; AIC = Akaike information criterion. The model is considered significant at $0.05 < P \leq 0.10$ and very significant at $P \leq 0.05$.

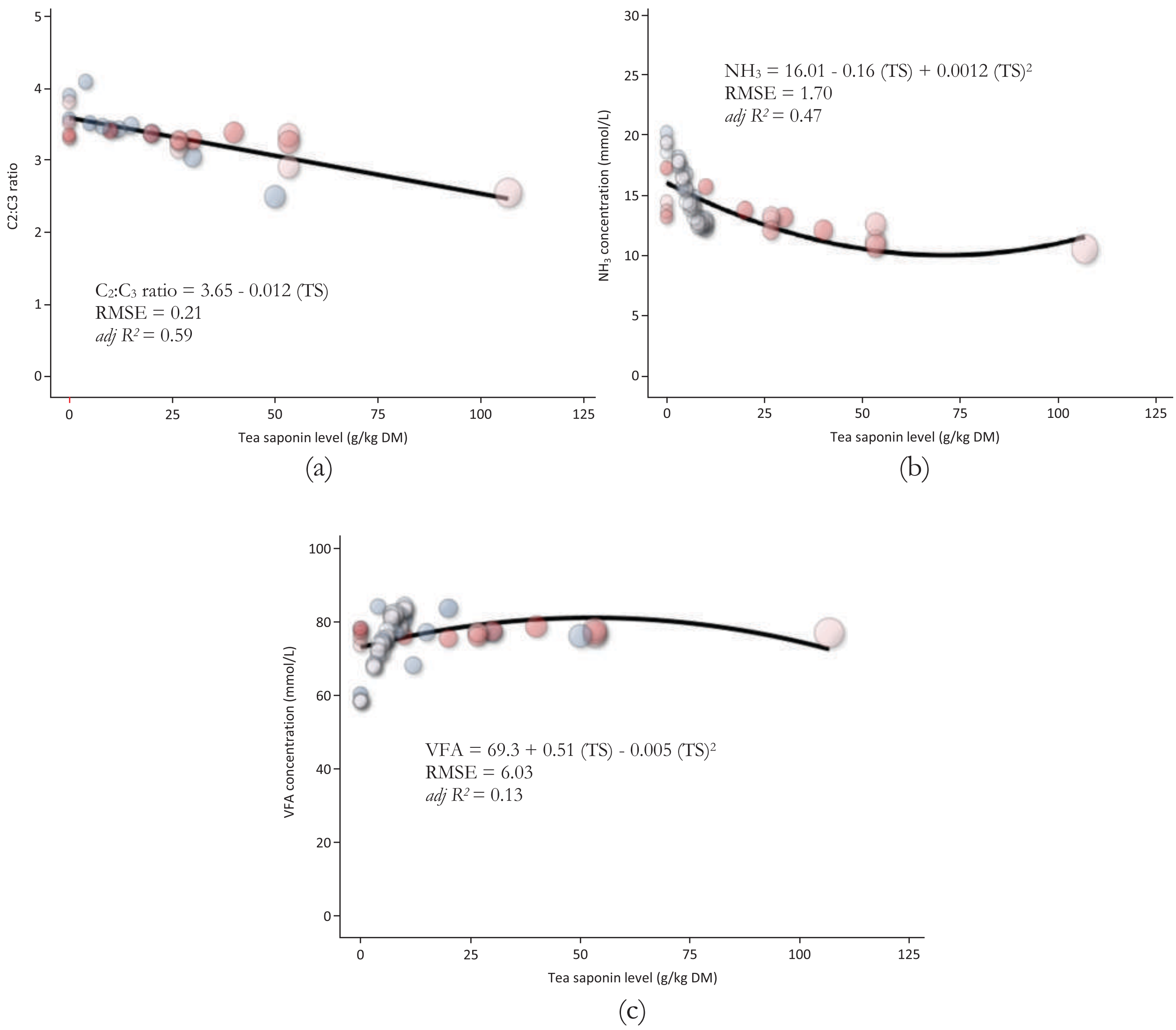


Figure 3 Relationship between the level of tea saponin (TS; g/kg DM) and (a) C_2:C_3 ratio; (b) NH_3 concentration (mmol/L); and (c) VFA concentration (mmol/L)

CONCLUSION

The present study proved that tea saponin suppresses *in-vitro* rumen enteric CH_4 production and modulates rumen fermentation. Suppressed *in-vitro* rumen enteric CH_4 production can be effective if tea saponin is supplemented below 100 g/kg of DM substrate. The suppressed *in-vitro* rumen enteric CH_4 production by tea saponin supplementation is directly related to the diminished protozoa population. The methanogenesis process was inhibited, while rumen fermentation products were modulated with the tea saponin supplementation.

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