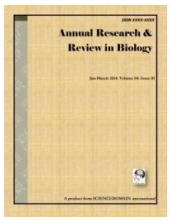

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The Influence of Ginger (*Zingiber Officinale*) on *In vitro* Rumen Fermentation Patterns

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

This study was carried out to assess the effect of Ginger (*Zingiber officinale*) on the *in vitro* rumen ecosystem of sheep. Rumen fluid was obtained from three male sheep with fistula and mixed with 0, 30, and 60 mg ginger plus a substrate which represented the basic diet of alfalfa hay and barley in a ratio of 70:30 which had been given to the sheep used in this study. In the experiments the ginger/substrate mixtures were incubated for intervals of 0, 2, 4, 6, 8, 10, 12, 18, 24, 36, and 72 h. A completely randomized design (CRD) was performed with four replicates per each treatment. The *in vitro* gas production (IVGP), methane emission, *in vitro* organic matter degradability (IVOMD), ammonia (NH3-N) concentration, partitioning factor (PF), microbial mass (MM), volatile fatty acid (VFA) concentrations and protozoan population were measured. The results showed that 60 mg ginger supplement significantly improved the potential gas production (*Linear (L)*; $P<0.001$). Cumulative gas production was also increased after 72 h (L; $P<0.031$). Methane production decreased by the addition of 30 and 60 mg of ginger compared with the control (Control vs ginger; $P=0.012$). The NH3-N concentration linearly declined in the presence of ginger (L; $P=0.000$). Total VFA concentrations were not influenced, but the acetate to propionate ratio declined (L; $P\leq 0.05$) and the branched fatty acids increased (L, $P<0.01$). The antiprotozoal activity was improved by ginger treatments especially on the Entodiniinae subfamily population (L, $P=0.028$) (Control vs ginger; $P=0.026$). Based on this study, it seems ginger supplementation could

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improve ruminal fluid fermentation due to NH3-N reduction, reduce methane losses and cause beneficial changes in protozoal population.

Keywords: Zingiber officinale; rumen fermentation; methane; gas production; protozoa.

1. INTRODUCTION

Additives that modify rumen fermentation such as organic acids, antibiotics and medicinal plants have been used to optimize performance in ruminant production systems [1]. Microbial degradation of feed in the rumen is characterized by losses of energy and ammonia N [2]. Methane (CH_4) production represents a loss of 2–12% of the gross energy consumed by ruminants depending on the type of diet [3]. Methane is a greenhouse gas which has been implicated as a contributor to global warming [4]. In the year 2010, 34, 24 and 15 percent of the global CH_4 emissions (100 Tera gram per year, Tera gram = 1 million tonne) from ruminant livestock came from Asia, Latin America and Africa, respectively [5]. Also from 75 to 85% of the N consumed by ruminants is excreted in feces and urine [6]. Therefore, scientists are interested in modifying the rumen microbial fermentation using herbal and medicinal plants in order to decrease methane and ammonia N production.

Protozoa could enhance methanogenesis, due to H_2 production, serve as hosts for methanogens and protect them from oxygen toxicity [3]. However, not all the protozoal genera have the same role in methanogenesis. There is still limited information on the individual protozoal genera contribution to methane emission. The *Polyplastron* is a weak producer, *Epidinium caudatum* is intermediate and I- *prostoma* and *Entodinium caudatum* are high producers [7,6].

Recent studies have shown that secondary metabolites of garlic powder [8,9], *Rheum officinale* and *Frangula alnus* [10], tannin rich legumes [11], and *Leucaena* containing tannin [12], can improve ruminal fermentation due to methane reduction and ammonia N production. Also, therapeutic effects of *Zingiber* against many diseases are well known [13]. Camphene (14.1%), neral (4.9%), geranial+bornyl acetate (8.1%), β -bisabolene (22.1%), ar-curcumene (14.5%) and β-eudesml were identified as the major secondary metabolites of ginger (*Zingiber officinale)* roots [14]. Therefore ginger could also manipulate the rumen microbial fermentation [15]. The information of the effect of ginger on ruminal fermentation parameters is contradictory; therefore, this study was conducted to evaluate the influence of ginger on the *in vitro* ecosystem of sheep. The fermentation kinetics, fermentation parameters (gas production*,* methane production*, In vitro* OM digestibility, ammonia (NH_3-N), partitioning factor, VFA concentrations) and protozoa population were investigated.

2. MATERIALS AND METHODS

2.1 *Zingiber officinale* Rhizome Used

Zingiber officinale, commonly known as ginger [14] is usually available on the phytotherapy market. The ginger was obtained from a grocery shop and was converted into finely ground powder by mill. Plant material was preserved in a dry, dark and cool place.

2.2 Animals Used

Three fistulated male Sanjabi sheep (50.8±1.9 kg) were used in the study. The animals were allocated to individual cages and 500 ml rumen fluid was obtained from each animal before the morning feeding. The animals were fed twice daily (08:30 and 16:30) with a basal diet containing 700 g kg^{-1} alfalfa and 300 g kg^{-1} concentrate (DM basis) (Table 1). Fresh water and minerals were available at all times [16].

2.3 Experiment Set Up

The study was conducted using an *in vitro* gas production method at incubation intervals of 0,2,4,6,8,10,12,18,24,36 and 72h. The experimental set up was a complete randomized design (CRD) with four replications per treatment. The treatments were control (0 mg), 30 mg or 60 mg of the ginger mixed in the substrate to which was added the rumen fluid obtained from the sheep. The substrate was a basal diet for the sheep comprised of alfalfa hay and barley at a ratio of 70:30. Two hundred mg of basal diet was added to 120 ml bottles and mixed with ginger powder for *in vitro* gas production and digestibility tests.

Table 1. Ingredients and nutrients composition (g/kg DM) and metabolizable energy (ME) for the experimental diets given to sheep

	g/Kg DM
Ingredients	
Alfalfa hay	68.5
Barley grain	30.0
Sodium bicarbonate	0.5
Commercial vitamin and mineral premix	0.5
Salt	0.5
Nutrients composition %	
Dry matter	92.85
Organic matter	85.3
Ether extract	2.83
Crude protein	14.3
Neutral detergent fiber	38.6
Acid detergent fiber	17.6
ME (MJ/KgDM)*	7.588

** ME was calculated using equations of Menke and Steingass [17] as: ME (MJ/kg DM) = 2.20 + 0.136 × Gp+ 0.0057 × CP + 0.00029 × XL2; Where CP is crude protein in g/100 g DM,Gp is the net gas production (ml) and XL2 is crude lipids from 200 mg DM after 24 h of incubation*

2.4 *In vitro* Gas Production (IVGP)

Twenty-four hour incubations were carried out with batch system. For IVGP experiments, 200 mg basal diet containing alfalfa hay and barley (70:30) was transferred into the Wheaton bottles (120 ml and four replicates for each treatment). *Zingiber officinale* was added to the medium at the levels of 0 mg (control), 30 mg or 60 mg. The rumen fluid was collected into a pre-warmed (39ºC) vacuum flask and filtered through four layers of cheesecloth under continuous flushing of CO_2. The buffer solution was prepared according to Menke and Steingass [17] Prior to adding rumen fluid, the medium had been extensively reduced with continuous bubbling of CO_2 and warmed at 39°C. Settlement time of 5 min was allowed after the pressure in the bottles was equilibrated by passing a needle through the stoppers to release the gas and the time recorded to mark the beginning of incubation.

2.5 Fermentation Parameters and Kinetics

Two sets of bottles were incubated: One set was to determine *in vitro* OM digestibility and fermentation parameters up to 24 h of incubation at 39ºC. At the end of incubation, the gas volume was recorded [18]. Another set was used to estimate kinetics of gas production which was examined for 72 h. A blank set comprised of buffered rumen fluid without samples was taken to correct for the presence of feed particles and microbial biomass in the rumen liquor.

To assess the kinetic of gas production, the gas volume was recorded at 0, 2, 4, 6, 8, 10, 12, 18, 24, 36, 48, 60 and 72 h. The index of fermentation kinetics (*a*, *b* and *c*) was calculated by Fitcurve 6.0 software. The kinetic parameters were estimated using the model of Ørskov and McDonald, [19] as follows:

$$P = a + b\,(1 - e^{(-ct)})$$

Where: P is the gas production at time *t*, *a* is the gas production from soluble fraction (ml g^{-1} OM), *b* is the gas production from insoluble fraction (ml g^{-1}.OM), *c is* the gas production rate constant (h), *a* + *b* the potential gas production (ml g^{-1}.OM) and *t* is the incubation time (h).

After 24 h incubation, the pressure of gas produced in the headspace of each bottle was recorded using a pressure transducer (Testo 512; Testo Inc., Germany) [20]. The produced gas due to fermentation of substrate was calculated by subtracting gas produced in a blank bottle from total gas produced in the bottle containing substrate and inoculums [18]. Then, the bottles were swirled on ice to stop fermentation and opened to take a sample of incubation medium for NH_3-N and protozoa enumeration and a supernatant (0.8 mL) for VFAs analysis.

Methane content was determined with injection of 4.0 ml of NaOH (10 M) to the bottle. Mixing of the contents with NaOH allowed absorption of CO_2, with the gas volume remaining in the syringe considered as CH_4 [21].

The samples of substrates were analyzed for dry matter (ID number 930.15), ash (ID number 924.05), total N (ID number 984.13), and ether extract using petroleum ether for distillation instead of diethyl ether (AOAC, 1990) [22]. Ether extracts using petroleum ether for distillation instead of diethyl ether (AOAC, 1990). The neutral detergent fibre (NDF) and acid detergent fibre (ADF) contents were determined as described by Van Soest et al. [23].

The OMD was estimated using equation of Menke et al. [24] as follows:

OMD % = 14.88 + [0.889 ×GP] + [0.045× XP] + [0.065 × XA]
Where: GP is the net gas production (ml), XP is crude protein (g Kg^{-1} DM) and XA is ash (g Kg^{-1} DM).

The NH_3-N concentration was determined by the phenol–hypochlorite method using a spectrophotometer as described by Broderick and Kang [25].

The ratio of substrate truly degraded (mg) to gas volume (ml) at different incubation times was expressed as the PF which was determined according to Vercoe et al. [18].

The VFAs were determined by a Shimadzu GC-14 B gas chromatograph (GC) (Shimadzu, Tokyo, Japan) equipped with a Carboxen TM 1000, 45/60, 2 m×1/8 column (Supelco, St. Louis, MO, USA) and a flame ionization detector. The VFAs were measured using 1 ml of the rumen fluid collected in a microfuge tube containing 0.20 ml metaphosphoric acid (25 ml/100 ml). An internal standard (2-ethyl-n-butyric acid) was used to help quantify VFA concentrations. The mixture was allowed to stand for 3 h at room temperature and centrifuged at 15,000×g at 4ºC for 15 min and supernatants were transferred to chromatography vials for VFA analysis and stored at −20ºC until analysis. For this purpose, 0.2 µl supernatant was injected into a gas chromatograph (Nucon-5765) equipped with a double flame ionization detector (FID) and chromosorb glass column (4 ft length and 1.8 mm diameter) as described by Cottyn and Boucque [26]. The gas flows for nitrogen, hydrogen and air were 30, 30 and 320 ml/min, respectively. Temperature of the injector oven, column oven and detector were 270, 172 and 270ºC, respectively.

Rumen ciliates on the basis of three subfamilies *Entodiniinae*, *Ophryscolecinae*, *Diplodiniinae* and family *Isotrichdae* were identified according to the method of Dehority [27]. All measurements were corrected for suitable blanks.

2.6 Statistical Analysis

The data from *in vitro* gas production (IVGP, methane emission, IVOMD, NH_3-N concentration, PF, VFAs) tests and subfamilies of protozoa were analyzed by one-way analysis of variance (ANOVA) using the Statistical Package for Social Science (SPSS 18.5) [28]. The completely randomized design (CRD) with four replicates was used and treatments means were compared by Duncan's test. Polynomial linear and quadratic contrasts were used to test the effect of treatments on traits.

The protozoan population was counted by the Kolmogorov-smirnov test for normal distribution before statistical analysis. The results were analyzed according to the following statistical model:

$$Y_{ij} = \mu + T_i + e_{ijk}$$

Where:

Yij represents the value of each individual observation, *µ* the average, *Ti* the effect (treatment) of the *i* th dose of additive (i = two level of *Zingiber officinale*) and e_{ijk} represents the residual error.

3. RESULTS

3.1 Effect on Kinetics of Gas Production

The immediately soluble (*a*) and the insoluble fraction (*b*) was not affected by ginger (Table 2). The rate of gas production (*c*) was decreased by addition of ginger to the basal diets (P<0.01). These changes ultimately led to improved the potential extent of gas production (*a+b*) (P<0.01).

3.2 Effect of Ginger on Fermentation Parameters

The methane production (µmol 200mg^{-1} DM) after 24 h incubation was reduced (P<0.01) due to supplementation with ginger (Control *vs* ginger = 0.012). The IVOMD (mg 200mg^{-1} DM) was not influenced following addition of ginger; but the ammonia N concentration (mg dl^{-1}) was reduced (P<0.01) (Table 2).

Ginger supplementation had no effect on the partitioning factor (PF), efficiency of microbial protein synthesis, [29] and microbial mass (MM) when compared with the control group. Therefore, the efficiency of microbial mass (EMM) was not changed at the end of fermentation in supplemented groups (Table 2).

The total VFAs (mmol L^{-1}) concentration and molar proportions of propionate, butyrate and valerate were unaffected by supplementation with ginger. The molar proportion of acetate

declined (30 mg of ginger; Q, $P<0.044$), isobutyrate and isovalerate increased (L, $P<0.018$ and Q, $P<0.035$) and the molar proportion of valerate was unaffected by addition of ginger. However, the C_2:C_3 ratio decreased (Control *vs.* ginger, $P< 0.028$) due to presence of ginger in the media (Table 2).

3.3 Effect on Rumen Protozoa

The number of total protozoa (L, $P<0.025$), and the subfamilies *Entodiniinae* (L, $P<0.028$) and *Diplodininane* (L, $P<0.045$) were reduced by ginger treatment (Table 3). The regression equation between these two variables (methane and protozoa) confirmed that methanogenic bacteria are associated with rumen ciliates and their metabolic activities yield H_2 which is a substrate for methanogenesis (Table 4).

4. DISCUSSION

4.1 Effect on Gas Production

Similar to Kongmun et al. [8] the secondary metabolites of plant additives had no effect on gas production of *'a'* and '*b'* fractions. The improvement in potential extent of gas production (*a+b*) by ginger was in agreement with Kongmun et al. [8] and Alipour and Rouzbehan [30]. It seems that plant secondary components of ginger enhanced the *'a+b*' fraction by reducing the rate of fermentation. Cumulative gas production at 72 h was significantly increased by inclusion of 60 mg of ginger. This may have been due to amplification of IVOMD as observed in the present study. This finding is in agreement with Kim et al. [31] who reported that ginger extract increased total gas production. The report of Patra et al. [32] also showed that the addition of ethanol and methanol extracts of Z*. officinalis* at low levels (0.25 and 0.5 mL) increased total gas production. The result showed that Z*. officinalis* could have the potential to improved gas production (*a+b*) and cumulative gas production at 72 h in sheep.

4.2 Effect of Ginger on Fermentation Parameters

Methane production was inhibited up to 21% and 12% by 30 mg and 60 mg of ginger, respectively, which might be due to defaunation of the subfamilies of *Entodininnae* [4]. The accompanying reduced methane emission with inhibition of the protozoan population indicated that methanogensis is associated symbiotically with the ciliates [7]. This result confirms previous findings [7,33,34]. In contrast, methane production was not inhibited by three types of ginger extracts (*e.g.,* methanol, ethanol and water) [9,32].

Ginger supplementation increased the IVOMD which is likely due to improvement of ATP^Y by methane production, which is in agreement with results of Mohammed et al. [35] and Patra et al. [32]. The inhibition of gas production was probably due to a reduction in the microbial activity [14,34] or VFAs reduction [34]. Declines in IVOMD and VFAs [15,36] due to ginger essential oils have been reported by several researchers.

The notable decrease in ammonia-N concentration, and increase in branched VFAs in the presence of ginger may have been due to decreased deamination of AA by ruminal bacteria [14,37,38] or protozoa [38]. Phenolic compounds have high antimicrobial activity due to the presence of a hydroxyl group within the phenolic structure [39]. Protozoa also possess proteolytic and deamination activities [40]. Thus, defaunation of protozoa from the rumen prevented recycling of N between bacteria and protozoa, which resulted in a decrease of ammonia-N in rumen. However, in contrast to the current study, NH_3-N concentration was increased by ginger essential oil at 300 mg L^{-1} [15], and at 2.0 mg L^{-1} of ginger extract [36]; but it was unchanged by 3, 30 and 3000 mg L^{-1} [15] The protozoa play a major role in protein degradation and engulf large molecules, carbohydrate, or even ruminal bacteria [41]. Also, protozoa play a role in regulating bacterial N turnover in the rumen, and they supply soluble protein to sustain microbial growth. Because protozoa are not able to use ammonia-N [42] a fraction of previously engulfed insoluble protein is later returned to the rumen liquid in the form of soluble protein [43]. This is one of the main reasons that defaunation can decrease ammonia-N concentration in the rumen.

The ginger treatment did not affect the PF and microbial mass (MM). Thus, the efficiency of microbial mass (EMM) was unchanged, which might have been due to a lack of synchronization of energy and N sources. Methane production inhibits the supply of energy to the rumen microbes, and reduces feed conversion into microbial mass [4], while in our study ginger treatment caused no remarkable reduction in

methane production. Therefore, as expected, the microbial mass had not improved by ginger secondary metabolites. Similarly, Alexander et al. [36] found that ginger (2 mg L^{-1}) did not improve EMM.

Methane production is usually associated with enhanced propionate and reduced acetate and C_2:C_3 ratio [44]. In the current study, the decrease in acetate may have been due to protozoa defaunation and depression in the C_2:C_3 ratio at both levels of ginger. Similar to our results, García-González et al. [10] and Hu et al. [45] observed that when methane production decreased, the acetate content decreased. However, acetate and propionate [36] and TVFA [15,31,32], were not influenced by ginger. Molar proportions of isobutyrate (Q, P< 0.041) and isovalerate (Q, P< 0.035) were influenced following administration of ginger (Table 2). The C_2:C_3 ratio decreased which was in agreement with Kim et al. [31]. However, Patra et al. [32] reported that ginger extract at any level had no effect on C_2:C_3 ratio. Methane emission in the rumen is closely related to the individual VFAs, and a decrease in methane emission led to a lower acetate to propionate ratio [46]. The formation of branched-chain VFAs in the current study would result in a lower availability of H_2 for methanogenesis.

Table 2. Effect of *Zingeber officinale* plant supplementation on kinetics of gas production, fermentation parameters and CH4 production

Parameters						*P*-Value		
	Zingeber levels (mg/200mg diet DM)				α	Contrasts		
	Control	30	60	SEM	Duncan	Control *vs.* ginger	L	Q
Fermentation kinetic values								
a	6.3	8.2	8.9	0.559	ns	0.056	0.065	0.535
b	58.01	58.79	61.47	0.978	ns	0.313	0.179	0.668
c	0.091^{b}	0.098^{b}	0.074^{a}	0.003	**	0.258	0.005	0.283
a+b	64.3^{a}	65.8^{a}	69.4^{b}	0.763	**	0.006	0.001	0.283
Gas 72 h	66.5^{a}	67.8^{ab}	70.3^{b}	0.664	*	0.257	0.031	0.601
Fermentation parameters								
Gas 24 h	41.1	41.0	45.5	0.990	ns	0.257	0.065	0.257
CH4 ml/200 mg DM	14.5	11.4	13.9	0.778	ns	0.012	0.762	0.124
CH4 ml/OMD_{mg}	7.6	6.0	7.8	0.450	ns	0.434	0.842	0.104
IVOMD %	52.3	52.5	56.3	0.889	ns	0.215	0.055	0.280
IVOMD mg	104.5	105.0	112.7	1.770	ns	0.215	0.055	0.280
Ammonia-N (mg/dl)	37.4^{b}	25.4^{a}	22.1^{a}	2.050	**	0.000	0.000	0.009
PF	2.54	2.55	2.47	0.020	ns	0.354	0.105	0.273
MM mg	12.9	16.2	12.6	0.406	ns	0.496	0.891	0.131
EMM %	12.7	15.19	11.2	0.607	ns	0.817	0.561	0.162
Total VFA(mmol/l)	74.0	62.1	58.7	4.68	ns	0.102	0.109	0.585
VFAs (mol/100 mol)								
Acetate	54.1^{a}	49.4^{b}	51.8^{a}	2.56	*	0.048	0.229	0.044
Propionate	20.8	20.8	21.4	0.697	ns	0.792	0.604	0.708
Butyrate	16.0	18.7	16.7	0.773	ns	0.269	0.638	0.166
Isobutyrate	2.7^{a}	4.7^{b}	4.3^{b}	0.619	**	0.005	0.018	0.041
Isovalerate	3.5^{a}	4.7^{b}	3.7^{a}	0.462	*	0.111	0.621	0.035
Valerate	2.9	3.9	2.7	0.514	ns	0.470	0.789	0.079
Acetate: Propionate (C_2:C_3)	2.6^{a}	2.3^{b}	2.4^{ab}	0.046	*	0.028	0.050	0.102

a=gas production from the immediately soluble fraction; a+b = potential extent of gas production; b=gas production from the insoluble fraction; c=gas production rate constant for the insoluble fraction (b); EMM= Efficiency of microbial mass; IVOMD= in vitro organic matter degradability; L= linear effect; MM= Microbial mass; NH3-N = ammonia-N; PF= Partitioning factor; Q= quadratic effects of supplemented treatments

Table 3. Protozoa population (×105/ml RF) subfamily from in vitro fermentation using sheep rumen fluid containing different levels of *Zingeber officinale* plant

Parameters	Zingeber levels (mg/200mg diet DM)				α	*P*-Value		
						Contrasts		
	Control	30	60	SEM	Duncan	Control *vs.* Zingeber	L	Q
Total Protozoa	2.667	1.583	1.250	0.237	*	0.029	0.025	0.359
Subfamily								
Entodiniinae	1.333	0.833	0.708	0.115	*	0.026	0.028	0.357
Ophryoscolecinae	0.000	0.000	0.125	0.042	ns	0.497	0.252	0.497
Diplodiniinae	0.444	0.275	0.257	0.082	*	0.771	0.045	0.859
Family								
Isotrichidae	0.492	0.375	0.250	0.075	ns	0.553	0.416	0.811

L= linear effect; Q= quadratic effects of supplemented treatments

Table 4. Protozoa population (×105/ml RF) (x) and methane (y) relationship by different levels of *Zingeber officinale* plant

Parameters	Zingeber levels (mg/200mg diet DM)		
	Control	30	60
Total Protozoa	Y=510.86-5.844x R^2=0.021(r =0.145)	Y=234.74+0.0011x R^2=0.810 (r =0.900)	Y=318.5+0.001x R^2=0.913 (r = 0.955)
Subfamily			
Entodiniinae	Y=510.86-5.844x R^2=0.021 (r =0.145)	Y=95.141+0.0038x R^2=0.953 (r = 0.976)	Y=391.885+0.0007x R^2=0.049 (r = 0.221)
Ophryoscolecinae	-	-	-
Diplodiniinae	Y=442.8+0.0014x R^2=0.464 (r =0.681)	Y=294.37+0.0029x R^2=0.724 (r = 0.851)	Y=317.3+58.336x R^2=0.950 (r = 0.975)
Family			
Isotrichidae	Y=438.35+0.00148x R^2=0.588 (r = 0.767)	Y=294.37+0.0029x R^2=0.724 (r = 0.851)	Y=426.33+0.00058x R^2=0.0491 (r = 0.221)

R2= Coefficient of determination; r = Correlation coefficient; RF= Rumen fluid

4.3 Effect on Rumen Protozoa

The literature indicated that decreasing the number of H_2 producers such as protozoa is an important way to reduce methane emission [3,14]. Since not all protozoan genera have the same role in methanogenesis [3], the role of various subfamilies of protozoa on fermentation parameters was evaluated in our study. A decrease in the number of total protozoa and the *Enotidininnae* subfamily was probably due to the presence of secondary metabolites and antiprotozoal activities of ginger components [33]. Many mechanisms are possible in explaining the effect of essential oil on protozoa: 1) the antimicrobial activity of ginger essential oil may increase fluidity and permeability of the cytoplasmic membrane [47], 2) disorder H^+ and K^+ ion gradients, and thus the proton motive force, leading to decreases in intracellular ATP concentration [48], 3) inhibition of glycolytic enzyme activity resulting in an inability of the microbes to utilize intracellular glucose [49], which leads to loss of cell contents and promotes cell lysis. Decreased rumen protozoa counts with some essential oil rich plants [33,50] have been reported, however, Patra et al. [9], demonstrated an increase in protozoa count by the addition of ginger extract.

The effect of defaunation on methane production is less clear; for example, the literature shows that there are contradictions in the effects of protozoa on methane production [8,12,32,33,51,52,53]. Morgavi et al. [3] reported that defaunation resulted in a 10.5% decrease in methane emission. In contrast, results obtained from a study by Goel et al. [52] showed that there was no relationship between methane production and protozoa. According to the results of the current study (Table 4), regression equations confirmed a positive relationship between these two variables. In other words, reducing protozoa resulted in less H_2 as a substrate for methane production [7]. The average correlation between the two variables of methane production and

protozoa numbers was 0.867, which indicates a high relationship. The evaluation of regression equations showed that the *Enotdininnae* and *Dplodininane* subfamilies had the greatest impact on the methane production. Whereas previous study indicated that rumen ciliates were apparently responsible for an average of 17% **(**between 9 and 25**)** of methanogenesis in the rumen fluid [7]. High regression was reported between these two variables concerning *E. amoneum, H. persicom, Eucalyptus and F. vulgare* [53], and tea saponin [47].

5. CONCLUSION

Zingiber officinale supplementation improved ruminal fermentation due to reduction in NH_3-N, methane and the protozoal population. The results showed that 60 mg of ginger significantly improved the potential extent of gas production. Methane production decreased in 30 and 60 mg of ginger treatments by 21.0 and 6.3%, respectively. *Entodiniinae* and *Dplodiniinae* subfamilies had the greatest impact on the production of methane and protozoa correlation was high. However, more research is needed to confirm the generally positive nutritional characteristics of ginger, especially on animal responses.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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