# Influence of Standard Tannin and Standard Saponin on Methane Mitigation and Rumen Fermentation Characteristics for Ruminants

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## ABSTRACT

*Methane is one of the important green house gas, which is normally emitted from ruminants and represents a loss of feed energy by 8-12 per cent. A huge number of feeding strategies are used to mitigate the methane emission from ruminants for sustainable animal production. Hence an experiment was conducted to study the influence of standard tannin (ST) and standard saponin (SS) on methane mitigation and rumen fermentation characteristics for ruminants by in vitro gas production technique (IVGPT). The IVGPT was carried out by incubating the Cumbu Nappier hybrid (CN-CO4) grass and rumen liquor with ST + SS at varying levels viz., 0, 1.03 per cent + 0.78 per cent, 2.06 per cent + 1.56 per cent, 3.09 per cent + 2.34 per cent and 4.12 per cent + 3.12 per cent of substrate in six replicates for a period of 24 hours in shaking water bath. After 24 hours the total gas production and pH were measured and methane was estimated in Gas Chromatography. The in vitro true dry matter digestibility (IVTDMD) was estimated and methane emission (ml) per 100 mg truly digested substrate was calculated. The rumen fermentation characteristics were also studied. The total gas production was significantly ($p<0.0$) decreased in all ST + SS added groups and the maximum reduction was observed in 3.09 per cent ST + 2.34 per cent SS added group than control. The methane emission was significantly ($p<0.01$) decreased by 26.03, 29.75, 38.02 and 40.91 per cent in 1.03 per cent + 0.78 per cent, 2.06 per cent + 1.56 per cent, 3.09 per cent + 2.34 per cent and 4.12 per cent + 3.12 per cent ST + SS supplemented groups respectively than control. The minimum level of ST + SS that reduced the methane emission per 100 mg truly digested substrate (35.64 per cent) was 3.09 per cent + 2.34 per cent when compared to control. The rumen fermentation characteristics viz. ammonia nitrogen, IVTDMD, bacterial and protozoal population was significantly decreased in ST + SS added groups than control. The pH of the fermented medium was not altered in all the treatment groups. The TVFA, propionic acid and butyric acid were significantly increased in standard tannin and saponin added groups than control. The acetic acid and acetate to propionate (A/P) ratio were significantly reduced in ST + SS treated groups when compared to control. It was concluded that at minimum concentration of 3.09 per cent ST + 2.34 per cent SS significantly reduced the methane emission and methane (ml) per 100 mg of truly digested substrate ($p<0.01$) than control without any adverse effect on rumen fermentation characteristics by IVGPT.*

**Key Words**: Tannin, saponin, methane, *in vitro* rumen fermentation, ruminants

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## INTRODUCTION

Methane ($CH_4$) is a fermented product of ruminants and normally emitted to the environment. 15-30 per cent of the methane was produced from rumen fermentation (Moss *et al.,* 2000) and causes global warming. The $CH_4$ emission also represents a loss of gross energy of feed by 8-12 per cent, leads to lowersing the animal production. Therefore, decreasing the methane emission is desirable for improved efficiency of the digested energy utilization for production (Johnson and Johnson 1995) in terms of growth, milk production and also reducing the negative effect on climate change (global warming). There are a number of feeding strategies used for reducing the methane emission for sustainable production in ruminants. The use of antibiotics, ionophore compounds and many chemical feed additives have been shown to decrease the methane emission in ruminants and these may cause resistance and residual effect in the product (Patra and Saxena, 2011). Alternative to these compounds are plant metabolites like tannins, saponin and organic acids that have been shown to selectively modulate the rumen microbial populations resulting in an improvement of rumen fermentation and nitrogen metabolism and a decrease in methane production (Bharathidhasan *et al.*, 2016 and Bharathidhasan*,* 2018).

Generally the tannins are combined together with proteins to form complexes due to the presence phenolic hydroxyl groups and have been found to be toxic for some of the rumen microbes, especially ciliate protozoa, fiber degrading bacteria and methanogenic archaea, and as a result methanogenesis in the rumen can be reduced. Earlier studies also reported reduction in methane emissions consequent to feeding high levels of tannins (Ramirez-Restrepo and Barry, 2005; Hess *et al*., 2006). Similarly plants rich in saponins are known to decrease the methane production in the rumen (Bharathidhasan *et al.*, 2013). The majority of research on saponin has been employed to exploit it for inhibition of rumen ciliate protozoa, which might improve the efficiency of microbial protein synthesis by reducing microbial protein turnover and enhance protein flow to the duodenum and finally reduce methane production. Saponin in *Yucca* extracts (Wallace *et al.,* 1994) and in tropical forage tree like *Sesbania grandiflora* (Newbold *et al.*, 1997) are natural defaunating agents and by reacting with cholesterol in protozoal cell membrane, these saponins decrease the availability of hydrogen ions for methanogens leading to decrease in methane production. Hence the present experiment was carried out with an objective to study the combined effect of standard tannin and standard saponin on mitigation of methane emission and rumen fermentation characteristics by *IVGPT* in forage based diet for ruminants.

## MATERIALS AND METHODS

The *in vitro* gas production technique (Menke and Steingass, 1988) was conducted to evaluate the influence of ST + SS at different levels viz. 0, 1.03 + 0.78 per cent, 2.06 per cent + 1.56 per cent, 3.09 + 2.34 per cent and 4.12 + 3.12 per cent, of substrate (*Pennisetum purpureum* x P*ennisetum glaucum* ) in six replicates on rumen methane mitigation for ruminants (**Table 1**). The standard tannin (CAS

No.1401-55- 4; EC No.215-753-2; MDL No.MFCD00066397) and standard saponin (CAS No.8047-15- 2; EC No.232-462-6; MDL No.MFCD00081981) was procured from M/s. Sigma Aldrich. The substrate Hybrid Cumbu Nappier (CN- CO4) grass (*Pennisetum purpureum* x P*ennisetum glaucum* ) was used for this study.

**Table 1. Experimental design to identify the level of standard tannin and standard saponin needed to reduce methanogenesis**

| Treatment* | Inclusion level of standard tannin + standard saponin (per cent of substrate) | Quantity of standard tannin + standard saponin included to 200 mg of substrate inoculated |
|---|---|---|
| 1 (Control) | 0 | 0 mg |
| 2 | 1.03 + 0.78 | 2.06 mg +1.56 mg |
| 3 | 2.06 + 1.56 | 4.12 mg + 3.12 mg |
| 4 | 3.09 + 2.34 | 6.18 mg + 4.68 mg |
| 5 | 4.12 + 3.12 | 8.24 mg + 6.24 mg |

*Each treatment was carried out with six replicates

The *in vitro* gas production study was carried out with rumen fluid collected by rumen extraction pump from three cattle maintained on grazing and it was squeezed through four layers of gauze in to an Erlenmeyer flask under continuous flushing with $CO_2$ and it was maintained at the temperature of 39 $^oC$. Then rumen fluid was mixed with media as described by Menke and Steingass (1988). The substrate Hybrid Cumbu Nappier grass (CN-CO4) was dried and milled to pass through 1 mm sieve and 200 mg was weighed and taken in 100 ml calibrated syringes and weighed quantity of standard tannin + standard saponin at various levels were added to the syringes in six replicates. Then 30 ml of rumen inoculum was anaerobically transferred to glass syringe and it was incubated in a shaking water bath at 39 $^oC$ for 24 hrs. At the end of the incubation period the total gas was measured and pH also determined in fermentation fluid. The gas samples were collected in vacuotainer for estimation of methane.

**Estimation of methane**

Methane concentration was estimated using Gas Chromatography (Perkin Elmer, Claurus 500 model) fitted with Flame Ionization Detector (FID) and capillary column (30 meter length and 250 micrometer diameter). Helium was used as carrier gas with oven temperature at 60$^o$ C, injector temperature at 100$^o$C and detector temperature at 110$^o$C. Methane concentration in samples (per cent) was calculated using the following formula.

$$\text{Methane concentration (per cent)} = \frac{\text{Peak area of sample gas}}{\text{Peak area of standard gas}} \times \text{Methane concentration in standard (per cent)}$$

$$\text{Methane emission (ml)} = \frac{\text{Methane concentration (per cent)}}{100} \times \text{Net gas production (ml)}$$

**Estimation of rumen fermentation characteristics**

The fermented fluid after 24 hrs incubation was collected for estimation of rumen fermentation characteristics. The ammonia nitrogen was estimated by steam distillation as per the method of Makkar and Becker (1996). The *in vitro* true dry matter digestibility (*IVTDMD*) was determined after the addition of Neutral Detergent Solution (NDS) with fermented dry matter in Fibretec ((Van Soest and Robertson, 1988). The volatile fatty acids were estimated as per the method of Chase, (1990). Bacterial (Gall e*t al.,* 1949) and protozoal (Moir, 1951) population were also counted by using the standard procedure.

**Statistical analysis**

The data collected on various parameters was statistically analyzed as per the method of Snedecor and Cochran (1989).

**RESULTS AND DISCUSSION**

**Effect of standard tannin and standard saponin on rumen methane mitigation**

The effect of standard tannin and standard saponin on total gas (ml), methane (ml), percentage of methane on total gas production and methane (ml) per 100 mg of truly digested substrate are presented in **Table 2**.

The total gas production was significantly ($p<0.01$) decreased in all standard tannin and standard saponin supplemented groups and it was lowered by 17.11, 17.93, 22.00 and 25.75 per cent in 1.03 per cent + 0.78 per cent, 2.06 per cent + 1.56 per cent, 3.09 per cent + 2.34 per cent and 4.12 per cent + 3.12 per cent ST + SS added groups respectively than control. Similarly, Getachew *et al*. (2008) observed that the addition of purified quebracho tannins to alfalfa hay decreased the rate of gas production significantly ($p<0.01$) with increased level of tannin from 0 to 150 mg/kg DM by *IVGPT*. The decrease in the rate of total gas production was due to the increasing the level of purified gallic acid and tannic acid which might suggest that the rumen microbes were decreased while degrading the gallic acid and tannic acid by *in vitro* (Getachew *et al*., 2008; Bharathidhasan *et al*., 2018). Vieira and Borba (2011) also reported that the effect of tannin using 2.5 per cent and 5.0 per cent *Quebracho* extracts significantly ($p<0.05$) decreased the total gas production than control. The addition of standard saponin in the present study further reduced the total gas production which might be due to the antiprotozoal effect of saponin (Istiqomah *et al.,* 2011). Further Makkar *et al*. (1998) also observed that saponin from *Acacia auriculoformis* decreased the total gas production. The addition of tannin and saponin in the present study might have inhibited the rumen microorganisms specially the protozoa and methanogens decreasing the rumen fermentation which lead to decreased total gas production (Jayanegara *et al*., 2010).

**Table 2.Effect of ST + SS in combination on total gas (ml), $CH_4$ (ml), percentage of $CH_4$ on total gas production and $CH_4$ (ml) per 100 mg of truly digested substrate by *IVGPT* (Mean# ± S.E)**

| Treatment | Inclusion level of ST + SS (per cent of substrate) | Total gas (ml) | $CH_4$ (ml) | per cent of $CH_4$ on Total gas production | $CH_4$ (ml) per 100 mg of truly digested substrate |
|---|---|---|---|---|---|
| 1 | 0 (Control) | $12.27 \pm 0.19^{c}$ | $2.42 \pm 0.01^{c}$ | $19.75 \pm 0.37^{c}$ | $2.02 \pm 0.01^{d}$ |
| 2 | 1.03 + 0.78 | $10.17 \pm 0.18^{b}$ | $1.79 \pm 0.06^{b}$ | $17.61 \pm 0.35^{b}$ | $1.52 \pm 0.04^{c}$ |
| 3 | 2.06 + 1.56 | $10.07 \pm 0.30^{b}$ | $1.70 \pm 0.09^{b}$ | $16.89 \pm 0.43^{ab}$ | $1.46 \pm 0.09^{bc}$ |
| 4 | 3.09 + 2.34 | $9.57 \pm 0.32^{ab}$ | $1.50 \pm 0.05^{ab}$ | $15.65 \pm 0.37^{a}$ | $1.30 \pm 0.04^{ab}$ |
| 5 | 4.12 + 3.12 | $9.13 \pm 0.20^{a}$ | $1.43 \pm 0.06^{a}$ | $15.63 \pm 0.30^{a}$ | $1.22 \pm 0.04^{a}$ |

# Mean of six observations; NS Not significant,

Means bearing different superscripts in the same column differ significantly ($p<0.01$)

The methane production was significantly ($p<0.01$) decreased in all the treatment groups than control. The decrease in methane production was lowered by 26.03, 29.75, 38.02 and 40.91 per cent in treatment 2, treatment 3, treatment 4 and treatment 5 respectively than control. The minimum level that reduced the maximum methane was 3.09 + 2.34 per cent of ST +SS.

In early, Bhatta *et al*. (2009) observed that the addition of *quebracho* tannin (7.62 per cent hydrolysable tannin and 1.33 per cent condensed tannin) at 5, 10, 15, 20 and 25 per cent of substrates like timothy hay (65): concentrates (35) decreased the methane production by 10.2 to 41.7 per cent in *IVGPT*. Jayanegara *et al*. (2010) also reported that the simple phenolics like cinnamic, caffeic, p-coumaric and ferulic acids decreased the methane production significantly ($P<0.05$) when added at 5 mM. They also reported that addition of purified chestnut and sumach (hydrolysable tannin) at 1mg/ml to *in vitro* rumen fermentation system containing hay: concentrate (70:30) decreased ($P<0.05$) methane production by 6.5 and 7.2 per cent respectively. The methane emission was decreased by 18 – 52 per cent by the addition of saponin from 1.2 mg to 3.2 mg/L (Lila *et al.,* 2003). Guo *et al*., (2008) observed that saponin at 0.4mg/ml significantly ($P<0.01$) reduced the methane release by 76 per cent than control by *in vitro*. Other scientists also reported a reduction in methane production by saponin rich plant such as *Yucca shidigera*, *Quillajia saponaria Acacia concinna* (Patra *et al*., 2006; Holtshausen *et al*., 2009) *Sapondis mukorassi* fruit pulps (Agarwal *et al*., 2006), *Knautia arvensis* leaves and *Sesbania sesban* leaves (Goel *et al*., 2008).

The highly significant ($p<0.01$) decrease in percentage of methane on total gas production was observed in all ST + SS added groups than control. The minimum level at 3.09 + 2.34 per cent of ST +SS was able to reduce the maximum methane on total gas production by 20.76 per cent when compared to control. On accordance to the study Pellikaan *et al*. (2011) who observed a reduction of methane emission on total gas production by 16.30 per cent and 15.85 per cent by the addition of condensed tannin and hydrolysable tannin at 100g/kg respectively when compared to control by *in vitro* study. Further, the addition of 0.3, 0.6, 0.9 g/litter of saponin significantly ($P<0.05$) reduced the methane levels by 23.43, 24.93 and 25.30 per cent respectively by *IVGPT* (Feng *et al*., 2012).

The methane (ml) per 100 mg of truly digested substrate was significantly ($p<0.01$) reduced by 24.75, 27.72, 35.64 and 39.06 per cent respectively in treatment 2, treatment 3, treatment 4 and treatment 5 than control. The minimum level at 3.09 per cent ST + 2.34 per cent SS was reduced the methane (ml) per 100 mg of truly digested substrate to the maximum extent when compared to control. Similar to the present study Castro –Montoya *et al*. (2011) also observed that the purified condensed tannin like *quebracho* tannin and mimosa tannin at 0.5, 0.75 and 1.0 mg/ml decreased ($P<0.001$) the methane emission per 100 mg true dry matter digestibility by 25, 30.77 and 36.54 per cent ($p=0.001$) and 23.08, 32.69 and 40.38 per cent respectively in in *quebracho* tannin and mimosa tannin than control. They also found that the purified hydrolysable tannin like sumach tannin and chestnut tannin at 0.5, 0.75 and 1.0 mg/ml decreased the methane emission (ml) per 100 mg true dry matter digestibility by 17.31, 23.08 and 30.76 per cent respectively in sumach tannin ($P=0.003$) and 13.46, 17.31 and 21.25 per cent respectively in chestnut tannin ($P = 0.007$) than control. Also the addition of condensed tannin and hydrolysable tannin at 10 per cent decreased the methane emission per gram of organic matter by 24.48 per cent and 17.88 per cent respectively than control (Pellikaan *et al*., 2011). The methane emission in mmol per 200 mg of dry matter addition of gross saponin of *Tribulus terrestris* supplementation at 0.30, 0.60 and 0.90 g/litre of incubation medium significantly ($P<0.05$) decreased by 26.67, 28.89 and 31.11 per cent than control (Feng *et al*.,2012).

The effect of tannin and saponin on reduction of methanogenesis in the present study could be expected since they affect the activities of rumen microbes, mainly inhibiting the bacterial and protozoal population. Further the antiprotozoal activities of tannin and saponin would decrease the methane production since a portion of methanogens are attached to the protozoa (Goel and Makkar, 2012; Bharathidhasan *et al.,* 2013; Bharathidhasan, 2018). Tavendale *et al*. (2005) suggested two modes of the action of tannin on methanogenesis: first directly affecting the activity or population of methanogens resulting in lower methane emission and second, indirectly by reduced hydrogen production by lowering the feed degradation. Similar suggestion was also reported by Jayanegara *et al*. (2011) for decreased methane emission while using tannin in the diet. Further saponin also decreases

the number of protozoal population as a result of cell death by forming complexes with cell membranes (Cheeke, 1999). The saponins modify the ruminal fermentation by suppressing ruminal protozoa and selectively inhibit some bacteria. The symbiosis of protozoa with methanogenic bacteria is well established in the rumen and selective suppression of protozoa has been suggested to reduce the methane production (Cheeke, 1999). Hence the combined effect of ST + SS was able to reduce the methane emission in the present study.

**Effect of standard tannin and standard saponin on rumen fermentation characteristics**

The effect of standard tannin and standard saponin on ammonia nitrogen, (mg/100ml), bacterial count, protozoal count, i*n vitro* true dry matter digestibility (*IVTDMD*) and pH are presented in **Table 3** and total volatile fatty acid (TVFA), acetic acid, propionic acid, butyric acid and acetate propionate (A/P) ratio are presented in **Table 4**.

The ammonia nitrogen content was significantly ($p<0.01$) reduced by 5.13, 10.18, 10.66 per cent in treatment 3, treatment 4 and treatment 5, respectively than control. Similarly, the addition of 2 mg/ml of *Moringa oleifera* aqueous methanol extract which contained 1.11 per cent of hydrolysable tannin decreased the total ammonia nitrogen by 13.63 per cent than control (Alexander *et al*., 2008). Pellikaan *et al*. (2011) also reported that the ammonia nitrogen was significantly ($P<0.01$) decreased by 35.18 and 33.38 per cent in condensed and hydrolysable tannin addition at 100g/kg substrate than control. Sliwinski *et al*. (2002) observed that the addition of *Yucca schidigera* extract containing saponin at 100 mg sarsaponin per kg dry matter to the basal diet containing grass silage, barley grain and grass hay decreased the ammonia nitrogen significantly ($P<0.05$) by 21.32 per cent than control. Hess *et al*. (2003) also reported that the effects of three saponin rich tropical fruits viz. 100 mg/g of *Sapindus saponaria* (Saponin 1.2 per cent and CT 0.32 per cent), *200 mg/g of Enterolobium cyclocarpum* (Saponin 0.39 per cent and CT 1.04 per cent) *or 200 mg/g of Pithecellobium saman* (Saponin 0.34 per cent and CT 1.22 per cent) or no tropical fruit supplemented diets decreased the ammonia nitrogen ($P<0.05$) by 17 per cent with *Pithecellobium saman* compared to other treatments. Hanim *et al*. (2009) also reported that that supplementation of saponin at 2 per cent and 3 per cent of substrate decreased ($P<0.01$) the ammonia concentration by 16.9 and 16.5 per cent respectively than control. The decreased ammonia nitrogen in the present study might be due to the binding of tannins with protein, which resists the rumen degradation, thereby reducing ammonia nitrogen in the rumen (Vieira and Borba, 2011).

There was a significant ($p<0.01$) difference in *IVTDMD* while supplementing the ST+SS in forage based diet. The reduction of *IVTDMD* was very limited from 1.94 per cent to 3.66 per cent in treatment groups than control. The decrease in *IVTDMD* in the present study was very less when compared to the earlier study by Alexander *et al*. (2008) who reported that the addition of 0.75 and 1.0 mg/ ml

of *Moringa oleifera* aqueous methanol extract significantly (P<0.05) decreased the apparent dry matter digestibility (but not the true dry matter digestibility) 7.94 and 13.53 per cent than control. Sliwinski *et al*. (2002) observed that the addition of *Castanea sativa* wood extracts containing the hydrolysable tannin at 0.5 and 2.5 g per kg dry matter did not influence the organic matter degradation in the treatment groups by *RUSITEC*. Further, the purified condensed tannin like quebracho tannin and mimosa tannin and hydrolysable tannin like sumach tannin and chestnut tannin at 0.5, 0.75 and 1.0 mg/ml of *in vitro* rumen fermentation study did not influence the *IVTDMD* in all treatment groups (Castro – Montoya *et al*., 2011).

**Table 3. Effect of SS + ST on ammonia nitrogen, (mg/100ml), bacterial count, protozoal count, *in vitro* true dry matter digestibility (*IVTDMD*) and pH (Mean[#] ± S.E)**

| Treatment | Inclusion level of ST + SS (per cent of substrate) | Ammonia Nitrogen (mg/100ml) | *In vitro* true dry matter digestibility (*IVTDMD*) | Bacterial count (X $10^8$) | Protozoal count (X $10^5$) | pH [NS] |
|---|---|---|---|---|---|---|
| 1 | 0 (Control) | 35.07 ± 0.22 [c] | 59.91 ± 0.03 [c] | 4.65 ± 0.04 [d] | 3.65 ± 0.06 [d] | 7.05 ± 0.08 |
| 2 | 1.03 + 0.78 | 34.17 ± 0.34 [bc] | 58.75 ± 0.06 [ab] | 4.13 ± 0.02 [c] | 3.30 ± 0.05 [c] | 6.87 ± 0.12 |
| 3 | 2.06 + 1.56 | 33.27 ± 0.22 [b] | 58.22 ± 0.18 [a] | 3.95 ± 0.04 [b] | 2.99 ± 0.06 [b] | 6.90 ± 0.06 |
| 4 | 3.09 + 2.34 | 31.50 ± 0.27 [a] | 57.72 ± 0.23 [a] | 3.69 ± 0.05 [a] | 2.67 ± 0.03 [a] | 6.73 ± 0.03 |
| 5 | 4.12 + 3.12 | 31.33 ± 0.40[a] | 58.60 ± 0.31 [ab] | 3.73 ± 0.02[a] | 2.68 ± 0.02 [a] | 6.77 ± 0.15 |

[#] Mean of six observations; [NS] Not significant, Means bearing different superscripts in the same column differ significantly (p<0.01)

Earlier reports suggested that the degradability of feed became decreased due to the inclusion of different plant extracts containing tannin and saponin in incubation media (Lila *et al*., 2003; Patra and Saxena, 2010; Santara *et al*., 2012). They concluded that the higher level of tannin and saponin might be detrimental to the rumen microbes leading to decrease in the rumen fermentation and digestibility to the maximum.

The bacterial count in all ST + SS treated groups was significantly (p<0.01) decreased than in control. The minimum dose with maximum reduction of bacterial load was by 20.65 per cent observed in 3.09 + 2.34 per cent ST + SS treated group than control. Similarly Sliwinski *et al*. (2002) reported that the addition of *Castanea sativa* wood extracts containing the hydrolysable tannin at 0.5 g and 2.5 g per kg dry matter to the basal diet with grass silage, barley grain and grass hay based diet reduced the bacterial count by 3.14 and 11.55 per cent respectively than control in *Rusitec*. The earlier work of Tagari *et al*. (1965) observed

that the cellulolytic and proteolytic bacteria growth was inhibited by pod tannins in an artificial rumen. It has been stated that tannins from carob pod extract changed the morphology of bacteria to produce antimicrobial activity (Heins *et al*., 1964). Hence inhibitory activity of tannins against bacteria has been implicated due to the ability of tannins to form complexes with the cell wall and membrane bacteria causing morphological changes of the cell wall and the extracellular enzymes secreted (Smith *et al*., 2005). Further the addition of saponin with tannin in the present finding agrees well with Wallace *et al*. (1994) who reported that saponins inhibit the growth of *Butyrivibrio fibrisolvens* and *Strptococcus bovis* bacteria. It seems that saponins show a more marked antibacterial activity against Gram positive than against Gram negative bacteria (Patra and Saxaena, 2010).

The protozoal count was significantly (P<0.05) reduced in all tannin and saponin treated groups than control. The reduction in protozoal count was 9.59, 18.08, 26.85 and 26.57 per cent in 1.03 + 0.78, 2.06 + 1.56, 3.09 + 2.34 and 4.12 + 3.12 per cent of ST + SS added groups, respectively than control. Similarly, Makkar *et al*. (1995) reported that the quebracho tannin significantly reduced the numbers of total protozoa, Entodiniomorph and holotichs, the effect being higher on Holotrichs which may increase the efficiency of microbial protein synthesis in the rumen. Also Patra *et al*. (2006) observed the tannin extracted with ethanol and methanol from *Terminalia chebula* decreased the numbers of total protozoa. Anti protozoal properties of tannins from *Lotus striata* and *Lotus cuneata* have been reported in many studies (Animut *et al*., 2008) Quebracho and mimosa tannin (Bhatta *et al.,* 2009).

In accordance to the present study, reduction in protozoal count by the addition of saponin was reported by Bharathidhasan *et al.* (2013). Hess *et al*. (2003) reported that the effects of *Sapindus saponaria containing s*aponin at 1.2 per cent and condensed tannin at 0.32 per cent decreased the protozoal population significantly (P<0.05) by 53.97 per cent than control. Hu *et al*. (2005) observed that the addition of tea saponin reduced the protozoal numbers significantly (P<0.05) by 19, 25, 45 and 79 per cent for 2, 4, 6 and 8 mg respectively of total saponin than control. Similarly, Guo *et al*. (2008) observed that tea saponin at 0.4 mg/ml reduced the protozoal count by 51 per cent (P<0.05) than control. Further, Feng *et al*.(2012) observed that the addition of gross saponin of *Tribulus terrestris* (GSTT) at 0.15, 0.30, 0.60 and 0.90 g/ litre of incubation medium significantly (P<0.05) reduced the protozoal population by 27.03, 37.03, 60.36 and 72.07 per cent respectively than control. One possible mechanism to explain the effect of saponins on protozoa is a change in cell membrane permeability (Klita *et al*., 1996) as they form complexes with cholesterol in protozoal cell membranes and result in cell lysis.

No significant difference of pH of fermented medium among the treatment groups in the present study was also agreed with the earlier reports by Hanim *et al*. (2009) who observed that the supplementation of saponin at 1, 2 and 3 per cent of substrate did not influence the pH of the fermented medium among the treatment groups. The addition of condensed tannin through

*Medicago sativa* and *Lotus pedunculatus* also did not influence the pH by *IVGPT* (Tavendale *et al*., 2005). The unaltered pH in the present study was due to the hydrogen accumulation during the inhibition of methanogens for methane reduction in which the carbohydrate fermenting bacteria utilize other mechanism of reducing equivalent particularly elimination of hydrogen ions and there by the pH is unaltered. (Kessel and Russel, 1996).

**Table 4. Effect of ST + SS on total volatile fatty acid (mg/dl), acetic acid (per cent), propionic acid (per cent), butyric acid (per cent) and A/P ratio (Mean# ± S.E)**

| Treatment | Inclusion level of ST + SS (per cent of substrate) | TVFA (mg/dl) ** | Acetic acid** (per cent) | Propionic acid * (per cent) | Butyric acid ** (per cent) | A/P ratio ** |
|---|---|---|---|---|---|---|
| 1 | 0 (Control) | 65.91 ± 0.32 $^{a}$ | 65.42 ± 0.20 $^{c}$ | 23.18 ± 0.22 $^{a}$ | 11.40 ± 0.28 $^{a}$ | 2.83 ± 0.03 $^{c}$ |
| 2 | 1.03 + 0.78 | 66.23 ± 0.08 $^{a}$ | 63.83 ± 0.21 $^{ab}$ | 23.85 ± 0.43 $^{ab}$ | 12.32 ± 0.50 $^{ab}$ | 2.69 ± 0.06 $^{bc}$ |
| 3 | 2.06 + 1.56 | 67.92 ± 0.68 $^{b}$ | 62.75 ± 0.17 $^{a}$ | 23.93 ± 0.26 $^{ab}$ | 13.32 ± 0.32 $^{b}$ | 2.63 ± 0.03 $^{ab}$ |
| 4 | 3.09 + 2.34 | 69.23 ± 0.29 $^{bc}$ | 62.33 ± 0.54 $^{a}$ | 24.57 ± 0.34 $^{b}$ | 13.52 ± 0.56 $^{b}$ | 2.53 ± 0.05 $^{a}$ |
| 5 | 4.11 + 3.12 | 69.10 ± 0.50 $^{c}$ | 62.08 ± 0.42 $^{a}$ | 24.08 ± 0.35 $^{ab}$ | 13.83 ± 0.54 $^{b}$ | 2.59 ± 0.05 $^{ab}$ |

# Mean of six observations; Means bearing different superscripts in the same column differ significantly ($p<0.01$)**, ($p<0.05$)*

The TVFA, propionic acid and butyric acid was significantly increased in ST + SS added groups than control. The TVFA and butyric acid was significantly ($p<0.01$) increased by 2.96, 4.8 and 4.62 per cent and 14.41, 15.68 and 17.57 per cent respectively in treatment 3, treatment 4 and treatment 5 when compared to control. The propionic acid was significantly ($p<0.05$) increased by 5.66 per cent in 3.09 + 2.34 per cent ST + SS supplemented group than control.

Similar to the present study, Hess *et al*. (2006) also observed that the TVFA significantly ($P<0.05$) increased by supplementation with low tannin legume (*Cratylia argentea)* or in mixture with high tannin legume (*Calliandra calothyrsus) or* in high tannin legume (*Calliandra calothyrsus)* alone than grass (without tannin) in *RUSITEC*. But Sliwinski *et al*. (2002) observed that the addition of *Castanea sativa* wood extracts containing the hydrolysable tannin at 0.5 and 2.5 g per kg dry matter to the basal diet with grass silage, barley grain and grass hay did not influence TVFA, propionic acid and butyric acid among the treatment groups in *RUSITEC*. Castro –Montoya *et al*. (2011) also observed that the propionic acid was increased in purified condensed tannin (CT) like quebracho tannin at 0.5- 1.0 mg/dl by 28.6 - 32.2 per cent and mimosa tannin at 0.5- 1.0 mg/dl by 29.4 - 31.9 per cent than

control (22.7 per cent) by *in vitro*. They also observed that the purified hydrolysable (HT) tannin like sumach tannin at 0.5 - 1.0 mg/ml increased the propionic acid by 22.5 - 26.6 per cent than control. Further they studied the effect of purified saponin from quillaja saponin at 0.5- 1.25 mg/dl increased (P= 0.001) the propionic acid by 17.75 – 29.5 per cent than control where as in gypsophilla saponin the increase was only by 1.25 mg /dl (2.99 per cent) than control.

Similar to the present experiment, Guo *et al*. (2008) also observed that the addition of tea saponin at 0.4 mg per ml of rumen liquor increased the total VFA marginally than control and the molar proportion of propionate was significantly ($p<0.05$) increased by 21.5 per cent to 24.1 per cent than control. The saponin based surfactant at 5, 10 and 20 µl/g DM increased ($P = 0.005$) the propionic acid by 4.35, 13.48 and 8.33 per cent, respectively and increased ($P = 0.241$) the butyric acid by 1.08, 11.96 and 3.66 per cent, respectively in barley grain based diet when compared to control by *in vitro* (Wang *et al*., 2011). Further, the propionic acid was significantly ($P<0.05$) increased by 8.44, 9.72 per cent while the addition of gross saponin of *Tribulus terrestris* (GSTT) at 0.60 and 0.90 g/litre of incubation medium respectively than control (Feng *et al*., 2012).

Alexander *et al*. (2008) reported that the addition of *Moringa oleifera* aqueous methanol extract which contained 1.11 per cent of hydrolysable tannin (HT) and 4.09 per cent of saponin increased ($p<0.01$) the propionic acid and butyric acid production by 6.8 per cent and 16.16 per cent respectively than control and however the TVFA production was significantly ($P<0.05$) decreased than control. The acetic acid and acetate to propionate ratio were significantly ($p<0.01$) decreased in ST + SS treated groups than control. The decrease in acetic acid and A/P ratio was 4.08 -5.11 per cent and 7.07 – 10.6 per cent respectively in ST + SS treated groups when compared to control.

Similarly Alexander *et al*. (2008) reported that the addition of *Moringa oleifera* aqueous methanol extract which contained 1.11 per cent of HT and 4.09 per cent of saponin decreased the total acetic acid production and acetate to propionate ratio significantly ($P<0.05$) by 13.83 per cent and 19.64 per cent respectively than control. Getachew *et al*. (2008) also observed that the addition of purified quebracho tannin reduced the total acetic acids and acetate to propionate ratio significantly ($P<0.01$) at 100 g/kg DM by 47.35 per cent and 27.59 per cent than control. Pellikaan *et al*. (2011) reported that the acetic acid production reduced significantly ($P<0.05$) by the addition of CT and HT at 100g/kg substrate than control. Castro –Montoya *et al*. (2011) also observed that the acetic acid and acetate propionate ratio was significantly ($p<0.01$) decreased in purified CT (quebracho tannin and in mimosa tannin), purified HT (sumach tannin and chestnut tannin) and purified saponin from quillaja saponin by *in vitro*.

Tea saponin reduced the acetate to propionate ratio from 3 per cent to 2.6 per cent significantly ($P<0.05$) (Guo *et al*., 2008). Wang *et al*. (2011) reported that the saponin based surfactant at 5, 10 and 20 µl/g DM decreased ($P = 0.063$) the acetic acid

by 0.83, 5.78 and 2.64 per cent respectively in barley grain based diet when compared to control by *in vitro*. The addition of gross saponin of *Tribulus terrestris* (GSTT) supplementation 0.90 g/litre of incubation medium significantly (P<0.05) decreased the acetic acid level (Feng *et al*., 2012).

The decrease in acetic acid and increase in propionic acid in the present study suggesting that the nutrients were partitioned more towards microbial protein synthesis in the presence of tannins (Makkar *et al*., 1995). Further the decrease in acetic acid may also be due to the stronger inhibitory effect over acetate producing bacteria (*Ruminococcus albus, Butyrivibrio fibrisolvens*) than on others, either by directly inhibiting them or by inhibiting the production of their preferred substrate (Castro-Montoya *et al*., 2011). The saponin may support faster growth of certain bacteria leading to increased propionate production in the rumen (*Selenomonas ruminantium*, *Succinomonas amylolytica*) (Pen *et al*., 2006). The reduced acetic acid to propionic acid ratio might have been combined consequence of the significant reduction of acetic acid and increased propionic acid levels by tannin and saponins (Castro-Montoya *et al*., 2011). The ideal compound to inhibit the methanogenesis would be one that is effective in reducing methane production but which also increases propionic acid (Tavendale *et al*., 2005). Ruminal VFA and methane production strongly correlate with acetic acid to propionic acid ratio (Russel and Vansoest, 1984). The strong inverse relationship between the molar proportions of propionic acid and methane production can be predicted from knowledge of interactions among ruminal microbial populations and compounds like tannin and saponin that promote higher production of propionic acid in the rumen which may also decrease methane production (Tavendale *et al*., 2005). The above explanation confirms the changes in the acetic acid, propionic acid and A/P ratio observed in the present study.

## CONCLUSION

It was concluded that the minimum concentration of 3.09 per cent ST + 2.34 per cent SS significantly reduced the methane, percentage of methane on total gas production and methane per 100 mg of truly digested substrate (p<0.01) by 38.02, 20.76 and 35.64 per cent respectively than control by *IVGPT.* The rumen ammonia nitrogen, *IVTDMD,* total bacteria and protozoa were significantly decreased in ST + SS treated groups than control. The TVFA, propionic acid and butyric acid were significantly increased and acetic acid and acetate to propionate ratio were significantly decreased in ST + SS treated groups. The decrease in methane emission recorded in this study was attributed to the suppression effects of tannin on rumen microbes especially on methanogen, protozoa and other bacteria which reduce the availability of hydrogen ions for methane production. Also the antiprotozoal activities of saponinn would decrease the methane production since a portion of methanogens attached to protozoa. Further the defaunation effect of saponin had synergistic with tannin justify the reduction of the methane emission in the present study without any adverse effect on rumen fermentation characteristics (Jayanegara *et al*., 2010). The energy saved

through decrease in methane emission can be used for sustainable animal production and may also curtail the global warming.

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