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Bee Propolis in Focus: *In Vitro* Fermentation of Napier Bajra Fodder Based Total Mixed Ration

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ABSTRACT

The present study investigated the effects of adding graded levels of bee propolis (0, 0.025, 0.05, 0.1, 0.15 and 0.2% on DM basis) on *in vitro* nutrient utilization and gas production parameters. The study also determined the chemical composition of bee propolis, conventional concentrate and TMRs with graded levels of bee propolis. The *in vitro* evaluation of napier bajra fodder based TMR incorporated with graded levels of bee propolis revealed no significant effect on net gas production, microbial mass production and efficiency, digestibility of DM, OM and NDF, SCFA production and ME availability. However, a numerically lower ($P>0.5$) value of ammonia nitrogen level was observed at 0.2% level of bee propolis as compared to control (0% level of bee propolis). Therefore, the study suggests that bee propolis has potential to lower the levels ammonia nitrogen in ruminants. However, more studies are needed to explore its potential using different feeds and fodders as well as different levels of bee propolis in different animal species.

HIGHLIGHTS

- Effects of incorporating bee propolis in the napier bajra fodder based TMR on fermentation characteristics were studied.
- NH_3-N levels decreased in response to increase in levels of bee propolis.

Keywords: Ammoniacal nitrogen, Bee propolis, *In vitro* evaluation, Napier bajra fodder

Livestock rearing is the key economic pursuit of rural population of India. Livestock sector supports livelihood of more than 70% of rural population in the nation, comprising landless labourers, small and marginal farmers. The sector provides employment to around 80 million people, especially helping marginal and at-risk communities (BAHS, 2024). India is a home to the largest population of livestock in the world with approximately 536.76 million animals (DAHD, 2024). India dominates the global milk production (BAHS, 2024).

Bee propolis also known as bee glue, is a resinous material collected by honeybees (*Apis mellifera*) from leaves and buds of plants and trees, combining it with pollen grains and enzymes secreted by bees (Soltan *et al*., 2016). Honey bees used bee propolis as a defense to protect hive, seal cracks and crevices of the hive, maintain temperature of the hive (35°C) during cold weather as well as protect hive against predator invasion (Jabbar *et al*., 2022). Bee propolis comprises of resins (50%) and wax (30%), essential oils (10%), pollen (5%) and other organic components (5%) (Kurek-Górecka *et al*., 2014). Bee propolis has numerous physiological effects likeantibacterial, antifungal, antiparasitic, antiviral, antioxidant, antimethanogenic, anti-inflammatory, immune modulatory and anticarcinogenic, attributed to natural presence of potent biologically active components like phenolic acids, flavonoids, fatty acids, alcohols, ketones and steroids (Morsy *et al*., 2013, 2015, 2016; Rufatto *et al*., 2018; Da Silva *et al*., 2019).

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Given the wide range of advantages of bee propolis, it has gained emmense popularity as a natural and novel feed additive in animal nutrition. Moreover, the constraints imposed by several countries on the use of antibiotics in animal nutrition have compelled scientists to explore natural, safer and sustainable alternative for animals. Many studies have been conducted to find out the alternative to the use of antibiotic in animal nutrition and bee propolis is one such emerging option and a viable alternative which can replace antibiotics to improve feed efficiency and performance of animals (Morsy *et al*., 2015; Soltan *et al*., 2016). Hence, the present study was carried out to determine the effects of adding bee propolis in napier bajra fodder based TMR on *in vitro* nutrient utilization and gas production parameters using buffalo rumen inoculum.

MATERIALS AND METHODS

The *in vitro* experiment was carried out at Department of Animal Nutrition, GADVASU, Ludhiana (Punjab) with the approval of Institutional Animal Ethics Committee (IAEC).

Experimental diets and treatments

The napier bajra fodder based total mixed ration (TMR) had roughage: concentrate ratio of 65:35. The roughage part contained napier bajra fodder and wheat straw in ratio of 50:50. The conventional concentrate used in the *in vitro* experiment comprised of maize (35%), soybean meal (15%), mustard cake (15%), wheat bran (9%), rice polish (7%), deoiled rice bran (15.5%), mineral mixture (2%), salt (1%) and urea (0.5%). TMR was supplemented with graded levels of propolis (0, 0.025, 0.05, 0.1, 0.15 and 0.2% on DM basis).

Chemical analysis

The chemical analysis of finely ground samples of TMR and bee propolis was carried out for proximate (AOAC, 2007) and cell wall constituents (Van Soest *et al*., 1991).

Collection of rumen inoculum

Male buffaloes were used as donor of rumen inoculum. The donor buffalo were maintained on ration containing 15 kg of fresh green forage, 3 kg of wheat straw and 2 kg conventional concentrate mixture. Rumen inoculum was collected in a thermos flask (maintained at 39°C) and flushed with carbon dioxide gas. The rumen inoculum was blended for 2-3 minutes at 39°C and then strained through four-ply muslin cloth.

As the reducing solution was added, carbon dioxide gas was subsequently pumped via a submerged tube. A slight bluish color appeared which turned pink and ultimately colourless in appearance. As the solution turned colourless, the strained rumen liquor (SRL) was then added to the buffer medium which contained a bicarbonate buffer solution, resazurin, macro and micro mineral solutions prepared as per (Menke *et al*., 1979; Menke and Steingass, 1988) in a 1:2 ratio. The medium was kept in a water bath maintained at 39ºC and flushed with CO_2. One hundred ml calibrated glass syringes containing 375 mg test feed were filled with 30 ml of buffered rumen fluid under anaerobic conditions. The syringes were clipped properly and placed in the water bath for 24 hours at 39ºC. A blank syringe containing only buffered rumen liquor was also run in triplicate with each incubation set. The volume of gas produced in each glass syringe was recorded after 24 hours of incubation and was used to calculate ME. The contents of all the syringes were collected in respective spoutless beakers and boiled with neutral detergent solution to estimate digestibility of OM and NDF (Van Soest and Robertson, 1988). A method described by France *et al*. (1993) was used to calculate partitioning factor (PF).

Determination of ME availability

An equation derived by Menke *et al*. (1979) was used to determine the substrate's ME value.

$$ME \text{ (MJ/kg)} = 1.24 + 0.146\, G \text{ (ml/200 mg DM)} + 0.007\, CP + 0.0244\, EE$$

where,

ME = Metabolizable energy, MJ/kg DM; G = Net gas production, ml/200mg DM; CP = Crude protein, g/ kg; EE = Ether extract, g/kg.

Statistical analysis

Data generated during the *in vitro* study were analysed by simple ANOVA, as described by Snedecor and Cochran

(1994), by using SPSS (2012) version 21. The differences in means were tested by Tukey's b.

RESULTS AND DISCUSSION

Chemical composition of bee propolis

The chemical profile of bee propolis used in the study consisted of organic matter 95.63%, total ash 4.37%, ether extract 1.10%, crude protein 2.51%, crude fibre 0.60% andtotal carbohydrates 92.02% on DM basis. The ingredient and chemical composition of the napier bajra fodder based TMR is presented in Table 1 and 2, respectively. In the TMR, the roughage portion was made up of wheat straw and napier bajra fodder in 50:50 ratio. The concentrate ingredients used in the TMR included maize 12.25%, soybean meal 5.25%, mustard cake 5.25%, wheat bran 3.15%, rice polish 2.45%, deoiled rice bran 5.425%, mineral mixture 0.7%, common salt 0.35% and urea 0.175%. The chemical profile of the TMR consisted of organic matter 91.57%, total ash 8.43%, crude protein 11.71%, ether extract 2.73%, neutral detergent fibre 64.90%, acid detergent fibre 36.35%, hemicellulose 28.55%, cellulose 25.50%, acid detergent lignin 5.50% and total carbohydrates 77.13%.

Table 1: Ingredient composition of the TMR used in the *in vitro* experiment (% DM basis)

Ingredient	Percent
Wheat straw	32.5
Napier bajra fodder	32.5
Concentrate Ingredients	
Maize	12.25
Soybean meal	5.25
Mustard cake	5.25
Wheat Bran	3.15
Rice polish	2.45
Deoiled rice bran	5.425
Mineral mixture	0.7
Common salt	0.35
Urea	0.175

Table 2: Chemical composition of the TMR used in the *in vitro* experiment (% DM basis)

Parameter	Percent
Organic matter	91.57
Total ash	8.43
Crude protein	11.71
Ether extract	2.73
Neutral detergent fibre	64.90
Acid detergent fibre	36.35
Hemicellulose	28.55
Cellulose	25.50
Acid detergent lignin	5.50
Total carbohydrates	77.13

The fermentation parameters of the TMR (napier bajra fodder based TMR) are presented in Table 3. NGP in napier bajra fodder based TMR did not differ significantly at different levels of bee propolis inclusion (0, 0.025, 0.05, 0.1, 0.15, and 0.2%); the corresponding NGP (ml/g DM/24h) values were 136.00, 134.67, 134.00, 137.33, 134.67, and 135.33 (Table 3), respectively. Our results are similar to those reported by Nascimento *et al*. (2020), who reported that addition of propolis extraction residue (100 g/cow/day) into bovine diets (with varying protein concentrate levels- 25, 50, and 75% DM) *in vitro* had no significant effect on total volume of gases produced at all concentrate levels (25, 50 and 75% DM) as compared to control (devoid of propolis extraction residue). Further, Morsy *et al*. (2015) used a semi-automatic *in vitro* gas production system to compare the effects of increasing levels (0, 125, 250, and 500µg per 500 mg of dietary dry matter) of ethanolic extracts of Brazilian red propolis and Egyptian brown propolis on ruminal degradation of nutrients and methane production, and found no significant differences in NGP among the propolis treatment (125, 250, and 500µg per 500 mg DM of both Brazilian red propolis and Egyptian brown propolis) groups as compared to negative control (without any supplementation). Moreover, Morsy *et al*. (2011) reported no significant ($P>0.05$) variation in gas production among both Brazilian propolis extracts, Brazilian green propolis (BGP) and Brazilian alamo propolis (BAP) at various doses (125, 250, and 500 µg/75 ml culture fluid) as compared to control.

PF in the TMRdid not differ significantly at different levels of bee propolis inclusion (0, 0.025, 0.05, 0.1, 0.15, and

0.2%); the corresponding PF (mg/ml) values were 3.90, 4.00, 4.20, 3.98, 3.93 and 3.89 (Table 3), respectively. Our findings are in line with Morsy *et al*. (2015), who used a semi-automatic *in vitro* gas production system to compare the effects of increasing levels (0, 125, 250, and 500µg per 500 mg of dietary dry matter) of ethanolic extracts of Brazilian red propolis and Egyptian brown propolis on ruminal degradation of nutrients and methane production, and found no significant differences in PF among the propolis treatment (125, 250, and 500µg per 500 mg DM of both Brazilian red propolis and Egyptian brown propolis) groups as compared to negative control (without any supplementation). In addition, Morsy *et al*. (2011) reported no significant variation in PF among both Brazilian propolis extracts, green (BGP) and alamo (BAP) at various dosages (125, 250, and 500 µg/75 ml culture fluid) as compared to control. The PF is defined as the ratio of OM degraded (mg) *in vitro* to the volume of gas (ml) produced. A higher PF means that proportionally more of the degraded matter is incorporated into microbial mass i.e. the efficiency of microbial protein synthesis is higher. The PF calculated *in vitro* provides useful information for predicting the dry matter intake (DMI), microbial mass production (MMP) in the rumen and methane (CH_4) emission of the entire ruminant animal. The PF of ruminant diets should lie in the range of 2.71 to 4.41 (Blümmel *et al*. 1997). The PF in the present study ranged from 3.89 to 4.20, which is within the suggested range.

Further, OM, NDF and DM digestibility also showed no significant difference across 0, 0.025, 0.05, 0.1, 0.15 and 0.2% levels of bee propolis inclusion in the TMR, with the respective OMD values as 57.92, 58.79, 61.41, 59.67, 57.77 and 57.48%, respectively; NDFD values as 40.63, 41.86, 45.56, 43.09, 40.42 and 40.01%, respectively; and DMD values as 59.73, 61.07, 63.73, 61.73, 59.73 and 59.73%, respectively. The findings of our study resembled with those of Mahmood *et al*. (2022), who studied the effects of different *moringa* by-products and raw propolis on ruminal fermentation using RUSITEC, reported that there was no significant (P>0.05) difference observed for degradation of DM, OM and NDF across the treatments (control diet without supplementation and the control diet top-dressed with *moringa* seed cake, *moringa* leaf powder, or raw propolis). Moreover, Nascimento *et al*. (2020), who studied the effect of adding propolis extraction residue (100 g/cow/day) into bovine diets with varying concentrate levels (25, 50, and 75% DM) on *in vitro* ruminal fermentation, reported that addition of propolis extraction residue into bovine diets had no significant effect on DMD at all treatments (25, 50, and 75% DM) as compared to control (without propolis extraction residue). Furthermore, Özturk *et al*. (2010), studied the effects of varying concentrations of propolis extract (0%, 20%, 60% ethanolic extract of propolis) using RUSITEC and revealed that propolis supplementation (at both 20% and 60% ethanolic extract) had no significant effect on DM digestibility.

The MMP and EMMP varied non significantly by the addition of graded levels of bee propolis to the TMR. Our results are similar to Valero *et al*. (2015), who studied the substitution of propolis for monensin in the diet of feedlots bulls, and revealed that both microbial protein synthesis as well as the efficiency of microbial synthesis were unaffected (P>0.05) by the supplementation of monensin or propolis. Moreover, Aguiar *et al*. (2014) studied the effect of supplementation of three variations of propolis based products (PBP) as PBP B1, PBP C1 and PBP C3 (differing in the concentrations of phenolic compounds as 3.81 mg/kg, 3.27 mg/kg, and 1.93 mg/kg of ingested DM, respectively) to the diets of Holstein dairy cattle, and reported that there was no significant (P>0.05) difference in the microbial protein synthesis and microbial efficiency on inclusion of propolis-based products (PBP B1, PBP C1 and PBP C3) in the diet as compared to control. Further, De Paula *et al*. (2016) studied the implications of giving propolis-derived phenolic compounds to water buffaloes in different doses (0, 16.95, 33.9, and 50.85 mg/d), reported that there were no significant differences (P>0.05) in microbial protein synthesis and microbial protein synthesis efficiency among propolis treatment groups and control.

Furthermore, adding graded concentrations of bee propolis (0, 0.025, 0.05, 0.1, 0.15, and 0.2%) to the TMR had no significant influence on the levels of SCFA production (0.60, 0.59, 0.59, 0.61, 0.59 and 0.60 mmole, respectively). Our results corroborated with the results noted by Mahmood *et al*. (2022), who investigated the effects of different *moringa* by-products and raw propolis on ruminal fermentation using RUSITEC and found that dietary treatment with propolis had no significant (P>0.05) effect on total SCFA concentration as compared to control (without propolis or *moringa* by-products). Moreover, Özturk *et al*. (2010),

Table 3: Effect of level of bee propolis on the in vitro gas production and digestibility of nutrients in napier bajra fodder based TMR (24 h)

Parameter	Level of bee propolis (% DM basis)						SEM
	0	0.025	0.05	0.1	0.15	0.2	
NGP,ml/g DM/24h	136.00	134.67	134.00	137.33	134.67	135.33	0.72
PF, mg/ml	3.90	4.00	4.20	3.98	3.93	3.89	0.04
OMD, %	57.92	58.79	61.41	59.67	57.77	57.48	0.50
NDFD, %	40.63	41.86	45.56	43.09	40.42	40.01	0.70
MMP, mg	86.68	90.78	100.33	91.58	87.28	85.73	1.87
EMMP, %	43.56	44.94	47.58	44.69	43.96	43.43	0.57
DMD, %	59.73	61.07	63.73	61.73	59.73	59.73	0.50
SCFA, mmole	0.60	0.59	0.59	0.61	0.59	0.60	0.00
ME, MJ/ kg DM	6.70	6.66	6.64	6.73	6.66	6.68	0.02
NH_3-N, mg/dl	20.24	20.24	19.09	20.39	20.82	18.66	0.26

NGP- Net gas production, PF- Partitioning factor, D- Digestibility, OM- Organic matter, NDF- Neutral detergent fibre, MMP- Microbial mass production, EMMP- Efficiency of microbial mass production, DM- Dry matter, SCFA- Short chain fatty acids, ME- Metabolizable energy, NH_3-N- Ammoniacal nitrogen, R:C ratio was 65:35 on dry matter basis.

studied varying concentrations of propolis extract (0%, 20%, 60% propolis ethanolic extract) using RUSITEC and revealed that propolis supplementation had no significant effect on SCFA production.

Furthermore, adding graded concentrations of bee propolis (0, 0.025, 0.05, 0.1, 0.15, and 0.2%) to the TMR had no significant influence on ME availability (6.70, 6.66, 6.64, 6.73, 6.66 and 6.68 MJ/kg DM, respectively). Our results resembled with the results of Da Silva *et al*. (2025) who explored the effects of supplementing increasing doses (0, 6, 12, 18, and 24 ml/day) of green propolis extract (GPE) in ration of feedlot rams for 85 days and found that ME in the rams' diet was unaffected (P>0.05) by the GPE supplementation across all treatment groups and was similar to control groups. However, in an *in vitro* study by Coşkuntuna *et al*. (2023), who studied the effects of adding 0.05% propolis extract to sorghum grain varieties (Es8z102, Albanus, Sugar Drip, Gül Şeker and Csr9303), it was reported that ME availability increased significantly (P=0.000) in response to addition of propolis extract to all the sorghum grain varieties as compared to the control (without addition of propolis extract).

Inclusion of graded concentrations of bee propolis (0, 0.025, 0.05, 0.1, 0.15, and 0.2%) to TMR 4 had no significant influence on the levels of NH3-N (20.24, 20.24, 19.09, 20.39, 20.82 and 18.66 mg/dl, respectively). However, NH3-N level was lower at 0.2% bee propolis level as compared to other levels, though the difference was not statistically significant. Morsy *et al*. (2015) also compared the effects of increasing levels of ethanolic extracts of Egyptian brown propolis and Brazilian red propolis on ruminal nutrient breakdown and methane production using a semi-automated in vitro gas production system and found that propolis treatments (125, 250, and 500µg per 500 mg DM of both Brazilian red propolis and Egyptian brown propolis) had no significant impact on the NH3-N levels in the incubation medium as compared to negative control (without any supplementation) and positive control (monensin). The basal diet in the study by Morsy *et al*. (2015) consisted of 500 g hay prepared from Bermuda grass (*Cynodon dactylon* (L.) Pers.; hybrid "Tifton-85"), 327 g ground maize, 150 g soybean meal, 10 g limestone, and 13 g mineral premix per kg of DM.

CONCLUSION

The *in vitro* evaluation of napier bajra fodder based TMR incorporated with graded levels of bee propolis revealed no significant effect on net gas production, microbial mass production and efficiency, digestibility of DM, OM and NDF, SCFA production and ME availability. However, a numerically lower value of ammonia nitrogen level was observed at 0.2% level of bee propolis as compared to control (0% level of bee propolis). Therefore, the study suggested that bee propolis has potential to lower the levels ammonia nitrogen in ruminants. However, more studies are needed to explore its potential using different feeds and fodders as well as different levels of bee propolis in the *in vivo* study.

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