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Research Article

Malic acid-heat treatment of oil cakes enhances rumen undegradable protein for effective protein utilization in buffaloes (*Bubalus bubalis*)

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Abstract

Protein nutrition in ruminants encompasses providing the optimum ratio of rumen-degradable and undegradable protein to strike a balance between optimizing rumen efficiency and maximizing animal productivity. The present study aimed to enhance the rumen undegradable proteins of groundnut cake, guar korma, mustard cake, and sunflower meal, and were sprayed with 1 M malic acid (400 mL/kg feed sample) and subjected to heat treatment at 60, 100, 120, and 150°C. Modified *in vitro* gas production technique (IVGP) was used to determine the *in vitro* rumen degradable protein of treated and untreated feeds. The malic acid heat treatment was found to be effective in increasing the rumen bypass fraction of feeds at all temperatures, with the highest increase occurring at 150°C. Sunflower meal was shown to be the most resistant to MAH treatment with only about 30% decrease in rumen protein degradability at 150°C as compared to groundnut cake, guar korma, and mustard cake, where a significant reduction (64-70%) was evidenced. Acid detergent insoluble crude protein (ADICP) analysis of feeds treated at 120°C and 150°C for assessing the availability of bypass protein to the small intestine showed no significant (P>0.05) heat damage in groundnut cake, guar korma, and sunflower meal, and thus were considered useful in ruminant rations. However, mustard cake at 150°C was severely heat damaged (41.02%, ADICP) and was considered suitable for use only up to 120°C. Therefore, malic acid-heat treatment of oil cakes could be an effective technology to enhance rumen bypass protein sources for animals and to reduce environmental pollution through faecal and urinary nitrogen excretion.

Keywords: Ammonia nitrogen, Bypass protein, Malic acid heat treatment, Oil cakes, Protein degradability

Highlights

- MAH treatment was highly effective in increasing the bypass protein fraction at all temperatures (60, 100,120,150°C) for groundnut cake, guar korma, and sunflower meal.
- The highest increase in bypass protein fraction was at 150°C and in groundnut cake.
- ADICP of feeds treated at 120°C and 150°C showed no significant heat damage in groundnut cake, guar korma, and sunflower meal; however, mustard cake at 150°C was severely heat damaged and considered suitable when treated up to 120°C.

INTRODUCTION

Protein is one of the most critical nutritional factors in ruminant feeds, as it is essential for the regulation of cell signaling, transport and storage of oxygen and nutrients, gene expression, regulation of muscle contraction, facilitation of enzyme-catalyzed reactions, maintenance of cell and tissue structures, buffering and colloidal properties, and hormone-mediated regulation of bodily processes. Nutrient requirements of growing and high-yielding animals during lactation, pregnancy, the transition period and stress are considerably higher compared to maintenance requirements (Drackley and Cardoso, 2014). In these conditions, the amount of protein supplied through synthesis by microbial biomass and rumen fermentation might fall short of the nutritional demands, which often coincides with a reduction in feed intake, especially in early lactation and stress.

Protein nutrition in ruminants encompasses providing the optimum ratio of rumen-degradable and undegradable protein to strike a balance between optimizing rumen efficiency and maximizing animal productivity by using a minimal amount of dietary crude protein. Feeding an increased ratio of highly degradable protein oil cakes results in excessive ammonia production in the rumen, which after absorption through the rumen wall, increases the formation of urea in the liver and ultimately enhances urinary nitrogen excretion

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with a loss of energy. Reduction in the ruminal protein degradation of high-quality proteins helps increase protein utilisation in the lower digestive tract and reduces nitrogen environmental emissions such as nitrous oxide from dung and urine. It has also been reported that protein supplements with higher rumen undegradable protein, such as cotton seed meal, tomato pomace, corn gluten meal, etc., have shown low methane production potential (Lamba *et al*., 2014). Ruminants have lower efficiency of N use compared to non-ruminants, and about 70-75% of the N ingested is excreted in manure (Huhtanen and Hristov, 2009). This inefficiency is primarily attributable to a wasteful use of N in the rumen. Protected proteins bypass the rumen and provide additional essential amino acids for absorption directly in the small intestine, therefore preserving the amino acid profile of high-quality protein. Protein supplements are high in rumen bypass protein and are known to increase the milk production performance of high yielders during the early lactation stage when the basal diet offered to cows is of low nutritive value (Tiwari *et al*., 2018).

One of the most common methods of converting rumen degradable feed protein to rumen bypass protein is heat treatment. Heat treatment involves decreasing the solubility of protein through the creation of cross linkages among peptide chains, which also become cross-linked with carbohydrates (Kumar *et al*., 2015). However, an increase in the temperature-time combination may cause changes in the protein, rendering it undigestible in either the rumen or small intestine. An additional benefit of heat treatment is the inactivation of various inherent heat labile anti-nutritional factors, such as lectins, trypsin inhibitors etc. The effective protein degradability was reported to decrease from 85.8% to 51.8% in the case of heat-treated groundnut cake at 150°C for 2 hours (Walli *et al.*, 2000). Extrusion cooking of mustard cake at 125°C for 3 hours led to a decrease in effective protein degradability from 86.56% to 33.89% (Kumar and Ravi, 2015). Heat treatment of soybean may convert about 50% to 55% of total protein into bypass protein (Osti *et al*., 2013). Acid treatment is another method of protecting feed protein from rumen degradation by facilitating denaturation and hydrolysis in the acidic conditions of the abomasum and subsequent assimilation in the small intestine. However, higher doses of acid may incur additional processing costs. Therefore, combining acid-heat treatment may result in a higher level of reduction in rumen protein degradability at a lower temperature-time combination with decreased risk of irreversible Maillard reaction leading to over-protection of protein, with the use of lower acid doses. The synergistic action of combined acid-heat treatment may reduce the disadvantages of either method (Arroyo *et al*., 2013). Studies have shown that malic acid-heat treatment at 150°C for 1 h resulted in effective protein protection of sunflower meal, while increased duration (3h) led to over-protection of the protein with sheep rumen liquor (Vanegas *et al*., 2017). In another study, incorporation of malic acid-heat treated sunflower seeds and sunflower meal led to decreased ammonia nitrogen production without negative effects on organic matter fermentation (Haro *et al*., 2018). The efficacy of this treatment at protecting protein from ruminal degradation in sources other than sunflower meal is yet to be determined. Protein protection from rumen degradation for commonly available protein sources in India, such as groundnut cake, mustard cake, guar korma, etc., is crucial to maximize the production potential of Indian livestock.

Various methods like *in-vivo* and *in-situ* techniques to measure rumen protein degradability are time-consuming, labour-intensive, and expensive. Determination of ammonia nitrogen production is another alternative, but since feed protein degradation and *de-novo* microbial protein synthesis occur simultaneously, estimating rumen protein degradability through ammonia release is a complex process. The modified *in-vitro* gas production (IVGP) technique, other than being a low-cost method with high analytical capacity, also eliminates the effects of *de novo* protein synthesis during fermentation. The present study aims to determine the protein degradability of various high-protein sources subjected to malic acid-heat treatment at various temperatures and to assess the extent of potential heat damage associated with high temperature treatments in view of developing a technology for reducing ruminal protein degradation for efficient protein utilization in buffalo and abatement of environmental pollution through nitrogen excretion.

MATERIALS AND METHODS

The experiment was conducted in the Division of Animal Nutrition and Feed Technologies, ICAR- Central Institute for Research on Buffaloes (CIRB), Hisar, Haryana, India (29.1203_N, 75.8069_E). The approval of the Institutional Animal Ethics Committee (IAEC-CIRB/16-17/A/09 dated 15 July 2016) for the care of the rumen-fistulated animals and for rumen fluid collection were taken for the experiment.

Selection of protein feeds and treatment: Four highly rumen degradable protein feeds were selected for the experiment, i.e., groundnut cake, guar korma, mustard cake, and sunflower meal (GNC, GK, MC, and SF). The feed samples were obtained from the Feed Unit of ICAR-

CIRB and were subjected to malic acid heat treatment as described by Vanegas *et al.* (2017). The feed samples, after grinding to a particle size of 1-2 mm, were sprayed with 1 M (134.09 g malic acid/l distilled water) food grade malic acid (Thirumalai Chemicals Ltd., Chennai, India) at a rate of 400 mL/kg feed. They were then kept at room temperature for 1 hour and were later subjected to heat treatment at 60°C, 100°C, 120°C and 150°C in a forced-air oven for 1 hour. Therefore, four untreated feeds (GNC, GK, MC, and SF) and sixteen treated protein feeds (GNC-60, GK-60, MC-60, SF-60, GNC-100, GK-100, MC-100, MC-100, GNC-120, GK-120, MC-120, SF-120, GNC-150, GK-150, MC-150, and SF-150) were obtained and subjected to the determination of *in vitro* crude protein degradability using the modified *in vitro* gas production technique.

Rumen fluid collection and preparation: Two adult male rumen-fistulated Murrah buffaloes (*Bubalus bubalis*) were used as donors to collect rumen fluid. The fistulated animals were provided with a maintenance ration (concentrate to roughage ratio of 40:60, with the roughage consisting of wheat straw and green fodder). The animals were housed in a semi-covered well-ventilated shed at the ICAR-CIRB Buffalo Farm with open access to water.

Rumen fluid was collected from both the rumen fistulated buffaloes in the early morning before feeding and watering. An equal volume was collected from each buffalo, pooled together and stored in a pre-warmed thermos flask. The flask was flushed with carbon dioxide (CO_2) beforehand to maintain an anaerobic environment. The sample was immediately taken to the laboratory, where it was strained through four layers of muslin cloth while constantly being bubbled with CO_2 to obtain strained rumen liquor (SRL) for further *in vitro* preparation.

Determination of *in vitro* rumen crude protein degradability: The *in vitro* rumen crude protein degradability (IVCPD) of treated and untreated feeds was determined using a modified *in vitro* gas production technique to assess the efficacy of malic acid-heat treatment (MAH) in reducing ruminal protein degradation. The filtered rumen fluid was conditioned by pre-incubation with added carbohydrates at 39°C for 3 hours. The carbohydrates added were 1.8 g of pectin (038052, Central Drug House Ltd., New Delhi, India); 3.5 g of maltose (RM 3050, HiMedia Laboratories Pvt. Ltd., Thane, India); 1.8 g of starch (RM 3029, HiMedia Laboratories Pvt. Ltd.) and 1.8 g of xylose (RM 111, HiMedia Laboratories Pvt. Ltd.) per litre of SRL. The added carbohydrates helped reduce background ammonia nitrogen levels and stimulate microbial activity.

After pre-incubation, the SRL was mixed with buffered mineral solution (1:2) to make buffered rumen fluid (BRF). Test feed (400 mg) and graded levels of carbohydrates (100, 200, 300 and 400 mg) consisting of maltose, starch, and xylose in a ratio of 2:1:1 were taken into ANKOM incubation bottles. 90 mL of BRF was added to each bottle under constant bubbling of CO_2 in three replicates (twelve bottles per sample) and incubated for 24 hours at 39°C. The gas pressure and temperature were monitored by ANKOM- RF Gas Production System, a radio frequency-based gas production equipment, with real-time graphing of pressure and temperature curves.

Varying levels of carbohydrates facilitated different levels of gas production and different ammonia nitrogen levels in the bottles. After the incubation period (24h), 5 mL of supernatant sample in duplicate were taken from each bottle, mixed with 1 mL of 25% metaphosphoric acid and stored overnight at -20°C for ammonia-nitrogen analysis.

Chemical analyses: Representative samples of protein feeds were taken and dried in a hot air oven at 100°C for 24 hours and then ground to pass through a 1 mm sieve. The standard methods of AOAC (2005) were followed to determine dry matter (DM), crude protein (CP; N × 6.25), ether extract (EE) and total ash (TA). Fibre fractions such as neutral detergent fibre (NDF) and acid detergent fibre (ADF) were assayed (Van Soest *et al.*, 1991). All analyses were performed in triplicate.

After the completion of *in vitro* incubations (24h), the supernatant samples were centrifuged at 11000-12000g for 20 minutes. The resulting supernatant was used for ammonia nitrogen analysis spectrophotometrically, using the reaction of alkaline hypochlorite and phenol reagent in the presence of the catalyst sodium nitroprusside (Alhidary *et al.*, 2019). The samples were read at an optical density (OD) of 550 nm using a microplate reader (BioTek Epoch, Agilent Technologies, Inc, USA).

Determination of irreversible damage due to heat treatment: The feed samples were exposed to heat treatments at 120°C and 150°C, and untreated feed samples were also included for comparison. The extent of heat damage was assessed by determining the acid detergent insoluble nitrogen (ADIN), which indicates the damage caused by the heat treatment. After analyzing the acid detergent fibre, the remaining ADF residues in the crucibles were analysed for ADIN using the Kjeldahl method of nitrogen analysis (AOAC, 2005).

Calculations: Cumulative gas pressure after 24 hours was converted to moles of gas by using the 'ideal' gas

law using the following equation:
Total gas production (mol) = P (V/RT)
Where:
P= Pressure in ANKOM RF reduction gas production bottle (KPa)
V= Headspace volume of ANKOM RF reduction gas production bottle (L)
T= Temperature of ANKOM RF reduction gas production bottle (Kelvin)
R= Gas constant (8.3144 L kPa/mol K)
Using Avogadro's law, gas production in moles was converted to gas measured in mL as follows:
Gas produced (mL) = n x 22.4 x 1000

Calculation of *in vitro* degradable CP (IVCPD) was done through extrapolation of the linear regression between ammonia-N (y, mg) and gas production (x, mL), a regression line equation and a y-intercept (b_0) were obtained, where the y-intercept represented the amount of ammonia-N at zero gas production when no fermentable carbohydrates are available, thus eliminating the effects of *de novo* microbial protein synthesis, and therefore indicative of the amount of ammonia released from protein and non-protein nitrogen compounds (NPN) present in the incubated feed.

The IVCPD was thus calculated using the following equation:
IVCPD = [Ammonia N at zero gas production (b_0) – Ammonia N of blank] / Total N of incubated feed

Statistical analysis: Data obtained were analysed as a completely randomized design (CRD) and were subjected to analysis of variance (ANOVA) using a general linear model procedure (IBM SPSS Statistics, Version 20). Differences among means were tested using Tukey's test, with significance declared if $p<0.05$.

RESULTS

Protein feeds with high rumen degradability were selected to assess the effectiveness of malic acid-heat treatment. Proximate analysis and fibre fractions of the protein feeds were conducted, and the results are presented in Table 1.

Following the modified *in vitro* gas production technique, the *in vitro* protein degradability of untreated and treated feeds was determined. A trend (p=) of linearly increasing gas production ($p<0.05$) was observed with the addition of graded levels of carbohydrates (Fig. 1), as evidenced by a higher level of fermentation.

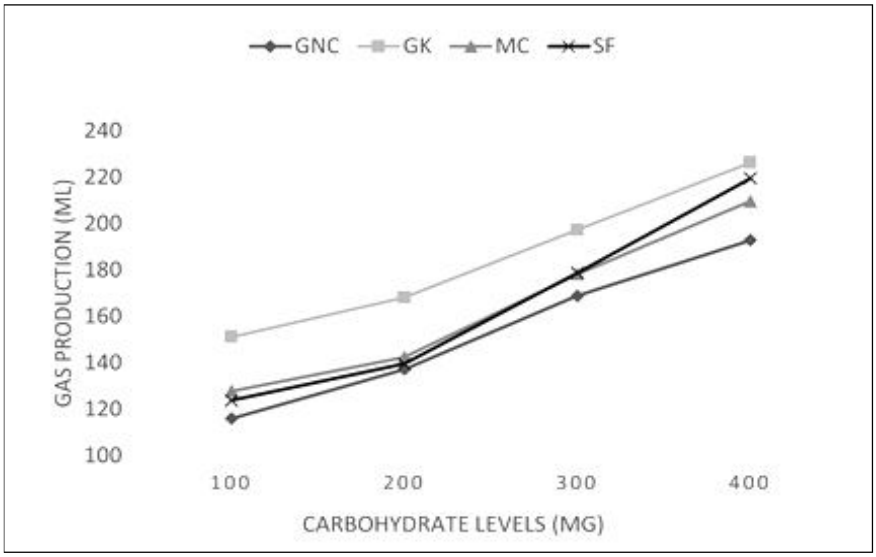

Fig.1. Relationship between carbohydrate levels and gas production from *in vitro* incubation of protein feeds

Table 1. Chemical composition (%DM basis) of protein feeds

Parameters	Groundnut cake	Guar korma	Mustard cake	Sunflower meal
Crude protein	44.98	49.55	35.02	32.40
Ether extract	5.54	6.69	9.54	0.85
Neutral detergent fibre	20.20	21.57	21.07	47.79
Acid detergent fibre	11.49	11.96	14.87	36.73
Total ash	7.04	5.35	8.26	8.03
Acid insoluble ash (%)	1.05	0.31	1.33	0.61

The regression coefficients for untreated GNC, GK, MC, and SF were 0.26, 0.25, 0.28, and 0.33, respectively. The y-intercepts obtained were different for each feed source, but the regression coefficients were in a similar range ($p>0.05$) across all twenty treatment feeds, i.e., 0.27±0.025 (Mean ± SD). This suggests that there was an increase of 0.27 mL in gas production/mg carbohydrates added. The regression equations between carbohydrate levels and gas production from untreated and MAH-150 treated protein feeds are presented in Table 2.

Ammonia nitrogen values for all the feeds at various carbohydrate levels were determined and plotted against gas production. An increase in gas production resulted in a significant increase ($p<0.05$) in the amount of nitrogen disappearing from the sample, as indicated by higher levels of ammonia nitrogen at lower gas production ($p<0.05$). This trend was observed in both treated and untreated protein feeds. The comparison of ammonia nitrogen and gas production between incubations of treated and untreated feeds is shown in Fig. 2 (a-e). Regression equations were developed based

Table 2. Regression equations and regression coefficients between gas production (mL) and carbohydrate levels (mg)

Protein feed	Regression equation	Correlation (R)	Regression coefficient (increase in mL gas production/mg carbohydrate added)
Groundnut cake (GNC)	y = 88.1447 + 0.2623 x	0.9973	0.2623
Guar korma (GK)	y = 122.054 + 0.2545 x	0.9933	0.2545
Mustard cake (MC)	y = 94.1788 + 0.2813 x	0.9872	0.2813
Sunflower meal (SF)	y = 83.7777 + 0.3265 x	0.9835	0.3265
GNC-150	y = 41.2866 + 0.2808 x	0.9954	0.2808
GK-150	y = 82.1473 + 0.2878 x	0.9872	0.2878
MC-150	y = 48.8056 + 0.2720 x	0.9985	0.2720
SF-150	y = 76.4732 + 0.2406 x	0.9975	0.2406

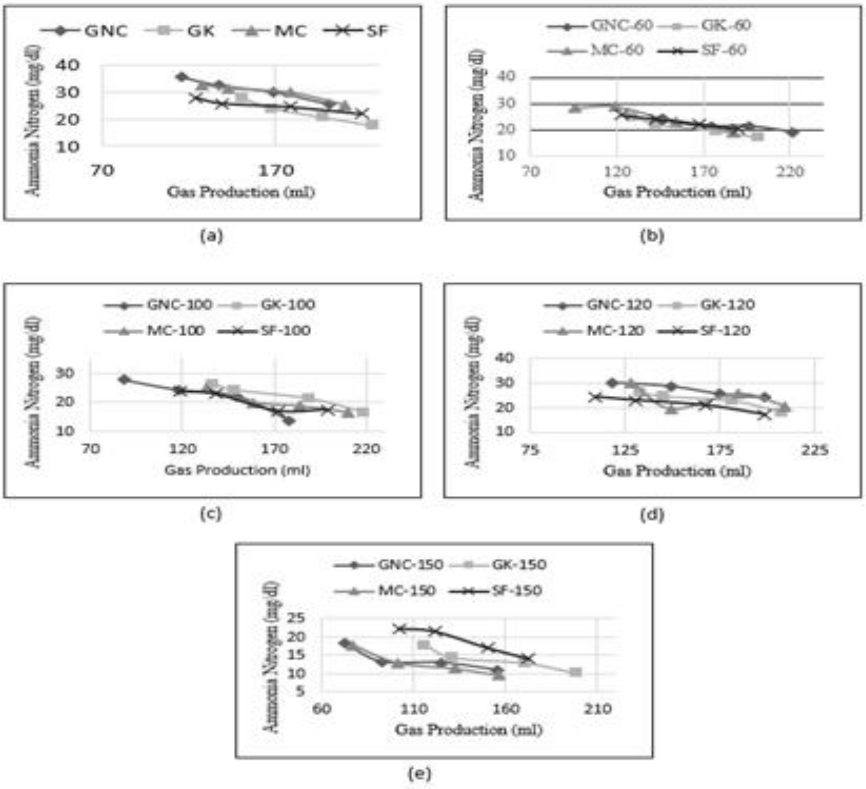

Fig. 2. Graphs representing the strong inverse relationship between ammonia nitrogen (mg/dL) and gas production (mL) from incubation of untreated and malic acid-heat treated protein feeds

on the inverse correlation between gas production and ammonia nitrogen values.

The regression lines of untreated and malic acid heat-treated GNC at 60, 100, 120, and 150°C are presented in Fig. 3, along with their regression equations. The correlation coefficients (indicating the rate of nitrogen disappearance with an increase in gas production) for all treatments of GNC, GK, MC, and SF were -0.083±0.016, -0.104±0.016, -0.092±0.014, and -0.086±0.021, respectively. On average, 0.091 mg of ammonia nitrogen disappeared with 1 mL of gas production, which was similar (p>0.05) across all feeds and treatments.

The *in vitro* crude protein degradability (IVCPD) of all protein feeds was determined (Table 3) and found that the protein degradability of GNC was highest (91.08%), followed by GK (81.90%), MC (81.61%), and SF (78.10%). A decreasing (P<0.05) trend of rumen IVCPD was obtained, irrespective of feed ingredients, as the temperature of treatment increased. In the present

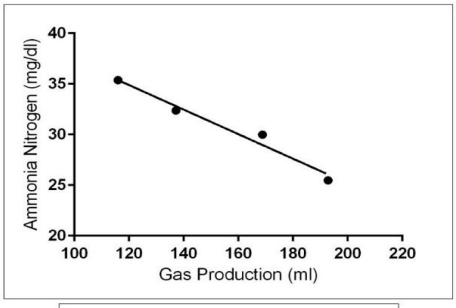

Fig.3. a. Relationship between gas production and ammonia nitrogen, untreated GNC
Regression Equation: y = 48.1528 - 0.1114 x
R^2 = 0.9855
Correlation = -0.9927

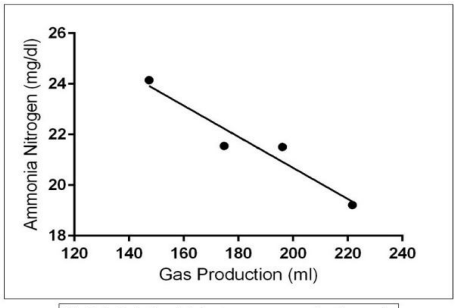

Fig.3. b. Relationship between gas production and ammonia nitrogen, GNC-60
Regression Equation: y = 32.9662 - 0.06141 x
R2 = 0.9275
Correlation = -0.9631

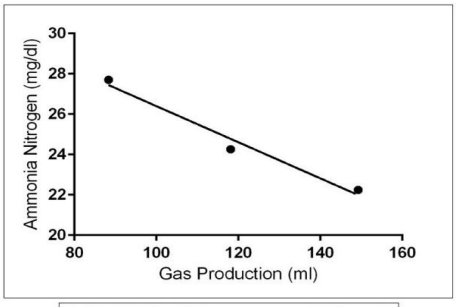

Fig.3. c. Relationship between gas production and ammonia nitrogen, GNC-100
Regression Equation: y = 35.3505 - 0.0895 x
R2 = 0.9736
Correlation = -0.9867

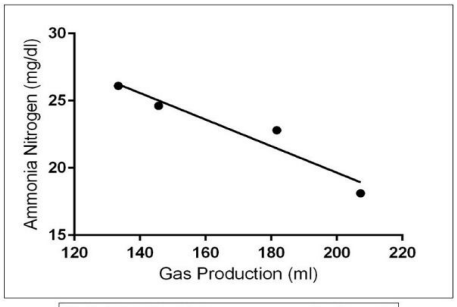

Fig.3. d. Relationship between gas production and ammonia nitrogen, GNC-120
Regression Equation: y = 39.554 - 0.07691 x
R2 = 0.9738
Correlation = -0.9868

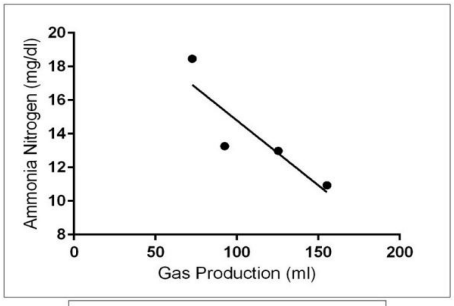

Fig.3. e. Relationship between gas production and ammonia nitrogen, GNC-150
Regression Equation: y = 22.5416 - 0.07739 x
R^2 = 0.7723
Correlation = -0.8788

Fig. 3. Relationship (Regression line) between gas production and ammonia nitrogen level on *in vitro* incubation of untreated and MAH treated GNC at different temperatures

Table 3. The IVCPD (%) of various protein feeds at different temperatures of the malic acid heat treatment

Samples	Untreated	60°C	100°C	120°C	150°C	SEM	P Value
Groundnut cake	91.08^{aA}	82.65^{abA}	75.91^{bA}	57.33^{cB}	28.70^{dB}	7.43	<0.001
Guar korma	81.90^{aAB}	75.32^{aA}	72.59^{abA}	62.12^{bA}	29.39^{cB}	6.22	<0.001
Mustard cake	81.61^{aAB}	78.77^{abA}	73.19^{bA}	54.45^{cB}	22.91^{dB}	7.27	<0.001
Sunflower meal	78.10^{aB}	75.63^{abA}	70.15^{abA}	63.12^{bcA}	54.71^{cA}	2.95	0.005
SEM	1.97	1.57	0.91	1.37	4.71		
P value	0.041	0.372	0.129	0.004	0.002		

* Means with different subscripts (a,b,c,d) in the same row differ significantly (p<0.05); # Means with different subscripts (A, B, C) in the same column differ significantly (p<0.05)

study, the MAH treatment was most effective in reducing (64-70%) IVCPD for GNC, GK and MC; however, SF was found to be less responsive with only about 30% reduction at the highest temperature treatment. The protein degradability of all the cakes examined (GNC, GK, MC, SF) remained similar (P>0.05) with MAH treatment up to 100°C; however, at 120°C, it remained comparable (P>0.05) for GNC and MC as well as GK and SF, although the IVCPD of GNC and MC is lower (P<0.01) than the GK and SF (Fig. 4). The study demonstrated that at highest temperature treatment (150°C) of oil cakes, there was significant (P<0.01) reduction in IVCPD of its counterparts from 120°C treatment, except for SF, where no (P>0.05) additional reduction was evidenced.

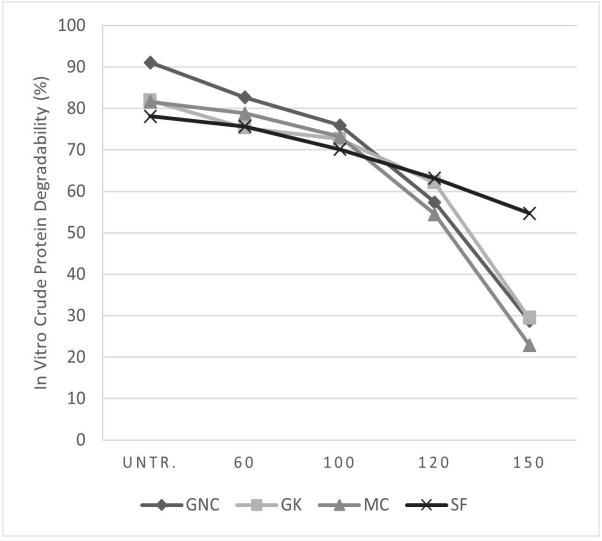

Fig. 4. Decrease in crude protein degradability of various protein feeds with increase in temperature of the malic acid heat treatment

Acid detergent insoluble crude protein (ADICP) was analysed for the determination of extent of heat damage due to high temperatures of MAH treatment, i.e., 120°C and 150°C (Fig. 5). The ADICP (% of CP) values, irrespective of oil cakes remained comparable (P>0.05) with the untreated control, when MAH treatment was performed up to 120°C; however, it remained high

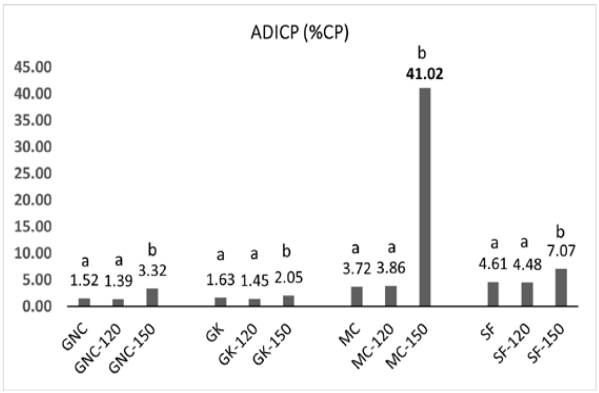

Fig. 5. A comparison of acid detergent insoluble crude protein [ADICP (% CP)] values of various treatments along with control (values with different letters: a,b, differ significantly (p<0.05)

(P<0.05) for MAH at 150°C treatments. Although the ADICP values for GNC, GK and SF at various temperatures remained lower (<10%), it was found to be very high (41.02%) in MC when treated at 150°C.

DISCUSSION

The chemical composition of various protein feeds selected for malic acid-heat treatment was determined through proximate analysis and fibre fractions, and all the parameters were found to be within the expected range (Ranjhan, 2001). Among the feeds, guar korma exhibited the highest protein content (49.55%), while sunflower meal had the lowest protein content (32.40%). The latter had a higher fibre content, which aligns with the composition of non-dehulled sunflower meal.

Gas production data from the *in vitro* incubations showed that increased fermentation due to added carbohydrates led to increased gas production (Average correlation R = 0.99) among all the feeds. The rate of increase in gas production due to added carbohydrates was not affected, although protein feeds treated at 150°C had lower gas production values compared to their untreated counterparts or protein feeds treated at lower

temperatures. The difference in y-intercepts (gas production at zero added carbohydrates) was due to variations in rumen fluid in different *in vitro* incubations, which was also supported by differences in blank ammonia nitrogen values from separate incubations that were considered when calculating IVCPD. However, the trend remained consistent across all treatments. In the present study, gas production increased at a rate of 0.27 mL/g of carbohydrates added. In an experiment, Raab *et al*. (1983) reported approximately 0.39 mL gas production/mg addition of starch. The difference in values could be attributed to the differences in the composition of carbohydrates used.

Lower ammonia nitrogen values observed in MAH-150 treated feeds indicated a lower availability of feed protein to rumen fluid microbes. However, this is not a complete indication of the effectiveness of protein protection due to MAH treatment. This is because ammonia nitrogen is an inconsistent and unreliable indicator for assessing nitrogen disappearance and protein degradability due to *de novo* microbial synthesis in the rumen and interference from background ammonia nitrogen values. Pre-incubation with added carbohydrates resulted in a decrease in background ammonia nitrogen values (from 10.5 mg/dL to 8.05 mg/dL). A similar pre-incubation medium with added buffers was reported to decrease background ammonia nitrogen from 8.1 to 5.3 mg/dL (Karlsson *et al*., 2009). Increased fermentation and subsequent increase in gas production led to a higher level of disappearance of ammonia nitrogen from the feed due to incorporation of nitrogen by rumen fluid microbes for *de novo* synthesis of protein. A strong inverse relationship between rumen fluid ammonia nitrogen and gas production was established (R = -0.94) through regression analysis. The rate of ammonia N disappearance followed a similar trend (0.091 mg/dL ammonia-N disappearance/mL gas production) for all feeds.

The *in vitro* crude protein degradability data revealed an increase in the rumen protected protein fraction across all protein feeds. The decrease in rumen degradability at MAH-60 and MAH-100 could be attributed to the efficacy of malic acid treatment, as these temperatures are unlikely to induce cross-linkage formation among peptide chains and carbohydrates to form bypass protein and were merely used for drying the sample. As shown in Fig. 4, the drop in rumen protein degradability from 60°C to 100°C is lesser compared to the decrease in degradability from 100°C to 120°C, indicating an increased level of cross linkages at 120°C. In a study, Suresh *et al*. (2011) suggested treating protein oilcakes at 120-150°C for 2-4 hours in a hot air oven to protect protein from rumen fermentation. A temperature-time combination of 150°C for 2 hours has been considered optimum for heat treatment of groundnut cake (Ganai *et al.*, 2019). As previously reported, treatment of GNC at 150°C led to more than a 50% decrease in protein solubility (Garg, 1998). Sunflower meal was the most resistant protein feed to MAH-150 treatment, which could be due to protein structure and their cross linkages (Table 3).

Among all untreated protein feeds, groundnut cake had the highest rumen degradability, which was significantly different ($P<0.05$) compared to its untreated counterparts. On the other hand, sunflower meal had the lowest protein degradability ($P<0.05$). After treatment of various protein samples with commercial proteases, the rumen degradable fractions of groundnut cake, mustard cake, guar korma, and sunflower meal were determined to be 75.03, 71.05, 69.13, and 75.97, respectively, resulting in estimation of the rumen undegraded nitrogen fractions (Mahesh *et al*., 2017). Sehgal and Makkar (1994) reported *in vitro* estimates of rumen protein degradability of groundnut cake (81.9%), mustard cake (78.3%), and guar korma (83.0%). The average *in vitro* nitrogen degradation of GNC was reported to be 0.921 for Murrah buffaloes (Dey *et al*., 2006). Arroyo *et al*. (2013) reported bypass crude protein of sunflower meal to be 15%. No significant difference ($p<0.05$) was observed between IVCPD values of MAH-60 and MAH-100 treated samples across all protein feeds, and there were no significant differences ($p<0.05$) regarding IVCPD among the four protein feeds treated at the same temperature in case of MAH-60 and MAH-100 treatments. A higher level of protein protection was observed in groundnut cake and mustard cake at 120°C ($p<0.05$) compared to guar korma and sunflower meal, which had statistically similar values. The IVCPD values at MAH-150 were similar for all the protein feeds except sunflower meal, which had a lower ($P<0.05$) degree of protein protection.

Although high levels of rumen protein protection were achieved through MAH treatment, heat damage due to irreversible Maillard reaction had to be eliminated to determine if the protected protein fraction was available for digestion in the small intestine. Acid detergent insoluble nitrogen has been used as an indicator to indicate the extent of heat damage (Vanegas *et al*., 2017). The Cornell Net Carbohydrate Protein System (CNCPS) assumes ADIN to represent the non-digestible protein fraction of a feedstuff, which is completely unavailable to the animal (Sniffen *et al*., 1992). According to Schroeder *et al*. (1996), ADICP (% total protein) values lower than 12-15% do not significantly decrease the digestibility of rumen undegradable protein (UDP-D), but values higher than

15% result in a progressive decrease in UDP-D, indicating significant heat damage and rendering a significant fraction of protein unavailable to the animal. In the present study, ADICP (ADIN*6.25, % of total CP) values for all MAH-120 treatments did not increase (P>0.05) compared to untreated feeds. While there was an increase in ADICP (% CP) fractions of GNC-150, GK-150, and SF-150, it was not enough to warrant heat damage (Schroeder *et al*., 1996), i.e., within the 15% limit of ADICP. However, MC-150 was found to be heat damaged as 41.02% of the total crude protein was present as ADICP, rendering a major fraction of feed protein unavailable to the animals.

Malic acid heat treatment proved highly effective in reducing the rumen degradable protein fraction and increasing the bypass protein fraction at all temperatures. The greatest protection was achieved at 150°C. The modified *in vitro* gas production technique holds promise as a reliable method for determining rumen crude protein degradability due to its simplicity, speed and cost-effectiveness. Malic acid-heat treatment at 150°C for groundnut cake, guar korma, and sunflower meal can be recommended to be incorporated into ruminant rations to increase the bypass protein fraction, while the mustard cake is suitable for malic acid-heat treatment at 120°C. Future research should focus on incorporating these protein feeds into ruminant rations and examining the efficacy of malic acid heat treatment on *in vitro* rumen fermentation of compound feeds and actual feeding value on *in vivo* feeding trials on growing and lactating buffaloes.

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Author's contributions: DA, BRS, TS: Conceptualization; TS: Data curation; TS, SRK: Methodology; TS: Validation; TS, SRK, DA: Investigation; TS: Writing -original draft; DA, LPC: Review and editing.

Ethical approval: Ethical clearance was obtained from the Institutional Animal Ethics Committee (IAEC) before commencing the experiment, and humane animal handling procedures were followed throughout the experiment.

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