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Effect of Dietary Supplementation of Rumen Protected Methionine and Lysine on Immunity Status and Trace Mineral Profile in Sahiwal Female Calves

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ABSTRACT

The experiment was conducted during the months of May-June in 2018 at the Bull Mother Experimental Farm (BMEF), College of Veterinary Science & Animal Husbandry, Anjora, Durg, and the Government Cattle Breeding Farm (CBF), Anjora, Durg to evaluate the effect of dietary supplementation of rumen-protected methionine (RPM) and rumen protected lysine (RPL) on immunity status and trace mineral profile in indigenous Sahiwal female calves. Twenty calves aged 7–12 months with body weights ranging from 80 to 111 kg were randomly allotted into four groups (n=5 each): T_1 (basal diet), T_2 (basal diet+RPM 5 g day^{-1}), T_3 (basal diet+RPL 15 g day^{-1}), and T_4 (basal diet+RPM 5 g+RPL 15 g day^{-1}) for a period of 90 days. Calves were maintained on a concentrate mixture and berseem hay (60:40 ratio) as per ICAR (2013) standards. Immunity was assessed using phytohaemagglutinin (PHA) skin test and lymphocyte proliferation assay, while serum concentrations of Fe, Cu, Zn, and Se were estimated by atomic absorption spectrophotometry. Results revealed no significant differences in PHA skin thickness or serum mineral concentrations among treatment groups, with values remaining within normal physiological ranges. However, RPM supplementation (T_2) significantly improved lymphocyte proliferation ($p \leq 0.05$), indicating enhanced immune competence. These findings suggested that RPM could be considered a functional feed additive to strengthen immune responses in Sahiwal calves without altering mineral balance.

KEYWORDS: Immunity, rumen protected lysine, rumen protected methionine, sahiwal cattle

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Data Availability Statement: Legal restrictions are imposed on the public sharing of raw data. However, authors have full right to transfer or share the data in raw form upon request subject to either meeting the conditions of the original consents and the original research study. Further, access of data needs to meet whether the user complies with the ethical and legal obligations as data controllers to allow for secondary use of the data outside of the original study.

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1. INTRODUCTION

Amino acids, the fundamental building blocks of proteins, play a pivotal role in cellular growth, metabolism, and overall physiological function. Beyond protein synthesis, they serve as substrates for numerous low molecular weight compounds such as nitric oxide, polyamines, and catecholamines, and act as precursors for small peptides, including glutathione and carnosine (Wu et al., 2014). In ruminants, adequate protein intake with a balanced amino acid profile is essential for optimal growth and health. Among essential amino acids, methionine and lysine are recognized as the most limiting under tropical feeding conditions, often contributing to poor performance in calves consuming forage-based diets (Merchen and Titgemeyer, 1992). Several studies have confirmed that methionine and lysine are the first two limiting amino acids in most dietary situations (Bequette et al., 1998; Doepel et al., 2004; Lapierre et al., 2006).

Methionine, a sulphur-containing amino acid, is multifunctional in vertebrate nutrition. In addition to its role in protein synthesis, it serves as a source of sulphur, a methyl donor, and a precursor for compounds such as cysteine, taurine, glutathione, metallothionein, carnitine, creatine, and S-adenosyl methionine (SAM), a universal methyl donor (Lehninger, 1977; Loest et al., 2002; Brosnan et al., 2007, Khan et al., 2023). SAM is very much essential for many biological processes including transsulfuration, polyamine biosynthesis etc (Lu and Mato, 2012). Methylation is critical for the biosynthesis of DNA, RNA, phospholipids, and proteins. Methionine also supports immune function by promoting lymphocyte proliferation, enhancing antioxidant activity, and maintaining hepatic and metabolic health (Flynn et al., 2002; Fang et al., 2002; Osorio et al., 2013; Zhang and Guo, 2008; Elmada et al., 2016; Ragupathi et al., 2024). Methionine alone or in combination with choline can protect from the oxidative stress by uplifting the glutathione transferase (GST) activity in the plasma (Tsiplakou et al., 2017).

Lysine, another essential amino acid, is vital for collagen formation and carnitine synthesis, which facilitates β-oxidation of fatty acids and energy production. It has been linked to calcium absorption and retention, while deficiencies in lysine can impair protein synthesis, cytokine production, and lymphocyte proliferation, ultimately weakening immune responses and increasing susceptibility to infection (Kidd et al., 1997; Konashi et al., 2000). Immune cell function is fundamental to the efficiency and duration of inflammatory processes triggered by infection or tissue injury (Medzhitov, 2008; Khan et al., 2023).

It is well established that the amino acid needs for high-yielding ruminant animals are not fully covered through dietary sources following microbial degradation (Ayyat et al., 2021). So, this must be met by protecting the proteins from ruminal breakdown. Hence, the dietary supplementation of rumen-protected proteins or amino acids, particularly methionine and lysine is important. These rumen-protected amino acids (methionine and lysine) have a positive role on the regulation of immunity, antioxidative, and anti-inflammatory status of the ruminants including cattle (Khan et al., 2023). Particularly during the periparturient period, the oxidative status affects the production performance and metabolism of the animals. In this state, supplementation of rumen protected limiting amino acids can be beneficial (Khan et al., 2023). Unofrtunately, limited information is available on the effect of rumen-protected methionine (RPM) and rumen-protected lysine (RPL) supplementation on immunity and mineral status in indigenous breeds such as Sahiwal calves. Therefore, the present study was undertaken to evaluate the impact of RPM and RPL, individually and in combination, on immunity status and trace mineral profile in Sahiwal female calves.

2. MATERIALS AND METHODS

2.1. Experimental animals and design

The experiment was conducted during May and June, 2018 at the Bull Mother Experimental Farm (BMEF), College of Veterinary Science and Animal Husbandry, Anjora, Durg, and the Government Cattle Breeding Farm (CBF), Anjora, Durg, to evaluate the effect of dietary supplementation of rumenprotected methionine (RPM) and lysine (RPL) on immunity status and trace mineral profile in indigenous Sahiwal female calves. A total of twenty calves, aged 7–12 months with body weights ranging from 80 to 111 kg, were selected from the purebred herds maintained at BMEF and CBF. The animals were randomly allotted into four groups of five calves each, following a Completely Randomized Design (CRD). The initial mean age and body weights of calves across the treatment groups were recorded and found to be statistically nonsignificant.

The initial mean age of calves in the four treatment groups was 10.41±0.73, 10.15±0.83, 10.12±0.43, and 10.08±0.87 months, with corresponding body weights of 95.8±7.4, 95.2±4.6, 95.2±2.5, and 95.4±2.9 kg, respectively. Differences in age and body weight among groups were statistically nonsignificant. Details of treatment groups are presented in Table 1.

2.2. Feeding management

During the 90day experimental period, calves were offered a concentrate mixture and berseem hay in a 60:40 ratio to meet protein and energy requirements for growth, as per ICAR (2013) standards. Fresh drinking water was provided ad libitum. The basal diet (concentrate and berseem hay)

Table 1: Details of different treatment group

Sl. No.	Group	Treatment
1.	T_1 (control)	Basal diet (concentrate mixture+ berseem hay)
2.	T_2	Basal diet with 5 g RPM/d
3.	T_3	Basal diet with 15 g RPL/d
4.	T_4	Basal diet with RPM 5 g+RPL 15 g d^{-1}

was analyzed for proximate composition and fibre fractions using (Anonymous, 2005) and Van Soest methods (Table 2).

Table 2: Chemical composition of concentrate, berseem hay and total mix feed (% on DM basis)

Particulars	Calf grower	Berseem Hay	Total mix feed*
DM	90	89	89.40
CP	20	13	15.80
CF	7.80	26	18.72
EE	0.79	1.30	1.10
TA	10.91	13.54	12.48
NFE	60.50	46.16	51.90
Ca	1.50	2.03	1.82
P	0.80	0.28	0.49
NDF	27.80	38.50	33.9
ADF	14.24	29.50	23.40

Commercially available rumen protected methionine (MetiPEARL™, Kemin Industries Inc.) and rumen protected lysine (LysiGEM™, Kemin Industries Inc.) were used as dietary supplements.

2.3. Immunity assays

2.3.1. Phytohaemagglutinin (PHA) skin test

Cellmediated immune response was assessed in vivo using the PHA skin test as described by Kelley (1982). On day 90, calves were injected intradermally with 150 µg of PHA in 0.1 ml of Dulbecco's Phosphate Buffered Saline (DPBS). Skin fold thickness was measured before injection and at 3, 6, 12, 24, and 48 hours postinjection using a digital Vernier caliper.

2.3.2. Lymphocyte proliferation assay

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (McCoy, 1997). Buffy coat was harvested, washed twice with DPBS, and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% fetal bovine serum (FBS) and 1% antibioticantimycotic solution. The culture medium was sterilized by filtration (0.2 µm) and incubated overnight at 37°C in 5% CO_2.

The lymphocyte suspension was adjusted to 1×10^6 cells/ml, and 100 µl of suspension was seeded per well in a 96well flatbottom tissue culture plate. Cells were stimulated with phytohaemagglutinin (PHAp) at 10 µg/ml, a concentration previously determined to provide maximal stimulation of bovine lymphocytes. Blank wells contained culture medium only. Cultures were incubated at 37°C in a humidified environment.

Lymphocyte proliferation was assessed using the MTT (tetrazolium) assay (Mosmann, 1983). The number of viable cells was directly proportional to the level of formazan product formed, which was quantified using an ELISA plate reader (Thermo Scientific, Multiskan EX).

2.4. Trace mineral estimation

Serum concentrations of copper (Cu), iron (Fe), zinc (Zn), and selenium (Se) were determined using an Atomic Absorption Spectrophotometer (ECIL AAS 4141). Samples were digested with triacid solution (HNO_3:H_2SO_4:$HClO_4$=9:2:1) and diluted to a known volume with distilled water prior to analysis. Singh et al. (2022) was additionally cited for mineral estimation in livestock.

2.5. Statistical analysis

Data were analyzed using one-way ANOVA under a Completely Randomized Design (Snedecor and Cochran, 1994). Treatment means were compared using Duncan's Multiple Range Test (DMRT), and the statistical procedures were further cross-validated with Gomez et al. (2022) to incorporate modern applications of ANOVA and DMRT in animal nutrition research.

3. RESULTS AND DISCUSSION

3.1. Phytohaemagglutinin (PHA) skin test

The results of the PHA skin test are presented in Table 3. Dietary supplementation with rumen protected methionine (RPM) and lysine (RPL) did not produce significant differences among treatment groups in skin fold thickness following intradermal injection of phytohaemagglutinin. The PHA skin test is a standard measure of Tlymphocyte proliferation in response to a mitogen (Smits et al., 1999). Although the present study did not reveal significant variation, earlier reports have shown that methionine supplementation can enhance immune responses. Li et al. (2007) observed improved innate and humoral immunity in chickens, while Batistel et al. (2018) reported enhanced neutrophil function in dairy cows supplemented with RPM. These findings suggest that methionine may exert immunomodulatory effects, though responses can vary depending on species, age, and physiological status.

Table 3: Effect of dietary supplementation of rumen protected methionine and lysine on PHA skin test

Hours	Treatment groups					
	I (Control)	II (RPM-5 g d^{-1})	III (RPL-15 g d^{-1})	IV (RPM 5 g+RPL-15 g d^{-1})	*p* value	Sig
3	6.14*±0.81	6.29±0.55	6.17±0.51	6.11±0.43	0.996	NS
6	5.42±0.56	5.65±0.56	5.31±0.31	5.37±0.27	0.951	NS
12	4.25±0.56	4.29±0.36	4.25±0.13	4.20±0.04	0.998	NS
24	3.36±0.21	3.39±0.24	3.23±0.15	3.26±0.06	0.911	NS
48	2.00±0.16	2.08±0.09	2.03±0.17	2.05±0.11	0.977	NS

* Increase in skin thickness (mm)

3.2. *Lymphocyte proliferation assay*

Results of the lymphocyte proliferation assay are shown in Table 4. Calves supplemented with RPM (T_2) exhibited significantly higher ($p<0.05$) PBMC viability compared to control and other treatment groups. This indicates that RPM improved lymphocyte proliferation capacity, thereby strengthening immune competence. Similar observations were reported by Soder and Holden (1999), who found increased proliferative responses of peripheral T lymphocytes with RPM supplementation. Woodward et al. (2004) also noted enhanced proportions of CD62L, CD25, and CD8 positive lymphocyte subgroups in cows fed RPM. Han et al. (2015) demonstrated that methionine improved cell viability under stress conditions, while Zhou et al. (2016) reported greater phagocytosis and oxidative burst activity in methioninesupplemented cows. Collectively, these findings support the immunomodulatory role of methionine in ruminants.

Table 4: Effect of dietary supplementation of rumen protected methionine and lysine on lymphocyte proliferation

Hours	Treatment groups					
	I (Control)	II (RPM-5 g d^{-1})	III (RPL-15 g d^{-1})	IV (RPM 5 g+RPL-15 g d^{-1})	*p* value	Sig
PHA+DMEM+ PBMC	120.34 ± 4.15^{ab}	128.07 ± 1.74^{a}	114.78 ± 2.14^{b}	120.42 ± 3.71^{ab}	0.032	*
DMEM+PBMC	114.93 ± 2.13	117.99±2.81	112.32 ± 1.89	115.16 ± 1.84	0.351	NS

Mean values bearing different superscript (a, b) in row differ significantly * ($p<0.05$)

3.3. *Trace mineral profile*

Serum concentrations of Fe, Cu, Zn, and Se are presented in Table 5. No significant differences were observed among treatment groups, indicating that RPM and RPL supplementation did not alter trace mineral status. All values remained within normal physiological ranges, consistent with Gadberry et al. (2003). Similar nonsignificant effects of amino acid supplementation on mineral metabolism have been reported in other ruminant studies, suggesting that RPM and RPL primarily influence immune parameters rather than mineral absorption or utilization.

Table 5: Effect of dietary supplementation of rumen protected methionine and lysine on serum mineral status

Hours	Treatment groups				
	I (Control)	II (RPM-5 g d^{-1})	III (RPL-15 g d^{-1})	IV (RPM 5 g+RPL-15 g d^{-1})	Sig
Fe (ppm)	1.34±0.11	1.37±0.11	1.16±0.07	1.19 ±0.08	NS
Cu (ppm)	0.87±0.04	0.87±0.02	0.84±0.01	0.81±0.01	NS
Zn (ppm)	1.11±0.12	1.04±0.11	1.13±0.11	1.21±0.05	NS
Se (ppm)	0.195±0.01	0.203±0.03	0.202 ±0.02	0.201±0.01	NS

4. CONCLUSION

RPM supplementation significantly enhanced lymphocyte proliferation, indicating improved immune competence, while no significant changes were observed in PHA skin thickness or serum mineral concentrations. Overall, RPM proved beneficial as a functional feed additive to strengthen immune responses in calves without altering mineral balance.

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