

Extra-Selenium Alleviated Epithelial Injury by Modulating the Oxidative Stress Intervened MYD88/TRIF Dependent TLR-4 Signalling Pathway in Rumen of Goat Fed High Concentrate Diet

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ABSTRACT

High concentrate (HC) diet consumption induced oxidative stress and rumen epithelial injuries associated with inflammation. Selenium (Se) regulates various physiological functions primarily through its incorporation into anti-oxidant enzymes. Thus the existing study was aimed to determine role of extra selenium supply against high concentrate (HC) diet provoked inflammatory response in ruminal epithelium of goat. Thirty goats allocated into 3 groups (n=10/groups) were fed low concentrate, LC (roughage: concentrate 65:35), HC (35:65) or Se supplemented HC (HCS, 35:65 + 0.5 mg.Se.kg⁻¹ diet) diets for 10 weeks. Short chain fatty acids (SCFAs), oxidative stress markers and genes expressions evaluated by gas chromatography; kits; and RT-qPCR correspondingly. The individual and total SCFAs were significantly higher ($P < 0.05$) with instantaneous dropped pH in HC and HCS groups in contrast to LC group. Addition of Se improved rumen fermentation by rising around 30% butyrate in HCS group than HC group. In comparison with LC, HC diet raised ($P < 0.05$) ruminal lipopolysaccharide (LPS) and decreased pH level. Histology of rumen wall revealed that HC diet exhibited severe epithelial damage that caused LPS leakage as evinced by increased plasma LPS concentration. Epithelial damage in HC group was found with lower glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (CAT) activities and higher ($P < 0.05$) malondialdehyde (MDA) in rumen suggesting the involvement of oxidative stress. Moreover, ruminal oxidative injury was associated with increased inflammatory response by upregulation of pro-inflammatory toll-like receptor-4 (TLR-4), cluster of differentiation-14 (CD-14), tumor necrosis factor- α (TNF- α), TNF receptor-associated factor-6 (TRAF-6), nuclear factor kappa B (NF- κ B), Myeloid differentiation primary response 88 (MYD88), interleukin-1 β (IL-1 β), IL-6, IL-8, chemokine ligand-5 (CCL5), LPS-binding protein (LBP), serum amyloid A (SAA) and downregulation of anti-inflammatory IL-10, IL-13 in the rumen. In contrast, inclusion of surplus Se in HC group lowered ruminal and plasma LPS concentration and alleviated OS intervened inflammatory response in rumen epithelium of HC-fed goats. In conclusion, extra-Se improved rumen wall histology by attenuating HC diet induced oxidative inflammatory lesions by regulation of MYD88/ TRIF dependent TLR-4 signalling pathway in the rumen of goat.

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RM: Accomplished the trial and protocols and conscripted the draft. MP: Conceptualized and monitored the research and ensured the novelty of manuscript. ABK and SAP: Aided in manuscript and analysis of data. SPS: Provide critical assistance in writing, reviewing and editing of the manuscript. UA: Assisted in designing and accomplishment of the research and help in data analysis. MAS, ABS and TS: Reviewed and ratified the article censoriously.

Key words

Selenium, Concentrates, Ruminal epithelium, Lipopolysaccharide, Inflammation

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INTRODUCTION

High concentrate (HC) diets are routinely fed for improving growth rate and productivity in small ruminants (Hua *et al.*, 2017; Solangi *et al.*, 2025). Nevertheless, long-term exposure to HC diet leads to multiple digestive and systemic disorders. HC diets induce a change in ruminal fermentation processes, causing

excessive formation and accumulation of short chain fatty acids (SCFAs). The accumulation results in acute pH reduction and a shift in the microbiome composition (Plaizier *et al.*, 2017). As a consequence, lipopolysaccharide (LPS), strong immunogens, are released into the rumen fluid. An increase in LPS concentrations can lead to impaired rumen epithelium structure, increasing its permeability and allowing LPS to enter systemic circulation (Abaker *et al.*, 2017; Shahid *et al.*, 2025).

The ruminal mucosa contains a four layered keratinized, stratified squamous epithelium (SSE), each layer performs precise physiological function. The stratum corneum layer forms a direct link to the rumen contents, playing an essential role in the physical barrier between rumen contents and lower layers (Böck *et al.*, 2023; Leite *et al.*, 2024). Parakeratosis prevalence is high due to abnormal osmolality and rumen pH levels (Steele *et al.*, 2011). Many studies revealed that a diet rich in grain in ruminants caused susceptibility to sub-acute ruminal acidosis (SARA) (Zhang *et al.*, 2016), the most prevalent disease found in high-producing dairy cattle that impact milk production and animal wellbeing (Plaizier *et al.*, 2017). Additionally, SARA causes gastrointestinal tract (GIT) injury, liver abscesses, and lameness. GIT injuries are linked to reduced ruminal barrier effectiveness and increased permeability, leading to LPS translocation into the peripheral bloodstream. The recent studies have shown that oxidative stress (OS) is associated with induction of inflammation and apoptosis in the GIT and liver (Shah *et al.*, 2022; Shahid *et al.*, 2026). The impairment of rumen epithelium caused LPS and pathogens to enter the bloodstream, which resulted in activation of pro-inflammatory cytokines and acute-phase proteins (APPs) (Pederzoli *et al.*, 2018). Upon recognition by the transmembrane receptor such as TLR-4, the LPS causes changes in APPs by activating the innate immune system. This results in an increase in plasma APP concentration indicating physiological and inflammatory changes (Shah *et al.*, 2022). LPS-binding protein (LBP), haptoglobin (Hp) and serum amyloid A (SAA) are the APPs whose levels were found to be affected by the consumption of high-concentrate diet (Chishti *et al.*, 2020; Ohtaki *et al.*, 2020). Of all these biomarkers, LBP is said to be one of the most reliable markers of systemic inflammation since it exhibits significant sensitivity to LPS stimulation. In a study involving ruminants that had been on HC diet for a long period of time, researchers noted that the level of LBP in plasma increased many fold (Chang *et al.*, 2015a; Shah *et al.*, 2022). Rumen-derived LPS causes the production of APPs by stimulating NF- κ B and TNF- α pathways through TLR-4-mediated signals (Ciesielska *et al.*, 2021; Kany *et al.*, 2019). Additionally, sustained consumption of HC

diet results in the expression of NF- κ B, thus promoting the secretion of the inflammatory cytokines such as IL-1 β , TNF- α , IL-6 and CCL-5, and retreating anti-inflammatory cytokines such as IL-8 and IL-10. These changes ultimately lead to variations in acute-phase protein synthesis in both hepatic and ruminal epithelial cells of cattle (Dai *et al.*, 2022; Shah *et al.*, 2022).

Selenium (Se) is an indispensable trace mineral that regulates various physiological functions in animals, primarily through its incorporation into anti-oxidant enzymes glutathione peroxidase (GSH-Px). These selenoproteins are widely distributed across body tissues, including the GIT, liver, pancreas, kidneys, spleen, lungs, ovaries, testis and skeletal muscles (Samo *et al.*, 2018; Zhang *et al.*, 2013). The bioavailability of Se in the body, and consequently the synthesis and activity of GSH-Px, largely hinge on both the concentration and form of Se present in animal ration. The absorption, metabolism, and retention of Se have been reported to be more efficient in animals fed HC diets compared to those on forage-based diets (Shah *et al.*, 2022). It has been shown that Se supplementation increases butyrate concentrations in rumen of goats fed HC diet (Samo *et al.*, 2020). Intraepithelial pH (pHi) maintenance depends upon the rate of SCFAs disappearance rate from rumen and their intraepithelial metabolic rates. Butyrate, being most preferred compound utilized by epithelial cells, yield large quantities of D-L-hydroxybutyric acid, followed by propionate which is converted into lactate, by rumen epithelium. The availability of ruminal butyrate in sufficient amount would prevent propionate utilization by epithelial cells due to the inhibition of the propionyl Co A synthetase activity (Nshanian *et al.*, 2025) and increase its flow towards liver to produce glucose. In addition, a risk of pH reduction due to lactate accumulation would be reduced.

Furthermore, supranutritional supplementation of Se, at levels 20–30 times higher than the recommended dietary requirement, has been shown to increase tissue Se deposition and enhance antioxidant capacity without inducing adverse effects (Del Razo-Rodriguez *et al.*, 2013). In goats fed HC diets, dietary Se levels above 0.6–0.7 mg/kg have been associated with reduced OS, attenuation of hepatic inflammation, and decreased apoptosis in the colon (Shah *et al.*, 2022; Samo *et al.*, 2020). Se supplementation has demonstrated beneficial effects under various stress conditions. For instance, in dairy cows, it enhances immune function and reduces the expression of APPs during the peri-parturient period (Gong and Xiao, 2021; Hall *et al.*, 2014). HC diets cause inflammation by disturbing the expressions of positive and negative APPs in goats and Se treatment attenuates the inflammation

by modulating APPs expressions (Solangi *et al.*, 2025; Shah *et al.*, 2022). Under heat stress, supplemental Se has been shown to improve immune responses and increase antioxidant status in pigs and sheep (Chen *et al.*, 2020; Liu *et al.*, 2021b). The rabbits stressed with bacterial infection diminished hepato-renal injury (Ujjan *et al.*, 2025) and pre-pubertal goat mitigates apoptotic gene expression in testes through Se supplementation (Soomro *et al.*, 2026). Moreover, in animals exposed to endotoxin challenges such as LPS, including heat- and drug-stressed pigs and poultry, Se exerts protective effects by modulating immuno-inflammatory gene expression, particularly through regulation of receptor proteins, adaptor molecules, transcription factors, and APPs within the TLR-4 signaling pathway (He *et al.*, 2022; Qu *et al.*, 2020).

It has already been observed that OS and inflammation induced in the rumen and liver tissue of animals consuming HC diets were due to the presence of LPS, which is produced by the rumen itself (Dai *et al.*, 2022; Shah *et al.*, 2022). LPS has been seen to induce OS and inflammation by leading to low levels of Se in both tissues and the blood of animals, reduced activity of GSH-Px enzymes in the tissues and blood, and disturbances in the metabolism of selenoproteins in the bodies of mice, goats and sheep (Cao *et al.*, 2006; Sherlock *et al.*, 2020; Wang *et al.*, 2017). Previous studies suggested that level of Se exhibits a pivotal role in regulating immune-inflammatory responses in animals experiencing LPS-induced stress because its higher utilization increases the demand for Se in the animal feed. Thus, based on the above information, it was predicted that dietary supplementation of extra Se would reduce HC diet-induced damage to the ruminal epithelium by improving the antioxidant response system and reducing inflammatory responses.

MATERIALS AND METHODS

The assortment of experimental animal

This research work was executed on thirty clinically vigorous goats which were crossbred, with an age group of 3-4 months and weighing 10-13 kilograms. Before beginning this study, a month-long acclimatization process for all these goats was done in separate enclosures that were 2.5 x 4 feet in size.

Experimental design and feed adjustment

All the experimental procedures were performed following ethical standards established by the Ethics Committee of Sindh Agriculture University, Tandojam. The trial goats were divided randomly into three groups (n=10 in each group). Group A was fed a low-concentrate (LC) ration having a ratio of roughage to concentrate

65:35. In contrast, Group B was offered a high-concentrate (HC) diet (ratio 35:65), whereas Group C received HC diet with a supplemental selenium yeast available as Selemex (Biorigin, Lençóis Paulista, São-Paulo, Brazil) dose at the rate of 0.5 mg Se/kg ration. Feeding was done twice in a day in morning at 08:00 h and afternoon at 17:00 h, respectively, while water availability remained ad libitum. For first two weeks of the adaptation period, all the groups were maintained on LC diet. Afterward, the concentrate percentage was gradually increased during next two weeks until reaching HC level. Similarly, dietary selenium concentration was gradually raised until achieving the desired level. The amount of selenium in the diet was quantified through the technique of inductively coupled plasma optical emission spectrometry (ICP-OES; Optima 2100 DV, PerkinElmer, USA) based on method described by Taylor *et al.* (2010). Feed contents and Se in diets are summarized in Table I. The trial period was designed for 2.5 months.

Table I. Feed formulation with Se level fed to trial animals.

Items	Treatment		
	LC	HC	HCS
Components (Dry Matter %)			
Corn	25.7	25.6	25.6
Wheat bran	-	30.6	30.6
Soybean meal	7.5	2.3	2.3
Rapeseed meal	-	4	4
Limestone	0.6	1.7	1.7
Digestible crude protein	0.9	0.8	0.8
Salt	0.5	0.5	0.5
Mineral premixes ¹	0.5	0.5	0.5
Se (mg kg⁻¹ feed)			
Se in the diet	0.035	0.15	0.15
Supplemented Se in diet	0.115	-	0.5
Total level	0.15	0.15	0.65

Animals were maintained on three experimental rations namely low-concentrate diet (LC), high-concentrate diet (HC), and high-concentrate supplemented with selenium (HCS) rations for ten weeks. ¹Vitamin & mineral premix composition per kilogram of ration included Vitamin A (6000 IU), Vitamin D₂ (500 IU), Vitamin E (80 mg), Cu (6.25 mg), Fe (62.5 mg), Zn (62.5 mg), Mn (50 mg), I (0.125 mg), Co (0.125 mg), Mo (0.125 mg). Addition of selenium in ration was done using selenium yeast (SY).

Animal slaughtering and sample collection

Shortly before slaughter, a 10 ml blood sample was obtained from each goat and allowed to stand at 25 °C for 30 min, followed by centrifugation at 3000 g for 15 min.

The clear supernatant fluid (serum) was then aspirated and kept at -20 °C for further analysis. After the experimental trial, the goats were slaughtered in slaughter-room a separate area within animal house eliminating any stress by pre slaughter transportation. Animals were restrained properly in lateral-recumbency and sacrificed through severing jugular vein by using razor sharp knife without being stunned. A trained, highly skilled and certified technician performed the procedure of slaughtering. After the slaughter, abdominal cavity was immediately opened and whole stomach was transferred in a sterile tub. The rumen tissue was separated out from others (omasum and abomasum). The digesta in the rumen was taken out using graduated cylinder. Digesta from rumen digesta was expressed using four layers of cheesecloth. An aliquot of 50 mL was collected in air tight containers. The pH value of rumen digesta was measured using portable pH meter (Sartorius, Goettingen, Germany). Rumen digesta samples were frozen (-20°C) for SCFAs analysis and LPS. HgCl_2 (10%, 1ml per 10 ml of rumen digesta) was added in each digesta sample. Then the digesta samples were washed with PBS. Finally, a small piece of rumen tissue was cut (5 cm²) and fixed in ethyl alcohol. The rumen epithelium was removed from an additional sample of rumen tissue (5 cm²), detaching the epithelial layer, and freezing it for enzyme analysis. Similarly, an additional portion of the rumen epithelium detaching layer was frozen at -80 °C for mRNA expressions.

Rumen histomorphology

Histomorphometric analyses of tissue sections harvested from the anterior part of the rumen of all goats were performed. Firstly, 4% paraformaldehyde was used to fix the samples for 24 h, then washed with distilled water. After that, the tissues underwent dehydration, clearing, and embedding into paraffin using the technique recommended by [Moolchand *et al.* \(2013\)](#). Sections of paraffin-embedded tissue samples were prepared with a thickness of 5-7 µm and stained with H&E. Ten measurements for each papilla of each goat were made.

SCFAs and LPS determination

The concentration of SCFAs was determined through the method described by [Samo *et al.* \(2020\)](#). The level of LPS were calculated through an endpoint chromogenic test using a Tachypleus Amebocyte Lysate (TAL) test kit produced by Chinese Horseshoe Crab Reagent Manufactory Co. Ltd., Xiamen, China. The solutions were properly diluted so that LPS concentrations were between 0.1 and 1.0 EU/mL, which was the same for endotoxin standards, as stated by [Dai *et al.* \(2022\)](#).

MDA and antioxidant activity determination

Homogenization of ruminal tissue samples was done by using phosphate buffered saline (PBS) whereupon the supernatants were then harvested in Eppendorf tubes and used in biochemical assays. The levels of MDA and the activities of the antioxidant enzymes GSH-Px, SOD, and CAT were quantified using ready-made ELISA kits from Nanjing Jiancheng Bioengineering Institute, China. All assays were conducted according to the manufacturer's directions.

Analysis of real-time RT-PCR

The tissue samples from the rumen were homogenized in order to extract total RNA utilizing TRIzol reagent (Takara Biotech Co., Dalian, China). Quantitative estimation and assessment of the integrity of isolated RNA samples were carried out via spectrophotometry using NanoDrop equipment and further verified using agarose-formaldehyde gel electrophoresis stained with ethidium bromide solution (1.4%). To synthesize the cDNA sample, 2 µg of isolated RNA were reverse-transcribed in a 25 µL reaction mix with 100 IU reverse transcriptase, 8 IU RNase inhibitor (Promega, USA), 5.3 mmol L⁻¹ random hexamer primers, and 0.8 mmol L⁻¹ deoxyribonucleotide triphosphates (dNTP) (TaKaRa, Dalian, China). Reverse transcription was performed at 37°C within one hour, and then the sample was exposed to thermal denaturing at 95°C for 5 minutes. The prepared cDNA sample was kept frozen at -20°C before use. For real-time qPCR experiment, 2 µL of the synthesized cDNA sample was mixed with 12.5 µL of PCR SuperMix (iQ SYBR Green, Bio-Rad Laboratories, Hercules, CA, USA) and 0.6–0.8 µM of gene-specific primers ([Table II](#)). Amplification conditions included one cycle at 95°C for 1 min and 40–45 cycles at 95°C for 20 sec, 60–64°C for 20–30 sec and 72°C for 30 sec. Expression levels of gene were calculated based on formula $2^{-\Delta\Delta\text{Ct}}$ with GAPDH as normalization control.

Statistical analysis

Data analysis was carried out by statistical method using one-way analysis of variance with the aid of GraphPad Prism (version 8.0.1). Data values are expressed as the mean ± SEM. Statistical significance between groups means was determined by considering $P < 0.05$.

RESULTS

Short chain fatty acids (SCFAs) and pH of ruminal fluid

The contents of acetate, propionate, butyrate, and total SCFAs in the ruminal fluid of the HC and HCS groups were greater ($P < 0.05$) in contrast to LC group ([Fig. 1](#)).

Table II. Real time RT-PCR primers for target genes.

Target gene	Primer sequence (5' to 3')	Size (bp)	Accession no.
<i>TLR4</i>	F: GTTTCCACAAGAGCCGTAA R: TGTTTCAGAAGGCGATAGAGT	195	JQ342090.1
<i>CD14</i>	F: CCGTTCAGTGATGGTTGCC R: TGCTTCGGGTCGGTGTT	239	NM_001077209.1
<i>MyD88</i>	F: ACAAGCCAATGAAGAAAGAG R: GAGGCGAGTCCAGAACC	98	98 JQ308783.1
<i>TRAF6</i>	F: GCGGCCTTCAAGTTAGGAGA R: TCATCAACTGCTCGTTCGGG	141	NM_001034661.2
<i>NF-κB</i>	F: ACGATCGTCACCGGATTGAG R: GGTGCTGAGAGATGGCGTAA	194	XM_005699996.1
<i>TNF-α</i>	F: CAAGTAACAAGCCGGTAGCCC R: CCTGAAGAGGACCTGCGAGTAG	173	AF276985.1
<i>IL-1β</i>	F: GAAGAGCTGCACCCAACA R: CAGGTCATCATCACGGAAG	172	D63351.1
<i>IL-6</i>	F: GGAGGAAAAGGACGGATGCT R: GGTCAGTGTTTGTGGCTGGA	226	EU276071.1
<i>IL-8</i>	F: CCTCTTGTTCAATATGACTTCCA R: GGCCCACTCTCAATAACTCTC	189	NM_173925.2
<i>IL-10</i>	F: TTAAGGGTTACCTGGGTTGC R: CCCTCTCTTGAGCATATTGA	178	DQ837159.1
<i>IL-13</i>	F: CAGTGTCATCCAAAGGACCAAG R: CGGACGTACTACTGGAAACC	248	NM_174089
<i>CCL5</i>	F: GAGTTGAGCTAGGGTGGAGG R: CGGAGCTTAGAGACCTCCTG	216	NM_175827.2
<i>LBP</i>	F: CAAGTAACAAGCCGGTAGCCC R: CCTGAAGAGGACCTGCGAGTAG	138	XM_004014566.1
<i>SAA</i>	F: CATCCTGCGTCTGGACCTGG R: TTCCTTGATGTCACGGACGATTT	121	AF540564.1
<i>Hp</i>	F: TAATGCCCATCTGCCTAC R: CGCCCTCATAGTGTTCAT	162	XM_004015111.1
<i>GAPDH</i>	F: GGGTCATCATCTCTGCACCT R: GGTCATAAGTCCCTCCACGA	180	HM043737.1

On the other hand, the rumen pH values in the HC and HCS groups were significantly lower ($P < 0.05$) than that in the LC group. Besides increasing overall activity of rumen fermentation, addition of Se resulted in higher concentration of butyric acid rising by around 30% in the HCS group than in the HC group.

Ruminal and plasma LPS concentration

Figure 2 shows ruminal and plasma LPS concentration. An increase ($P < 0.05$) in LPS was recorded in goats on HC and HCS feed compared with those on LC feed (Fig. 2). However, the addition of Se in HCS feed significantly lowered ($P < 0.05$) the LPS content caused

by HC feed in the rumen. The similar drift was observed in plasma LPS content where there was an increase ($P < 0.05$) in LPS content in the HC than in the LC group. However, the addition of Se in HCS feed significantly lowered ($P < 0.05$) LPS content in the plasma.

Histo-morphology of rumen papillae

Histological examination of rumen papillae showed severe damages by loss of epithelial integrity in HC group denoted by arrow (Fig. 3B) as compared to excessive keratinization in LC group (Fig. 3A). Nevertheless, Se supplementations prevented such detrimental effects of HC diet on rumen papillae (Fig. 3C).

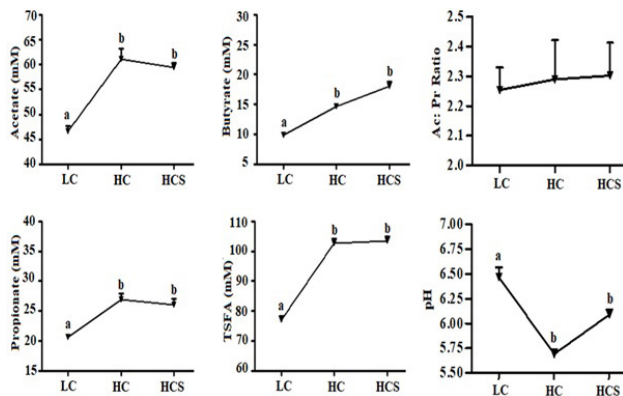


Fig. 1. The impact of dietary supplementation on fermentation profile of ruminal fluid from goats in the trial. Three different diets, low and high concentrate (LC and HC), and high concentrate supplemented alongside selenium yeast (HCS) diet groups were fed to goats for 2.5 months. Selenium yeast was added to the HC diet at 0.5 mg Se per kg of diet. Data represent means $\pm$ SEM. Bars carrying diverse letters (a, b, c) are significantly dissimilar ($P < 0.05$).

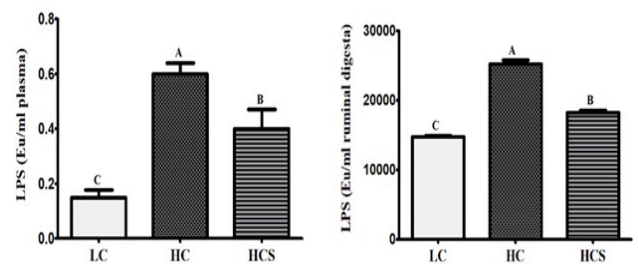


Fig. 2. The impact of various dietary treatment on the concentration of lipopolysaccharides (LPS) in the rumen fluid and plasma of goats. Lipopolysaccharide concentrations were stated in EU/mL. Three different diets, low and high concentrate (LC and HC), and high concentrate supplemented alongside selenium yeast (HCS) diet groups were fed to goats for 2.5 months. Selenium yeast was added to the HC diet at 0.5 mg Se per kg of diet. Values are stated as mean $\pm$ SEM. Superscripts with different letters (A, B, C) in a row show alterations among dietary groups ($P < 0.05$).

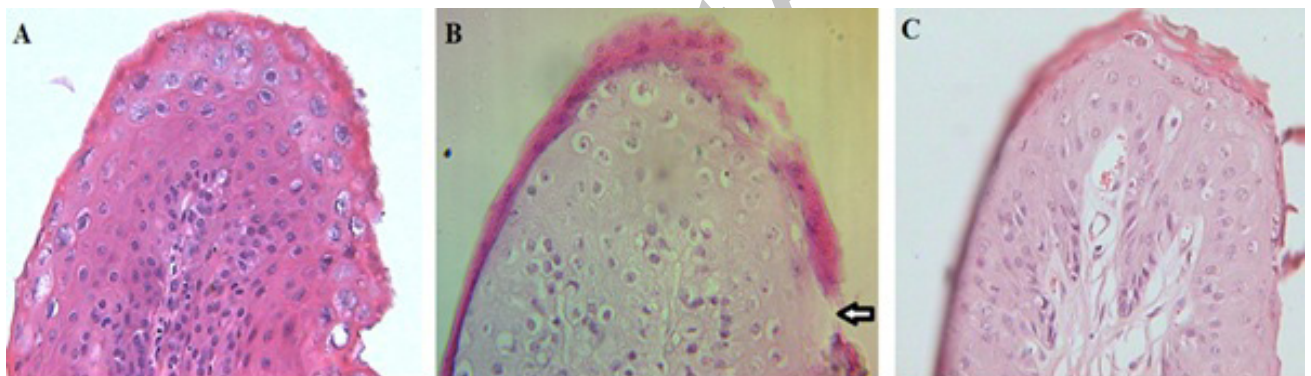


Fig. 3. Morphological alterations in the histological structure of ruminal papillae of goats fed three different diets, low and high concentrate (LC and HC), and high concentrate supplemented alongside selenium yeast (HCS) for 2.5 months. Selenium yeast was added to the HC diet at 0.5 mg Se per kg of diet (LC; A, HC; B and HCS; C). Stain: Hematoxylin and eosin (H & E) magnification 10 $\times$ (scale bar = 100 μ m).

Oxidative stress markers and inflammatory gene expression in rumen epithelium

Figure 4 shows the activity of GSH-Px enzyme showed a significant decrease ($P < 0.05$) in HC animals in comparison with the LC group. Nevertheless, there was a significant elevation ($P < 0.05$) in GSH-Px activity in the HCS group as compared with HC goats. The same trend was observed for the activity of SOD, which showed a substantial reduction ($P < 0.05$) in HC but an elevation ($P < 0.05$) in HCS group than in the LC group. On the other hand, the activity of CAT decreased significantly ($P < 0.05$) in both HC and HCS groups in contrast with the LC group, although a significant increase ($P < 0.05$)

was recorded in the HCS compared with the HC group. MDA concentration was considerably increased ($P < 0.05$) in HC and HCS groups in contrast with the LC group, nevertheless Se supplementation substantially decreased ($P < 0.05$) MDA concentration in HCS animals compared with the HC group.

Figure 5 shows the pattern of inflammatory genes expression such as transcription factors, cytokines, and acute-phase proteins (APPs) in the ruminal epithelial tissues of goats. The mRNA expression levels of TNF α , MYD88, TLR-4, NF- κ B, CD-14 and TRAF-6, were significantly upsurged ($P < 0.001 - P < 0.05$) in goats under HC diet than those of goats under LC diet. The

level of mRNA expression of such transcription factors significantly dropped ($P < 0.001$) in goats supplemented with selenium in the HC diet (HCS) than in those of goats without selenium supplementation (HC); nevertheless, the expression of TRAF-6 was not significantly different ($P > 0.05$) between these LC and HCS diets.

The expression level of pro-inflammatory (IL-1 β , IL-6, IL-8 and CCL-5) cytokines were significantly augmented ($P < 0.001$) in HC diet-fed goats than the ones fed with LC diets, while anti-inflammatory cytokines IL-10 and IL-13 mRNA expression were significantly downregulated. Contrariwise, Se supplementation significantly ($P < 0.001$) attenuated such results in the HCS versus HC group (Fig. 5B).

The mRNA expression levels of acute-phase proteins such as lipopolysaccharide-binding-protein (LBP), serum-amyloid-A (SAA) and haptoglobin (Hp) were upregulated ($P < 0.001$ to 0.05) in HC and HCS compared to LC. In the goats supplemented with Se (HCS), the expression level of all respective genes was lowered ($P < 0.01$) compared to goats of HC group. However significant difference ($P < 0.05$) was found among three groups (Fig. 5C).

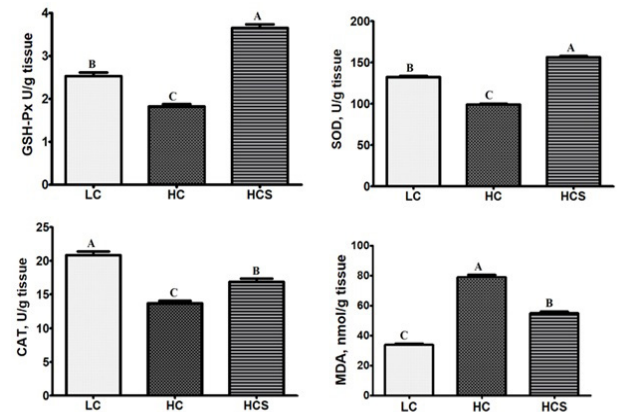


Fig. 4. Impact of various dietary interventions on stress biomarkers in the ruminal epithelium of goats. The variables analyzed are MDA, GSH-Px, SOD, and CAT. Three different diets, low and high concentrate (LC and HC), and high concentrate supplemented alongside selenium yeast (HCS) diet groups were fed to goats for 2.5 months. Selenium yeast was added to the HC diet at 0.5 mg Se per kg of diet. Data are presented as mean $\pm$ SEM. Diverse letters (A, B, C) indicate significant variances among groups ($P < 0.05$).

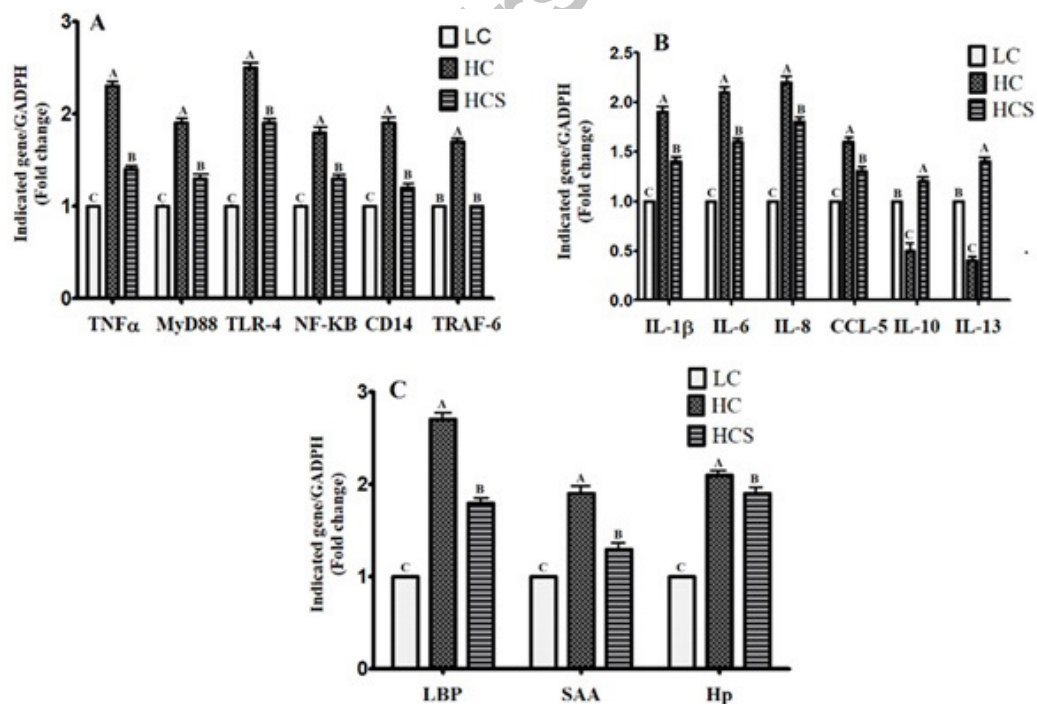


Fig. 5. Influence of various dietary interventions on expression of inflammatory genes in the rumen of goats: (A) transcription factors, (B) cytokines, and (C) acute phase proteins. Three different diets, low and high concentrate (LC and HC), and high concentrate supplemented alongside selenium yeast (HCS) diet groups were fed to goats for 2.5 months. Selenium yeast was added to the HC diet at 0.5 mg Se per kg of diet. Data represent mean $\pm$ SEM. Superscript letters (A, B, C) indicate statistically significant differences between groups at $P < 0.05$.

DISCUSSION

Rumen fermentation pattern and LPS level

The use of HC diet in ruminants leads to increased fermentation and SCFAs synthesis, resulting in decreased ruminal pH and produced acidic conditions. Moreover, continued HC diet ingestion negatively influences rumen fermentation kinetics, and the degree of change is positively correlated with the period of HC diet intake (Shah *et al.*, 2022; Wang *et al.*, 2018). Current study reveals that, after 2.5 months of ingestion of the HC diet, there were higher molar amounts of each type of SCFAs such as acetate, propionate, and butyrate in goats than in goats on the LC diet. These results are similar to previous studies in which HC diets were fed to goats (Lu *et al.*, 2018; Shen *et al.*, 2016), sheep (Garcia Diaz *et al.*, 2018; Kuzinski *et al.*, 2011; Wencelová *et al.*, 2015) and cattle (Agle *et al.*, 2010; Steele *et al.*, 2011). Intraepithelial pH (pHi) maintenance depends upon the rate of SCFAs disappearance rate from rumen and their intraepithelial metabolic rates. Butyrate; being most preferred compound utilized by epithelial cells, prevent propionate utilization by epithelial cells due to the inhibition of the propionyl Co A synthetase activity (Nshanian *et al.*, 2025) and increase its flow towards liver to produce glucose and avoids a risk of pH reduction due to lactate accumulation. Present study revealed that, adding selenium to the HC diet led to more activity of rumen fermentation, with the concentration of butyric acid rising by around 30% in the HCS group when compared to the HC group. Increased butyrate in the rumen is believed to be related to higher numbers of rumen protozoa, resulting in improved fermentation profiles (Abubakr *et al.*, 2013). In this case, it can be assumed that the high level of butyric acid observed could be attributed to the increase in the concentration of ruminal protozoa in goats receiving HC plus Se diets since, according to some researchers, selenium addition to the food at a concentration of 0.3 mg/kg increased the total number of protozoa in the rumens of lambs (Mihaliková *et al.*, 2005) and goat (Shahid *et al.*, 2020). A significant decrease in pH in ruminal fluid has been observed in goats receiving HC and HC plus Se diets. However, goats on HC diet showed much lower pH levels in ruminal fluid compared with goats receiving HC plus Se diets. This phenomenon may be caused by the fact that increased butyric acid concentration helped to reduce the pH to a less extent. By substitution of 2:1 (2 acetates to 1 butyrate) would be useful in maintenance of ruminal fluid pH through three main processes namely first by lowering osmotic pressure which may impede absorption of SCFAs in rumen (Schweigel *et al.*, 2005), second by preventing acetate build up as it would have occurred due to slow absorption at low pH of rumen and finally by lessening H+

production (Aschenbach *et al.*, 2010).

Lipopolysaccharides (LPS) are essential components of outer layer of membranes of Gram-negative bacteria with both immunogenic and antigenic activities (Ciesielska *et al.*, 2021). Some studies have revealed that LPS concentration is increased in the gut, particularly the rumen (Dai *et al.*, 2022) and plasma (Solangi *et al.*, 2025) of ruminants after chronic intake of concentrate diets. This observation is corroborated by our findings, which show an increase in concentration of LPS in rumen fluid and plasma of HC diet served goats. In addition, other studies have indicated that serum and colonic digesta LPS concentrations are increased in animals consuming a diet composed of 60-90% grains (Tao *et al.*, 2017; Wang *et al.*, 2021). Likewise 65% grain diet in male goats implies the drip of LPS with 0.860.20 Eu/mL LPS concentration in the plasma (Liu *et al.*, 2013). The rationale behind LPS elevation has been attributed to a decline in ruminal pH resulting from HC diet, which causes the death and rupture of Gram-negative bacteria, thereby releasing LPS into the rumen fluid and damaging ruminal epithelium (Zhang *et al.*, 2020). In contrast, the existing investigation has found a significant decrease in the level of ruminal and plasma LPS content in the goats given the selenium-enriched HC diet (HCS). Such findings were similarly documented by previous studies involving the use of goat models (Samo *et al.*, 2020; Shah *et al.*, 2022). The exact pathway responsible for the lower LPS content in HCS animals is yet to be discovered; however, this could be due to a reduction in bacterial lysis since selenium supplementation was found to lower the amount of LPS in high-concentrate intervened stress conditions (Shah *et al.*, 2022). According to Čobanová *et al.* (2017), the reason for such protection against bacteriolysis might be due to the role played by selenium in incorporating itself into microbial systems, thus boosting antioxidant defense and dwindling bacteriolysis that ultimately encourage them to endure detrimental acidic milieu.

Epithelial injury and oxidative stress

It is already reported that the ruminants served concentrate rich ration are at high possibility of damages to gastrointestinal integrity (Liu *et al.*, 2013). Current outcome represented that 2.5 months lasts feeding of HC diet resulted in histo-morphological alterations described by severe epithelial injury in the rumen epithelium of goats as compared to the excessive keratinization showed by the goats offered LC ration. Acidic lumen and the concentration of LPS are two interconnected aspects responsible for the maintenance of epithelial integrity and the severity of the ruminal and colonic injury (Emmanuel *et al.*, 2007). Several studies have described

that declined pH post high concentrate feeding causes epithelial disruption in cecum (Klevenhusen *et al.*, 2013) and colon (Tao *et al.*, 2015) ensuing the leakage of LPS to the blood. As stated by previous researchers, high grain diet in ruminants increases the serum level of LPS due to the high level of LPS passage through damaged rumen epithelial tight junctions (Liu *et al.*, 2013). Furthermore, topical studies have reported that high-concentrate diets can induce severe damage to the colonic mucosa in goats (Samo *et al.*, 2020) as well as pathological alterations in hepatic tissue (Shah *et al.*, 2022). In contrast, additional Se supplement in association with HC diet reduced the harmful effect of HC diet on rumen epithelium of goats. According to previous reports, Se supplementation in the diet protected rabbits against injuries in aorta after high fat diet consumption (Mehta *et al.*, 2002), and had defensive influence against damages in bursa and jejunum of chickens (Hu *et al.*, 2018). Since Se supplementation attenuated LPS concentration in the ruminal lumen and hence translocation in present study, the minor injury to the epithelium of Se supplemented concentrate rich ration might be due to dropped pH in the rumen.

Oxidative stress can be described as an imbalance between physiologically generated pro-oxidants and antioxidants (enzymatic and non-enzymatic) in their nature and that may upswing the number of highly reactive free radicals ($O_2^{\cdot-}$, H_2O_2 , $ROO^{\cdot}$, NO, and $ONOO^{\cdot}$) (Abaker *et al.*, 2017). This will result in DNA mutation and oxidizes several lipids and proteins in tissues ensuing in cellular harms (Nita and Grzybowski, 2016). In brief, MDA, a three-carbon dialdehyde, is a naturally produced compound that is formed during lipid peroxidation process and induces destructive alterations leading to cell damage (Ma *et al.*, 2018; Sharma *et al.*, 2012). Levels of MDA content correspond to the magnitude of oxidative stress since it reflects the intensity of the process of lipid peroxidation. Oppositely, the enzymatic defense of anti-oxidation system consist of the presence of SOD and GSH-Px which play a fundamental part in the neutralization of excessive reactive oxygen species and preventing oxidative damage to tissue organs. Firstly, superoxide radicals are converted into hydrogen peroxide (H_2O_2) and oxygen (O_2) by the enzyme SOD while GSH-Px reduces H_2O_2 to water and lipid hydroperoxides into alcohols (Ighodaro and Akinloye, 2018). The current findings confirmed the development of OS in HC offered group via showing the rise in MDA content and declined GSH-Px, SOD and CAT activities in ruminal tissue than in the goats offered LC. Our results have shown the affinity with previous studies with similar results in blood, liver, colonocytes, rumen fluid and epithelium of goats reared on HC feed (Ma *et al.* 2018; Tao *et al.*, 2014). According

to the previously developed facts, the pH-induced acidity and exaggerated LPS concentration results in increased MDA and decreased antioxidant status hence develops OS in gut epithelial tissue of ruminants (Solangi *et al.*, 2025; Shahid *et al.*, 2025). Conversely, Se addition reversed HC diet-related changes via showing suppressed MDA and elevated enzymatic anti-oxidant status in the ruminal epithelium of goats in current study. The increased activity of GSH-Px and lowered MDA content has already been unveiled due to Se concentration in gut microbiota (Čobanova *et al.*, 2017). Besides, Shahid *et al.* (2020) re-counted 25% augmentation in the level of GSH-Px in the goat supplemented with Se yeast. The cyto-epithelial injuries in rumen of goats served HC diet is a consequence of LPS induced OS (Abaker *et al.*, 2017). However, suppressed concentration of LPS after Se addition mitigates ruminal (Shahid *et al.*, 2025) and colonic (Samo *et al.*, 2020; Solangi *et al.*, 2025) hence assisting anti-oxidation progression (Mousaie, 2021). Therefore, Se supplementation to the goat instigated alleviation in OS intervened by the consequences of concentrate rich diet and thus diminished ruminal epithelial injury in current study.

Rumen inflammation

Increased LPS is known to trigger an acute phase response (APR) via the induction and release of APPs; hence, alterations in their peripheral blood levels of LPS and APPs serve as markers for inflammatory response (Shah *et al.*, 2022). Primarily, the interaction between LPS and LBP is central in activating the inflammatory response mediated by LPS. LBP is an APP with molecular weight of 60 kDa that is highly specific and sensitive to LPS. Other classic biomarker for inflammatory response are serum amyloid A (SAA) and Hp that are raised during different stressed circumstances such as shock, bacterial/ viral infection, and fermentable diets. Hence, they are communally known as the inflammatory indicators in ruminant (Yaghoobpour *et al.*, 2025). Concomitant with increased plasma LPS, concentrate rich diet amplified the expression of already mentioned APPs in the rumen of goat under experiment in current study. Formerly, the dairy cows challenged with HC feed stimulates APR represented by boost of LBP, SAA and Hp, complemented with translocation of LPS (Ma *et al.*, 2024). In dissimilarity, the goats supplemented with Se-added HC ration allayed diet persuaded APPs in rumen epithelium. The perpetual supplementation of Se beyond the suggested levels (≥ 0.5 mg kg^{-1} diet) develops immune response by the suppressing APPs during postpartum stress in cows (Gong and Xiao, 2021), heat stress in pigs (Liu *et al.*, 2021a) and diet- prompted stress in goat (Shah *et al.*, 2022), respectively.

The changes in concentration of APPs in small ruminants relied on HC diets are also accompanied by changes in the concentration of pro- and anti-inflammatory cytokines (Guo *et al.*, 2017; Ohtaki *et al.*, 2020). Our findings suggests, the expressions of the pro-inflammatory TNF- α , IL-1 β , IL-8 and CCL-5 were increased, but the expression of anti-inflammatory IL-10 and IL-13 cytokines were reduced in goats consuming HC in contrast to LC. It is established that LPS produced in the rumen triggers an increase in APP by activating a sequence of signalling molecules such as TNF- α , IL-1, and IL-6 along with NF- κ B (Kany *et al.*, 2019). NF- κ B controls cytokines and chemokines synthesis at the cellular level by targeting immune-response genes in the nuclear compartment (Dai *et al.*, 2022; Zhang *et al.*, 2020). A number of studies have shown that HC feeding stimulates the transcription and expression of NF- κ B mRNA and proteins that act through LPS activation and affect the transcription and expression of inflammatory cytokines, such as TNF- α , IL-1 β , IL-6, and IL-10, thus changing the levels of APPs in goat and cows (Duanmu *et al.*, 2016; Guo *et al.*, 2017). The anti-inflammatory cytokines (IL-10 and IL-13) exert a protective effect on inflammatory tissues and play a regulatory role in preventing tissue damage, such as hepatic ischemia in animals (Kato *et al.*, 2003). Another study has reported that the TLR-4/MyD88 pathway is responsible for inflammatory resolution by stimulating anti-inflammatory factors like IL-10 (Ciesielska *et al.*, 2021). The current research shows that IL-10 transcription was decreased in goats consuming the HC diet, but selenium supplementation upregulated the transcription of IL-10 and IL-13. Therefore, selenium can be considered to promote inflammatory recovery from HC diet exposure in the rumen epithelium. Chang *et al.* (2015b) found that the mRNA expression of hepatic IL-10 did not show substantial increase in goats provided with HC diet for 1.5 months. On the other hand, the administration of Se in a dose-dependent manner was shown to induce IL-10 production along with suppression of inflammation, where protective properties were seen under supplemented conditions (Shah *et al.*, 2022; Zhang *et al.*, 2017). The activation of the TLR-4 pathway has a pivotal role in LPS-induced inflammation through activating and transporting NF- κ B in rumen-targeted epithelial cells. Once LPS binds to LBP, it is further transported to CD14 before being delivered to the TLR-4/Md-2 complex for activation. Subsequently, this activates the adapter protein MyD88, thereby triggering the signalling pathway from TRAF-6 to nuclear translocation of NF- κ B and inflammatory gene transcription (Ciesielska *et al.*, 2021; Kany *et al.*, 2019). According to the current investigation, there was an observable increase in mRNA expression of CD14,

TLR-4, and TRAF-6 and MYD88 in the rumen tissue under the influence of HC diet. Such outcomes have been reported in studies conducted on goats and other ruminant species consuming high-grain diet, specifically in the liver tissue (Duanmu *et al.*, 2016). Interestingly, enhanced mRNA expression of TLR-4 may be caused by the hypomethylation of the TLR-4 promoter region and decreased condensation of the chromatin, indicating epigenetic control during HC feed intake (Chang *et al.*, 2015a, b). Another essential mediator involved in the process of signal transduction in the TLR-4/MyD88 pathway is tumor necrosis factor receptor-associated factor 6 (TRAF-6). It is responsible for activating the NF- κ B transcription factor, which is then accountable for the transcription of the cytokines (Ciesielska *et al.*, 2021). Ma *et al.* (2021) have demonstrated increased mRNA and protein levels of NF- κ B and TRAF-6 in the ruminal mucosa of goats on HC diet. This implies that activation of ruminal inflammation through LPS exposure activates the TLR-4/MyD88 signaling pathway mediated by NF- κ B. This conclusion is reinforced by several studies in cattle, demonstrating increased mRNA expression of TNF- α , IL-1 β , IL-6, MyD88, and the p65 subunit of NF- κ B in response to HC diet or LPS administration (Jiang *et al.*, 2008).

In opposition, in the current research, Se supplementation in diet has ameliorated ruminal inflammation because of the down-regulation of TLR-4, CD14, TRAF-6, MYD88 and NF- κ B expression. There has been documented evidence from past literature that Se supplementation in diets can prevent the disruption of epithelial integrity and reduce inflammatory responses because of TLR-4 modulation in liver, thymus, and jejunum of growing pigs under drug and heat-induced stress (He *et al.*, 2022; Liu *et al.*, 2021a). Likewise, in case of chickens and rats challenged with LPS, similar results were obtained in relation to Se supplementation in protecting the uterus and liver tissues (Chen *et al.*, 2020; Qu *et al.*, 2020). From the present study, it is evident that consumption of HC diet increases LPS levels in rumen and blood, which cause histopathological changes related to oxidative stress and inflammation in the rumen. The increased oxidative stress and inflammation caused by HC diet consumption activate the MyD88 and TRIF-dependent signalling pathway of TLR-4. Conversely, Se in diet can protect ruminal tissues from damages by reducing oxidative stress and inflammation.

CONCLUSIONS

HC diet elevated LPS and resulted in oxidative stress and Inflammation, which were related to the up-regulation

of pro-inflammatory cytokines through the TLR-4 signalling pathway, causing damage to epithelial tissue in rumen of goats. Extra-Se improved rumen wall histology by attenuating HC diet induced oxidative inflammatory lesions by regulation of MYD88/ TRIF dependent TLR-4 signalling pathway in the rumen of goat.

DECLARATIONS

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IRB approval

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Ethical approval

All the experimental procedures such as caring of animals, handling, slaughtering and sample collection were performed in current study granted earlier approval in 154th meeting of BASR (approval No. DAS/952/ of 2024).

Generative AI and AI assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Statement of conflicts of interest

The authors have declared no conflict of interest

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