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Effects of Selenium Supplementation on Hematological Profile, Gut Microflora Composition, *in Vitro* Biofilm Formation Assay and Serum IgG Concentration in Goats

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ABSTRACT

The present study was carried out to investigate the effects of selenium (Se) supplementation on gut microflora and immune parameters in goats. Twelve female cross breed goats (BW: 10-12 kg) were equally divided into two groups i.e., Control that offered basal diet and Se supplemented group that offered basal diet + selenium yeast (0.15mg/kg BW). In basal diet concentrate was fed at the rate of 2% body weight while roughage (hay)was given as *ad-libitum*. The dietary treatments were continued for 2 months. The results showed that microbial count (*E. coli* and *Lactobacillus*) was higher ($p<0.05$) in goats offered basal diet supplemented with organic selenium compared to goats fed on basal diet with no Se supplementation. However, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were significantly higher ($p<0.05$) in control group goats compared to goats in supplemented group. Significantly higher ($p<0.05$) IgG concentration was found in Se supplemented goats as compared to control group. The bacterial species produced weak positive biofilm formation ($p<0.05$) in Se supplemented goats as compared to control group. The hematological indicators revealed that the values of hemoglobin, hematocrit, erythrocytes, mean cell hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin concentration, leukocytes, neutrophils, lymphocytes and monocytes count were significantly higher ($p<0.05$) in the Se treated group compared to control. It was concluded that Se supplemented goats have better gut microflora composition and immune response compared to goats fed on basal diet without Se supplementation.

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MA conducted the experiments and wrote the manuscript. AAK conceived the experiments and proofread the manuscript. MJA supervised the lab experiments and helped in statistical analysis.

Key words
Selenium, Goats, Microflora, Hematology, Immunity, Biofilm formation

INTRODUCTION

It has been described in recent studies that minerals supplementation have an important and vital role in animals' performance and also have a significant association with physiology, wellbeing, health and production parameters (Argüello, 2011; Patel *et al*., 2017). Fundamental and basic trace elements are structural, functional and regulatory constituents of a number of biomolecules that play an imperative role in the metabolism of living organisms (Czauderna *et al*., 2020).

Selenium (Se) is an essential trace element/ micronutrient that plays a vital role in various biological activities related to health, performance and disease prevention in farm animals (Čobanová *et al*., 2016). Selenium is essential for the progression and expression of humoral and cell-mediated immune responses (Zhou *et al*., 2009; Zhou and Wang, 2011). Selenium supplementation to animals via feed increases antibody levels, enhances the phagocytic activity of neutrophil granulocytes and macrophages, and when stimulated in myogens, increases T lymphocyte counts (Hoffman, 2007; Kamada *et al*., 2007). As an antioxidant, Se function all over the cells including extracellular space, the cytoplasmic matrix, and being associated with cell membranes particularly in the gastrointestinal tract, eventually affecting the immune system (Miller *et al*., 2001). The data showed that dietary selenium influences both the composition of intestinal microflora as well as the colonization of gastrointestinal tract, thus, impact the host selenium status and selenoproteome expression (Kasaikina, 2011).

In addition to the above functions, selenium also acts as an antibacterial agent. Researchers contributed to prove the antibacterial effects of numerous selenium compounds. For instance, selenium supplemented probiotics appeared to obviously inhibit the growth of pathogenic *E. coli in vitro* and *in vivo* (Yang *et al*., 2009). The manufactured

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organ selenium compounds appeared to be as potent as penicillin in having an inhibitory effect against the growth and multiplication of *S. aureus in vitro* (Pietka-Ottlik *et al*., 2008). Selenium nanoparticles are considered to possess distinctive mechanisms that possibly resist the bacterial growth and biofilm formation, for instance, modulation in attachment activity of surface hydrophobicity averting bacteria (Tran *et al*., 2010).

Available literature shows that there is a controversy in results regarding supplemental effects of Se on hematology and humoral immune responses in animals. Some studies have reported the positive effect of Se on hematological indices (Raza *et al*., 2018) and humoral immunity (Rossi *et al*., 2017; ChuanRong *et al*., 2009) by improving the heterophil to lymphocyte ratio, red blood cell count, packed cell volume, hemoglobin, and lymphocyte percentages. While others have reported no or deleterious effects on hematology (Mohri *et al*., 2011) and humoral immunity (Moeini *et al*., 2011) by showing the decreased levels of creatinine kinase and aspartate aminotransferase and no effect on humoral immune parameters. Moreover, no data is available on the biofilm formation activity of intestinal bacteria of Se supplemented goats. Therefore, the current study was designed to evaluate the effects of dietary selenium on humoral immunity, gastrointestinal microflora, hematology and biofilm formation activity of gut bacteria in goats.

MATERIALS AND METHODS

Experimental animals

All experimental procedures were approved by Directorate of Advanced Studies, SAU Tandojam. Twelve female cross breed goats of an approximately 3-4 month of age having 10-12 kg body weight were used for this experiment. All animals were purchased from nearby goat farm and housed at Livestock Experimental Station, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University, Tandojam. Animals were given the duration of four weeks for adaptation to experimental surroundings. The feed and water were provided *ad-libitum*. All goats were vaccinated against contagious diseases including goat pox, contagious caprine pleuropneumonia, enterotoxaemia etc., and drenched with anthelminthic.

Feeding of goats and selenium treatment

After familiarization period of 4 weeks, the goats were randomly divided into two groups (n=6) i.e., Control and selenium yeast treatment group. All animals were fed with same basal diet i.e., concentrate at the rate of 2% body weight and hay that was offered *ad-libitum*. Water was also given *ad-libitum*. In group A, goats were fed basal diet (Table I) with no Se supplementation. In group B, goats received basal diet supplemented with organic selenium (Selemax™, Lençóis, Paulista, São Paulo, Biorigin®, Brazil) at the rate of 0.15mg/kg body weight. The dietary treatments were continued for 2 months.

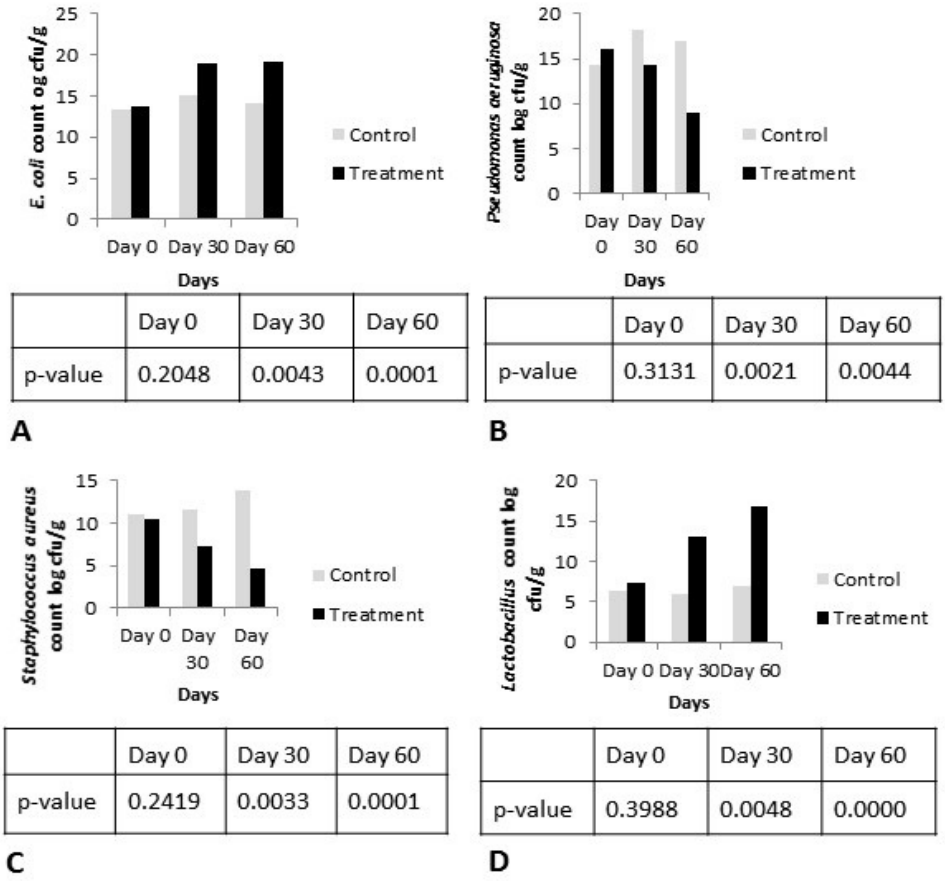
Quantitation of gut microflora

Fecal samples (about 5 g) were collected aseptically from rectum of both the experimental and control group goats at three different intervals i.e., day 0, 30 and 60 after treatment. Samples were analyzed for the quantitation of major species of gut microflora using Standard Plate Count Method. In brief, 1gm of fecal sample was diluted in 1ml of distilled water to dilute the original solid sample. Ten-fold serial dilutions were made from the diluted sample. A 100µl of each dilutions were plated on different media using spread plate method and incubated at 37°*C* for 24h. After incubation the visible bacterial colonies were counted manually. The CFU/g were calculated by multiplying the number of colonies with dilution factor and the mean count was expressed as log cfu/g. All the isolates were identified based on the morphological, cultural and biochemical characteristics following the Bergey's manual of systematic bacteriology (Whitman *et al*., 2012).

Table I. Chemical analysis of feed offered to goats during experiment as a basal diet.

Ingredients	%	Quantity (g)	Chemical composition*						
			DM	CP	TDN	CF	Ash	Ca	P
Berseem	ad lib.	1000	185.00	15.45	35.88	26.6	20.25	1.60	0.33
Corn	54.56	545.40	490.86	3.40	36.96	18.35	4.40	0.26	0.15
Wheat bran	30.20	307.80	273.94	5.16	23.06	3.25	1.80	0.80	0.90
Soybean meal	12.62	120.60	65.12	5.01	10.58	0.72	0.47	0.04	0.07
Lime stone	0.60	6.00	--	0.009	--	--	--	--	--
DCP**	1.08	10.80	10.80	0.007	--	0.002	0.0009	0.38	--
Salt	0.32	3.20	3.20	0.004	--	0.0008	0.0002	0.11	--
Mineral premix	0.62	6.20	6.20	--	--	--	--	--	--

*DM, dry matter; CP, crude protein; TDN, total digestible nutrients; CF, crude fiber; Ca, calcium; P, phosphorus; ** Di-calcium phosphate.



	Day 0	Day 30	Day 60
p-value	0.2048	0.0043	0.0001

A

	Day 0	Day 30	Day 60
p-value	0.3131	0.0021	0.0044

B

	Day 0	Day 30	Day 60
p-value	0.2419	0.0033	0.0001

C

	Day 0	Day 30	Day 60
p-value	0.3988	0.0048	0.0000

D

Fig. 1. Effect of Se supplementation in goats on microbial count of *E. coli* (A), *Pseudomonas aeruginosa* (B) *Staphylococcus aureus* (C) and *Lactobacillus* (D).

Immunomodulatory analyses

A 2 ml of blood sample was collected in duplicate tubes from both the experimental and control group at the end of experiment using sterilized syringe. One sample was used for hematological analysis while, other investigated for quantification of antibodies using ELISA procedure. Sample for hematological investigation were collected in commercial tubes containing anticoagulant whereas for antibody quantification samples were collected without anticoagulant.

In order to investigate the Se effects on white blood cells and other blood components, hematological screening was done using hematology analyser (SINNOWA Medical Science and Technology Co., Ltd., Nanjing, China). For ELISA, serum was separated from blood samples by centrifugation method and was stored at -20°C in till further analyses. The sera samples were tested using Goat Total Immunoglobulin G (Total IgG) ELISA Kit (Biont, Shanghai YL Biotech Co. Ltd. Shanghai, China) for the quantification of IgG using the prescribed protocol of manufacturer.

Biofilm formation assay

Biofilm formation was determined using a microtiter plate following the procedures of Perez *et al*. (2011) with few modifications. In brief, fresh cultures were prepared from the isolates (*Pseudomonas aeruginosa* and *Staphylococcus aureus*) obtained from the control, treatment group and ATCC's (Reference strain). Five colonies from each plate were then dispensed in 5ml of Trypticase Soy Broth (TSB) and incubated for 24 h at 37°C without shaking. After incubation the stationary-phase culture of all the bottles vortexed and then diluted 1:100 in TSB with 0.25% glucose. 200µl of this solution was dispensed and incubated in 96-well microtiter plate for 24 h at 37°C, suspended bacterial media was then discarded. The plate was washed four times carefully with water and allowed to be air dried. About 200µl of 0.9% crystal violet solution was added in the wells for staining and left for 15 min. The dye solution then discarded and washed with water. The remaining attached dye was solubilized with 95% ethanol. At 450/630nm the optical

density of adherent biofilms was measured twice using spectrophotometer (Multiskan FC Microplate Photometer, Thermo Scientific, Madrid, Spain). In this experiment TSB containing 0.25% glucose was used as a negative control and ATCC's of the respective isolates as positive control. All isolates were analysed in triplicates.

Statistical analysis

All data was analyzed using JMP statistical package software (version 5.0.1.a, SAS Institute Inc., Cary, NC). The difference between the groups was compared by Student's t test at significance level of $p< 0.05$.

RESULTS

Microbial count (log cfu/g)

Se treatment exhibited a significant ($p<0.05$) increase in *E. coli* count (log cfu/g) on day 30 (25.6%) and 60 (36.8%) compared to control group (Fig. 1A). *Pseudomonas aeruginosa* count (log cfu/g) was found to decrease ($p<0.05$) by the dietary supplementation of Se on day 30 (26.3%) and 60 (87.9%) compared to control group (Fig. 1B). Moreover, dietary supplementation of Se significantly ($p<0.05$) reduced the *Staphylococcus aureus* count (log cfu/g) on day 30 (59.6%) and 60 (204.2%) compared to control group (Fig. 1C). On the other hand, *Lactobacillus* count (log cfu/g) was significantly ($p<0.05$) improved by the dietary supplementation of Se on day 30 (120.9%) and 60 (139.2%) compared to control group (Fig. 1D). No difference was recorded ($p>0.05$) between the control and Se supplemented groups for microbial count of *E. coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Lactobacillus* count on 0day.

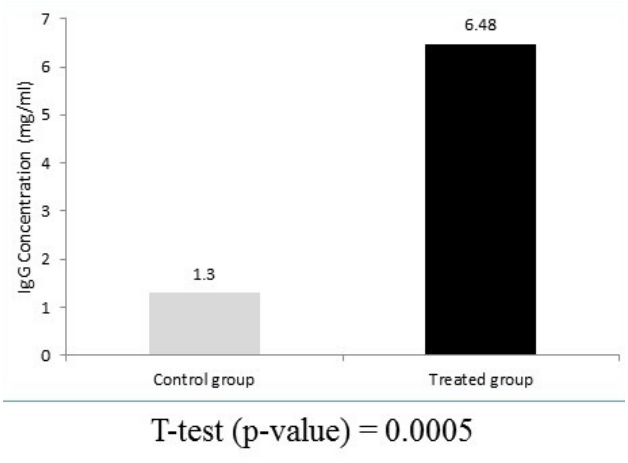
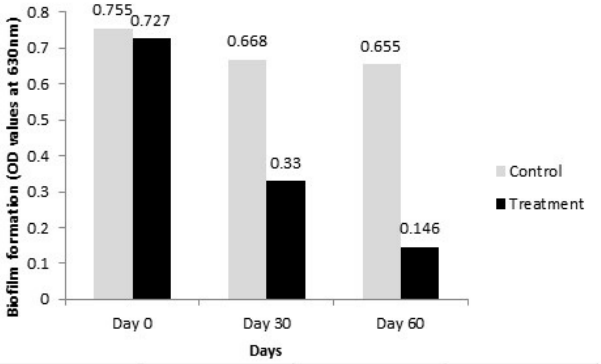


Fig. 2. Effect of Se supplementation in goats on serum IgG concentration.

IgG concentration

Result regarding IgG concentration in control and Se supplemented goats is presented in Figure 2. The data indicates that Se supplementation increased ($p<0.05$) the IgG concentration about 5 times (from 1.3 to 6.48 mg/ml) compared to control group.



	Day 0	Day 30	Day 60
p-value	0.0533	0.0079	0.0041

Fig. 3. Effect of Se supplementation in goats on biofilm formation activity. The results indicate the mean of triplicate measurements of each animal absorbance $_{630}$ after crystal violet staining of biofilms adhered to the wells of microtiter plate.

Table II. Influence of Se supplementation in goats on blood parameters.

Parameters	Control group	Treated group
Haemoglobin (g/dL)	7.65	10.80*
Haematocrit (%)	3.08	6.80*
RBC (10^6/L)	1.40	3.65*
MCV(fL)	30.13	36.90*
MCH (Pg)	48.7	56.37*
MCHC (g/dL)	150.75	166.28*
WBC (10^6/µL)	15.89	21.11*
Neutrophils (%)	9.65	15.73*
Lymphocytes (%)	70.62	78.35*
Monocytes (%)	6.05	10.75*
Eosinophils (%)	0.10	0.43*
Basophils (%)	0.18	0.20*

RBC, red blood cells; MCV, mean corpuscular volume; MCH, mean cell hemoglobin; MCHC, mean corpuscular hemoglobin concentration; WBC, white blood cells.

Biofilm formation

Results showed that biofilm formation in control group was recorded as 0.755, 0.668 and 0.655 on day 0, 30 and 60, respectively (Fig. 3). Surprisingly, Se supplementation significantly ($p < 0.05$) reduced the biofilm formation of bacterial pathogens at day 30 (102.4%) and 60 (352.0%) post-treatment as compared to control group. However, there was non-significant ($p > 0.05$) difference in biofilm formation among the control and treatment groups on 0 day.

Hematological values

The results of hematological picture in goats revealed that the dietary supplementation of Se in goats significantly improved ($p<0.05$) the red blood cell (RBC), while blood cell (WBC), lymphocytes, monocytes and neutrophils count; hemoglobin and haematocrit concentration; and mean corpuscular volume (MCV), mean cell hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) level as compared to control group. However, the eosinophil and basophil count was not affected ($p>0.05$) by the dietary supplementation of Se in goats (Table II).

DISCUSSION

Findings of the present study showed that *E. coli* and *Lactobacillus* count was significantly higher in goats fed on basal diet supplemented with organic selenium (0.15mg/kg.BW) compared to goats in control group. Whereas, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were significantly higher in goats fed on basal diet with no Se supplementation compared to Se supplemented goats. Ren *et al*. (2011) stated that dietary trace elements have a positive influence on the composition of gastrointestinal microbiota and gut colonization. This finding is also supported by the study of Kasaikina (2011) who worked on mice and reported the beneficial role of Se on the modulation of gut microflora. The study used different selenium diets that were given to both the germ free and conventionalized mice. All treated mice showed an increased diversity of gut microbiota. High through-put sequencing of mice revealed distinctive selenium effects across certain phylotypes within a single genus in addition to a modified selenoproteome expression. Selenium-enriched probiotics are evident in inducing a strong inhibitory effect against pathogenic form of *E. coli in vivo* and *in vitro* (Yang *et al*., 2009). According to Pietka-Ottlik *et al*. (2008) the integrated organo selenium compounds proved to implicit marked antagonizing effects as penicillin against *Staphylococcus aureus, in vitro*. Probiotic supplementation to piglets and sows during the period of lactation and subsequent to weaning resulted in a modified microbial diversity of intestine by enhancing helpful bacterial population and reducing pathogenic microbes including genterotoxigenic *E. coli* (Daudelin *et al*., 2011). It has been reported that quantitative increase of helpful bacteria in the gut may modify normal gut microflora in two ways: competitive exclusion and antagonism. Once benefical bacteria reach the gut and establish themselves, they may produce bactericidal compounds (bacteriocins) such as organic acids, hydrogen peroxide, and lactoferrin. These substances reduce the pH in the gut and this creates an environment where pathogenic bacteria cannot grow (Deraz, 2018; Kamada *et al*., 2013; Nehru *et al*., 2017).

Se is known to significantly affect the T and B -cell activity. The B-cells are very important lymphocytes that proliferate and produce plasma cells that synthesize antibodies. Selenium deficiency may affect B-cells and reduce their proliferation (McKenzie *et al*., 2002). Selenium supplementation is known to improve humoral immunity (antibody-mediated immunity) in various animals like lambs (Kumar *et al*., 2009), calves (Reffett *et al*., 1988) and kids (Shokrollahi *et al*., 2013). In current study dietary supplementation of Se exhibited a stimulatory effect on the plasma concentration of total IgG antibody in goats. This finding agreed with some previous researches who reported improved serum levels of IgG antibody by supplementation of dietary Se in ponies (Knight and Tyznik, 1990) and cows (Hall *et al*., 2014). Similarly, Kamada *et al*. (2007) reported that the serum IgG concentration dramatically elevated in suckling calves by dietary inclusion of selenium. Previous reports have highlighted that Se supplementation could improve the lymphocytes proliferation (Hall *et al*., 2013), that could be a better explanation of increased IgG titre in our study because antibodies are synthesized by the B-lymphocytes via plasma cells.

Biofilms are aggregates of microorganisms that adhere to non-biological surfaces, such as stream rocks, plant surfaces (roots) or in animals (epithelium). They are often enclosed in an outer polymer layer or matrix. This represents a protected mode of growth that not only allows cells for their survival in hostile environment, but also to colonize new niches by dispersal of microorganisms from the microbial clusters (Hall-Stoodley *et al*., 2004). Biofilms are most common cause of wide-spread infections (Costerton, 1999) such as infections in artificial implants induced by biofilms through catheters (Auler *et al*., 2010) and formation of dental plaques (Rogers, 2008). The biofilm bacteria are more resistant to antibiotics as compared to planktonic because of well protected by EPS or exopolysaccharide. This resistant effect against antibiotics can increase a thousand-fold in some cases (Stewart and Costerton, 2001). Therefore, bacterial biofilms are usually linked with persistent infections in body (Nataro *et al*., 2000). Findings of current study indicated that the bacterial species (*Pseudomonas aeruginosa* and *Staphylococcus*

aureus) produced weak positive biofilm formation in goats received basal diet supplemented with organic selenium (0.15mg/kg.BW) on day 30 and 60. The current results are in conformation with Tran and Webster (2011) who observed the antibacterial property of selenium for *Staphylococcus aureus* specifically by utilizing it in the form of nanoparticles *in vitro* resulted in a reduced growth at different intervals by the induction of different quantities of nanoparticles. Consequently, authors recommend employing Se nanoparticles for the prevention and treatment of infections caused by *Staphylococcus aureus*. Similar findings were observed by Wang *et al*. (2015) who coated the surface of paper towels with Se nanoparticles to analyze their potential in preventing biofilm forming ability of certain bacteria through biofilm formation assays. Ninety percent bacterial inhibition was observed for gram positive such as *S. epidermidis* and *S. aureus* at 24 to 72 h of treatment. In response to gram –ve bacteria this treatment resulted 84% decline in biofilm formation of *E. coli* while 57% for that of *P. aeruginosa* after 72 h.

In our study the concentrations of blood parameters such as hemoglobin, haematocrit, RBC, MCV, MCH, MCHC, WBC, neutrophils, lymphocytes and monocytes were found higher in goats supplemented with organic selenium (0.15mg/kg BW) as compared to goats fed basal diet without Se supplementation. Previous studies have demonstrated that trace elements supplementation have remarkable effects on blood parameters. Results of the present study are parallel with the findings of previous findings which reported elevated RBC (Faixova *et al*., 2007) and WBC (Shokrollahi *et al*., 2013) level in Se supplemented lambs as compared to control. The hemoglobin concentrations and MCHC values were found higher in the rainbow trout received 0.1-0.2 mg/l AgNP solution than control group. In another study, selenium enriched diet (4 mg/kg DM basis) improved the WBC, monocytes and lymphocytes counts in Taihang goats as compared to control group (Shi *et al*., 2018). Hematological investigations are vital index for monitoring health conditions as various physiological functions including growth, immunity and wellbeing are affected when trace elements are absorbed from the blood or vice versa (Iqbal *et al*., 2017). It is very difficult to predict a health indicator based on variation in blood parameters produced by dietary or any other mean. Because a number of factors such as species, age, health, environmental conditions, nutrition and maturation are reported to affect the blood and physiological parameters (Iqbal *et al*., 2017).

CONCLUSION

It was concluded from the findings of this study that, goats fed on organic selenium (0.15mg/kg BW) supplemented diet showed better immunity and hematological values compared to goats fed on basal diet without Se supplementation. Moreover, Se supplementation positively modulated the gut microflora composition and reduced the biofilm formation ability of gut pathogens.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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