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Supplementation of Selenium Nanoparticles-Loaded Chitosan Improves Production Performance, Intestinal Morphology, and Gut Microflora in Broiler Chickens

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The current study aimed to evaluate the efficacy of selenium nanoparticles (SeNPs), combined or loaded with chitosan (COS), in broiler chickens reared under standard management protocols. The parameters under investigation were production performance, organ development, components of the intestinal barrier, and ileal microbial count. Two hundred and forty day-old chicks were raised in five groups, with each group containing eight replicates ($n=6$/replicate). The control group received a basal diet whereas the other four groups received basal diets supplemented with SeNPs (0.5 mg/kg), COS (200 mg/kg), SeNPs+COS (0.5 mg/kg SeNPs + 200 mg/kg COS), and SeNPs-loaded COS (SeNPs-L-COS) (200 mg/kg) respectively. On day 35, two birds/replicate were sampled to collect the viscera under investigation. The results revealed that dietary inclusion of SeNPs-L-COS increased ($p<0.05$) the body weight gain and improved ($p<0.05$) feed conversion ratio. Similarly, SeNPs-L-COS supplementation increased ($p<0.05$) the small intestinal villus surface area as well as the count of acidic goblet cells and intraepithelial lymphocytes when compared with the control group. Whereas the total goblet cell count was higher ($p<0.05$) in the small intestines of both the SeNPs+COS and SeNPs-L-COS groups. Microbial analysis of ileal contents also revealed an increase ($p<0.05$) in *Lactobacilli* species count with a concurrent decrease ($p<0.05$) in *Escherichia coli* count in the SeNPs-L-COS group when compared with the COS and control groups. Based on the results of the current trial, we can conclude that supplementation with SeNPs-L-COS is a superior combination for promoting the gut health and performance of broilers.

Key words: gut health, microbiota, poultry, prebiotic, trace minerals

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Introduction

Selenium is an important trace element that exhibits antiviral and antibacterial properties. It plays a significant role in enhancing the production performance and cellular integrity of tissues. Moreover, it has also shown promising results in regulating the immune system and antioxidant status in chickens (Ahmadi *et al*., 2018). However, inorganic Se has a narrow margin between beneficial and toxic doses when added to broiler feed, and therefore there is a need for efficient delivery and distribution of a minimal dose. Nanomaterials are small-sized particles that provide a larger surface area, lower chances of toxicity, expansible catalytic potency, more surface-active centers, and promising biological effects, both as pharmaceutical and nutraceutical agents. Pharmaceuticals are used as transport-specific curative agents to improve the biological availability of drugs that affect higher uptake (Hu *et al*., 2012). Selenium nanoparticles (SeNPs) play a vital role in the formation of several types of selenoproteins, such as thioredoxin reductases and glutathione peroxidase, which protect against free radicals produced

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during oxidative stress (Habibian *et al*., 2014; Mahmoud *et al*., 2016).

In poultry feed, the inclusion of nano-selenium results in its higher absorption, which is reported to reflect the increased Se concentration in serum and tissue (Hu *et al*., 2012; Zhou and Wang, 2011). This higher retention results in improved production performance, intestinal microarchitecture, gut microbiota count, immunological activity, antioxidant status, fatty acid profile in skeletal muscles, and meat quality (Saleh, 2014; Zhou and Wang, 2011; Gangadoo *et al*., 2018). The health-promoting benefits of orally administered SeNPs can be further enhanced by loading them onto biological carriers, which improves their stability and biological availability (Bai *et al*., 2017).

COS is a naturally occurring, non-toxic, and biodegradable polysaccharide polymer derived from chitin. It can be used as a carrier to efficiently deliver drugs and trace nano-minerals that otherwise have lower bioavailability in the gastrointestinal tract (Han *et al*., 2012). SeNPs carry a positive charge and can be loaded onto the surface of COS, thus producing SeNPs-L-COS. SeNPs loaded on chitosan have antioxidant activity and have a significant impact on performance traits and biological activities (Bai *et al*., 2017).

To the best of our knowledge, no comparable research has been conducted on the use of SeNPs-L-COS in broiler feed. Therefore, the current study was designed to determine the effect of SeNPs-L-COS supplementation on the production performance, gut morphology, and microbiota of broilers.

Table 1. **Ingredients and nutritive value of the basal diets**

Ingredients	Percentage
Corn	58.5
Canola meal	8.0
Vegetable oil	1.5
Sunflower meal	3.5
Soybean meal 44%	25
Limestone	1.5
Dicalcium phosphate	0.9
Common salt	0.5
Vitamin Premix	0.13
D-L Methionine	0.21
L-lysine HCl	0.12
Micro min premix	0.13
Total	100
Molasses	4.0
Nutrient contents	
ME (MJ/kg)	12.2
CP (%)	20.7
Ca (%)	0.91
P (%)	0.61

[1] Feed (kg) contained vitamins (Vit) and minerals: Vit A (11,000 IU), Vit B12 (0.0132 mg), Vit D3 (2200 IU), Vit E (22 IU); pantothenic acid (22 mg), folic acid (1.1 mg), choline Cl (440 mg), menadione (2.2 mg), riboflavin (8.8 mg), ethoxyquin (250 mg), thiamine (4.4 mg), pyridoxine (4.4 mg), biotin (0.22), Zn (200 mg), Fe (20 mg), Mn (240 mg), Ca (170 mg), Cu (20 mg), and I (0.91 mg).

Materials and Methods

Synthesis of SeNPs-L-COS

SeNPs-L-COSs were synthesized using the method reported by Zhang *et al*. (2004). First, a selenious acid solution (0.25 M) was prepared by dissolving selenium dioxide (Sigma-Aldrich) in deionized water. The COS solution (0.5%) was prepared by dissolving COS (78% DDA; Qingdao BZ Oligo Biotech Co. Ltd. China) in 2% acetic acid solution. The COS and the selenious acid solutions were mixed under constant stirring. Ascorbic acid (Sigma-Aldrich) solution (0.05 M) was added dropwise under constant mechanical stirring to initiate the reaction. The change in the color of the solution from colorless to red indicates the formation of SeNPs. The mother liquor was stirred constantly for 30 min at room temperature. In the next step, a sodium hydroxide solution (1 M) was added to this mixture to neutralize the protonated amine group of COS. The COS formed a coating around the SeNPs as the pH of the solution rose above 6.0, resulting in the formation of SeNPs-L-COS. The mixture was centrifuged to separate the SeNPs-L-COS from the solution, which was then washed thoroughly with deionized water. SeNPs-L-COS was dried and stored in a clean vial for further use. SeNPs were synthesized using the same protocol without the addition of a COS solution.

Experimental Design

Two hundred and forty day-old birds (Hubbard) were assigned to five groups with eight replicates each, with six birds per replicate (six square feet of floor space/replicate). The total duration of the experiment was 35 days, and the birds were kept in an environmentally controlled experimental shed. A temperature of 35±1℃ was maintained on the 1st day and was gradually lowered (2.8℃ per week) to 26±1℃ by the end of the 3rd week. From day 22nd to the end of the experiment, the temperature was maintained at 26℃. The relative humidity was maintained at 65±5% throughout the trial. From day 1 to 35, the chicks were offered a commercial corn soya-based diet (Table 1) according to the formulation recommended by the National Research Council (NRC 1994) guidelines to fulfill the nutrient requirements of poultry. The control group was fed with a corn soya-based basal diet (BD) whereas the SeNPs group was given BD + 0.5 mg/kg SeNPs; the COS group was given BD + 200 mg/kg COS; the SeNPs +COS group was given BD + 0.5 mg/kg SeNPs + 200 mg/kg COS, and the SeNPs-L-COS group was given BD + 200 mg/kg SeNPs-L-COS. Both the feed and freshwater were provided *ad libitum*. The initial weight of the chicks was recorded upon arrival and was subsequently documented weekly for average body weight gain (BWG) together with recording of feed intake (FI) of individual replicates to calculate the feed conversion ratio (FCR). This study was approved by the Ethical Review Committee of the University of Veterinary and Animal Sciences, Lahore, Pakistan. DR/393, dated 12-04-2019.

Sampling Protocols

On the 35th day, two birds were sampled from each replicate

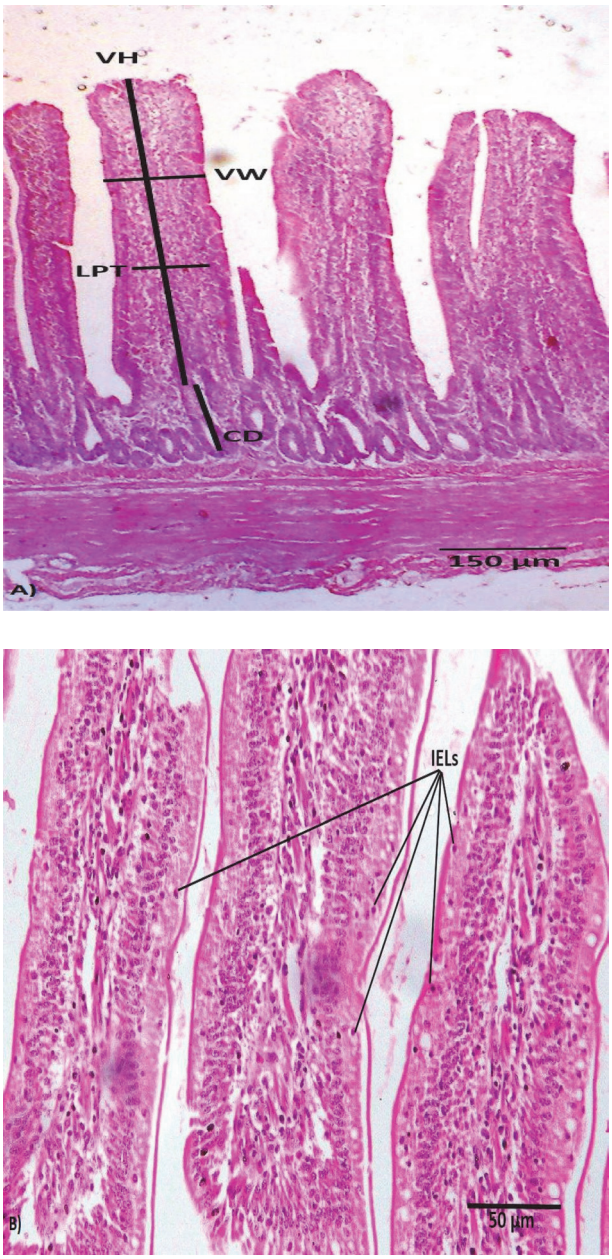

Fig. 1. **Cross section of the small intestine (Ileum), either hematoxylin–eosin stained (A, B) or Alcian blue and periodic acid Schiff stained (C), showing the measured parameters.** (**A**) Villus height (VH), villus width (VW), crypt depth (CD) and lamina propria thickness (LPT) (4×). (**B**) Intraepithelial lymphocyte count (IELs) (10×). (**C**) Acidic goblet cells (AGCs) and mixed goblet cells (MGCs) (40×).

(16 birds/group). The final body weight of the birds was measured using a digital balance (Libra Scales®). After cervical dislocation, the organs (proventriculus, liver, spleen, pancreas, gizzard, heart, intestine, and bursa of Fabricius) were collected and weighed to calculate the relative weights (RW) of the viscera. The small intestine and cecum were weighed with and without contents. The lengths of the small intestine and cecum were also recorded using a measuring tape. The duodenum, jejunum, and ileum were sampled at the duodenal loop, mid-jejunal point, and terminal part of the ileum, respectively, and 2 cm long segments were removed for histological analysis. The collected samples were washed with 0.9% sodium chloride solution to remove ingesta, debris, and blood. All tissue samples were fixed in 10% formalin solution (Bancroft *et al*., 2018).

Intestinal Histomorphometry

The paraffin embedding technique has been used for tissue processing (Bancroft *et al*., 2018). Three tissue sections were prepared for each sample. Hematoxylin and eosin (H&E) staining was performed on the intestinal sections. Intestinal histomorphometric parameters, including villus height (VH), villus width (VW), villus surface area (VSA), lamina propria thickness (LPT), and crypt depth (CD) were studied in all three segments of the small intestine. All images were captured with a 4× objective lens using a bright field microscope and subsequent histomorphometric measurements were made using a commercial program (Prog Res®2.1.1 Capture Prog Camera Control Software). For the measurement of VH, VW,

and CD, five intact and well-oriented villi were considered in each cross-section of the intestinal slides. The VH was measured from the tip of the villus to the villus-crypt junction (Fig. 1A). The VSA (μm²) was determined using the following formula: (2×3.14)×(VW÷2)×(VL) (Ashraf *et al*., 2013).

Intraepithelial Lymphocyte (IEL) Count

For IEL count, the H&E-stained slide preparations were examined under a bright field microscope (Labomed® LX-400, USA) using a 40× objective lens. Three tissue sections from each intestinal sample were analyzed. Within each section, five intact and well-oriented villi were randomly selected for IEL counting (Fig. 1B). Later, the average of 15 values was calculated and presented as the IEL count per VH. IELs were identified in the intestinal epithelium as rounded cells with a spherical nucleus (Ashraf *et al*., 2013).

Goblet Cell Classification

For goblet cell (GC) identification, three tissue sections from each intestinal segment were stained using the Alcian Blue-Periodic Acid Schiff (AB-PAS) method (Bancroft *et al*., 2018). The stained slides were then observed under a bright-field microscope using a 10× objective lens. Five intact and well-oriented villi per tissue section were selected to count the different types of goblet cells. Under a microscope, GCs appear as narrow bases with wide apical portions. Based on their staining characteristics with AB-PAS staining, GCs are classified as blue-stained acidic goblet cells (AGCs), purple-stained mixed goblet cells (MGCs), and magenta-stained neutral goblet cells (NGCs) (Ashraf *et al*., 2013). The average of 15 values of AGCs and MGCs per VH was calculated (Fig. 1C). The total goblet cell (TGC) count was calculated as the sum of the AGC and MGC counts per VH.

Gut Microflora

Samples of gut digesta were collected from two birds per replicate to count the selected microbial species using conventional culturing techniques. The findings were reported as a colony-forming units (Nabizadeh, 2012).

Statistical Analyses

Data are presented as mean±standard error of the mean (SEM) and were analyzed using SPSS (Version 20.0). The group means were analyzed using a one-way analysis of variance. Tukey's test was used to compare the group differences. The significance level was set at $p<0.05$.

Results

Production Performance

The effect of SeNPs with COS supplementation on different aspects of broiler production performance are presented in Table 2. Production performance parameters were not affected by supplementation during the first three weeks of the trial. At the end of the 4th week, the body weight was higher ($p<0.05$) in birds supplemented with SeNPs-L-COS group than in the control group. At the end of the 5th week, the body

Table 2. **Effect of supplemental selenium nanoparticles and chitosan on production performance in broilers**

Parameters	Week	Control[1]	SeNPs[2]	COS[3]	SeNPs+COS[4]	SeNPs-L-COS[5]	SEM	*p*-Value
Body weight (g)	1st	130	132	124	127	131	2.5	0.22
	2nd	338	349	342	349	367	5.4	0.11
	3rd	684	701	691	711	728	8.4	0.06
	4th	1171^{b}	1199^{b}	1175^{ab}	1225^{ab}	1254^{a}	9.8	0.02
	5th	1636^{d}	1690^{c}	1652^{d}	1740^{b}	1804^{a}	6.6	0.01
Body Weight Gain (g)	1st	83	85	76	79	83	2.2	0.16
	2nd	208	218	218	222	236	5.1	0.11
	3rd	347	352	350	357	361	4.2	0.37
	4th	487	498	484	513	526	8.5	0.14
	5th	466^{b}	491^{ab}	477^{ab}	515^{a}	550^{a}	9.6	0.02
Feed Intake (g)	1st	124	127	117	123	127	3.1	0.38
	2nd	345	335	340	330	337	2.8	0.16
	3rd	596^{a}	573^{ab}	607^{a}	561^{b}	555^{b}	9.0	0.04
	4th	802^{a}	788^{ab}	795^{ab}	810^{a}	779^{b}	6.5	0.03
	5th	1059^{a}	995^{ab}	1018^{ab}	977^{ab}	934^{b}	9.3	0.04
FCR	1st	1.51	1.50	1.54	1.55	1.55	0.03	0.94
	2nd	1.70	1.54	1.57	1.49	1.43	0.02	0.05
	3rd	1.72^{ab}	1.62^{abc}	1.74^{a}	1.57^{bc}	1.54^{c}	0.03	0.02
	4th	1.66^{a}	1.58^{ab}	1.65^{ab}	1.57^{ab}	1.48^{b}	0.03	0.04
	5th	2.31^{a}	2.04^{abc}	2.14^{ab}	1.90^{bc}	1.70^{c}	0.05	0.01

[1] Control=Basal diet only.
[2] SeNPs=Selenium nanoparticles.
[3] COS=Chitosan.
[4] SeNPs+COS=Selenium nanoparticles plus chitosan.
[5] SENPs-L-COS=Selenium nanoparticles loaded to chitosan.
[6] Different values illustrate the mean±SEM of eight replicates.
Significant difference ($p<0.05$) is represented employing different superscripts($^{a-d}$) in same row.

Table 3. **Effect of supplemental selenium nanoparticles and chitosan on organs weight (g) and length (cm) in broilers**

Parameters[6]	Control[1]	SeNPs[2]	COS[3]	SeNPs+COS[4]	SeNPs-L-COS[5]	SEM	*p*-Value
Liver	2.4	2.6	2.6	2.6	2.5	0.06	0.52
Pancreas	0.39^{a}	0.29^{ab}	0.38^{a}	0.24^{b}	0.25^{b}	0.03	0.02
Pro_FW	0.54	0.51	0.58	0.56	0.49	0.02	0.14
Pro_EW	0.45	0.44	0.50	0.50	0.44	0.02	0.26
Giz_FW	2.8^{a}	2.5^{bc}	2.6^{b}	2.4^{bc}	2.4^{c}	0.05	0.01
Giz_EW	1.8	1.9	1.7	1.7	1.8	0.05	0.04
Spleen	0.12^{ab}	0.11^{b}	0.13^{ab}	0.15^{a}	0.11^{b}	0.01	0.04
Heart	0.63^{ab}	0.65^{a}	0.58^{abc}	0.54^{c}	0.55^{bc}	0.02	0.01
Bursa of Fabricius	0.12^{b}	0.12^{b}	0.12^{b}	0.15^{a}	0.12^{b}	0.01	0.02
SI_FW	5.5	5.6	5.6	5.6	5.3	0.16	0.70
SI_EW	3.6	3.7	3.6	3.6	3.6	0.08	1.00
Cm_FW	0.66	0.63	0.61	0.80	0.62	0.05	0.30
Cm_EW	0.42	0.43	0.38	0.52	0.44	0.03	0.15
SI_L	164	167	165	174	181	4.80	0.08
Cm-L	21	22	21	23	23	0.50	0.12

[1] Control=Basal diet only.
[2] SeNPs=Selenium nanoparticles.
[3] COS=Chitosan.
[4] SeNPs+COS=Selenium nanoparticles plus chitosan.
[5] SENPs-L-COS=Selenium nanoparticles loaded to chitosan.
[6] Parameter, Giz=gizzard Pro=proventriculus. SI=small intestine. L=length. EW=empty weights. Cm=caecum. FW=filled weights.
[7] Different values illustrate the mean±SEM of eight replicates
Significant differences ($p<0.05$) are represented by different superscripts($^{a-c}$) in the same row.

weight and BWG were higher ($p<0.05$) in the SeNPs-L-COS and SeNPs+COS groups than in the control group. At the end of the 3rd and 5th weeks, feed consumption was lower ($p<0.05$), and FCR was improved ($p<0.05$) in the SeNPs-L-COS and SeNPs+COS groups when compared with the control group.

Weight/length of Visceral Organs

The effect of SeNPs with COS supplementation on the weights and lengths of visceral organs in broilers are presented in Table 3. The RW of the bursa of Fabricius was higher ($p<0.05$) in the SeNPs+COS group than in all other supplemented and control groups. The RWs of the pancreas and filled gizzard were lower ($p<0.05$) in the SeNPs+COS and SeNPs-L-COS groups than in the control group. However, the RWs of the liver, filled and empty proventriculus, empty gizzard, filled and empty small intestine, and caecum did not differ among groups. The lengths of the small intestine and cecum also did not vary among the groups.

Morphometry of Small Intestine

The effect of SeNPs with COS supplementation on the morphometric assessment of the small intestine in broilers are presented in Table 4. In the duodenum and ileum, VH was higher ($p<0.05$) in the SeNPs-L-COS and SeNPs+COS groups than in the control group. In all segments of the small intestine, VSA and VH/CD were greater ($p<0.05$) in the SeNPs-L-COS group than in the control group. The VH/CD was also greater ($p<0.05$) in the duodenum and ileum in the SeNPs and SeNPs+COS groups than in the control group. However, the VW (all segments of the small intestine), LP (duodenum, ileum), and CD (duodenum) did not vary among the experimental groups.

Goblet Cell Classification

The effect of SeNPs with COS supplementation on the goblet cell count in different segments of the small intestine of broilers are presented in Table 5. In the duodenum and jejunum, supplementation with SeNPs+COS and SeNPs-L-COS resulted in higher ($p<0.05$) AGC and TGC counts compared to the control group. In the ileum, the AGC count was higher ($p<0.05$) in the SeNPs and COS groups than in the control group. The MGC count was also higher ($p<0.05$) in the duodenum of the SeNPs-L-COS group than in the control group. In the jejunum, MGC count did not vary among the groups. NGCs were not detected in the intestinal sections.

IEL Count

The effect of SeNPs with COS supplementation on the IEL count of the small intestine in broilers are presented in Table 6. The IEL count was higher ($p<0.05$) in the ileum of the SeNPs and SeNPs+COS groups than in the control group. In the duodenum and jejunum, the IEL count did not vary between the control and supplemented groups.

Gut Microflora

The effect of SeNPs with COS supplementation on the gut microflora in the ileum of broilers are presented in Table 7. *Lactobacilli* CFU count was higher ($p<0.05$) in the SeNPs +COS and SeNPs-L-COS groups than in the control group. *Escherichia coli* CFU count was lower ($p<0.05$) in the SeNPs-L-COS group than in the control group.

Table 4. **Effect of supplemental Selenium nanoparticles and Chitosan on histomorphometry of small intestine in broilers**

Parameters[6]	Control[1]	SENPs[2]	COS[3]	SeNPs+COS[4]	SeNPs-L-COS[5]	SEM	*p*-Value
				Duodenum			
VH (μm)	1010^{d}	1210^{c}	1027^{d}	1337^{b}	1535^{a}	8.6	0.01
VW (μm)	140	114	139	123	131	5.4	0.06
VSA (mm)^{2}	0.44^{b}	0.43^{b}	0.45^{b}	0.51^{b}	0.63^{a}	0.02	0.01
CD (μm)	210	189	204	173	180	6.3	0.05
LPT (μm)	83	79	89	91	86	3.2	0.18
VH:CD	5.0^{c}	6.8^{b}	5.3^{c}	7.9^{ab}	8.9^{a}	0.3	0.01
				Jejunum			
VH (μm)	851^{b}	997^{ab}	883^{b}	1007^{ab}	1159^{a}	9.1	0.02
VW (μm)	141	114	131	104	123	9.8	0.85
VSA (mm)^{2}	0.35^{b}	0.33^{b}	0.35^{b}	0.32^{b}	0.43^{a}	0.02	0.02
CD (μm)	125^{a}	92^{b}	119^{ab}	110^{ab}	101^{b}	4.2	0.02
LPT (μm)	77^{b}	88^{b}	108^{a}	81^{b}	98^{ab}	4.5	0.01
VH:CD	7.3^{b}	10.8^{ab}	8.0^{b}	10.1^{b}	12.5^{a}	0.8	0.02
				Ileum			
VH (μm)	591^{c}	734^{b}	639^{c}	776^{b}	852^{a}	9.9	0.00
VW (μm)	122	106	131	100	114	6.5	0.07
VSA (mm)^{2}	0.22^{b}	0.24^{b}	0.26^{ab}	0.24^{b}	0.30^{a}	0.01	0.02
CD (μm)	128^{a}	92^{c}	119^{ab}	110^{abc}	101^{bc}	3.8	0.01
LPT (μm)	61	77	66	74	69	4.2	0.18
VH:CD	4.7^{c}	8.1^{a}	5.7^{bc}	7.5^{ab}	8.9^{a}	0.4	0.01

[1] Control=Basal diet only.
[2] SeNPs=Selenium nanoparticles.
[3] COS=Chitosan.
[4] SeNPs+COS=Selenium nanoparticles plus chitosan.
[5] SENPs-L-COS=Selenium nanoparticles loaded to chitosan.
[6] Parameters: VH=Villus height VW=Villus width. VSA=Villus surface Area. LPT=Lamina propria thickness. CD=Crypt-Depth. VH:CD=Villus height:Crypt-depth.
[7] Different values illustrate the mean±SEM of eight replicates
Significant differences ($p<0.05$) are represented by different superscripts ($^{a-d}$) in the same row.

Table 5. **Effect of supplemental selenium nanoparticles and chitosan on different types of goblet cells counts/ VH (μm) in small intestine of broilers**

Intestinal sections	Goblet Cell[6]	Control[1]	SENPs[2]	COS[3]	SeNPs+ COS[4]	SeNPs-L-COS[5]	SEM	*p*-Value
Duodenum	AGC	57^{c}	79^{b}	63^{c}	92^{a}	99^{a}	2.4	0.01
	MGC	43^{b}	56^{a}	37^{b}	50^{ab}	57^{a}	4.5	0.02
	TGC	100^{b}	134^{a}	100^{b}	142^{a}	156^{a}	6.7	0.01
Jejunum	AGC	75^{d}	96^{cd}	120^{bc}	144^{ab}	156^{a}	6.2	0.01
	MGC	43	35	48	38	41	3.2	0.18
	TGC	118^{c}	131^{bc}	167^{ab}	181^{a}	197^{a}	8.4	0.02
Ileum	AGC	89	167^{a}	182^{a}	113^{b}	135^{b}	5.3	0.01
	MGC	51^{ab}	42^{ab}	35^{b}	50^{ab}	57^{a}	4.8	0.03
	TGC	139^{c}	209^{a}	217^{a}	163^{b}	192^{ab}	9.1	0.01

[1] Control=Basal diet only.
[2] SeNPs=Selenium nanoparticles.
[3] COS=Chitosan.
[4] SeNPs+COS=Selenium nanoparticles plus chitosan.
[5] SENPs-L-COS=Selenium nanoparticles loaded to chitosan.
[6] Goblet cells: AGC=acidic goblet cells. MGC=mixed goblet cells. TGC=total goblet cell
[7] Different values illustrate the mean±SEM of eight replicates.
Significant differences ($p<0.05$) are represented by different superscripts ($^{a-d}$) in the same row.

Table 6. **Effect of supplemental selenium nanoparticles and chitosan on IEL counts/VH of small intestine in broilers**

Intestinal sections	Control[1]	SENPs[2]	COS[3]	SeNPs+COS[4]	SeNPs-L-COS[5]	SEM	*p*-Value
Duodenum	89	86	88	71	85	5.0	0.06
Jejunum	72	61	73	70	66	2.9	0.20
Ileum	56^{b}	65^{a}	48^{b}	69^{a}	60^{ab}	4.4	0.02

[1] Control=Basal diet only.
[2] SeNPs=Selenium nanoparticles.
[3] COS=Chitosan.
[4] SeNPs+COS=Selenium nanoparticles plus chitosan.
[5] SENPs-L-COS=Selenium nanoparticles loaded to chitosan.
[6] Different values illustrate the mean ± SEM of eight replicates.
Significant differences ($p<0.05$) are represented by different superscripts ($^{a-b}$) in the same row.

Table 7. **Effect of supplemental selenium nanoparticles and chitosan on gut microflora of broilers**

Bacterial popu-lation	Control[1]	SENPs[2]	COS[3]	SeNPs+COS[4]	SeNPs-L-COS[5]	SEM	*p*-Value
Lactobacilli	7.7^{c}	7.8^{bc}	7.8^{bc}	8.3^{ab}	8.4^{a}	0.3	0.02
Bifidobacteria	7.4	7.8	8.0	8.5	8.5	0.3	0.17
Escherichia coli	7.5^{a}	7.1^{ab}	7.4^{a}	7.0^{ab}	6.4^{b}	0.3	0.03

[1] Control=Basal diet only.
[2] SeNPs=Selenium nanoparticles.
[3] COS=chitosan.
[4] SeNPs+COS=Selenium nanoparticles plus chitosan.
[5] SENPs-L-COS=Selenium nanoparticles loaded to chitosan.
[6] Different values illustrate the mean±SEM of eight replicates.
Significant differences ($p<0.05$) are represented by different superscripts ($^{a-c}$) in the same row.

Discussion

Se inclusion in diets has been reported to significantly improve the growth traits of broilers (Selim *et al*., 2015). However, commercial broiler diets have either negligible or no Se, which can have health implications for chickens. The bioavailability of dietary Se can be improved by converting it to nanoparticles and by providing it a carrier (Gangadoo *et al*., 2018), as investigated in this study.

In the current study, the inclusion of SeNPs, COS, and SeNPs-L-COS did not influence the growth performance of broilers at the end of the first and second weeks, respectively. These results corroborate the findings of Zhou and Wang (2011) and Selim *et al*. (2015), who reported that SeNPs did not affect the average BWG, FI, and FCR early in the trial. After hatching, the intestinal tract is not fully developed and undergoes significant morphological changes until day 14 (Nabizadeh, 2012; Jung and Batal, 2012). Structurally, the mucosal surface of the intestinal tract consists of numerous folds, villi, and microvilli, which serve to increase the surface area for absorption of nutrients; thus, their development with age has been shown to significantly improve the utilization of nutrients and growth performance in broilers (Jung and Batal, 2012; Hu *et al*., 2012). Supplementation of SeNPs+COS and SeNPs-L-COS resulted in higher BWG at the end of the 5th week, while improvement in FCR was observed at the end of the 3rd and 5th weeks. Multiple studies have reported the positive impact of selenium nanoparticles on the growth traits of broilers. The mechanism leading to enhanced growth in broilers can be multifaceted. A possible explanation for these improvements can be linked to the presence of COS, in combination or loaded with SeNPs, which might have improved the bioavailability of Se. Se is an auxiliary factor and activator of the key enzyme iodothyronine 5′-deiodinase. The latter converts thyroxine (T4 prohormone) to its active metabolite triiodothyronine (T3), which is necessary for the growth and development of poultry (Selim *et al*., 2015; Safdari-Rostamabad *et al*., 2017).

Supplementation with SeNPs+COS increased the RWs of immune organs such as the spleen and bursa of Fabricius. The developmental status of immune organs is usually evaluated using RW measurements (Chen *et al*., 2014). Boostani *et al*. (2015) reported the positive effect of nano-selenium on the RWs of the spleen, thymus, and bursa of Fabricius. However, Se deficiency is known to reduce the RWs of immune organs and cause lymphocyte depletion (Saleh, 2014; Cai *et al*., 2012). The observed increase in the RW of the bursa of Fabricius may be attributed to the enhanced proliferation of B-cells (Ekino *et al*., 2012) with a concomitant reduction in their apoptosis. The former may be linked to a physiological increase in the secretion of IL-1, while the latter may be attributed to a decrease in the expression of Bax and caspase-3 and an increase in the expression of Bcl-2 (Huang *et al*., 2007; Chen *et al*., 2014). The observed increase in the

RW of the spleen has also been reported previously (Mahmoud *et al*., 2016; Malgorzata *et al*., 2019) and may be attributed to physiological increases in the secretion of IL-1 and IL-2, resulting in the proliferation of B and T cells, respectively (Huang *et al*., 2007). The RWs of the liver, empty gizzard, proventriculus, small intestine, and cecum were not affected by supplementation. Limited information is available in the literature regarding the effect of SeNP supplementation on the RWs of these viscera. However, we assume that the supplementation level of SeNPs and COS in the current study did not have deleterious effects on the visceral organs of broilers.

The VSA and VH/CD in the small intestine (duodenum, jejunum, ileum) improved with the inclusion of dietary SeNP-L-COS. Dietary Se has been reported to have a positive effect on the VH (Dalia *et al*., 2020), VSA, and mucosal function (Saleh, 2014) in the small intestine of broilers. Intestinal integrity and health are linked with VSA and VH/CD, which are the main indicators of intestinal function, such as digestion and absorption (Khambualai *et al*., 2009). Supplementation with organic Se has been reported to induce hyperplasia of enterocytes (Dalia *et al*., 2020) which explains the increased VSA observed in the current study. SeNPs delay apoptosis and increase enterocyte viability by scavenging reactive oxygen species (Moghaddam *et al*., 2017). Moreover, SeNPs increase the count of gut bacteria (Gangadoo *et al*., 2018). COS supplementation also improves villus morphology in broilers by increasing the volatile fatty acids necessary for enterocyte proliferation (Han *et al*., 2012; Xu *et al*., 2013) as well as by reducing the population of harmful bacteria (Khambualai *et al*., 2009). The stability of SeNPs is reported to improve when they are loaded onto the COS (Bai *et al*., 2017). This stability might contribute to their complementary role in the SeNPs-L-COS, which explains the increased VH, VH/CD, and VSA observed in our study.

Supplementation of SeNPs+COS and SeNPs-L-COS resulted in higher TGC counts in the small intestine. Alkhudhayri *et al*. (2018) reported that the inclusion of SeNPs in the diet significantly increased the TGC count after an infection-induced decline in their count. Intestinal GCs are an integral component of the innate gut immune system and indicate the mucin production potential of the intestine (Dawood *et al*., 2019). Se supplementation stimulated the differentiation of GCs through the upregulation of Math-1. Math-1 supports the differentiation of epithelial cells to GCs and increases the production of mucin mediated by MUC-2 protein (Saxena *et al*., 2017). Similarly, the inclusion of COS in feed has been reported to improve the expression of IL-4. IL-4 induces hyperplasia of GCs, resulting in increased production of mucin (Latif *et al*., 2016). Similarly, SeNPs+COS and SeNPs-L-COS supplementation increased AGC count in the small intestine. This increase appears to be at the expense of NGCs because the maturation cycle of GCs involves the conversion of NGCs into AGCs. Acidic mucin provides better protection against pathogenic bacteria and strengthens the intestinal mucosal barrier (Ashraf *et al*., 2013).

Supplementation with SeNPs+COS increased the IEL counts in the jejunum. IELs are a part of the gut-associated lymphoid tissue, and any alteration in their count is subject to physiological and environmental stressors. This study is apparently the first to report the effect of SeNPs on the IEL count in the intestine of broilers. The possible mechanism behind this increase could be that Se suppresses the hyper-production of oxidants in T-cells, which leads to T-cell proliferation (He *et al*., 2020). COS supplementation has also been reported to increase the IEL count in the small intestine (Xiong *et al*., 2015). Therefore, we assume that in the SeNPs+COS group, SeNPs and COS could have augmented each other's effect.

SeNPs-L-COS increased the *Lactobacilli* CFU count and lowered the *E. coli* CFU count in ileal digesta. The literature suggests that SeNPs shift the balance of gut microflora in a positive direction by preventing the colonization of harmful bacteria such as *E. coli* and *Salmonella* spp. (Gangadoo *et al*., 2018). SeNPs mediate antimicrobial activity in three ways. First, SeNPs interact with the acetylcholine receptor of the bacterial cell membrane and disturb their permeability. Second, SeNPs passing through the nuclei of microorganisms inhibit protein synthesis and mRNA expression. Third, the formation of the outer barrier results in the reduction of essential nutrients for bacterial growth (Huang *et al*., 2016). In addition to Se, COS nanoparticles have also been reported to display remarkable antimicrobial activity against gram-negative bacteria, such as *Salmonella* spp., *E. coli*, and *Staphylococcus aureus* when loaded with metal ions. COS increases the permeability of bacterial cell membranes, thus disrupting their integrity (Du *et al*., 2009). These functions of Se and COS help to explain the findings of our study.

In conclusion, supplementation with SeNPs-L-COS is a superior combination for improving the production performance, intestinal architecture, mucosal protection, and beneficial gut microflora count. However, further studies are required to elucidate the mechanisms through which dietary SeNP-L-COS stimulates the development of broiler gut and optimization of its local environment.

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Conflicts of interest

The authors declare no conflict of interest.

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