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Original Research Article

Influence of Feed Restriction and Zinc Oxide Nanoparticles Supplementation on Growth Performance, Blood Biochemistry, Intestinal Morphology and Cecal Fermentation Parameters of Growing Rabbits

Key words

feed restriction;
growth;
rabbits;
Zn oxide nanoparticles

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Abstract: The present study investigated the response of growing rabbits in terms of growth performance, serum biochemical, intestinal morphology, and caecal fermentation parameters to feed restriction and zinc oxide nanoparticles (ZnO-NPs) supplementation. A total of 60 New Zealand male rabbits were randomly distributed into 6 groups: AL-0 (fed *ad libitum* + fresh water as control); AL-15 and AL-30 (*ad libitum* + water supplemented with ZnO-NPs in water, 15 and 30 mg/L, respectively); and R-0, R-15 and R-30 were the same as the first 3 groups but with restricted feeding regime. Rabbits fed *ad libitum* and supplemented with ZnO-NPs (15 mg/L) showed the highest body weight with no significant difference from AL- fed groups or R-0. Feed conversion ratio (FCR) showed no difference among the different experimental groups ($P > 0.05$). ZnO-NPs supplementation reduced the serum lipid profile parameters, catalase enzyme in R-30, superoxide dismutase in AL-15 and AL-30 while increased serum malondialdehyde (MDA) in both *ad libitum* and restricted rabbits. ZnO-NPs administration resulted in lower caecal ammonia in AL-30 compared to its control (AL-0) as well as the content of individual volatile fatty acids (VFAs) (acetate, butyrate and propionate) ($P < 0.05$). Ileum morphological parameters (mucosal length, villi length, and goblet cell number) were modified in response to the feed restriction and ZnO-NPs addition. In conclusion, feed restriction program applied in this experiment altered rabbit growth performance (final body weight and weight gain with no differences in FCR), improved ileum morphology while had no significant effect on caecal fermentation (VFAs profile) or microbiological parameters. ZnO-NPs supplementation in both levels (15 and 30 mg/L) differently modulated serum lipid profile, antioxidant enzymes and MDA, VFAs profile in cecum and ileal morphology with no differences in rabbit growth performance.

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Introduction

In mammals, the peri-weaning period is considered one of the main stress periods in animal life as the young animal shifts from mother's milk to the solid feed. During this critical period, fast growth occurs accompanied by many problems such as high incidence of metabolic disorders, high morbidity and mortality, and digestive disturbances, which have deleterious effects (1), causing economic losses in the commercial rabbit production. Antibiotics have been used to control these disturbances, but the emergence of

antibiotic resistance has heightened the need to find other solutions to this problem. Several approaches have been considered to protect rabbit health during this stressful period, such as nutritional modulation and hygienic control. One of these approaches is the feed restriction, which can be done in two ways: quantitatively or qualitatively. Qualitative restriction involves limiting the amount of nutrients like protein and energy in feed (2). The quantitative restriction of feed can be done by limiting the feeder access time or reducing the amount of offered feed (2). Previous trials informed beneficial effects of feed restriction as it stimulated compensatory growth, improved feed efficiency utilization (3), improved digestibility of nutrients, lowered fat accumulation in carcasses (4, 5), and reduced the post-weaning digestive disturbance as the epizootic rabbit enteropathy (6). Beside these beneficial effects, also some negative effects such as reduced final weight and dressing out percentage in feed restricted rabbits, were reported (7, 8).

Another different approach is through the supplementation of additives in feed or water. Zinc (Zn) is an essential trace element which plays a vital role in cell division, synthesis, and stabilization of DNA (9). Moreover, it has many beneficial effects on different physiological functions such as acid-base balance, nutrient metabolism, and immune response (10, 11). MacDonald (12) reported that Zn improved feed utilization through participating in the metabolism and assimilation of different nutrients such as carbohydrates, proteins, and lipids. Recently, manufactured nanoparticles (NP) have shown new characteristics such as great specific surface area, high surface activity, a lot of surface-active centers, and high catalytic efficiency (13). Due to the advantage of small size and high surface reactivity, nanoparticles showed higher transport, uptake and increased absorption efficiencies, enhancing their bioavailability inside the animal body (14). The inclusion of nano additives especially nano minerals in animal nutrition has been widely adopted to enhance the growth and production of livestock. Zinc nanoparticle supplementation was found to be beneficial in rabbits (15, 16), piglets (17) and poultry (18, 19). Therefore, the aim of the present study was to investigate the effect of feed restriction and ZnO-NPs supplementation in the drinking water on the growth performance, some blood biochemical parameters, intestinal morphology, caecal microbiology, and volatile fatty acids fermentation in growing rabbits.

Materials and methods

Ethical statement

Animal management procedures were undertaken in accordance with the requirements of the Animal Care and Ethics Committee of the Faculty of Veterinary Medicine, Alexandria University, Egypt (AU 013-2022/10/12-3-149).

Rabbit care and experimental design

Sixty New Zealand male weaned rabbits; 33-35 days old (average body weight 770 ± 5.96 g) were randomly distributed into 6 experimental groups (10 rabbits/group) with 3 replicates/group (3-4 rabbits/each). Experimental groups were arranged as follows: AL-0 (rabbits were fed *ad libitum* + fresh water as control); AL-15 and AL-30 (rabbits fed *ad libitum* + water supplemented with ZnO-NPs, 15 and 30 mg/L respectively); R-0 (restricted feed + fresh water); R-15 and R-30 (restricted feed + water supplemented with ZnO-NPs, 15 and 30 mg/L respectively). The restriction program applied throughout a 2-month experimental period was done as rabbits were fed *ad libitum* (AL) at the first day, next day they were fed at a level of 93% of AL, then again AL and next day 93% of AL of the day before (20). The zinc oxide water dispersion nanoparticles used were <100 nm particle size (TEM), (Sigma-Aldrich, catalog No.721077). Growing rabbits were kept on a commercial pelleted diet illustrated in table 1. The commercial diet used was formulated to meet the nutrient requirements of the growing rabbits according to (21). Rabbits were kept in wire-galvanized batteries that had feeders and drinkers. All the animals were kept under the same management and hygienic conditions.

Growth performance: To implement the feed restriction program and ZnO-NPs supplementation, rabbits were weighed every 2 weeks, and feed intake (FI) was measured daily for all the experimental groups. Feed conversion ratio (FCR) and weight gain (WG) were calculated in accordance.

Sample collection: At the end of the experiment, three rabbits from each group were chosen at random and used for sample collection. Blood was drawn from the rabbit ear vein for serum separation and biochemical parameter analysis. Rabbits were then sacrificed with an overdose of pentobarbital sodium at 60 to 70 mg/kg live weight. The ileum specimen was preserved in 10% formalin for histopathological examination. Caecal content (one gram) was collected under aseptic conditions for microbiological examination, while the remainder was used for VFAs fermentation analysis.

Blood biochemical parameters: Blood samples were collected in clean vials without anticoagulant. The serum was separated by centrifugation at 3000 rpm for 10 min. Samples were used for the analysis of some serum biochemical parameters including total protein, albumin, glucose, triglycerides (TG), total cholesterol (TC), low and high-density lipoproteins (LDL and HDL), some liver function enzymes as aspartate amino transferase (AST) and alanine amino transferase (ALT), some kidney function parameters (uric acid and creatinine), some antioxidant enzymes as superoxide dismutase (SOD) and catalase, serum zinc concentration, and malondialdehyde (MDA) using commercial kits produced by Bio-diagnostic Co. (Diagnostic and Research reagents).

Intestinal morphology: The fixed ileal tissue was embedded in paraffin blocks, sectioned (5 µm), stained with haematoxylin and eosin (H&E) as previously described by Bancroft et al. (22), and then examined using a light microscope. The morphometric measurement of various parameters of intestinal villi and their associated crypt was performed quantitatively using image J software (Bethesda, MD, USA) according to Abràmoff et al. (23).

Table 1: Diet ingredient composition used during the experiment

Ingredients	**g/kg**
Berseem hay	305
Yellow corn	90
Barely	203.5
Wheat bran	180
Soybean Meal (44%)	160
Wheat straw	20
Molasses	20
Dicalcium phosphate	8
Calcium carbonate	7
Common Salt	3
Mineral and vitamin premix[1]	3
Dl-methionine	0.5
Chemical composition (%)	
Dry matter	87.32
Crude protein	17.01
Ether extract	2.60
Crude fiber	12.18
Nitrogen free extract	49.02
Ash	6.51
Digestible energy (DE) MJ/kg diet	10.46

[1]Mineral and vitamin premix composition (per 3kg): Vitamin A = 12,000,000 IU, D3 = 2,000,000 IU, E = 10,000 mg, K3 = 1,000 mg, B1 = 1,000 mg, B2 = 5,000 mg, B6 = 1,500 mg, B12 = 10 mg, Niacin = 30,000 mg, Biotin = 50 mg, Folic acid = 1,000 mg, Pantothenic acid = 10,000 mg, Choline chloride = 500,000 mg, Zinc = 50,000 mg, Manganese = 60,000 mg, Iron = 30,000 mg, Copper = 10,000 mg, Iodine = 1,000 mg, Selenium = 100 mg, Cobalt = 100 mg, Calcium carbonate to 3kg

Caecal microbiology and volatile fatty acids: According to Mountzouris et al. (24), the collected cecal contents were serially diluted ten times. The total bacterial and total coliform counts were calculated using the method described by Bivolarski et al. (25). The remaining cecal contents were immediately collected, squeezed with sterile gauze to obtain the caecal filtrate, and the residues were discarded. The caecal filtrate was diluted with an equal volume of diluted sulfuric acid before the pH was determined (using a digital pH meter). The prepared filtrate samples were frozen at -20°C for subsequent analysis of ammonia and VFAs concentrations according to the methods described by Alvarenga et al. (26).

Statistical analysis: The obtained data were subjected to two-way ANOVA to test the effects of different levels of ZnO-NPs, feed restriction as well as their interaction. Statistical analysis was conducted using GraphPad Prism 6 (GraphPrism Software, La Jolla, CA, USA). The obtained data were presented as mean ± standard error (SE) and significance was considered at P< 0.05.

Results

Growth performance of growing rabbits

Table 2 illustrates the rabbit's growth performance in response to ZnO-NPs supplementation and feed restriction. Applying the feed restriction significantly affected the rabbit's growth performance in terms of final body weight (BW), weight gain (WG), and feed intake (FI) ($P < 0.05$). The final BW and WG of rabbits under restriction and supplemented with ZnO-NPs (15 mg/L) were significantly reduced ($P < 0.05$) compared with the same group of rabbits freely fed and receiving the same dose of ZnO-NPs. The ZnO-NPs administration in the water had no significant effect ($P > 0.05$) on BW or WG either in the *ad libitum* or restricted rabbits. However, rabbits fed *ad libitum* and supplemented with ZnO-NPs (15 mg/L) showed the highest BW and WG ($P > 0.05$).

A non-significant reduction of FI ($P > 0.05$) was obtained in restricted rabbits compared with their relative control rabbits. The effect of the restriction regime was clear in those supplemented with ZnO-NPs (30 mg/L) as FI was significantly reduced ($P < 0.05$) compared with their control group, which received the same dose but fed freely. Neither feed restriction nor Zn supplementation affected the FCR ($P > 0.05$) of growing rabbits, although it was non-significantly improved in *ad libitum* fed rabbits supplemented with the lower dose of ZnO-NPs (15 mg/L).

Blood biochemical parameters

Blood serum concentrations of total protein and glucose showed no difference ($P > 0.05$) among different experimental groups; however, glucose concentration

Table 2: Effect of feed restriction and Zinc nanoparticles (ZnO-NPs) supplementation on rabbit growth performance

ZnO-NPs (mg/L)		Initial body weight (g/ rabbit)	Final body weight (g/ rabbit)	Total weight gain (g/ rabbit)	Feed intake (g/ rabbit)	Feed conversion ratio
AL-0	*Ad libitum*	774.50±14.92	2142.50±88.40^{AB}	1368.00±83.98^{AB}	7148.29±53.10^{A}	5.23±0.36
AL-15		763.50±16.33	2251.00±44.90^{A}	1487.50±39.99^{A}	7104.00±60.58^{AB}	4.77±0.10
AL-30		766.50±17.51	2065.56±71.18^{AB}	1299.06±73.12^{AB}	7239.85±190.42^{A}	5.57±0.36
R-0	Restricted	776.50±17.65	2077.50±69.99^{AB}	1301.00±72.38^{AB}	6924.10±212.14^{AB}	5.32±0.39
R-15		775.00±14.26	2042.50±105.37^{B}	1257.50±110.61^{B}	6970.32±247.79^{AB}	5.54±0.39
R-30		764.00±17.43	1971.50±21.63^{B}	1207.50±25.82^{B}	6697.78±108.04^{B}	5.54±0.16
Two-way Anova (*P-value*)						
Feed offering regime		0.785	0.039	0.031	0.026	0.230
ZnO-NPs level		0.821	0.187	0.246	0.886	0.544
Interaction		0.909	0.573	0.482	0.414	0.164

Values shown in table are mean ± SE. Uppercase letters correspond to statistical significance ($P < 0.05$) between *ad libitum* and restricted fed groups

was numerically increased in feed restricted rabbits irrespective of ZnO-NPs supplementation (Table 3). The interaction between the feed restriction regime and ZnO-NPs supplementation significantly altered the serum albumin and globulin concentrations ($P < 0.05$). Albumin concentration was significantly reduced in *ad libitum*-fed rabbits supplemented with 15 mg/L of ZnO-NPs, while showed no differences ($P > 0.05$) in the restricted ones.

Reducing the amount of feed significantly increased ($P < 0.05$) the globulin concentration when compared with rabbits fed freely without added ZnO-NPs (control). Unlike albumin, serum globulin concentration was significantly increased in full-fed rabbits supplemented with 15 mg/L of ZnO-NPs, while showed no differences ($P > 0.05$) in the restricted ones.

Table 3: Effect of feed restriction and Zinc nanoparticles (ZnO-NPs) supplementation on some blood serum biochemical parameters of growing rabbits

ZnO-NPs (mg/L)		Total Protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)	Albumin/ globulin ratio	Glucose (mg/dL)
AL-0	*Ad libitum*	5.96±0.05	5.20±0.09^{aA}	0.76±0.14^{bB}	7.49±1.77	93.07±2.41
AL-15		6.01±0.04	4.85±0.11^{bB}	1.16±0.14^{aA}	4.30±0.56	99.56±1.14
AL-30		6.03±0.02	4.97±0.03^{abAB}	1.06±0.05^{aA}	4.69±0.25	102.90±5.12
R-0	Restricted	6.01±0.03	4.97±0.12^{aAB}	1.04±0.09^{aA}	4.84±0.49	104.36±6.38
R-15		6.02±0.01	5.12±0.01^{aA}	0.90±0.00^{aAB}	5.71±0.02	107.35±4.39
R-30		6.02±0.04	5.08±0.02^{aAB}	0.94±0.04^{aAB}	5.41±0.25	106.06±3.77
Two-way Anova (*P-value*)						
Feed offering regime		0.538	0.422	0.654	0.795	0.053
ZnO-NPs level		0.444	0.482	0.367	0.292	0.378
Interaction		0.667	0.019	0.029	0.055	0.639

Values shown in table are mean ± SEM. Lowercase letters correspond to the statistical differences ($P < 0.05$) between different ZnO-NPs levels, while uppercase letters correspond to statistical significance ($P < 0.05$) between *ad libitum* and restricted fed groups

Table 4: Effect of feed restriction and Zinc nanoparticles (ZnO-NPs) supplementation on the serum concentrations of antioxidant enzymes (catalase, superoxide dismutase), malondialdehyde and serum zinc concentration in growing rabbits

ZnO-NPs (mg/L)		Catalase (U/ml)	SOD (U/ml)	MDA (nmol/ml)	Zinc (mg/dL)
AL-0	*Ad libitum*	145.16±8.69^{aA}	427.67±36.67^{a}	9.56±0.40^{b}	3.59±0.22^{bB}
AL-15		124.37±12.15^{aAB}	304.37±12.40^{b}	12.69±0.38^{a}	5.11±0.14^{aA}
AL-30		107.52±1.50^{aA}	292.60±9.05^{b}	14.37±0.81^{a}	4.81±0.32^{abAB}
R-0	Restricted	89.95±6.97^{aB}	346.31±38.28^{a}	10.66±0.36^{b}	3.94±0.16^{bB}
R-15		120.92±4.98^{aAB}	314.10±14.64^{a}	12.98±0.52^{a}	4.28±0.20^{bAB}
R-30		42.24±18.32^{bB}	274.00±19.21^{a}	13.75±0.24^{a}	5.43±0.22^{aA}
Two-way Anova (*P-value*)					
Feed offering regime		0.0004	0.160	0.519	0.781
ZnO-NPs level		0.001	0.003	<0.0001	0.0001
Interaction		0.023	0.207	0.247	0.013

Values shown in table are mean ± SEM. Lowercase letters correspond to the statistical differences ($P < 0.05$) between different ZnO-NPs levels, while uppercase letters correspond to statistical significance ($P < 0.05$) between *ad libitum* and restricted fed groups.

Table 4 shows the effects of experimental treatments on the serum concentrations of antioxidant enzymes (SOD and catalase), MDA, and serum Zn concentration. Catalase enzyme activity was significantly altered with ZnO-NPs addition, feed restriction applied, and the interaction between them ($P < 0.05$). Lower enzyme activity was observed in restricted rabbits compared with those freely fed. With ZnO-NPs supplementation, reduced catalase activity was found in restricted rabbits received 30 mg/L. SOD enzyme activity nearly followed the same trend of reduction which

Table 5: Effect of feed restriction and Zinc nanoparticles (ZnO-NPs) supplementation on some serum liver and kidney function related parameters of growing rabbits

ZnO-NPs (mg/L)		AST[1] (U/L)	ALT[2] (U/L)	Uric acid (mg/dL)	Creatinine (mg/dL)
AL-0	*Ad libitum*	38.33±2.60^{aB}	27.00±4.00	6.17±0.03	1.97±0.04
AL-15		43.00±1.00^{aAB}	31.00±4.58	6.08±0.07	1.95±0.04
AL-30		40.67±1.45^{aAB}	29.00±1.73	6.09±0.05	1.91±0.04
R-0	Restricted	47.33±3.84^{aA}	32.67±2.03	6.14±0.02	1.98±0.01
R-15		37.67±0.88^{bB}	32.00±1.73	6.00±0.06	1.98±0.04
R-30		39.00±1.73^{bB}	27.00±1.53	6.14±0.02	1.94±0.06
Two-way Anova (*P-value*)					
Feed offering regime		0.714	0.519	0.625	0.511
ZnO-NPs level		0.367	0.496	0.084	0.437
Interaction		0.017	0.430	0.396	0.920

Values shown in table are mean ± SEM. Lowercase letters correspond to the statistical differences ($P < 0.05$) between different ZnO-NPs levels, while uppercase letters correspond to statistical significance ($P < 0.05$) between *ad libitum* and restricted fed groups. [1]AST: aspartate amino transferase, [2]ALT alanine amino transferase.

Table 6: Effect of feed restriction and Zinc Nanoparticles supplementation (ZnO-NPs) on serum lipid profile of growing rabbit

ZnO-NPs (mg/L)		Cholesterol (mg/dL)	Triglyceride (mg/dL)	HDL[1] (mg/dL)	LDL[2] (mg/dL)	VLDL[3] (mg/dL)
AL-0	*Ad libitum*	86.63±3.50^{aA}	110.03±1.92^{a}	21.37±0.77^{a}	43.26±2.36^{a}	22.01±0.38^{a}
AL-15		70.87±1.86^{bAB}	92.97±1.65^{b}	17.13±0.38^{b}	35.14±2.26^{b}	18.59±0.33^{b}
AL-30		72.77±2.10^{bAB}	96.10±4.37^{b}	19.30±0.93^{ab}	34.25±3.66^{b}	19.22±0.87^{b}
R-0	Restricted	80.70±1.81^{aA}	108.33±2.19^{a}	20.23±0.3^{a}	38.80±2.45^{a}	21.67±0.44^{a}
R-15		78.27±1.40^{abAB}	99.00±1.74^{ab}	18.57±0.70^{a}	39.90±1.40^{a}	19.80±0.35^{b}
R-30		69.50±1.92^{bB}	89.97±2.64^{b}	17.00±0.86^{b}	34.51±1.24^{a}	17.99±0.53^{c}
Two-way Anova (*P-value*)						
Feed offering regime		0.736	0.781	0.263	0.092	0.781
ZnO-NPs level		0.0002	<0.0001	0.002	0.048	<0.0001
Interaction		0.020	0.098	0.054	0.192	0.098

Values shown in table are mean ± SEM. Lowercase letters correspond to the statistical differences ($P < 0.05$) between different ZnO-NPs levels, while uppercase letters correspond to statistical significance ($P < 0.05$) between ad libitum and restricted fed groups. [1]HDL: high density lipoprotein, [2]LDL: low density lipoprotein, [3]VLDL: very low- density lipoprotein

was observed also with increasing ZnO-NPs level in the water especially in the *ad libitum* rabbits ($P < 0.05$). MDA as an important indicator of oxidative stress, was significantly increased with ZnO-NPs supplementation (P< 0.05) compared to rabbits received fresh water without Zn. ZnO-NPs supplementation altered the serum Zn concentration, which was significantly increased ($P < 0.05$) in either *ad libitum* or restricted rabbits. The highest serum Zn concentration was shown in the 15 mg/L ZnO-NPs supplemented *ad libitum* rabbits, and the 30 mg/L restricted fed rabbits.

As presented in table 5, kidney function-related parameters such as uric acid and creatinine were not affected by the feed offering regime or ZnO-NPs administration or the interaction

Table 7: Effect of feed restriction and Zinc Nanoparticles (ZnO-NPs) supplementation on volatile fatty acids in caecum of growing rabbits

ZnO-NPs (mg/L)		Ammonia (mg/dL)	Total volatile fatty acids (mM)	Acetate (% of total VFAs)	Propionate (% of total VFAs)	Butyrate (% of total VFAs)
AL-0	*Ad libitum*	14.90±0.38^{a}	50.40±3.72	71.73±1.16^{aA}	6.03±0.33^{a}	24.23±1.19^{a}
AL-15		13.00±0.42^{ab}	45.67±1.17	63.47±0.81^{bAB}	4.80±0.35^{b}	20.80±0.80^{b}
AL-30		12.20±0.29^{b}	40.67±6.23	63.80±1.00^{bAB}	4.90±0.42^{b}	19.17±0.73^{b}
R-0	Restricted	13.67±0.64^{a}	49.93±3.44	67.07±0.86^{aAB}	5.33±0.03^{a}	22.57±0.55^{a}
R-15		13.23±0.24^{a}	40.50±4.65	67.27±0.80^{aAB}	5.03±0.24^{a}	23.07±1.23^{a}
R-30		12.30±0.74^{a}	41.90±1.05	62.90±1.50^{bB}	4.93±0.20^{a}	20.03±0.41^{b}
Two-way Anova (*P-value*)						
Feed offering regime		0.462	0.613	0.505	0.552	0.505
ZnO-NPs level		0.004	0.077	0.0003	0.031	0.003
Interaction		0.284	0.962	0.006	0.274	0.114

Values shown in table are mean ± SEM. Lowercase letters correspond to the statistical differences ($P < 0.05$) between different ZnO-NPs levels, while uppercase letters correspond to statistical significance ($P < 0.05$) between *ad libitum* and restricted fed groups.

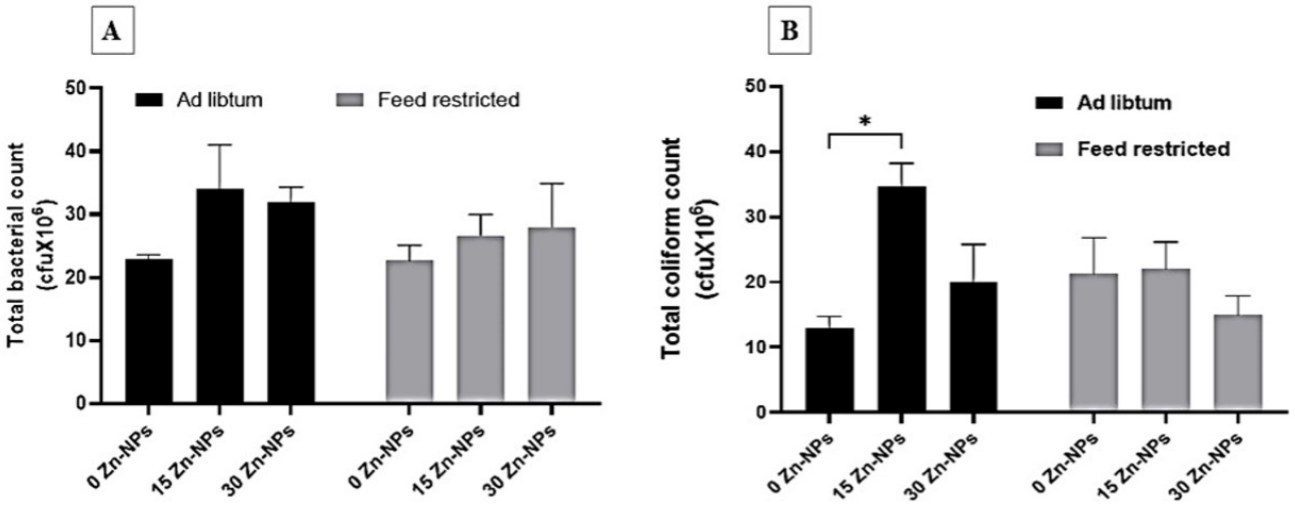

Figure 1: Effect of feed restriction and Zinc Nanoparticles supplementation on cecal microbiology (total bacterial count and total coliform count) in growing rabbits. Results expressed as means ± SEM

between them. The same response was obtained with the ALT enzyme activity. On the other hand, AST enzyme activity was significantly modified by the interaction of our treatments ($P < 0.05$). Zn supplementation lowered the AST concentration ($P < 0.05$) in feed restricted rabbits compared with their control rabbits without Zn addition. As illustrated in table 6, all the measured parameters of the serum lipid profile were significantly ($P < 0.05$) modified by ZnO-NPs addition in water. ZnO-NPs administration (both levels) to the full-fed rabbits resulted in lower ($P < 0.05$) TC, TG, LDL, VLDL, as well as reduced serum HDL only with 15 mg/L when compared to their control without Zn addition. Additionally, the same trend of reduction was observed in TC, TG, LDL, and VLDL in feed restricted rabbits which received the higher dose of ZnO-NPs (30 mg/L).

Volatile fatty acid concentration and caecal microbiology

In the freely fed rabbits, ZnO-NPs supplementation significantly reduced ($P < 0.05$) ammonia (AL-30) and individual VFAs concentrations (acetate, propionate, and butyrate) in the cecal contents at both levels (AL-15 and AL-30) compared to AL-0 (Table 7). The same response of

Table 8: Effect of feed restriction and Zinc Nanoparticles (ZnO-NPs) supplementation on morphology of small intestine (ileum) of growing rabbits

ZnO-NPs (mg/L)		Mucosal length (µm)	Villi length (µm)	Villi width (µm)	Crypt depth (µm)	Goblet cell (number/mm²)
AL-0		556.44±27.22^{bB}	290.57±19.27^{bB}	171.14±37.22^{aA}	72.04±10.62^{b}	2.78±0.25^{cD}
AL-15	*Ad libitum*	781.54±22.05^{aAB}	527.21±54.64^{aAB}	114.15±9.61b^{AB}	111.39± 4.46^{a}	8.71±0.04^{bB}
AL-30		826.11±12.33^{aAB}	239.07±115.45^{bB}	111.67±1.85b^{AB}	115.85±5.04^{a}	11.41±0.34^{aA}
R-0		673.19±32.19^{bB}	380.08±21.23^{bB}	85.30±4.80^{bB}	86.44±3.36^{b}	4.81±0.18^{cC}
R-15	Restricted	936.33±25.92^{aA}	709.36±35.20^{aA}	122.23±13.48^{bAB}	117.07±2.33^{a}	10.48±0.62^{bAB}
R-30		1000.93±83.95^{aA}	567.18±115.64^{abA}	126.37±10.03^{bAB}	104.89±2.44^{a}	11.48±0.32^{aA}
Two-way Anova (*P-value*)						
Feed offering regime		0.0021	0.015	0.162	0.565	0.0014
ZnO-NPs level		<0.0001	0.009	0.815	0.0002	<0.0001
Interaction		0.821	0.547	0.022	0.167	0.052

Values shown in table are mean ± SEM. Lowercase letters correspond to the statistical differences ($P < 0.05$) between different ZnO-NPs levels, while uppercase letters correspond to statistical significance ($P < 0.05$) between *ad libitum* and restricted fed groups

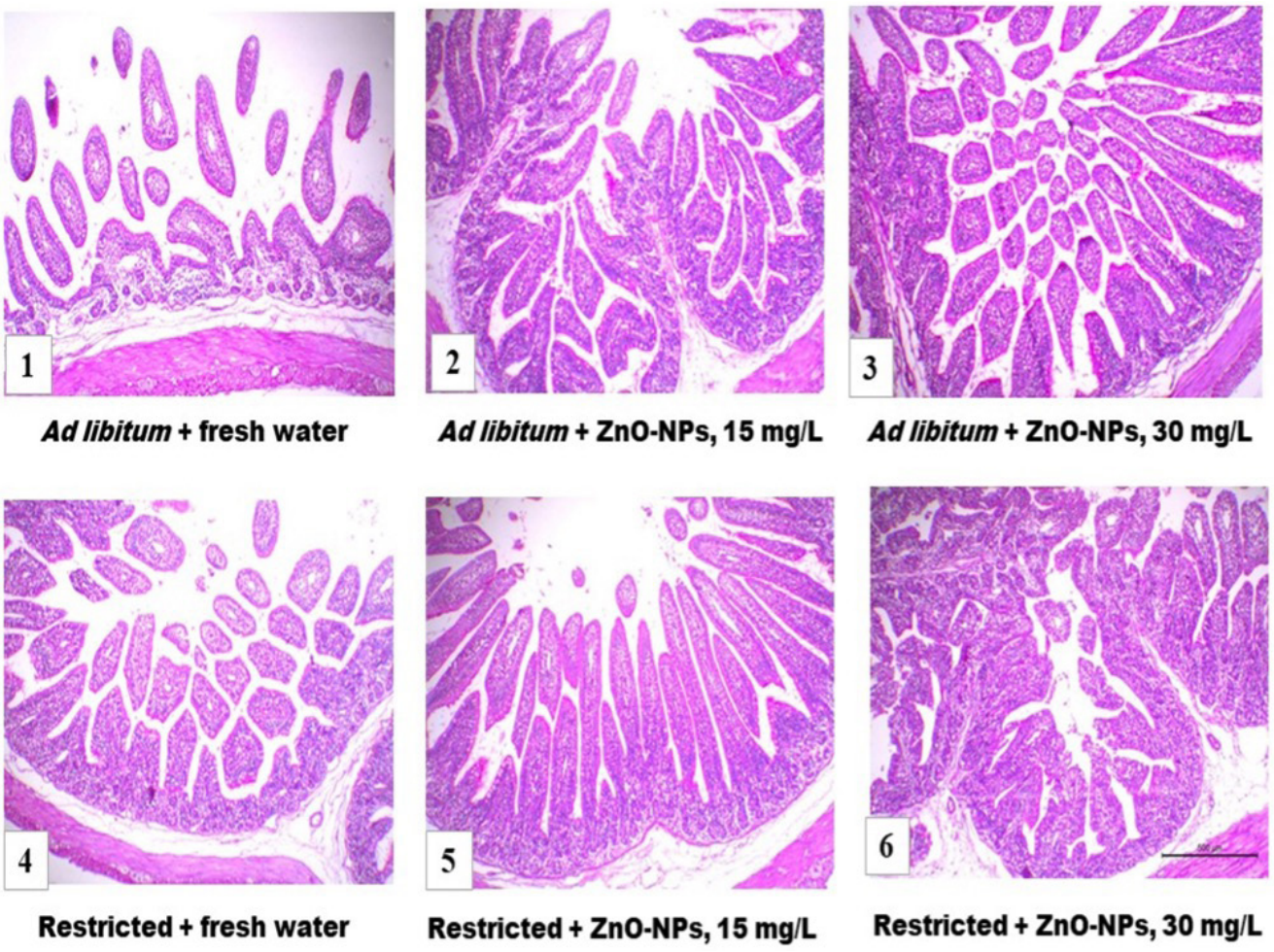

Figure 2: Morphology of rabbit ileum in response to different feeding regimes and ZnO-NPs supplementation As shown in 1) normal mucosal lining with no pathological changes; 2) an increase in the villi length; 3) an increase in mucosal length; 4) increase in the villi branches; 5) increase in the villi length; and 6) marked increase in the mucosal villi number and branches

reduction was obtained with cecal acetate and butyrate content in the feed restricted rabbits supplemented with 30 mg/L (R-30) when compared to control restricted rabbits (R-0).

The total bacterial count showed no differences among groups ($P > 0.05$) (Figure 1). On the other hand, the total coliform count was significantly altered ($P < 0.05$) with ZnO-NPs administration. In the freely fed rabbits, ZnO-NPs addition (15 mg/L) increased the total coliform count while reduced it ($P > 0.05$) when a higher dose of Zn was used (30 mg/L). In the restricted fed rabbits, non-significant difference was obtained ($P > 0.05$) when compared with the control receiving fresh water.

Intestinal Morphology

Intestinal morphometry parameters of the ileum are shown in Table 8 and Figure 2. Feed restriction and ZnO-NPs supplementation significantly affected the mucosal length, villi length, and goblet cell number. When compared to the control group, it increased ($P < 0.05$) mucosal length, villi length, crypt depth, and goblet cell number while decreasing villi width. Villi length was significantly increased in both rabbits (freely fed or restricted) and supplemented with 15 mg/L. On the other hand, villi length was reduced ($P < 0.05$) in the *ad libitum* fed rabbits supplemented with 30 mg/L of ZnO-NPs compared to rabbits supplemented with 15 mg/L ZnO-NPs. Goblet cells showed the highest number with the highest level of Zn added to the water. Besides, villi width was significantly changed in response to the interaction of the two factors ($P < 0.05$). In *ad libitum* rabbits, Zn administration reduced the villi width ($P < 0.05$), while showed no difference in the restricted rabbits ($P > 0.05$) compared with their control group without Zn addition.

Discussion

Feeding strategy for growing animals considers an important factor affecting their growth performance, lean body mass obtained and could help in avoiding problems associated with the early life fast growth rate such as increased body fat deposition, high incidence of metabolic disorders and high mortality. In recent years, global interest in using nanotechnology has been increased

as nanoparticles including Zn-NPs demonstrated a great potential as mineral supplements in livestock diets.

In the present study, feed restriction reduced rabbit growth performance in terms of final weight and gain while showed no effect on FCR. Different result was reported with Yakubu et al. (27), who found that feed restriction (8 hrs. per day feeding (7.00 a.m-3.00 p.m.) or a skip-a-day system) of weaned rabbits for five weeks resulted in no difference in their weight. Also, Meo et al. (5) found that both restricted (90 % of the *ad libitum*) and *ad libitum* rabbits showed similar weight at the slaughter time however, reported improved FCR in the restricted group. Birolo et al. (20) found that the application of 93% of *ad libitum* intake during the first growing period (from weaning, 37 d until slaughter (at 73 d and 80 d of age) enhanced rabbit health status without affecting growth performance. Moreover, feed restriction negatively affected the WG of rabbits, which could be associated with the reduced amount of feed consumed per day, which in turn resulted in inadequate intake of nutrients needed to support the rapid growth and development of the growing rabbit (28). No statistical difference was observed in the FCR between the different experimental groups. The reported results were consistent with Tumova et al. (29) who documented that feed restriction systems for growing rabbits had no effect on the feed efficiency. As reported before, the impact of feed restriction depends on its intensity, duration, digestive physiology, and age of rabbits when applied (30). Our results suggested that the restriction was not severe (mild restriction) to affect rabbit growth, resulting in restricted rabbit weight nearly approaching the weight of a full fed rabbit and reflected on the FCR at the end of the experiment.

Moreover, ZnO-NPs administration had no significant effect on rabbit growth performance, however, freely fed rabbits received 15 mg/L showed the highest body weight among groups. This improvement could be attributed to the better bioavailability and absorption of Zn, which has an important role in the metabolism of nutrients, in addition to the enhanced intestinal villi length. Unlike the obtained results, Hassan et al. (16) found that dietary supplementation of rabbits with nano-Zn at 30 and 60 mg /kg diet increased BW and FI. In support, Tag-El Din (15) reported that BW was insignificantly larger in rabbits that received different Nano-Zn compared with their control. Inconsistency in the obtained results between experiments could be associated with the difference in the experimental design, such as the route of Zn administration, nanoparticle size, number of samples used for measurements and the feeding regime applied in the current experiment.

Serum biochemical parameters are considered important diagnostic tools which help in assessing the metabolic condition of an animal and reflect its physiological response to different external and internal conditions, including nutrition (31). The interaction between the feed offering regime and Zn supplementation altered the albumin and globulin serum concentrations. The current results showed lower serum albumin concentration in the freely fed rabbits supplemented with ZnO-NPs (15 mg/L) compared to the same group subjected to feed restriction. In the same regard, Hassan et al. (16) found that diet fortification with nano Zn elevated total protein and globulin with no clear changes in albumin concentration of growing rabbits. Increased serum globulin in freely fed rabbits supplemented with ZnO-NPs was consistent with weaning piglets supplemented with nano Zn (32) and (33) in broiler chickens. Differences in the obtained results in terms of these parameters between restricted and freely fed rabbits could be associated with the reduced FI, which is correlated with the water consumption and consequently affected the dose of Zn administrated.

In the current experiment, a non-significant difference in glucose concentration in response to feeding restriction and ZnO-NPs addition was obtained. Likewise, Van Harten and Cardoso (34) found that the limitation of voluntary intake didn't reduce glucose concentration. They explained that animals under restriction require no more catabolism of glucose, and this was supported by the level of the glucose-6-phosphate, which didn't show any changes. Similarly, Ebeid et al. (35) stated that plasma glucose concentration showed no difference in growing rabbits subjected to feed restriction.

Regardless of the feed offering regime applied, serum MDA concentration was increased with increasing ZnO-NPs level added in water, suggesting that the addition of ZnO-NPs could have an oxidative stress effect on the growing rabbits. This finding was supported by Sharma et al. (36), who found that ZnO-NPs induced oxidative stress effects and increased the liver function enzymes, AST and ALT. In agreement, Ismail and El-Araby (37) found a significant increase in hepatic MDA levels in rabbits that received ZnO-NPs in their diet. Zn plays a significant role in the antioxidant defense system as an important part of the antioxidant enzyme SOD (38). The antioxidant defense system is formed of three levels of defense with SOD, CAT, and glutathione peroxidase forming the first level, as their main function is the prevention of free radical formation through scavenging their precursors (39). In our study, SOD and catalase serum activities were reduced by increasing the ZnO-NPs level, with the lowest concentration recorded at 30 mg/L, suggesting that the supplemented Zn failed to enhance the antioxidant system. Likewise, Ismail and El-Araby (37) found depressed renal and hepatic catalase activity in ZnO NPs supplemented rabbits, which could be associated with oxidative stress induced by nanoparticles. While this contradicts the findings of Hassan et al. (16) who reported elevated SOD activities in growing rabbits given 30 and 60 mg nano-Zn /kg diet.

Moreover, the interaction between the 2 factors affected the activity of the liver enzyme, AST. In *ad libitum* fed rabbits, the activity of this enzyme was increased with increasing Zn

levels, while the opposite result was obtained in restricted rabbits. Fazilati (40) reported that liver enzyme activity was increased in rat serum treated with ZnO nanoparticles (50 ppm, 100 ppm and 200 ppm). The measured kidney function related parameters, creatinine and uric acid, showed no changes between the different groups, suggesting no adverse effects of experimental treatments in the present trial. These results are consistent with Peris and Abd El-Latif (41), who found that ALT, urea-N, and creatinine serum concentrations showed no significant difference between restricted and *ad libitum* fed rabbits. Furthermore, ZnO-NPs supplementation at both levels was associated with a significantly increased serum Zn concentration in the Zn-supplemented rabbits, which suggests high availability and absorption of ZnO-NPs. Similar results were reported by Hassan et al. (16).

Regarding the lipid profile, it was altered with ZnO-NPs supplementation. In the full-fed rabbits, different lipid profile parameters, including TC, TG, HDL, LDL, and VLDL, were significantly reduced with Zn addition. The response of reduction in these parameters appeared with administration of ZnO-NPs (30mg/L) in the restricted rabbits. In the same direction, Tag-El Din (15) detected an insignificant reduction in plasma TG and TC of rabbits supplemented with Nano-Zn (30 and 60 mg/kg diet). Also, Ismail and El-Araby (37) who found a significant decrease in serum TG, TC, and VLDL-C in ZnO-NPs supplemented rabbits. Zinc consider as an important component of several enzymes (metalloenzymes) involved in lipid digestion and absorption (42). Additionally, cholesterol concentration responded to the interaction between the Zn added and the feed restriction regime as it showed the lowest level in restricted rabbits supplemented with 30 mg/L of ZnO-NPs. In support, El-Speiy et al. (30) documented lower TC, total lipids, and TG in rabbits under feed limitation. Our results suggested that ZnO-NPs added had an important regulatory role in the lipid metabolism, which was reflected by changes in the lipid profile parameters measured and growth performance of growing rabbits. The obtained lipid profile in response to ZnO-NPs administration needs further studies to understand the mechanism behind especially at the genetic level.

The fermentation process occurs in rabbit cecum and their end products as VFAs plays an essential role in feed utilization efficiency and rabbit performance. Gidenne (43) reported that the VFA covers about 30-50 % of the maintenance energy requirements of the adult rabbit. In the freely fed rabbits, ZnO-NPs addition in water altered the concentration of ammonia and different individual VFAs (acetic, butyric, and propionic) in the cecal content, as they were reduced compared with the control rabbits which received fresh water. A different result was obtained by Chrastinová et al. (44), who found no differences in VFAs profile between control and experimental groups supplemented with different sources of Zn. The obtained results confirm the importance of Zn as an essential microelement involved in metabolic processes in the body.

Rabbits subjected to a feed limitation regime showed a non-significant change in the total bacterial count or coliform count in the cecal content compared with the freely fed rabbits. Similarly, Martignon et al. (45) found that the FI level had no effect on the bacterial community structure (the number of bacterial 16S rDNA copies per gram of cecum). The constant composition of ingested feed material as well as the buffering capacity of the cecal contents were suggested by Michelland et al. (46) as the main explanation for the lack of effect of different FI levels on the cecal bacterial profile. In either full fed or restricted rabbits, ZnO-NPs administration showed a numerical increase in total bacterial count, with the higher count in the full fed ones. In support, Diao et al. (47) found a similar result when 100 mg/kg of zinc was supplemented in the diet of weaned piglets.

Besides, the total coliform count was increased with 15 mg/L ZnO-NPs administration, while it was reduced with 30 mg/L ZnO-NPs in the full-fed rabbits. In the restricted fed rabbits, non-significant difference was obtained when compared with the control receiving fresh water. In the same regard, Mahmoud et al. (18) found a significant reduction in the coliform count in the cecal content of broilers with different levels of Zn nanoparticles. The alteration in the cecal microbiota in response to the ZnO-NPs supplementation could be associated with its antibacterial activity (48). The latter author documented that the antibacterial activity of nanoparticles is dependent on their surface area and concentration, as the smaller the size, the larger the surface area, which becomes more reactive against bacteria, increasing their antibacterial activity. Therefore, the inconsistency obtained between the current study and the previous studies could be associated with the characteristics of nanoparticles used as size, concentration, form, and duration of exposure (18), species under the study, techniques that could give a complete picture of the microbial profile in the cecum and the host gut health and number of samples taken during the experiment. In the current study, the limited number of samples taken could contribute to the unclear picture of the experimental treatment response.

Nutrition including the amount of feed offered, is considered an important factor affecting the physiological function of the gastrointestinal tract as it relates to absorption (49). Gidenne et al. (2) assumed that the limitation of voluntary intake could change the morphology of the intestinal mucosa. In the present study, the ileal morphology of growing rabbits in terms of mucosal length, villi length, crypt depth, and goblet cell number were increased in response to feeding restriction and ZnO-NPs administration in water. In the same regard, Tůmová et al. (50) revealed that one-week feed restriction of growing rabbits modified the intestinal morphology as it showed longer small intestine villi with deeper crypts. In contrast, Martignon et al. (45) found that a three-week intake limitation applied (25% reduction of the

intake) did not affect the morphometric development of the ileal mucosa. The discrepancy in experimental outcomes might be associated with the restriction regime applied, duration, and intensity. Moreover, Oliveira et al. (51) stated that young animals have higher nutrient requirements and the intestinal mucosa recovers rapidly during refeeding. Dou et al. (52) reported that duodenal morphometric changes that occurred in response to starvation were normalized by refeeding on the second day. This could explain our finding of increased intestinal morphological parameters in restricted rabbits as they were freely fed in the first day then they were restricted in the next day (fed at the level of 93 % of *ad libitum* fed the day before), then again *ad libitum* followed by restriction.

Izadi et al. (53) reported that the increased intestinal absorptive capacity was associated with longer intestinal villi, and consequently beneficial effects on growth performance. This finding was shown in the present study as ZnO-NPs supplementation at the lower dose (15 mg/L) increased the intestinal villi length as well as deeper crypt, which was reflected by increased rabbits' final weight in the freely fed rabbits. In the same direction, El-Katcha et al. (33) reported that nano- Zn addition to the broiler diet increased the jejunal villi length. Increasing the Zn dose to 30 mg/L had a negative impact as it reduced the intestinal villi length in the freely fed rabbits or showed non-significant changes in the restricted rabbits when compared with the lower dose of ZnO-NPs (15 mg/L). These changes with the higher dose of Zn might be associated with reduced intestinal absorptive capacity and reduced body weight of rabbits obtained at the end of the experiment. Goblet cells contribute to the maintenance of the intestinal barrier by secreting mucin. In the present study, increasing ZnO-NPs levels were associated with an increased goblet cell number in the intestine, which could have a favorable protective effect on rabbit intestine. This result is in quite agreement with studies, which documented higher goblet cell numbers with Zn supplementation in mouse (54) and weaned piglets (55).

Conclusion

Under the conditions of the current experiment, it could be summarized that the feed restriction program applied in this experiment (rabbits were fed *ad libitum* for one day, then on the next day, they were fed at the level of 93 % of the intake fed the day before) altered rabbit growth performance (final body weight and weight gain with no differences in feed conversion ratio), improved the ileum morphology while had no effect on caecal fermentation (VFAs profile) or microbiological parameters. ZnO-NPs supplementation in both levels (15 and 30 mg/L water) differently modulated serum lipid profile, antioxidant enzymes and MDA, VFAs profile in cecum and ileal morphology with no differences in rabbit growth performance.

Disclosure statement

No conflicts of interest, financial, or otherwise, are declared by the authors.

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Vpliv omejitve krme in dodajanja nanodelcev cinkovega oksida na rastno zmogljivost, biokemijo krvi, črevesno morfologijo in parametre cekalne fermentacije rastočih kuncev

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Izvleček: V tej študiji smo proučevali odziv rastočih kuncev na omejitev krme in dodajanje nanodelcev cinkovega oksida (ZnO-NP) v okviru uspešnosti rasti, biokemičnih parametrov v serumu, morfologije črevesja in fermentacije v slepem črevesu. Skupno 60 samcev novozelandskih kuncev je bilo naključno razdeljenih v 6 skupin: AL-0 (krmljenje *ad libitum* + sladka voda kot kontrola); AL-15 (krmljenje *ad libitum* + voda z dodatkom 15 mg/l ZnO-NP) in AL-30 (krmljenje *ad libitum* + voda z dodatkom 30 mg/l ZnO-NP). Skupine R-0, R-15 in R-30 so bile enake prvim trem, vendar z omejenim režimom krmljenja. Kunci, hranjeni *ad libitum* z dodatkom ZnO-NP (15 mg/L), so imeli največjo telesno maso brez statistično značilnih razlik v primerjavi s skupinami AL-0, AL-30 in R-0. Razmerje pretvorbe krme (FCR) se med različnimi poskusnimi skupinami ni razlikovalo ($P > 0,05$). Dodajanje ZnO-NP je vplivalo na zmanjšanje parametrov lipidnega profila v serumu, tj. encim katalaza pri R-30 in superoksid dismutaza pri AL-15 in AL-30, medtem ko se je vsebnost serumskega malondialdehida (MDA) povečala tako pri kuncih, krmljenih *ad libitum*, in kuncih z omejenim režimom krmljenja. Dajanje ZnO-NP je pri AL-30 v primerjavi s kontrolo (AL-0) povzročilo znižanje vsebnosti amonijaka v slepem črevesu ter vsebnosti posameznih hlapnih maščobnih kislin (acetata, butirata in propionata) ($P < 0,05$). Kot odgovor na omejitev krme in dodajanje ZnO-NP so se spremenili morfološki parametri ileuma (dolžina sluznice, dolžina resic in število čašastih celic).

Ključne besede: omejitev krme; rast; kunci; nanodelci Zn oksida