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Nitrate detoxification using antioxidants and probiotics in the water for rabbits¤

Detoxificación de nitrato usando antioxidantes y probióticos en el agua para conejos

Detoxificação de nitrato usando antioxidantes e probióticos na água para coelhos

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Abstract

Background: Agricultural practices increase groundwater pollution from nitrate. High nitrate intake could negatively affect animal growth. **Objective:** To determine the effects of different levels of nitrate in drinking water on digestive, liver, and kidney functions, and on water and feed intake, and to determine the ability of vitamin C, vitamin E + selenium (Se) or probiotics to overcome the effects of nitrate in New Zealand White rabbit bucks. **Methods:** Forty-two male rabbits were randomly distributed into six treatment groups: 1) control group (with no nitrate); 2) 350 ppm nitrate; 3) 700 ppm nitrate; 4) 700 ppm + 200 ppm vitamin C; 5) 700 ppm + 200 ppm vitamin E + Se; and 6) 700 ppm + 1000 ppm probiotic. Productive performance, digestive, liver, and kidney functions, and hepatic and renal histology were evaluated. **Results:** Water intake was reduced ($p<0.05$) by the 350 ppm nitrate treatment. Rabbits given 700 ppm nitrate showed lower ($p<0.05$) dry matter intake, nutrient digestibility, and increased ($p<0.05$) water and nitrate intake, as well as urea concentration, and aspartate aminotransaminase (AST) and alanine aminotransferase (ALT) activity. Vitamin C, vitamin E + Se and probiotics improved ($p<0.05$) feed intake and nutrient digestibility, and reduced ($p<0.05$) water

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and nitrate intake, urea concentration, and AST and ALT activities. **Conclusion:** Rabbits may tolerate up to 350 ppm of nitrate, but 700 ppm of nitrate negatively affect digestive, liver, and kidney functions, which are improved by vitamin C, vitamin E + Se and probiotic supplementation.

Keywords: *additives, animal nutrition, water composition and quality, water pollution, water purification.*

Resumen

Antecedentes: Las prácticas agrícolas aumentan la polución de aguas subterráneas con nitratos. Una alta ingestión de nitratos posiblemente afecta negativamente el crecimiento animal. **Objetivo:** Determinar los efectos de diferentes niveles de nitrato en el agua de bebida sobre las funciones digestivas, hepáticas y renales, ingestión de agua y alimento, y determinar la habilidad de la vitamina C, vitamina E + Selenio (Se) o probióticos en superar los efectos del nitrato en conejos Nueva Zelanda Blanco. **Métodos:** Cuarenta y dos conejos machos se distribuyeron al azar entre seis tratamientos: 1) grupo control (sin nitrato), 2) 350 ppm nitrato, 3) 700 ppm nitrato, 4) 700 ppm + 200 ppm vitamina C, 5) 700 ppm + 200 ppm vitamina E + Se, y 6) 700 ppm + 1000 ppm probiótico. Se evaluó el desempeño productivo, las funciones digestivas, hepáticas y renales, y la histología renal y hepática. **Resultados:** La ingestión de 350 ppm de nitrato disminuyó ($p<0,05$) la ingestión de agua. Los conejos que consumieron 700 ppm de nitrato presentaron menor ($p<0,05$) ingestión de materia seca y digestibilidad de nutrientes, y mayor ($p<0,05$) ingestión de agua y nitrato, concentración de urea, y mayores actividades de aspartato aminotransferasa (AST) y alanina aminotransferasa (ALT). Las vitaminas C, E + Se y el probiótico mejoraron ($p<0,05$) el consumo de alimento y la digestibilidad de nutrientes, y redujeron ($p<0,05$) la ingestión de agua y nitrato, la concentración de urea, y las actividades de AST y ALT. **Conclusión:** Los conejos pueden tolerar hasta 350 ppm de nitrato, pero 700 ppm de nitrato afectan negativamente las funciones digestivas, hepáticas y renales, las cuales mejoran con la suplementación de vitamina C, vitamina E + Se y del probiótico.

Palabras clave: *aditivos, composición y calidad del agua, nutrición animal, polución del agua, purificación del agua.*

Resumo

Antecedentes: As práticas agrícolas aumentam a contaminação de águas subterrâneas a partir de nitrato e a alta ingestão desta substância pode afetar negativamente o crescimento animal. **Objetivo:** Determinar os efeitos de diferentes níveis de nitrato na água de bebida sobre as funções digestivas, hepáticas e renais, ingestão de água e ração e determinar a habilidade da vitamina C, vitamina E + Selênio (Se) ou probiótico em sobrepor os efeitos do nitrato em coelhos Nova Zelândia Branco. **Métodos:** Quarenta e dois coelhos machos foram distribuídos ao acaso em seis tratamentos: 1) grupo controle (sem nitrato), 2) 350 ppm de nitrato, 3) 700 ppm de nitrato, 4) 700 ppm + 200 ppm de vitamina C, 5) 700 ppm + 200 ppm vitamina E + Se e 6) 700 ppm + 1000 ppm de probiótico. Foram avaliados o desempenho produtivo, as funções digestivas, hepáticas e renais além da histologia hepática e renal. **Resultados:** A ingestão de 350 ppm de nitrato diminuiu ($p<0,05$) a ingestão de água. Coelhos que receberam 700 ppm de nitrato apresentaram menor ($p<0,05$) ingestão de matéria seca, menor digestibilidade de nutrientes, e maior ($p<0,05$) ingestão de água e de nitrato, maior concentração plasmática de ureia e maiores atividades de aspartato aminotransferase (AST) e alanina aminotransferase (ALT). A adição de vitaminas C ou vitamina E + Se e probiótico melhorou ($p<0,05$) o consumo de ração, a digestibilidade de nutrientes e reduziram ($p<0,05$) a ingestão de água e de nitrato, a concentração de ureia e as atividades de AST e ALT. **Conclusão:** Coelhos podem tolerar até 350 ppm de nitrato, mas 700 ppm de nitrato afeta negativamente as funções digestivas, hepáticas e renais, as quais são melhoradas a partir da suplementação de vitamina C, vitamina E + Se ou ainda probiótico.

Palavras-chave: *aditivos, composição e qualidade da agua, nutrição animal, poluição da agua, purificação da água.*

Introduction

Agricultural practices may increase groundwater pollution from nitrate (Burrow *et al.*, 2010), and high nitrate intake adversely affects rabbit productive performance (Shehata, 2005). Ingestion of high concentrations of nitrates builds up nitrite, and the major biological effect of nitrite is its involvement

in the transformation of normal hemoglobin to methemoglobin, which is unable to transport oxygen to the tissues (Ndounla and Pulgarin, 2014). In ruminants, the rumen is well suited for nitrate reductase activity, and only 10-20% of nitrite is absorbed in the blood stream, as the bulk is rapidly metabolized by rumen microflora into ammonia (Cockburn *et al.*, 2013). However, non-ruminants are unable to carry out this conversion (Robson, 2007).

The common symptoms of chronic nitrate poisoning are a reduction in weight gain, decreased milk production, depressed appetite, and greater susceptibility to infections in cattle (Gulmezoglu *et al.*, 2010). Symptoms include a bluish color in the mucous membranes, rapid and difficult breathing, rapid pulse, tremors, staggering, collapse, and death (Hancock, 2010). In addition, nitrites react with secondary amines to form nitrosamines, many of which are carcinogenic in experimental animals (Hord *et al.*, 2009). Sharma *et al*. (2011) evaluated the effect of nitrate levels (45, 100, 200, 400, and 500 mg/L) in drinking water on the lungs of rabbits and found lungs with no congestion, inflammatory cells, and breakdown of alveoli, and normal bronchiole with concentrations of only 45 mg/L.

Sharma *et al.* (2013; 2014) reported that 200, 400, or 500 ppm of nitrate in drinking water caused cyanosis and lethargy in rabbits. Tachycardia and tachypnea preceded cyanosis and were proportional to the nitrate concentration in water. Nitrate intake at 200 and 400 ppm resulted in focal collection of lymphocytes in interstitial tissues of the kidney. Congestion of blood vessels and damage to tubular epithelium was observed with 500 ppm nitrate intake.

Shehata (2005) showed vitamin C (Vit C) efficacy in nitrate and mercury detoxification. Selim *et al.* (2008) reported antioxidant effects of vitamins E (Vit E) and Vit C. Probiotics can also convert nitrite into ammonia and, ultimately, to amino acids and proteins (Robson, 2007).

Vitamin E is a fat-soluble vitamin with antioxidant properties. Ascorbic acid, or Vit C, is a potent water-soluble antioxidant (Attia *et al.*, 2011a). Selenium (Se) is the active center of glutathione peroxidase, and this catalyzes the breakdown of hydrogen peroxide (Attia *et al.*, 2010). Moreover, dietary probiotics demonstrate antioxidant activity and protective effect against sperm damage induced by high-fat diets (Chen *et al.,* 2013).

Considering that high nitrate concentration in drinking water has negative effects on animal health and performance, and the importance of water detoxification to maintain the health and productivity of rabbits, this study was carried out to determine the effects of different levels of nitrate in drinking water on digestive, liver and kidney functions (digestibility coefficients, nutritive values), water and feed intake, and also the ability of Vit C, Vit E + Se or probiotics to overcome the effects of nitrate in New Zealand White (NZW) rabbit bucks.

Materials and methods

Animals

Forty-two, 16-week-old New Zealand White (NZW) male rabbits, with an average body weight of 2,180 ± 51 g, were randomly distributed among six treatment groups of seven rabbits each, at 16 to 61 weeks of age. The experiment was conducted at the Rabbit Research Unit, Al-Bostan, Faculty of Agriculture, Damanhour University, during the period of June to July. The rabbits were kept under the same hygienic (health and vaccination program), and environmental (27˚C temperature, 58% relative humidity, and day length) conditions during the experimental period. The light-dark cycle was 14:10 h daily.

The treatments included three main groups, in which nitrate concentrations were 0 (tap water), 350 or 700 ppm. Due to expected negative effects (Attia *et al.,* 2013), only the higher nitrate dose (700 ppm) was supplemented with vitamins and probiotics to test the effects on nitrate detoxification. Within the 700 ppm group, there were four subgroups, from which one group was used as the control and the water for the other three was supplemented with either 200 ppm Vit C (United Company for Chemicals and Medical Preparation, Cairo, Egypt), 200 ppm Vit E + 0.2 ppm of Se as sodium selenite (United Company for Chemicals and Medical Preparation, Cairo, Egypt), and 1000 ppm of a probiotic containing *Lactobacillus*

acidophilus, *Saccharomyces cerevisiae,* sodium chloride, monobasic potassium phosphate, sodium bicarbonate, and dextrose (Vetapharm Company, Cairo, Egypt). Nitrate was supplemented as sodium nitrate (Misr Company for Chemical and Medical Preparation, Cairo, Egypt). The commercial pelleted diet used was composed of barley hay, wheat bran, maize, soybean meal, dicalcium phosphate, sodium chloride, and vitamin and mineral premix. Chemical analysis of the diets on as-fed basis was conducted according to the AOAC (2004) specifications, except by the contents of NDF, ADF (Van Soest and Wine, 1967), and hemicellulose (by subtraction of ADF from NDF; Table 1).

Table 1. Chemical composition of the commercial pelleted diet.

Parameter	Level (%)
Dry matter	89.74
Organic matter	90.59
Crude protein	16.17
Crude fiber	13.71
Ether extract	2.51
Nitrogen-free extract	58.20
Ash	9.41
Neutral detergent fiber	32.31
Acid detergent fiber	15.90
Hemicellulose	16.41

The selected nitrate doses were based on the results obtained by other researchers (Gilman *et al.*, 1998; Shehata, 2005), who showed that nitrate concentration at 600-700 ppm had deleterious effects on rabbit performance. Nitrate content in the drinking water and feed was determined according to Pappenhagen (1958), and values were 14 ppm in tap water, 7 ppm in well water, 12 ppm in agricultural drainage, and 50 ppm in the feed. Nitrate intake was calculated with the determined values of feed and water, and the supplemented nitrate dose.

The rabbits were raised in an open-system rabbitry and housed in a galvanized wire cage battery (50 cm width × 40 cm depth × 45 cm height). All cages were provided with a manual feeder and clean fresh water that was continuously available through an automatic system of nipple drinkers. Each rabbit was offered a fixed amount of water (500 mL) at the same time every day. The amount consumed was calculated by subtracting the residual water from the amount offered.

Both rabbits and rations were weighed at the beginning and at the end of the experimental period to determine the body weight and ration consumption, respectively. At 32 and 60 weeks of age, a digestibility trial was performed to determine the digestibility coefficients and nutritive values using six animals per treatment. The animals were housed individually in metabolic cages that allowed collection of feces throughout the digestibility trial (5 d). Quantitative collection of feces was started at 24 h after offering the daily feed. The feces of each rabbit were collected once a day, at 9:00 am. Feed intake was recorded at the same time every morning for a 5-d collection period. All collected feces of each rabbit were mixed for homogeneity, then subsampled and stored at -18 °C until analysis. Feed samples were also collected and prepared for proximate analysis. Fecal samples were dried in a forced-ventilation oven at 60 °C for 72 h and then ground through a 1 mm screen on a Wiley grinder. Chemical analyses (dry matter, crude protein, ether extract, and crude fiber) of the feeds and feces were conducted according to AOAC (2004) specifications. The obtained mean was used to calculate the digestibility coefficient, expressed on dry matter (DM).

At 32 and 56 weeks of age, six blood samples per treatment were randomly collected from the marginal ear vein under vacuum in clean heparinized tubes for determination of biochemical constituents in plasma. Blood samples were centrifuged for 20 min at 2000 × *g* and the plasma was frozen at -18 °C for analysis. Plasma urea and creatinine concentrations were determined using commercial kits (Diamond Diagnostics, Cairo, Egypt; N.S. BIOTEC, Alexandria, Egypt), respectively. The activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) as (U/L) in plasma were determined with commercial kits produced by Pasteur Lab (Tehran, Iran). Total antioxidant capacity (TAC) was determined according to Koracevic *et al.* (2001).

Statistical analysis

Statistical analysis was carried out using one-way analysis of variance (Ferreira, 2009), with orthogonal contrasts: 0 vs 350 ppm; 0 vs 700 ppm; 350 vs 700 ppm;

750 ppm vs. Vit C + Vit E+Se + Prob; Vit C vs Vit E + Se; Vit C vs Prob; and Vit E+Se vs Prob.

Results

Neither nitrate level nor additives affected ($p>0.05$) body weight. However, compared to the control group, 350 ppm ($p<0.05$) reduced water intake and increased nitrate intake, and 700 ppm ($p<0.05$) increased water and nitrate intake. Comparing both nitrate levels, 700 ppm increased ($p<0.05$) water and nitrate intakes. For nitrate addition at 700 ppm +, all additives resulted in higher ($p<0.05$) feed intake and lower water and nitrate intakes. Water and nitrate intake were reduced ($p<0.05$) when Vit C was added compared to the Vit E + Se addition, but comparing the addition of Vit E + Se to probiotic, water and nitrate intakes were increased ($p<0.05$) due to the probiotic (Table 2).

Table 2. Effect of different nitrate levels in drinking water and the supplementation with Vit C or Vit E + Se or probiotic on productive performance, water, and nitrate intake of New Zealand White rabbit bucks during 16-61 weeks of age.

Nitrate (ppm)	Supplementation	Body weight at 61 weeks (g)	Feed intake (g/d)	Water intake (mL/d)	Nitrate intake (mg/d)
0	-	2634	103	326	8.11
350	-	2733	104	312	114
700	-	2727	95	365	256
700	Vit C	2799	105	324	229
700	Vit E + Se	2934	107	284	206
700	Probiotic	3033	108	325	232
SEM		186.7	3.76	2.45	1.52
		Orthogonal contrast – p value			
Control vs 350 ppm		0.211	0.818	0.001	0.001
Control vs 700 ppm		0.647	0.117	0.001	0.001
350 vs 700 ppm		0.358	0.074	0.001	0.001
700 vs 700 + all additives		0.764	0.005	0.001	0.001
700 Vit C vs Vit E + Se		0.156	0.736	0.001	0.001
700 + Vit C vs Probiotic		0.155	0.612	0.757	0.720
700 + Vit E + Se vs Probiotic		0.920	0.865	0.001	0.001

n = 7 rabbits per treatment. SEM: Standard error of the mean.

For both age classes, no treatment effect ($p>0.05$) was observed on ether extract digestibility. Compared to the control group, 350 ppm nitrate intake reduced ($p<0.05$) crude protein (CP) digestibility, and at 700 ppm it reduced ($p<0.05$) dry matter intake, and digestibility of CP and crude fiber (CF). Increasing nitrate levels from 350 to 700 ppm resulted in lower ($p<0.05$) digestibility of CP and CF. However, when all additives were used, DMI and digestibility of CP, CF, and DM (the latter only at 32 weeks) were increased ($p>0.05$). Supplementation with Vit E + Se reduced ($p<0.05$) CP and CF digestibility compared to supplementation with Vit C or probiotic. Compared to Vit C supplementation, probiotic addition increased CP digestibility (Table 3).

There was no effect of the treatments on creatinine level at 32 weeks of age. Nitrate addition and increasing nitrate in water from 350 ppm to 700 ppm resulted ($p<0.05$) in higher urea concentration, AST and ALT activities, and lower TAC. The 700 ppm + all additives treatment reduced ($p<0.05$) urea and creatinine concentrations, and AST and ALT activities, but increased ($p<0.05$) TAC. At 56 weeks of age, probiotic supplementation resulted in higher ($p<0.05$) creatinine levels and TAC compared to Vit C inclusion (Table 4).

Table 3. Effect of different nitrate levels in drinking water and the supplementation of Vit C or Vit E + Se or probiotic on dry matter intake and digestibility coefficients of NZW rabbit bucks at 32 and 60 weeks of age.

Nitrate (ppm)	Supplementation	DMI	Digestibility coefficient (%)				DMI	Digestibility coefficient (%)			
		(g/d)	DM	CP	EE	CF	(g/d)	DM	CP	EE	CF
		32 weeks of age					*60 weeks of age*				
0	-	110	64.4	75.4	48.6	43.8	108	60.7	71.4	44.5	39,6
350	-	101	63.3	72.7	48.6	42.0	98	69.3	68.7	44.9	38,0
700		92	60.5	63.2	45.6	33.4	88	56.4	59.3	41.7	29,6
700	Vit C	103	68.5	76.6	48.6	45.4	109	64.1	72.7	44.7	41,0
700	Vit E + Se	111	66.7	74.4	46.0	39.3	109	62.6	70.3	42.0	35,4
700	Probiotic	113	66.7	78.7	47.5	49.1	101	62.7	74.6	43.3	45,0
SEM		3.24	1.63	0.56	1.57	2.28	6.02	4.81	0.58	1.63	2,29
		Orthogonal contrasts – p value									
Control vs 350 ppm		0.083	0.622	0.005	1.000	0.581	0.278	0.230	0.008	0.876	0.617
Control vs 700 ppm		0.002	0.116	0.001	0.204	0.007	0.042	0.536	0.001	0.237	0.009
350 vs 700 ppm		0.073	0.259	0.001	0.204	0.021	0.278	0.082	0.001	0.186	0.023
700 vs 700 + all additives		0.001	0.003	0.001	0.366	0.001	0.026	0.247	0.001	0.393	0.001
700 Vit C vs Vit E + Se		0.106	0.467	0.016	0.273	0.082	1.000	0.829	0.015	0.270	0.114
700 + Vit C vs Probiotic		0.043	0.467	0.022	0.631	0.274	0.347	0.840	0.034	0.546	0.234
700 + Vit E + Se vs Probiotic		0.619	1.000	0.001	0.524	0.010	0.347	0.988	0.001	0.602	0.012

n = 3 animals per treatment. DMI: Dry matter intake; DM: Dry matter; CP: Crude protein; EE: Ether extract; CF: Crude fiber. SEM: Standard error of the mean.

Discussion

The lack of a significant effect of nitrate on body weight of rabbit bucks is because animals become adapted to the high nitrate concentrations and may be able to use a portion of nitrate as a non-protein nitrogen source. The supplements decreased nitrate intake as a result of the lower water intake and increased feed intake. The supplementation with Vit C, Vit E + Se, and probiotic reduced water intake in 11, 22, and 11%, respectively, compared to the control treatment with 700 ppm of nitrate, showing that Vit E + Se had a stronger effect than Vit C and probiotic on water and nitrate intakes. This could be due to the synergetic effects of Vit E and Se as antioxidants (Attia *et al.*, 2010). Kritas *et al.* (2008) noted that probiotic supplementation improved growth performance and decreased morbidity and/or mortality of rabbits, which could be attributed to improved gut function. Differences among the above-mentioned results could be attributed to the age of the rabbits, the type of supplementation and hygienic conditions.

The results of this study are also in agreement with those reported by Abd El-Khalek *et al.* (2008), who found that Vit C with or without Vit E significantly increased feed intake. This could partially explain the role of antioxidant agents in overcoming the negative effects of nitrate and eliminating free radicals, in agreement with the increased total antioxidant capacity in blood plasma found in the present study with Vit C, Vit E + Se or probiotic supplementation. Similar results were observed by Attia *et al.* (2011b) with probiotic supplementation, and Dokoupilova *et al.* (2007) with Vit C (200 or 400 mg/L) in drinking water supplemented with 729 mg nitrate/L in rabbits.

High nitrate intake, such as 350 ppm or 700 ppm, may inhibit the hypothalamic-pituitary-thyroid axis and contribute to reduced blood plasma concentrations of T3 and T4 (Zaki *et al.*, 2004). Resting metabolic rate and energy expenditure might be reduced by high nitrate intake, as demonstrated by Larsen *et al.* (2014) in humans, which may explain the lower DMI and consequent lower nutrient digestibility in animals

Table 4. Effect of different nitrate levels in drinking water and the supplementation of Vit C or Vit E + Se or probiotic on blood plasma constituents of NZW rabbit bucks collected at 32 and 56 weeks of age.

Nitrate (ppm)	Supplementation	Urea-N (mg/dL)	Creatinine (mg/dL)	AST (U/L)	ALT (U/L)	TAC (µMol/L)	Urea-N (mg/dL)	Creatinine (mg/dL)	AST (U/L)	ALT (U/L)	TAC (µmol/mL)
		32 weeks of age					*56 weeks of age*				
0	-	43.16	0.90	24.98	23.42	1.30	42.98	1.14	26.40	27.51	1.13
350	-	49.74	1.02	30.42	30.64	1.00	45.43	1.00	34.51	31.08	0.83
700		57.33	1.03	47.07	42.05	0.34	53.80	0.72	41.50	38.41	0.58
700	Vit C	38.70	1.30	30.45	29.12	1.57	39.80	1.32	27.31	24.38	1.32
700	Vit E + Se	40.78	1.18	29.12	25.24	1.60	39.23	1.64	26.38	21.88	1.64
700	Probiotic	39.56	1.28	26.26	25.00	1.50	39.21	1.74	27.03	23.51	1.74
SEM		0.89	0.19	1.52	1.79	0.09	1.92	0.11	2.19	2.41	0.13
		Orthogonal contrasts – p value									
Control vs 350 ppm		0.0001	0.6700	0.0187	0.0090	0.0273	0.3757	0.4085	0.0137	0.3048	0.1198
Control vs 700 ppm		0.0001	0.6360	0.0001	0.0001	0.0001	0.0004	0.0137	0.0001	0.0033	0.0062
350 vs 700 ppm		0.0001	0.9622	0.0001	0.0002	0.0001	0.0045	0.0850	0.0318	0.0400	0.1893
700 vs 700 + all additives		0.0001	0.3423	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
700 Vit C vs Vit E + Se		0.1116	0.6700	0.5431	0.1395	0.8463	0.8367	0.0557	0.7655	0.4700	0.1014
700 + Vit C vs Probiotic		0.5012	0.9433	0.0638	0.1177	0.5622	0.8320	0.0137	0.9278	0.8015	0.0338
700 + Vit E + Se vs Probiotic		0.3423	0.7223	0.1971	0.9256	0.4410	0.9952	0.5343	0.8354	0.6361	0.5975

n = 6 samples per treatment. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; TAC: Total antioxidant capacity. SEM: Atandard error of the mean.

receiving nitrate in water, mostly at 700 ppm. These results are similar to those reported by Shehata (2005), who found that addition of nitrate significantly decreased feed intake and nutrient digestibility.

Digestibility coefficients of CP and CF were reduced in animals receiving nitrate in drinking water, and this coincided with increased water intake at the highest nitrate dose by 21%. The increase in water intake at the highest nitrate level could be due to frequent micturition (Cockburn *et al.*, 2013) indicating that rabbits try to excrete the extra nitrogenous compounds through the urine. According to Cockburn *et al.* (2013), high nitrate intake results in more peristaltic movements and, consequently, less time for nutrient absorption. In addition, nitrate and nitrite may decrease the number of clonogenic cells, which initiate mucosal cell regeneration and can cause apoptosis in the intestinal crypt and atrophy of intestinal villi (Grudzinski and Law, 1998ab). However, no changes in the relative weight of the small intestine were seen. Vit C, Vit E + Se or probiotic supplementation overcame the negative effects of nitrate on feed and digestibility coefficients, improving gut function.

Although nitrate had no negative effect on organ weights, changes in liver enzymes (ALT and AST), kidney function (urea concentration) and antioxidant status (TAC) were observed. The increase of AST and ALT in the 700-ppm nitrate group indicates hepatocyte necrosis, which was confirmed by histopathology. The negative effect on kidney function was confirmed by the increase in blood plasma urea and the change in the urea/creatinine ratio, and further confirmed by histopathological examination. Similarly, Shehata (2005) found that NZW female rabbits that drank water supplemented with 729 mg nitrate/L showed higher blood urea concentrations. These findings agree with those by Sharma *et al.* (2013; 2014) who indicated that nitrate impairs liver metabolism and kidney function.

Vitamin C, Vit E + Se, and probiotics overcome the negative effect of 700 ppm nitrate on liver enzymes (ALT and AST) and kidney (urea, creatinine concentration) function, and these results agree with those reported by Selim *et al.* (2008). The improved liver and renal function due to Vit C, Vit E + Se, and probiotics coincided with increased total antioxidant capacity, showing their role as antioxidants. These boosting effects of probiotics agree with those reported by Selim *et al.* (2008), and could be attributed to the improvement in gut microbiota, which might have utilized or degraded nitrate for growth and performed nitrate detoxification. Moreover, the effects of Vit C, Vit E + Se, or probiotics on gut, liver, and kidney functions coincided with the decreasing severity of pathologic changes in the liver and kidney, supporting the hypothesis that Vit C, Vit E + Se and probiotics improve the structure and function of liver and kidney.

In conclusion, rabbits may tolerate up to 350 ppm nitrate, but 700 ppm nitrate negatively affect digestive, liver, and kidney functions, which are improved with Vit C, Vit E + Se, and probiotic supplementation.

Conflicts of interest

The authors declare they have no conflicts of interest with regard to the work presented in this report.

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