Spanish Journal of Agricultural Research
18 (3), e0612, 12 pages (2020)
eISSN: 2171-9292
https://doi.org/10.5424/sjar/2020183-16146
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)

RESEARCH ARTICLE

OPEN ACCESS

Maintaining intestinal microflora balance in heat-stressed broilers using dietary creeping wood sorrel (*Oxalis corniculata*) powder and chromium (chromium picolinate)

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Abstract

Aim of study: To determine the effect of dietary creeping wood sorrel powder (*Oxalis corniculata*) and chromium supplemented to broilers (1-42 days) exposed to heat stress, on their performance and on the intestinal and caecal microbiota.

Area of study: Ilfov, Romania

Material and methods: The feeding trial was conducted on 60, day-old Cobb 500 broilers, divided equally in two groups, homogenous in terms of body weight: 46.36 ± 2.96 g (C), 46.36 ± 2.93 g (E). Each group consisted in six replicates (5 chicks/replicate). The broilers were housed in an experimental hall at 32 °C constant temperature and 23 h light regimen. Unlike the dietary control diet (C), the experimental diet (E) was supplemented with 1% creeping wood sorrel powder and 0.2 mg chromium picolinate/kg diet. Six birds (1 per each replication) were slaughtered on days 28 and 42, and samples of caecal and intestinal content were collected for bacteriological analysis.

Main results: The dietary creeping wood sorrel powder and chromium supplements for heat-stressed broilers had no significant influence on their growth performance (1-42 d). Overall, the E diet had a beneficial effect on the balance of the caecal microflora ($p<0.05$); however, in the intestine, the E diet had a positive influence ($p<0.05$) on the balance of the intestinal microflora, only for the samples collected at 28 days.

Research highlights: Dietary creeping wood sorrel powder and chromium supplements can be an efficient tool for maintaining a proper balance of intestinal microflora of heat-stressed broilers at grower stage.

Additional key words: antioxidants; chicks; heat stress; microbiota

Abbreviations used: AA (ascorbic acid); ADFI (average daily feed intake); ADWG (average daily weight gain); BW (body weight); C (control diet); CFU (colony forming units); DM (dry matter); DW (dry weight); E (experimental diet); FCR (feed conversion ratio); ROS (reactive oxygen species); TAC (total antioxidant capacity).

Authors' contributions: Conceived and designed the experiments: RDC, TDP. Performed the experiments: TDP, CS; Analysed the data: MS, CT, AU, IV, AW. Wrote the paper: MS, RDC. Critical revision of the manuscript for important intellectual content: RDC. All authors read and approved the final article.

Citation: Saracila, M; Panaite, TD; Tabuc, C; Soica, C; Untea, A; Varzaru, I; Wojdyło, A; Criste, RD (2020). Maintaining intestinal microflora balance in heat-stressed broilers using dietary creeping wood sorrel (*Oxalis corniculata*) powder and chromium (chromium picolinate). Spanish Journal of Agricultural Research, Volume 18, Issue 3, e0612. https://doi.org/10.5424/sjar/2020183-16146

Received: 04 Dec 2019. **Accepted:** 30 Sep 2020.

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Funding agencies/institutions	Project / Grant
Romanian Ministry of Education and Research	PN 19 09 0102

Competing interests: The authors have declared that no competing interests exist.

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Introduction

Heat stress is one of the factors that affect significantly overall animal physiology, health, and productivity. In poultry, adverse effects on the metabolic status and physiological balance (Rhoads *et al.*, 2013), on the morphology and integrity of the intestinal barrier (Song *et al.*, 2014) and on performance parameters (Al-Fataftah & Abdelqader, 2014; Song *et al.*, 2014; Sarica *et al.*, 2019) rank among the main influences of the heat stress. The gastrointestinal tract is particularly sensitive to stressors, which can cause a variety of changes, including the alteration of the normal protective microbiota (Bailey *et al.*, 2004), and may allow pathogens like *Salmonella* the opportunity to bind to and colonise the intestinal epithelium (Burkholder *et al.*, 2008). Commensal intestinal bacterial populations can protect the host from colonisation by pathogens, by competing for epithelial binding sites and nutrients, by strengthening the intestinal immune response, and by producing antimicrobial bacteriocins (MacDonald & Monteleone, 2005).

During the period of heat stress, lower circulating levels of vitamins and minerals have been determined in broilers, which can be associated to lower feed intake and high water intake. Beneficial effects are generated by supplemental vitamins and minerals given to broilers reared under heat stress (Khan *et al.*, 2012), which reduce the reactions of cell oxidation. Hence, it has been reported that the negative effects of environmental stress could be prevented by the use of some phytoadditives, minerals and vitamin supplements, such as vitamin C and Cr alone or in combinations (Sahin & Sahin, 2001; Sahin & Kuçuk, 2001; Yoo *et al.*, 2016; Vlaicu *et al.*, 2017; Panaite *et al.*, 2018; Saracila *et al.*, 2018; 2019; Abd El-Hack *et al.*, 2020).

Oxalis corniculata L. (Family: *Oxalidaceace*), commonly known as creeping wood sorrel, has a wide range of biological activities (Sharma & Kumari, 2014; Siddiqui *et al.*, 2017). Native to Southern Europe, the creeping wood sorrel spread to the other continents as well. In Romania, it grows in the plain, and up to 1500 m altitude. A wide range of phytochemical compounds has been isolated from *O. corniculata*, such as flavonoids, tannins, phytosterols, polyphenols, glycosides, fatty acids (Sharma & Kumari, 2014). Siddiqui *et al.* (2017) showed that most of these compounds, found in the *Oxalis corniculata* leaves, possess antibacterial activity, the polyphenols being the most effective (Raghavendra *et al.*, 2006), and they are involved in the growth and viability of lactic acid bacteria (Viveros *et al.*, 2011; Gwiazdowska *et al.*, 2015; Brenes *et al.*, 2016). These phenolic compounds contained by *O. corniculata* show strong activity in the scavenging of free radicals (Sharangouda & Patil, 2007; Badwaik *et al.*, 2011; Sharma & Kumari, 2014; Kaur *et al.*, 2017). Given these activities, an attempt could be made to use wood sorrel as a source of antioxidant, to counteract the effects of heat stress.

Although the multiple beneficial effects of the creeping wood sorrel in traditional medicine are well established, the literature is poor in studies regarding its effect on animals. For example, in normal condition of temperature, *O. corniculata* has been reported to exert anxiolytic effects in mice (Gupta *et al.*, 2012), antioxidant and hepatoprotective potential in rats (Sreejith *et al.*, 2014). To our knowledge, there are no previous studies on broiler under heat stress.

Chromium is an essential mineral for the activation of certain oxidative enzymes and for stabilisation of proteins (Rao *et al.*, 2012), reducing lipid peroxidation and modulating the expression of stress-related nuclear transcription factors (Orhan *et al.*, 2012). Moreover, antioxidant minerals such as Cr can be used to prevent the effects of environmental stress (NRC, 1997; Toghyani *et al.*, 2012). In heat stressed-broilers, chromium supplements are part of a nutritional strategy to improve growth performance (Ghazi *et al.*, 2012), nutrient metabolism (Huang *et al.*, 2016), the immune response (Oba *et al.*, 2012), the antioxidant function and the response to stress measured as stress hormones level (Khan *et al.*, 2014).

Thus, it might be highly useful for researchers in the poultry sector and in the pharmaceutical industry to widen their knowledge in promoting plants, like creeping wood sorrel, and minerals like Cr, and its antioxidant combination usage. In this context, we conducted a feeding trial to determine the effects of the dietary creeping wood sorrel (*O. corniculata*) and chromium (as chromium picolinate) on broilers reared under heat stress (32 °C) to maintain a proper balance of the intestinal microbiota.

Material and methods

The feeding trial was conducted in an experimental hall of the National Institute for Animal Nutrition (Ilfov, Romania) according to an experimental protocol, approved (case no. 4775/02.08.2019) by the Ethics Commission of the Institute. Sixty chicks, 1 day old (unsexed), of the Cobb 500 hybrid, purchased from a local hatchery were evaluated in a completely randomised design, with two homogenous groups in terms of bodyweight: 46.36 ± 2.96 g (control diet, C), 46.36 ± 2.93 g (experimental diet, E). Each group consisted in six replicates (5 chicks/replicate). The chicks were housed randomly in three-tiered digestibility cages (5 chicks/cage), having the following dimensions: height of front =455 mm, height of back =375 mm, total depth =550 mm, height between tiers =582 mm and tilt =14%, allowing the daily recording of the feed intake and excreta. Throughout the experimental period, the environmental temperature of the experimental hall was kept constant at 32 ºC. The light regimen was adequate to broiler age, *i.e.* 23 h light/ 1 h darkness. Starting from the age of 1 day, broilers received a corn and soybean meal-based (46.8% crude protein) C diet. Compared with the C (Table 1), the E diet included an additional 1% creeping wood sorrel powder (*O. corniculata*) and 0.2 mg Cr /kg diet. The diet formulations (Table 1) were developed by using dedicated software (Hybrimin® Futter 2008, Germany), in agreement with the feeding requirements (NRC, 1994) and the nutritional requirements of the Cobb 500 hybrid (The Management Guide of Cobb 500 Hybrid, 2015).

The creeping wood sorrel material was harvested when plants were in their late vegetative stage (44.62° N, 26.12° E). All parts of the plant (leaves, stem, flowers and roots) were dried for three weeks, under shade, at ambient temperature (20 °C), finely chopped and ground

Table 1. Nutrient composition of experimental basal diets (%)

Ingredient	Starter (1-14 d)		Grower (15-28 d)		Finisher (29-42 d)	
	C	E	C	E	C	E
Corn	32.73	31.73	36.63	35.63	40.64	39.64
Wheat	20.00	20.00	20.00	20.00	20.00	20.00
Corn gluten (CP 59%) [1]	2.00	2.00	4.00	4.00	6.00	6.00
Soybean meal (CP 46.8%)	36.17	36.17	30.20	30.20	23.95	23.95
Creeping wood sorrel powder (CP 12.25%)	-	1.00	-	1.00	-	1.00
Sunflower oil	3.85	3.85	4.30	4.30	4.72	4.72
Monocalcium phosphate	1.68	1.68	1.52	1.52	1.43	1.43
Calcium carbonate	1.50	1.50	1.38	1.38	1.31	1.31
Salt	0.39	0.39	0.38	0.38	0.33	0.33
Methionine	0.33	0.33	0.25	0.25	0.21	0.21
Lysine	0.30	0.30	0.29	0.29	0.36	0.36
Choline	0.05	0.05	0.05	0.05	0.05	0.05
Vitamin-mineral premix [2]	1.00	1.00 [3]	1.00	1.00 [3]	1.00	1.00 [3]
Total	100	100	100	100	100	100
Calculated metabolizable energy, kcal/kg	3039.79		3128.99		3217.72	
	Chemical composition- calculated					
Crude protein	23.00		21.50		20.00	
Ether extractives	5.48		6.01		6.49	
Crude fibre	3.77		3.57		3.36	
Calcium	0.96		0.87		0.81	
Phosphorus	0.77		0.70		0.65	
Available phosphorus	0.48		0.43		0.41	
Lysine	1.44		1.29		1.19	
Methionine	0.69		0.61		0.57	
Tryptophan	0.25		0.22		0.19	

[1] CP: crude protein. [2] 1 kg premix contains: 1100000 IU/kg vit. A; 200000 IU/kg vit. D3; 2700 IU/kg vit. E; 300 mg/kg vit. K; 200 mg/kg Vit. B1; 400 mg/kg vit. B2; 1485 mg/kg pantothenic acid; 2700 mg/kg nicotinic acid; 300 mg/kg vit. B6; 4 mg/kg Vit. B7; 100 mg/kg vit. B9; 1.8 mg/kg vit. B12; 2000 mg/kg vit. C; 8000 mg/kg manganese; 8000 mg/kg iron; 500 mg/kg copper; 6000 mg/kg zinc; 37 mg/kg cobalt; 152 mg/kg iodine; 18 mg/kg selenium. [3] Vitamin-mineral premix +20 mg Cr picolinate/kg premix

(at about 1 mm) to obtain creeping wood sorrel powder. The drying process was made for a longer period of time at ambient temperature, as Shi (2006) showed that it ensures the maximum retention of vitamin C. The drying method used was in agreement with Hossain *et al.* (2010). The dose used in the study was in accordance with the European Food Safety Authority Panel on Dietetic Products, Nutrition and Allergies (EFSA, 2014). The chromium supplement was used in the premix as chromium picolinate (Cr $(C_6H_4NO_2)_3$) (Santa Cruz Biotechnology, CA, USA). Feed and water were provided for ad *libitum consumption.* None of the groups (C, E) had coccidiostat in the premix. All diets were fed as mash. Throughout the experimental period (1-42 days, broiler age) the following variables were monitored: body weight, BW (g); average daily feed intake, ADFI (g feed/broiler/day); average daily weight gain, ADWG (g/broiler/day) and feed conversion ratio, FCR (g feed/g gain). The individual BW was recorded on a weekly basis.

One bird from each replicate (6 birds per treatment) with BW within ± 10 g deviation in relation to the mean treatment weight, was slaughtered on days 28 and 42 by cervical dislocation, then immediately bled. After this, the gut was carefully excised, from the oesophagus to the cloaca. Intestinal and caecal contents (2 caeca per bird) were collected aseptically in sterilised plastic tubes and preserved at -20 ºC until the bacteriological tests (*Enterobacteriaceae*, *Escherichia coli*, staphylococci, lactobacilli, *Salmonella* spp.). Any digesta remaining in the two caeca was emptied by applying gentle pressure.

Feed samples were taken from each batch of compound feeds and assayed for the chemical proximate composition, by using the chemical methods specified by Regulation (CE) no. 152/2009 (Methods of sampling and analysis for the official inspection of feeds). Dry matter (ISO 6496/2001), crude protein (ISO 5983-2/2009), ether extractives (SR ISO 6492/2001), crude fibre (ISO 6865/2002) and ash (ISO 2171/2010) were determined.

The extracts of creeping wood sorrel and feed samples were obtained by adding 1 g of sample in 10 mL of 80% methanol; the samples were kept on a rotary shaker, in the dark, for 24 h. The extract obtained was centrifuged at 1500 × g for 10 min, and the supernatant was considered for analysis. The vitamin C content of the creeping wood sorrel was determined by the titrimetric method, as described by Kolniak-Ostek *et al.* (2013). In brief, 5 mL of plant extract obtained as described previously were added to 5 mL of oxalic acid (2%). The solution was titrated with 2,6-dichloroindophenol until a pink color was seen. The content of ascorbic acid (AA [mg/L]) was calculated as follows: AA= (Vi) × 63) / Vp, where V_i is the volume of 2,6-dichlorophenylindophenol used in the titration, and Vp is the volume of the sample taken for titration.

The total phenol content of creeping wood sorrel and feed samples was measured spectrophotometrically according to the Folin-Ciocalteu's method, described by Untea *et al.* (2018). The results were expressed as mg gallic acid equivalent (GAE)/g DW.

The total antioxidant capacity (TAC) of the creeping wood sorrel and feed samples was evaluated by the phosphate-molybdenum method of Prieto *et al.* (1999), based on the reduction of molybdenum(VI) to molybdenum(V) in the presence of a reducing agent (antioxidant) in the samples analytes and the further formation of a phosphate/molybdenum(V) green complex with acid pH. The results were expressed as mmol AA equivalent /kg DW and as mmol vitamin E equivalent /kg DW.

The classical medium of isolation, G.E.A.M. or Levine, was used to determine the *Enterobacteriaceae* and the *E. coli*, as described previously by Criste *et al.* (2017). The samples were first soaked in the medium with lauryl-sulphate (enrichment medium); then they were homogenised and left for 20-30 minutes at room temperature (23-24 °C). Decimal dilutions were made up to 10-5 in the medium with lauryl-sulphate. The dilutions of 10^{-2}-10^{-5} were used to seed 2 Petri dishes each per dilution, on Levine medium. The Petri dishes were incubated for 48 h at 37 °C, and the colonies were counted. *E. coli* formed characteristic colonies on this medium (dark violet with metallic shine). The other *Enterobacteriaceae* formed either dark red opaque colonies (lactic-positive species) or pale pink semi-transparent or colourless colonies (lactic-negative species). The analysis for staphylococi consists of immersing 1 g sample in 9 mL hyper-chlorinated liquid medium and innoculating in successive dilutions (10^{-3}-10^{-8}) on solid hyper-chlorinated medium. The samples were incubated at 37 °C for 24-48 h, followed by the counting of the developed colonies. The lactobacilli were determined on selective mediums (MRS broth and MRS agar), characteristic for the isolation, and by the counting of these bacteria. The *Salmonella* spp. was determined according to SR EN ISO 6579/2003/A1:2007. The colony counter Scan 300, Interscience (France) was used to determine the colony count of *Enterobacteriaceae*, *E. coli*, staphylococci, and lactobacilli. The results were expressed as log base 10 colony-forming units (CFU) per gram of caecal contents.

The complete randomised model was used to analyse the data for growth performance and intestinal and caecal microbiota. The effects of treatments were tested by analysis of variance, using the GLM procedure of the Minitab software, v. 17 (Minitab, 2015), with treatment as fixed effect according to the model $Y_i = T_i + e_i$, where Y_i is the dependent variable, T_i is the treatment, and ei is the error. When the overall F-test was significant, the differences between means were declared significant at $p<0.05$ using the test of Tukey.

Results

Table 2 shows the chemical composition of the powder of creeping wood sorrel, highlighting a rather high level of crude protein (12.25%) and crude fibre (10.64%). The

Table 2. Analysis of creeping wood sorrel powder (*Oxalis corniculata*)

Variable	Creeping wood sorrel powder (n=2)
Dry matter, %	89.26
Crude protein, % DM	12.25
Ether extractives, % DM	1.69
Crude fibre % DM	10.64
Ash, % DM	9.98
P (%) DM	0.50
Cu (mg/kg) DM	6.40
Fe (mg/kg) DM	243.68
Mn (mg/kg) DM	41.00
Zn (mg/kg) DM	92.10
Vitamin C (mg/100g DW)	11.77
Total polyphenols, mg GAE/g DW	4.96
Total antioxidant capacity, mmol AA equivalent/kg DW	31.60
Total antioxidant capacity, mmol vitamin E equivalent/kg DW	31.22

DM: dry matter; DW: dry weight; GAE: gallic acid equivalents; AA: ascorbic acid.

Table 3. Chemical composition of the compound feeds

Variable	Starter compound feed (1–14 days)		Grower compound feed (15–28 days)		Finisher compound feed (29–42 days)	
	C	E	C	E	C	E
Dry matter, %	90.03	90.16	90.29	90.23	90.51	90.45
Total polyphenols, mg GAE/g DW	1.71	2.08	1.89	1.96	1.71	2.51
Antioxidant capacity, mmol AA equivalent/kg DW	42.71	44.79	43.28	44.40	42.83	45.30
mmol vitamin E equivalent/kg DW	45.15	47.43	44.54	47.02	48.14	51.10

C: dietary control diet; E: the dietary control diet supplemented with 1% creeping wood sorrel powder and 0.2 mg chromium picolinate/kg diet; GAE: gallic acid equivalents; DW: dry weight; AA: ascorbic acid.

product also has an important concentration of polyphenols and vitamin C.

The analysis of the dietary polyphenols (Table 3), for all three growth stages, showed a higher level of total polyphenols in group E (supplemented with powder of creeping wood sorrel and Cr) than in group C (conventional diet formulation). The compound feed with creeping wood sorrel and Cr also had a higher antioxidant capacity than the conventional diet formulation.

The BW of broilers in group E was not significantly (p >0.05) different from that of those in group C (Table 4). Although not statistically significant, the ADFI (1-42 d) was numerically greater in the broilers from E when compared to C group (Table 4). There was no effect (p >0.05)

Table 4. Effects of dietary treatments on growth performance (1-42 days) of heat-stressed broiler chickens

Variable	C	E	SEM	*p*-value
BW (g)				
1	46.36	46.36	0.345	0.9975
14	446.10	463.78	5.141	0.0856
28	1212.57	1238.06	16.807	0.4524
42	1987.97	2015.47	30.589	0.7660
ADWG (g/broiler/day)				
1-14	28.56	29.82	0.259	0.3066
15-28	54.75	55.31	0.793	0.9839
29-42	55.39	55.53	2.637	0.8497
1-42	46.23	46.88	0.728	0.7660
ADFI (g feed /broiler/day)				
1-14	34.87	35.38	0.415	0.9105
15-28	77.38	79.50	1.856	0.9846
29-42	102.02	101.34	3.564	0.9948
1-42	71.42	72.07	1.900	0.9969
FCR (g feed/g gain)				
1-14	1.22	1.19	0.007	0.1703
15-28	1.42	1.44	0.006	0.3596
29-42	1.84	1.82	0.036	0.8643
1-42	1.54	1.54	0.010	0.7039

C: dietary control diet; E: the dietary control diet supplemented with 1% creeping wood sorrel powder and 0.2 mg chromium picolinate/kg diet; SEM: standard error of the means; BW: body weight; ADWG: average daily weight gain; ADFI: average daily feed intake; FCR: feed conversion ratio.

Table 5. Effects of the dietary treatments on intestinal and caecal bacterial populations (log10 CFU/g wet intestinal/caecal content)

Variable	Intestinal content				Caecal content			
	C	E	SEM	*p*-value	C	E	SEM	*p*-value
				Determination at 28 days				
Enterobacteriaceae	7.465[a]	7.415[b]	0.008	<0.0001	11.142[a]	11.119[b]	0.005	0.0005
E. coli	6.103[a]	6.070[b]	0.006	<0.0001	9.944[a]	9.875[b]	0.014	0.0003
Staphylococci	5.796[a]	5.689[b]	0.021	<0.0001	8.130[a]	7.924[b]	0.039	<0.0001
Lactobacilli	7.106[a]	7.181[b]	0.014	<0.0001	9.898[a]	10.965[b]	0.202	<0.0001
Salmonella spp.	absent	absent	NA	NA	absent	absent	NA	NA
				Determination at 42 days				
Enterobacteriaceae	7.461	7.456	0.002	0.1739	11.390[a]	11.357[b]	0.003	<0.0001
E. coli	6.140	6.142	0.003	0.5650	10.159[a]	10.126[b]	0.004	<0.0001
Staphylococci	5.852	5.848	0.003	0.7650	8.919[a]	8.738[b]	0.021	<0.0001
Lactobacilli	7.368[a]	7.381[b]	0.002	0.0002	10.991[a]	11.104[b]	0.011	<0.0001
Salmonella spp.	absent	absent	NA	NA	absent	absent	NA	NA

[a,b] Means in the same column with different superscripts differ significantly ($p<0.05$). n=6; CFU: colony forming units; C: dietary control diet; E: the dietary control diet supplemented with 1% creeping wood sorrel powder and 0.2 mg chromium picolinate/kg diet; SEM: standard error of the means; NA: non-adequate.

of diet supplementation with creeping wood sorrel powder (1%) and Cr picolinate (0.2 mg/kg diet) on FCR (1-42 days). In our study, there were no mortalities in any of the two experimental groups.

The dietary supplements of 1% creeping wood sorrel and Cr significantly influenced the *Enterobacteriaceae*, *E. coli* and staphylococci count in the intestinal and caecal content of the broilers (28 days). Thus, all the tested pathogenic bacteria colony forming units were lower ($p<0.05$) in the intestinal and caecal content of E broilers (28 days), compared to C broilers (Table 5).

At the end of the trial, the diet supplementation with 1% creeping wood sorrel and Cr had an effect against the tested pathogens only in the caecum (Table 5). Both at 28 and at 42 days, the lactobacilli populations were higher ($p<0.05$) in the intestinal and caecal content of E broilers than in C broilers (Table 5).

Discussion

The proximate composition of the plant (Table 2) revealed a significant content of fibre, which can be an important factor to be considered when establishing the inclusion of sorrel in the diets of monogastric animals. Regarding the mineral content, the creeping wood sorrel contained important levels of iron and zinc, according to Yang *et al.* (2011). These minerals are essential for broiler growth and are involved in many digestive, physiological and biosynthetic processes in the body.

The characterisation of creeping wood sorrel powder highlighted an important concentration of polyphenols and vitamin C, the results being in the same range of values reported in the scientific literature (Borah *et al.*, 2012). The chemical composition of plants depends on the geographical area and soil and climate conditions, part of the plant, drying temperature, exposure to oxygen in the air, solvent and method of extraction and analytical method (Zechmann *et al.*, 2011; Suzuki *et al.*, 2014). Both polyphenols and vitamin C can react with free radicals (Grune *et al.*, 2004; Durdun *et al.*, 2011), thereby reducing their amounts and preventing the deleterious consequences of oxidative stress in birds. Chromium is not directly capable of preventing or reducing the formation of reactive oxygen species (ROS) (Króliczewska *et al.*, 2004). If we consider that there is a relationship between insulin resistance and oxidative stress, in this situation, Cr act as indirect antioxidant, by decreasing the high insulin level and preventing glucose auto-oxidation, a reaction that generates ROS (Roussel *et al.*, 2007). The same authors showed that, in the presence of Cr, much lower amounts of insulin are required, and insulin sensitivity is improved.

The antioxidant capacity of plants, measured by the TAC method, provided information about lipophilic and hydrophilic antioxidant compounds. Research has shown that there is a strong correlation between the phenolic content and the antioxidant capacity (Aryal *et al.*, 2019). The overall antioxidant activity is not due to only one chemical compound, since the presence of non-phenolic antioxidants (vitamin C, vitamin E, carotenoids, etc) also plays an important role (Untea *et al.*, 2018). The data reported in Table 2 confirmed the valuable concentrations of vitamin C and polyphenols; thus, wood sorrel can be considered an important source of natural antioxidants. The antioxidant potential was studied, by using a different method proposed by Kumar *et al.* (2012); this showed that a concentration of 30 mg/mL methanolic extract of creeping wood sorrel was necessary to inhibit 50% of DPPH radical (IC_{50}), lower than for the AA, highlighting that the extract had a higher antioxidant power than the AA. There is a positive correlation in terms of the content of polyphenols in wood sorrel and in the supplemented feed samples (E diet). The supplementation with wood sorrel improved the polyphenol content and, thus, the antioxidant capacity of the experimental feed samples. In this regard, Hu *et al.* (2019) acknowledged that phytochemicals with antioxidant activity offer great hope as a dietary solution for heat stress in poultry.

Although there was a slight improvement in the performance variables (final BW, ADFI, ADWG) of broilers fed with the E diet, compared with those fed with the C diet, these results were not statistically assured (p>0.05). Thus, diet supplementation with Cr and wood sorrel did not affect (p>0.05) the performance of heat-stressed broilers in the trial period (1-42 days).

In contrast, Toghyani *et al.* (2012) reported 4% higher FCR in broilers exposed to high ambient temperatures (33 °C) to which diets supplemented with 1.50 mg/kg Cr-nicotinic were fed, when compared with those on diets without Cr supplementation. Subhani *et al.* (2018) conducted a study on unsexed Hubbard broilers (7-42 days), by using a dietary ethanolic extract of *O. corniculata* (250 mg/kg and 500 mg/kg respectively), alone or combined with aflatoxin B1 (350 ppb). In contrast with our study, Subhani *et al.* (2018) showed that the use of creeping wood sorrel extract (250 mg/kg and 500 mg/kg, respectively) given to broilers reared in normal environmental conditions resulted in a BW (42 days) and cumulative feed consumption (42 days) that were significantly lower than those of the broilers treated with the conventional diet. These differences can be due to the dietary form of inclusion, the inclusion level, the broiler hybrid, the rearing conditions, and to the fact that our study used a combination of creeping wood sorrel and chromium. Some studies support the synergistic action of Cr and other antioxidants in stress conditions; by reciprocally amplifying their actions, they lead to enhanced performance in poultry (Sahin *et al.*, 2001; Haq *et al.*, 2017; Al-Sultan *et al.*, 2019). Except for performance, a positive effect of the dietary combination of vitamin C and Cr was related to their potent antioxidant property against oxidative stress (Al-Sultan *et al.*, 2019). There is a relation between Cr supplementation and AA intracellular availability. It has been claimed that a higher level of glucose decreases the intracellular AA content (Seaborn *et al.*, 1994), and that Cr supplementation reduces plasma glucose concentrations in broilers. Consequently, Cr plays indirectly a role in the increase of the intracellular availability of vitamin C, by intensifying the action of insulin on the cellular uptake of glucose (Sahin *et al.*, 2002).

The data obtained in this study revealed that dietary creeping wood sorrel powder and Cr had an effect against the tested pathogens (*Enterobacteriaceae*, *E. coli,* staphylococci) in the caecum of heat- stressed broilers (Table 5). This fact is important, as heat stress is known to favour the increase of harmful bacteria over beneficial ones, with bacterial density reaching a maximum in a different section of gut intestinal tract within the first week of age (Yadav & Jha, 2019). Interestingly, only in the early stage of growth (28 days) did the supplemented diet (E) decrease significantly the CFU of *Enterobacteriaceae*, *E. coli,* staphylococci in both caecum and intestinal segment, while no effect was recorded in the intestine at 42 days (Table 5). Yadav & Jha (2019) stressed the fact that the microbiota becomes more diverse and tends to be relatively stable in older age. The early growth period is important for microbial balance and consequently for broiler health because gut intestinal health problems in broilers typically occur between the ages of 20 to 30 days (Teirlynck *et al.*, 2011). It is likely that the dose of 1% creeping wood sorrel powder was insufficient, or maybe the polyphenols did not possess bioavailability in the broilers' intestines to cause susceptibility to pathogenic bacteria at 42 days. Even though the compound shows strong antioxidant or other biological activities *in vitro*, it would have little biological activity in vivo if little or none of the compound gets to the target tissues (D'Archivio *et al.*, 2010). Caecum is the part of large intestine where the final stages of nutrient and water absorption occur, with the synthesising of certain vitamins, accumulation of residues in order to eliminate. Probably, in our case, polyphenols resulting from creeping wood sorrel had a stronger bioavailability in the caecum than in the intestine, thus reflecting in its antimicrobial effect, reducing the pathogens populations in caecum. An explanation in this regard may be that, in the small intestine, the ingested polyphenols are absorbed only in a share of 5%–10%, while the remaining unmodified polyphenols (~90%–95%) cross the intestinal tract and accumulate in the large intestine (Santhakumar *et al.*, 2018). Thus, in the present study, the different results on microflora at 42 days between caecum and intestine might be due to the degree of bioavailability of polyphenol in several segments of the gastrointestinal tract and to broiler growth stage.

Furthermore, in our study, this antibacterial effect could be enhanced by the combination of the creeping wood sorrel with Cr, which is involved in the antioxidant mechanism. Chromium as an insulin cofactor, it acts as a secondary antioxidant (Króliczewska *et al.*, 2004; Farag *et al.*, 2017; Krol *et al.*, 2017), and it also enhances the immune system of heat stressed-broilers (Dalólio *et al.*, 2018). A strong immune system leads to broiler gut health because the gastrointestinal tract is not only a site for digestion and absorption of nutrients but it also acts as a metabolic and immunological organ, maintaining the intestinal integrity and thus, preventing bacterial adherence to the mucosa (Adedokun & Olojede, 2019). *In vitro* studies showed that the extracts of different parts of *O. corniculata* are a good source of secondary metabolites demonstrating natural antioxidant and antibacterial activities (Kaur *et al.*, 2017). Other investigations related to the antimicrobial effects of creeping wood sorrel (Mohan & Pandey, 2016; Panda *et al.*, 2016; Kaur *et al.*, 2017). This is also supported by the results reported by Meghla *et al.* (2016), who revealed that the ethanolic, methanolic, and aqueous extracts of *O. corniculata* showed to be effective against the tested Gram-positive and Gram-negative bacteria. On the other hand, Criste *et al.* (2017) noticed that the use of phytoadditives known for their antioxidant and antibacterial properties (1% oregano powder or 1% rosehip powder) given to broiler chicks (14-35 d) exposed to heat stress (32 °C) depressed the pathogen bacteria from the ileum and supported the replication of the lactic acid releasing bacteria. Stress has an important role in determining the extent and type of bacteria colonisation (Hamidi *et al.*, 2019). They also showed that some of the bacteria can modulate the expression of genes in host epithelial cells, thus creating a favourable habitat for themselves, and can prevent the growth of other bacteria introduced later. This is the reason why one must make sure of gut population with beneficial bacteria, such as *Lactobacillus* species, much more so under conditions of heat stress.

Both at 28 and at 42 days, the lactobacilli populations were higher (p<0.05) in the intestinal and caecal content of E broilers than in those of C broilers (Table 5). Some reports stated that phenolic compounds can also selectively stimulate the growth of beneficial bacteria, such as *Lactobacillus* and *Bifidobacterium* and may, therefore, modulate gut microbiota (Tzonuis *et al.*, 2008; Gwiazdowska *et al.*, 2015; Lipiński *et al.*, 2017). However, the explanation of phenolic compounds selectivity for beneficial bacteria growth is still unclear. A possible explanation for the stimulatory effect of polyphenolic compounds have on bacterial growth is that some microorganisms are able to use these compounds as nutritional substrates (Ghorbani *et al.*, 2014). In the particular case of lactobacilli, these bacteria can metabolise phenolic compounds supplying energy to cells and positively affecting the bacterial metabolism (Garcia-Ruiz *et al.*, 2008). The present results showed that creeping wood sorrel had an effect mostly on lactobacilli as gram-positive bacteria rather than gram-negative bacteria. Papuc *et al.* (2017) reported that gram-positive bacteria are more resistant to the action of polyphenol than gram-negative bacteria. This is due to differences in cell wall composition; the outer hydrophilic membrane of Gram-negative bacteria is mainly composed of lipopolysaccharides (Nohynek *et al.*, 2006) and it hinders polyphenol connections to the peptidoglycan layers of these microorganisms (Cui *et al.*, 2012). Moreover, Cr can interact with gut microbiota. Feng *et al.* (2019) showed that the use of Cr as a nutritional supplement and micronutrient may provide significant protection to the gut microflora, particularly *Lactobacillus*, against some of the commonly used antibiotics. The data revealed that a combination of 1% creeping wood sorrel and Cr had a beneficial effect on the lactobacilli populations in the intestine and caecum of chickens during heat stress condition, maintaining the body's homeostasis.

In conclusion, dietary creeping wood sorrel (*O. corniculata*) and chromium supplements for broilers reared under heat stress (32 °C) had no significant influence on their growth performance (1-42 d). The inclusion of 1% creeping wood sorrel and 0.2 mg Cr picolinate/kg diet in broiler diet had a positive effect on the preservation of a proper balance of bacteria species (in caecum and intestine) only at the grower stage of the heat-stressed broiler. However, no effect was recorded on the pathogenic bacteria tested in the intestinal microflora of broiler at finisher stage.

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