Open Veterinary Journal, (2024), Vol. 14(3): 830-839
ISSN: 2226-4485 (Print)
ISSN: 2218-6050 (Online)
Original Research
DOI: *10.5455/OVJ.2024.v14.i3.10*

Submitted: 03/12/2023 Accepted: 10/02/2024 Published: 31/03/2024

The effect of dietary eugenol nano-emulsion supplementation on growth performance, serum metabolites, redox homeostasis, immunity, and pro-inflammatory responses of growing rabbits under heat stress

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Abstract

Background: Heat stress (HS) is a main abiotic stress factor for the health and welfare of animals. Recently, the use of nano-emulsion essential oils exhibited a promising approach to mitigate the detrimental impacts of abiotic and biotic stresses, ultimately contributing to the global aim of sustainable livestock production.

Aim: The current study was piloted to assess the impact of eugenol nano-emulsion (EUGN) supplementation on growth performance, serum metabolites, redox homeostasis, immune response, and pro-inflammatory reactions in growing rabbits exposed to HS.

Methods: A total of 100 male weaning rabbits aged 35 days were divided into 4 treatments. Rabbits were fed the diet with EUGN at different concentrations: 0 (control group; EUGN0), 50 (EUGN50), 100 (EUGN100), and 150 (EUGN150) mg/kg diet for 8 weeks under summer conditions.

Results: Dietary EUGN levels significantly improved ($p < 0.05$) the body weight, body weight gain, carcass weights, and improved feed conversion ratio of rabbits. EUGN supplementation significantly increased Hb, platelets, and red blood cells , while the mean corpuscular hemoglobin and eosinophils were significantly decreased compared to the control one. Compared with EUGN0 stressed rabbits, all EUGN-experimental groups had a reduction in levels of total glycerides ($p < 0.01$), uric acid, total bilirubin, direct bilirubin, and gamma-glutamyl transpeptidase ($p < 0.01$). Total antioxidant capacity and glutathione peroxidase were significantly improved by EUGN treatment when compared to the control one ($p < 0.01$), while the EUGN100 exhibited the greatest levels of catalase. Lipid peroxidation (malondialdehyde) was significantly decreased in EUGN-treated groups. All pro-inflammatory cytokines serum interleukin 4, Interleukin 1β, and tumor necrosis factor alpha were considerably decreased after dietary EUGN supplementation ($p < 0.05$). The serum concentrations of immunoglobulins (IgG and IgM) were significantly improved in rabbits of the EUGN150 group.

Conclusion: This study shows that EUGN can be used as a novel feed additive to enhance the growth performance, immune variables, and antioxidants, and reduce the inflammatory response of growing rabbits exposed to thermal stress.

Keywords: Eugenol nano-emulsion, Heat stress, Growing rabbit, Immunity, Health.

Introduction

As global warming intensifies, heat stress (HS) is rapidly becoming a global health concern for both humans and animals (Abdelnour *et al.*, 2019). In recent years, there has been a growing demand for rabbit meat as a valuable source of protein for human consumption (Siddiqui *et al.*, 2023; Zamaratskaia *et al.*, 2023). During summer periods, especially in tropical and sub-tropical areas, rabbit producers face challenges due to a significant reduction in reproductive and production capacity (Abdelnour *et al.*, 2021; Madkour *et al.*, 2021). This is primarily because rabbits (*Oryctolagus cuniculus*) are highly sensitive to HS (Oladimeji *et al.*, 2022). Due to their fur, elevated metabolic rate, and lack of sweat glands, they are exceptionally susceptible to the adverse consequences of HS (Marai *et al.*, 2001; Alagawany *et al.*, 2016, 2017; Khafaga *et al.*, 2019; Saeed *et al.*, 2019; Abdelnour *et al.*, 2020a). As a result, rabbits serve as an ideal animal model for researching the impacts of HS and developing strategies to mitigate its adverse effects. HS can trigger many physiological dysfunctions, comprising endocrine syndromes,

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electrolyte imbalance, and metabolic defects in growing rabbits (Mutwedu *et al.*, 2021; Liang *et al.*, 2022), thereby increasing animal susceptibility to certain diseases. In addition, HS induces a considerable reduction in feed intake, intestinal barrier function, nutrient digestibility, and absorption, ultimately leading to impaired growth performance in rabbits (Marai *et al.*, 2001; Madkour *et al.*, 2021; Liang *et al.*, 2022).

HS disrupts the intricate interplay between the redox and immune systems in rabbits, resulting in oxidative stress (OS), inflammation in immune organs, and subsequent impairment of their immune capabilities (El-Raghi *et al.*, 2023; Saghir *et al.*, 2023). Recently, there has been significant attention given to the use of essential oils (EOs), or their bioactive constituents encapsulated in nano-formulations as alternative supplements, particularly in organic animal farming (Pavoni *et al.*, 2019; Heydarian *et al.*, 2020). Nanoparticles possess greater solubility, surface area, and reactivity compared to bulk materials (Pavoni *et al.*, 2019; Ashour *et al.*, 2020; Swelum *et al.*, 2020; Wasef *et al.*, 2020; 2021; Abd El-Hack *et al.*, 2022), making them a promising approach to mitigate the detrimental impacts of abiotic and biotic stress, ultimately contributing to the global aim of sustainable agriculture. Multiple previous reports have indicated that the nano-emulsion of certain EOs added to animal diets can help mitigate HS and improve the immunity and health status of rabbits (El-Raghi *et al.*, 2023; Ismail *et al.*, 2023; Saghir *et al.*, 2023). In the past decade, co-innovative approaches have offered promising solutions to overcome environmental stresses and enhance livestock sustainability. In the case of eugenol, incorporating it into a nano-emulsion form represents a novel strategy to enhance its stability and bioavailability, thereby amplifying its growth-promoting and antimicrobial effects.

Eugenol (4-allyl-2-methoxyphenol) is the primary active compound found in clove EO. It has garnered significant attention in poultry farming due to its strong antioxidant, anti-inflammatory, and antibacterial properties (Zhao *et al.*, 2022). Numerous studies conducted on various poultry species have shown that eugenol acts as an appetite and digestion stimulant. It also promotes the growth of beneficial intestinal microflora, maintains the integrity of the intestinal mucosal barrier, and enhances immune capacity (Mohammadi, 2021). Eugenol nano-emulsion (EUGN) has been effectively used in broiler diets as a growth promoter and to combat coccidiosis (Youssefi *et al.*, 2023). It has also been shown to reduce the severity of *Escherichia coli* infections in broilers (Ibrahim *et al.*, 2022). These effects are likely due to EUGN's ability to enhance antioxidant capacity, strengthen immune function (Youssefi *et al.*, 2023), and reduce OS and inflammation (Mohammadi, 2021; Ibrahim *et al.*, 2022). Despite the existence of numerous studies on the use of eugenol in animal diets, its protective effects during HS conditions in growing rabbits have not been explored. Based on the aforementioned data, we hypothesize that adding EUGN to the diet would improve heat tolerance in growing rabbits kept under summer conditions, thanks to its biological activity. Therefore, this study was designed to investigate the effectiveness of EUGN supplementation on growth performance, serum metabolites, redox homeostasis, immunity, and pro-inflammatory responses in growing rabbits exposed to HS.

Material and Methods

Preparation and characterization of EUGN

Eugenol (E51791) was acquired from Sigma-Aldrich (Germany). EUGN was created using the ultrasonication emulsification technique (Rodríguez-Burneo *et al.*, 2017). Specifically, the formulation consisted of 15% (*w*/*w*) clove essential oil (CEO) or EUGN, with a mixture of Span 80 and Tween 80 surfactants at a weight ratio of 56:44%, with a hydrophilic–lipophilic balance. The remaining portion comprised MilliQ water. All ingredients were combined at room temperature (25°C) and mixed for 3 minutes on a vortex mixer to form the initial emulsion. Subsequently, the samples were subjected to ultrasonic treatment using an ultrasonic UP400S processor (Hielscher Ultrasonics GmbH, Teltow, Germany) for 10 minutes at 24 kHz and 0.8 w/cm^2. The application of ultrasonic waves facilitated a more uniform mixing of the phases and resulted in smaller droplet sizes (Modarres-Gheisari *et al.*, 2019). The particle size, zeta potential, and polydispersity index of the prepared nano-emulsion (Fig. 1) were assessed using the Nano-ZS ZEN analyzer (Malvern, UK).

Animal's management and experimental design

Growing New Zealand male rabbits (*n* = 100, average body weight 642.72 ± 02.5 g, and 35-day-old) were included in this study (*n* = 25 in each group, and each animal was considered as a replicate). The animals were randomly assigned to four groups from 35 days (weaning) until 91 days of age (slaughtering). The animals were fed basal diets with gradual levels of EUGN at 0 (EUGN0), 50 (EUGN50), 100 (EUGN100), and 150 (EUGN150) mg/kg diet. The mineral premix was mixed with the EUGN, and this mixture was then combined with the feed components. The resulting pelleted total mixed meals were given to the rabbits.

Twenty-five replicate cages were enrolled in each group of this study. The study period was conducted for 8 weeks in summer conditions (July and August months). Galvanized wire net cages, measuring size 44 cm in width, 50 cm in length, and 35 cm in height, were used to house individual rabbits. These cages were equipped with a manual feeder and an automatic drinker. The rabbits were kept in similar management, sanitary, and environmental conditions throughout the entire 56-day trial period. They had unrestricted access

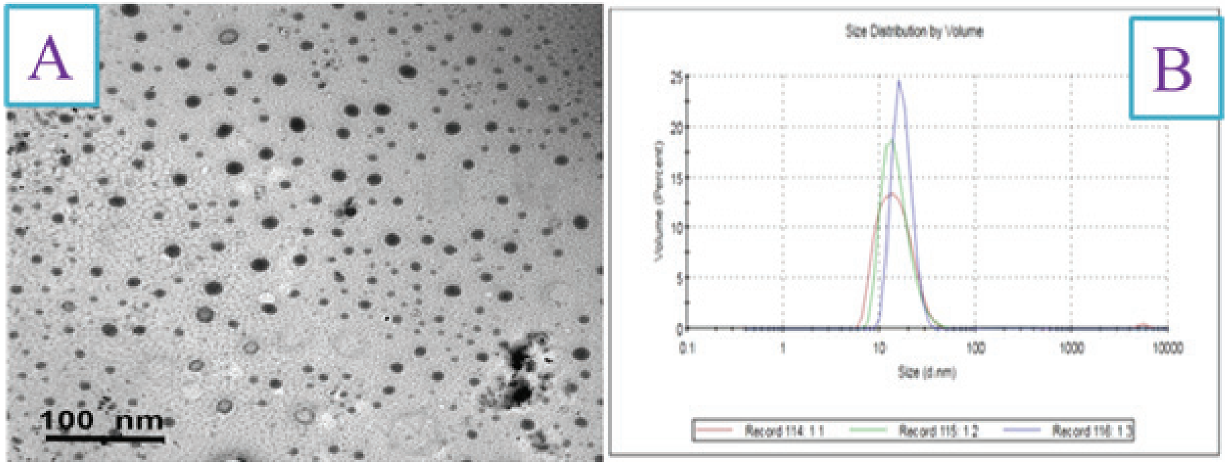

Fig. 1. The characterization of EUGN characterized by Zetasizer and transmission electron microscope (type JEOL-JEM 2100) for size distributions and TEM morphology, respectively.

to pelleted meals, and freshwater was provided through automatic nipple drinkers.

The analytical chemical composition of the diet used in this experiment consisted of crude protein (17.5%), metabolized energy (7.95 MJ/kg), crude fiber (11.36%), and calcium (0.88%). The growing animals were reared under an open house system (a naturally ventilated space with windows and ceiling fans) with natural light conditions. The average ambient temperature was 34.5°C–36.5°C, a relative humidity of 50%–55%, and the values of temperature–humidity index (THI) were 31.38–33.42 for the entire experimental period according to the equation following natural environmental condition. The THI was determined using the following formula:

$$THI = db\ °C - [0.31 - 0.31RH/100]\ (db\ °C - 14.4)$$

where db ºC stands for dry bulb temperature in Celsius and RH for relative humidity in percentage.

Growth performance and feed utilization

Measurements of growth performance [final body weight (FBW, g) and average daily gain (ADG, g/day)] and feed intake were assessed after 8 weeks of treatment. The changes in average feed intake, g/day (AFI) were also assessed. The feed conversion ratio (FCR) was determined by subtracting the average daily body weight growth from the daily feed consumption. During the experiment period, no mortality rate was recorded.

Blood sampling

At the end of the experiment and after fasting for 12 hours, six rabbits were randomly selected for collecting blood samples at 91 days of age. According to Floris *et al.* (2010) procedure, no anesthesia is required for blood samples. The samples were collected into two tubes; the first one comprised EDTA for assessing the hematology variables. The other one was kept for an hour at room temperature to allow the blood to coagulate. Then, the serum was separated by centrifuging for 15 minutes at 3,000 g for biochemical analysis.

Blood hematology

Hematological variables of growing rabbits kept under summer conditions and fed diets with gradual levels of EUGN (0, 50, 100, and 150 mg/kg diet) were assessed (n = 6, each group). The blood hematological parameters were assessed by ProCyte Dx Hematology Analyzer (Idexx Laboratories Inc., Westbrook, ME) for veterinary uses.

Serum metabolites assessments

The serum liver functions including total glycerides (TG), gamma-glutamyl transpeptidase (GGT), total protein (TP), albumin (ALB), globulins (GLU), total bilirubin (TB), and direct bilirubin (DB) were assessed. Kidney function, including uric acid and creatinine, was also assessed in the rabbit serum. All serum metabolites of growing rabbits were assessed calorimetrically using commercial kits (Biosystems S.A. Costa Brava, Barcelona, Spain).

Redox status

Total antioxidant capacity (TAC; MBS2540515), catalase (CAT; MBS1601538), glutathione peroxidase (GSH-Px) (MBS727520), and malondialdehyde (MDA; MBS739495). All commercial kits for determining the TAC, CAT, GSH-Px, and MDA were acquired from MyBioSource company following the methods of (Benzie and Strain, 1996), (Goth, 1991), (Zhang *et al.*, 2011), and (Ohkawa *et al.*, 1979), respectively.

Pro-inflammatory cytokines and immunity

Serum interleukin 4 (IL-4) concentration was quantified using a commercially available ELISA kit for rabbits (catalogue number CSB-E06902Rb; Cusabio, USA). Interleukin 1β (IL-1β) concentration in the rabbit serum was analyzed using a commercially available ELISA kit for rabbits (catalogue number

CSB-E06900Rb; Cusabio, Houston, TX). The serum concentration of tumor necrosis factor alpha (TNF-α) was quantified using a commercially available ELISA kit for rabbits (catalogue number EL RB0011; Elabscience, Houston, TX). The serum levels of immunoglobulins (Ig G and Ig M) were assessed using quantitative Sandwich ELISA kits (Ohkawa *et al.*, 1979).

Statistical analysis

A one-way analysis of variance was conducted in SPSS version 21 for Windows (SPSS, Inc., Chicago, IL) to perform statistical tests. The normal distribution of the data was confirmed, and *post hoc* Tukey honestly significant difference (HSD) multiple comparisons were then used to identify statistically significant differences among the different parameters in all experimental replicates. A p-value of less than 0.05 was considered as the threshold for statistical significance. The data were presented as means ± standard error of the mean (SEM).

Ethical approval

The Animal Welfare Ethics Committee accepted the investigational process of Tabuk University and supported it by following the endorsements of the European Group on Rabbit Nutrition (Fernández-Carmona *et al.*, 2005).

Results

Effects on growth performance and carcass traits

Heat-stressed rabbits exhibited lower FBW, ADG, higher AFI, and FCR compared to the other groups ($p < 0.05$; Table 1). The EUGN50 group did not have a significant effect on FBW and ADG compared to the EUGN100 group ($p > 0.05$). Growing rabbits fed with a diet containing 150 mg/kg of EUGN had better results in terms of FBW and ADG. All EUGN-supplemented groups had lower values of AFI and FCR compared to the EUGN0 group ($p < 0.05$). The treatment did not have any statistical effects on the weights of the kidney, liver, heart, lung, and spleen compared to the EUGN0 group ($p > 0.05$). The inclusion of dietary EUGN significantly improved carcass weights compared to the control group ($p < 0.05$). The EUGN150 group had the highest carcass weights compared to the other groups ($p < 0.05$).

Effects on blood hematology

There were non-significantly differences in the values of white blood cells (WBC) ($p = 0.18$), hematocrit ($p = 0.84$), basophils ($p = 0.81$), monocytes ($p = 0.69$), mean corpuscular volume (MCV) ($p = 0.44$), lymphocytes ($p = 0.08$), and MCHC ($p = 0.51$) among all experimental groups, as shown in Table 2. The treated groups showed higher Hb levels ($p < 0.05$) and lower MCH levels ($p = 0.01$) compared to the control group. Both the EUGN100 and EUGN150 groups had lower eosinophils ($p = 0.03$) and higher platelets ($p = 0.02$) compared to the other groups. red blood cells (RBCs) were highest in the EUGN150 group compared to the other groups ($p < 0.05$).

Table 1. Changes in the growth and carcass traits of growing rabbits fed diets with gradual levels of EUGN (0, 50, 100, and 150 mg/kg diet) for 8 weeks during summer season.

Items	EUGN (mg/kg diet)				Pooled SEM	*p*-value
	EUGN0	EUGN50	EUGN100	EUGN150		
Growth performance						
IBW, g	645.00	640.00	641.25	645.00	2.19	0.83
FBW, g	1,952.50[c]	2,017.50[b]	2,062.50[b]	2,151.25[a]	22.96	0.00
ADG, g	23.35[c]	24.60[b]	25.28[a]	25.38[a]	0.40	0.00
Feed utilization						
AFI, g	72.14[a]	67.46[b]	68.44[b]	68.57[b]	0.77	0.14
FCR, g feed/g gain	2.94[a]	2.74[b]	2.70[b]	2.68[b]	0.03	0.00
Carcass traits						
Carcass weights	1,073.75[c]	1,132.50[b]	1,155.00[b]	1,216.25[a]	15.62	0.00
Kidney	11.75	11.00	11.25	11.50	0.27	0.82
Liver	49.50	50.25	50.25	50.25	1.09	0.99
Heart	7.50	9.25	7.50	8.00	7.38	0.17
Lung	17.00	17.50	17.50	17.25	16.41	0.98
Spleen	0.47	0.51	0.49	0.51	0.45	0.90

[a, b, c] Means within a row without a common superscript letter differ at $p < 0.05$. IBW; initial body weight, FBW; final body weight, ADG, average daily gain, AFI; average feed intake, FCR; feed conversion ratio. Rabbits fed diets containing EUGN at various levels 0 (EUGN0), 50 (EUGN50), 100 (EUGN100), and 150 (EUGN150) mg/kg diet during summer season.

Table 2. Blood hematology of growing rabbits fed diets with gradual levels of EUGN (0, 50, 100, and 150 mg/kg diet) for 56 days during summer season.

Items	EUGN (mg/kg diet)				Pooled SEM	*p*-value
	EUGN0	EUGN50	EUGN100	EUGN150		
Hb	9.56[b]	12.01[a]	11.53[a]	11.88[a]	0.32	<0.001
RBCs	3.86[b]	3.35[c]	3.77[bc]	4.14[a]	0.10	0.02
WBCs	5.71	5.63	5.90	5.32	0.10	0.18
Platelets	854.67[c]	898.33[b]	935.67[a]	952.33[a]	13.46	0.02
Hematocrit, %	37.17	37.31	37.09	37.26	0.08	0.84
Basophils, %	0.27	0.27	0.28	0.28	0.00	0.81
Eosinophils, %	0.41[a]	0.40[a]	0.34[b]	0.35[b]	0.01	0.03
Lymphocytes, %	83.58	86.91	85.28	84.54	1.50	0.08
Monocytes, %	0.95	1.00	0.95	0.96	0.02	0.69
MCV	89.51	88.75	91.18	90.38	0.53	0.44
MCH	32.01[a]	28.75[b]	28.04[b]	29.55[b]	0.53	0.01
MCHC	26.67	28.10	28.41	28.31	0.43	0.51

[a, b, c] Means within a row without a common superscript letter differ at $p < 0.05$. Hemoglobin (Hb), red blood cells (RBCs), white blood cells (WBCs), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). Rabbits fed diets containing EUGN at various levels 0 (EUGN0), 50 (EUGN50), 100 (EUGN100), and 150 (EUGN150) mg/kg diet during summer season.

Table 3. Serum metabolites of growing rabbits fed diets with gradual levels of EUGN (0, 50, 100, and 150 mg/kg diet) for 56 days during summer season.

Items	EUGN (mg/kg diet)				Pooled SEM	*p*-value
	EUGN0	EUGN50	EUGN100	EUGN150		
Liver function						
TG (mg/dl)	110.00[a]	86.67[b]	90.00[b]	87.00[b]	2.99	0.000
GGT (mg/dl)	45.00[a]	34.33[b]	35.67[b]	35.33[b]	1.40	0.001
TP (mg/dl)	7.31	7.89	7.92	7.74	0.13	0.332
TB (mg/dl)	1.54[a]	1.29[b]	1.36[b]	1.42[ab]	0.03	0.021
DB (mg/dl)	1.24[a]	1.05[b]	1.08[b]	1.05[b]	0.03	0.058
ALB (mg/dl)	4.03	4.10	4.28	4.45	0.09	0.393
GLU (mg/dl)	3.28	3.80	3.64	3.30	0.12	0.376
Kidney function						
Creatinine (mg/dl)	2.85	2.92	2.88	2.89	0.01	0.313
Uric acid (mg/dl)	10.90[a]	8.95[b]	8.54[b]	10.72[ab]	0.37	0.015

[a, b, c] Means within a row without a common superscript letter differ at $p < 0.05$. Total glycerides (TG), gamma-glutamyl transferase (GGT), total protein (TP), albumin (ALB), globulins (GLU), total bilirubin (TB), and direct bilirubin (DB). Rabbits fed diets containing EUGN at various levels 0 (EUGN0), 50 (EUGN50), 100 (EUGN100), and 150 (EUGN150) mg/kg diet during summer season.

Effects on serum metabolites

Compared to EUGN0-stressed rabbits, all EUGN-experimental groups showed a reduction in TG ($p < 0.01$) and GGT ($p < 0.01$) levels (Table 3). The dietary inclusion of EUGN did not result in statistical differences ($p > 0.05$) in serum protein fractions, including TP, ALB, and GLU, although these treatments tended to increase the protein fraction in rabbits. Creatinine levels were not affected by the inclusion of EUGN in the diet ($p = 0.313$). The levels of TB, DB, and uric acid were significantly decreased in the serum of rabbits that were fed diets containing 50, 100, and 150 mg/kg of EUGN.

Table 4. Antioxidant markers, immunological variables, and pro-inflammatory response of growing rabbits fed diets with gradual levels of EUGN (0, 50, 100, and 150 mg/kg diet) for 56 days during summer season.

Items	EUGN (mg/kg diet)				Pooled SME	*p*-value
	EUGN0	EUGN50	EUGN100	EUGN150		
Redox status						
MDA (nmol/ml)	0.41[a]	0.38[b]	0.34[c]	0.33[c]	0.02	0.001
GSH-Px (U/ml)	14.46[b]	20.07[a]	22.05[a]	24.95[a]	2.36	<0.001
CAT (U/ml)	19.03[b]	20.07[b]	21.24[a]	20.18[b]	0.18	0.030
TAC (U/ml)	26.89[b]	40.95[a]	41.94[a]	41.38[a]	1.96	<0.001
Pro-inflammatory cytokines						
IL-4 (ng/ml)	75.00[a]	39.67[c]	41.00[bc]	44.33[bc]	4.41	<0.001
TNF-α (ng/ml)	85.15[a]	71.10[b]	71.64[b]	56.97[c]	3.02	<0.001
IL-1β (ng/ml)	13.51[a]	10.58[b]	10.55[b]	10.66[b]	0.41	0.001
Immunity response						
IgM (ng/ml)	50.67[b]	111.00[a]	113.00[a]	113.33[a]	8.09	<0.001
IgG (ng/ml)	134.00[b]	201.67[a]	204.00[a]	205.00[a]	9.15	<0.001

[a, b, c] Means within a row without a common superscript letter differ at $p < 0.05$. Total antioxidant capacity (TAC), catalase (CAT), glutathione peroxidase (GSH-Px), malondialdehyde (MDA), interleukin-4 (IL-4), Interleukin 1 β (IL-1 beta), interferon gamma (IFN-α). Rabbits fed diets containing EUGN at various levels 0 (EUGN0), 50 (EUGN50), 100 (EUGN100) and 150 (EUGN150) mg/kg diet during summer season.

Effects on redox status, immunological variables, and pro-inflammatory responses

TAC and GSH-Px levels were significantly improved by EUGN treatment compared to the control group ($p < 0.01$; Table 4). Among all the groups, EUGN100 showed the highest levels of CAT in rabbit serum ($p = 0.03$). Lipid peroxidation (MDA) was significantly reduced in the EUGN-treated groups. The dietary supplementation of EUGN led to a significant decrease in all pro-inflammatory cytokines IL4, IL-1β, and TNF-α ($p < 0.05$). The EUGN50 and EUGN150 groups had the lowest serum levels of IL-4 and TNF-α, respectively. The control group (EUGN0) exhibited higher levels of IL-4, IL-1β, and TNF-α compared to the treated groups ($p < 0.05$). The serum concentrations of immunoglobulins (IgG and IgG) were significantly improved in rabbits fed diets containing EUGN up to 150 mg/kg diet.

Discussion

HS often causes a decline in growth and feed efficiency and promotes immune dysfunction, leading to significant economic losses in rabbit production. The use of certain phytochemical EOs has been shown to enhance the heat resistance and productivity of rabbits greatly (El-Raghi *et al.*, 2023; Ismail *et al.*, 2023; Saghir *et al.*, 2023). In this study, we investigated the effects of dietary administration of EUGN on the growth, antioxidant-immune responses, and inflammatory responses of growing rabbits under HS. Our goal was to mitigate the negative effects of HS and fully improve the rabbits' growth performance.

The present study reveals that EUGN significantly improved growth performance (FBW, ADG), feed utilization (lower FCR), and carcass weights. It has been indicated that HS can cause shrinkage in the growth of growing rabbits. This is because it reduces the metabolism and absorption of essential nutrients necessary for growth (Liang *et al.*, 2022; Oladimeji *et al.*, 2022). In addition, this event may cause hormonal disturbance, especially growth hormone and thyroid hormones, by increasing the cortisol level in the bloodstream (Liang *et al.*, 2022). Many studies have demonstrated that HS (Ebeid *et al.*, 2023; El-Raghi *et al.*, 2023; Ismail *et al.*, 2023) reduces feed efficiency, as noticed in the current study. The use of nano-emulsion EOs has been evidenced to improve the heat resistance of growing rabbits, such as cardamom nano-emulsion (Ismail *et al.*, 2023), *Origanum majorana* nano-emulsion (El-Raghi *et al.*, 2023), and bergamot oil nano-emulsion in rabbit bucks (Saghir *et al.*, 2023). These studies indicate that nano-emulsified EOs can improve growth and feed utilization (lower FCR), as well as the health status and welfare of rabbits.

Moreover, the current findings align with the consequences stated by Abdelhadi *et al.* (2022), who demonstrated that incorporating EOs and nano-emulsified EOs (such as tea tree, pomegranate, and garlic EOs) into rabbit diets had significant effects on growth performance and feed consumption. The observed improvement in growth performance in our study can be attributed to enhanced health status and decreased inflammation induced by HS, which helps to increase the digestibility of nutrients (El-Gindy *et al.*, 2021; Li *et al.*, 2021). EOs play a crucial role in enhancing digestion by stimulating the secretion of digestive enzymes and fluids and promoting a healthier gut environment (Tiihonen *et al.*, 2010). The potential impact of EUGN on rabbit performance during thermal stress can be better understood by enhancing the availability and effectiveness of eugenol. This is achieved through deeper tissue penetration and easier cellular absorption in the gastrointestinal tract (El-Gindy *et al.*, 2021). As a result, the genes responsible for digestive enzymes are upregulated in an organized manner, leading to improved rabbit performance.

Furthermore, the positive effects of EUGN on broilers' performance persist even after exposure to *E. coli* infection in broilers (Abou-Kassem *et al.*, 2021), which can be accredited by its ability to modulate the immune response and reduce infection severity without causing antimicrobial struggle (Ibrahim *et al.*, 2022). Moreover, the supplementation of EUGN at 150 mg/kg diet was found to significantly increase the relative weight of the carcass. However, the other carcass traits did not show statistical differences among all groups.

Hematological parameters serve as reliable indicators of the overall health status, immune function, presence of infectious diseases, and environmental factors like HS in rabbits. Elevated temperatures can disrupt hematological variables, particularly the counts of WBCs (leucocytes) and RBC (erythrocytes) (Abdelnour *et al.*, 2019), rendering animals more susceptible to infections and diseases (Bashar *et al.*, 2023). Abdelnour *et al.* (2023) highlighted that HS can reduce the levels of leucocyte counts, leading to impaired immune function due to the decline in WBC counts in rabbits' blood. However, the presence of EUGN in the diet resulted in a substantial increase in erythrocyte count, including platelet and RBC counts, as well as hemoglobin (HGB) concentration. Furthermore, dietary supplementation of EUGN led to a significant decrease in WBC count, which remained within the normal range, indicating an enhanced health profile of growing rabbits subjected to elevated temperatures. These findings suggest that incorporating EUGN into rabbit diets can enhance the liver's health, which is essential for synthesizing blood proteins and other enzymes associated with heat tolerance (Ibrahim *et al.*, 2022; Zhao *et al.*, 2022; Youssefi *et al.*, 2023).

HS can cause impairment to hepatocytes, which are expressed by the increase of the liver enzymes (GGT) and accumulate the TG in the blood. GGT is an enzyme found on the outer surface of cell membranes. It plays a significant function in regulating the normal levels of cytoplasmic glutathione and protecting cells from OS (Hanigan, 2014). It achieves this by breaking down extracellular glutathione and increasing the availability of amino acids for its synthesis inside the cells. Maintaining normal levels of GGT is critical for indicating the health of the liver in animals (Abdelnour *et al.*, 2020b). HS caused an increase in GGT levels in the blood of growing rabbits. However, adding certain nano-emulsion EOs (El-Raghi *et al.*, 2023; Ismail *et al.*, 2023; Saghir *et al.*, 2023) or pigments (Abdelnour *et al.*, 2020a) can effectively reduce these elevations induced by HS. Interestingly, there were no changes in protein fractions among all groups ($p > 0.05$). Multiple authors (Floris *et al.*, 2010; El-Raghi *et al.*, 2023; Ismail *et al.*, 2023) have indicated that HS leads to a significant increase in total and indirect bilirubin, which impairs liver function during HS. In our study, we demonstrated that EUGN significantly decreased the elevated levels of direct and indirect bilirubin due to its hepatoprotective effect (Fathy *et al.*, 2022). Uric acid and creatinine are the markers of kidney function, and they were significantly elevated in the serum of animals exposed to thermal stress (Ebeid *et al.*, 2023). In our study, only the uric acid was increased in the HS group, while the EUGN can effectively reduce this elevation. Under high-temperature conditions, it is important to note that ROS were excessively produced, leading to OS and damage to vital molecules such as proteins, lipids, and DNA structures (Ebeid *et al.*, 2023). The antioxidant defensive system, consisting of enzymes such as superoxide dismutases (SOD) and GSH, usually balances the production of ROS. Previous scientific studies have shown that HS condition typically increase OS by promoting lipid oxidation and/or protein carbonylation while disrupting the production of antioxidant enzymes (Bai *et al.*, 2022; Ebeid *et al.*, 2023).

In this study, the inclusion of EUGN in the diet improved markers of antioxidant activity, including TAC, CAT, and GSH-PX. The EUGN (100 mg/kg diet) exhibited higher levels of CAT, while all EUGN-supplemented groups showed improvements in GSH-PX and TAC compared to the control group. MDA, a marker of lipid peroxidation, was found to increase in the serum of growing rabbits exposed to HS (Bai *et al.*, 2022). However, the addition of 100 or 150 mg of EUGN/kg resulted in a decrease in MDA compared to the control. This study supports previous findings that EOs can alleviate the adverse effects of HS by inhibiting oxidation reactions, delaying lipid and/or protein oxidation, and preserving the stability of other nutrients (Ebeid *et al.*, 2023; El-Raghi *et al.*, 2023). Several studies have demonstrated that including dietary nano-emulsion EOs significantly reduce MDA levels in stressed rabbits, such as cardamom oil nano-emulsion (Ismail *et al.*, 2023), nano-emulsion of *Origanum majorana* (El-Raghi *et al.*, 2023), and bergamot oil nano-emulsion (Saghir *et al.*, 2023).

Elevated ambient temperature can lead to increased levels of pro-inflammatory biomarkers, such as IL-1β, TNF-α, and IL-4, in the bloodstream, which can enhance intestinal permeability to pathogens (Ahmad *et al.*, 2022). However, our study demonstrated significant reductions in the levels of pro-inflammatory biomarkers (IL-1β, TNF-α, and IL-4) in rabbits supplemented with EUGN compared to the control group, indicating the strong anti-inflammatory properties of EUGN. The anti-inflammatory effect of EUGN can be attributed to its ability to suppress cyclooxygenase-2 expression (Kim *et al.*, 2003; Barboza *et al.*, 2018). Therefore, EUGN can regulate OS and potentially decrease key mediators involved in inflammatory processes, acting as an indirect anti-inflammatory agent (Barboza *et al.*, 2018). Recently, two studies, Ibrahim *et al.* (2022) and Youssefi *et al.* (2023) reported that EUGN has great potential in reducing pathogenic bacteria in broilers. Several research teams have presented evidence showcasing eugenol's anti-inflammatory and antioxidant properties, suggesting that it may be particularly effective in reducing inflammation (Barboza *et al.*, 2018; Ibrahim *et al.*, 2022).

It is worth noting that the rabbits supplemented with EUGN showed higher levels of IgM and IgG in their blood serum. This is consistent with the increased levels of antioxidant markers (GSH-px, CAT, and TAC), indicating that the Ig concentrations (IgG and IgM) remained within the normal range. Interestingly, dietary management resulted in a significant increase in the levels of immunoglobulins, which play a crucial role in protecting against pathogens and heat tolerance in elevated temperatures (Chauhan *et al.*, 2021; Mohammadi, 2021). Overall, the nano form of eugenol demonstrated remarkable effectiveness in improving heat tolerance in growing rabbits. This suggests that it has potential as a therapeutic agent to counteract the harmful effects of HS, possibly due to its antioxidant and anti-inflammatory properties. Further investigations are needed to understand the molecular effects of EUGN in growing rabbits fully.

Conclusion

The use of eugenol in the form of nano-emulsion (100 or 150 mg/kg diet) effectively regulated and maintained its release in the intestinal tract. This resulted in enhanced bioavailability, improved growth-promoting efficiency, and increased antioxidant and anti-inflammatory activity against the negative effects of HS. These positive effects were especially significant when EUGN was included in the diet at a concentration of 150 mg/kg. These effects are likely due to the ability of EUGN to stimulate immunity and antioxidant capabilities, ultimately contributing to maximizing the growth of rabbits in farming practices.

Acknowledgment

The authors extend their appreciation to the Deanship of Research and Graduate Studies at the University of Tabuk for funding this work through Research no.0071-1444-S.

Conflict of interest

The authors declare that there is no conflict of interest.

Funding

This research received no external funding.

Authors contribution

SSA, Conceptualization, Methodology, Formal analysis, and drafted the manuscript. HAA, and JGE, Supervision, Funding acquisition, and Writing–Review and Editing. All authors have read and agreed to the published version of the manuscript.

Data availability

Data is available upon request from the corresponding author.

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