Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/jptcp.v31i7.6692

IMMUNOMODULATORY EFFECTS OF DIETARY SUPPLEMENTED *ROSMARINUS OFFICINALIS* ESSENTIAL OILS ON CYTOKINE PROFILE OF CHICKENS CHALLENGED WITH *EIMERIA MAXIMA* AND *E. TENELLA*

Saba Badar[1*], Gulbeena Saleem[2*], Asim Aslam[3] and Kamran Ashraf[4]

[1*,2*,3]Department of Pathology, Faculty of Veterinary Sciences, University of Veterinary & Animal Sciences, Lahore, Pakistan
[4]Department of Parasitology, Faculty of Veterinary Sciences, University of Veterinary & Animal Sciences, Lahore, Pakistan

***Corresponding author:** Gulbeena Saleem, Saba Badar
*Department of Pathology, Faculty of Veterinary Sciences, University of Veterinary & Animal Sciences, Lahore, Pakistan, Email: gulbeena.saleem@uvas.edu.pk
*Department of Pathology, Faculty of Veterinary Sciences, University of Veterinary & Animal Sciences, Lahore, Pakistan, Email: sabamominjaan@gmail.com

Abstract
Chickens frequently contract the parasite disease coccidiosis, which is expensive for the industry. This study shows that immune modulatory substances like active constituents in Rosemary essential oil (REO) have a unique immune-potentiating effect on broiler chickens' immunity challenged with Eimeria maxima and Eimeria tenella. A total of 700 one-day-old broiler chicks (Ross-Male) were randomly distributed into six treatment groups (Positive control *Eimeria maxima*, Positive control *Eimeria tenella*, *E. maxima* challenged and treated with sulpha drug, *E. tenella* challenged and treated with sulpha drug and REO fed *E. maxima* challenged, REO fed *E. tenella* challenged) and one negative control group. Two groups fed with diet supplemented with 100 ppm of rosemary essential oil. Coccidiosis free status was determined twice at 14 days of age. On day 14 of age, unchallenged birds were inoculated by gavage with sporulated oocysts of genus *Eimeria tenella* and *Eimeria maxima*. Intestinal tissue samples from two birds per sampling day, from each group were collected on day of challenge, 5th, 7th and 9th day post infection (dpi). DNA extraction and Quantitative Polymerase Chain Reaction (Q-PCR) analysis was conducted for gene expression of cytokines after Eimeria challenge in the challenged and control groups. The REO groups exhibited increased gene expression of interferon (IFN), interleukin (IL), Tumor Growth Factor (TGF) and tumor necrosis factor (TNF). The REO modulated the inflammatory response against *Eimeria* spp. In the absence of the parasite, there was no stimulation of genes involved in the inflammatory response, demonstrating that the REO is an effective tool in specifically modulating the immune system of birds afflicted with coccidiosis.

Keywords: Chicken; Ceaca; Coccidiosis; Essential oil; Intestine; T-cell immunity

Introduction
The affliction identified as coccidiosis in chickens, which is brought about by the presence of protozoan parasites belonging to the genus *Eimeria*, has been observed to result in more substantial

economic ramifications compared to similar occurrences in other species of domesticated poultry. Notwithstanding the implementation of anticoccidial drugs, the subclinical coccidiosis in broiler poultry that accounts for substantial worldwide financial losses, with estimations reaching as high as US$ 750 million. The estimated annual expenditure for the prevention of coccidiosis in poultry worldwide amounts to a substantial sum of USD 300 million (Saif, 2003; Dalloul and Lillehoj., 2004; Habtamu and Gebre., 2019).

Clinical coccidiosis is most common in young birds because they have not fully developed immune systems; adults are often asymptomatic carriers (Toah *et al.,* 2021). Immune systems in broiler chicken have been the focus of recent research as increased production has necessitated an expansion of our understanding of these processes (Stojiljkovic *et al.,* 2018; Moraes *et al.,* 2019; Bremner *et al.,* 2021; Oliveira *et al.,* 2021). When confronted with a pathogen, the body deploys two primary immune response mechanisms: the innate immune system and the cell-mediated immune system. Systemic and metabolic changes are facilitated by the acute phase response, the first line of defence in the immune response includes cytokines notably Tumor necrosis factor (TNF) and Interferon gamma (IFN-γ) (Liu *et al.,* 2022). These cytokines regulate the progression of the immunological response to infection. The well-being of broiler chickens is unquestionably impacted by an inadequate immune response. Exploring cytokine expression during coccidial infection is a step in deducing the underlying immunoregulatory mechanism. Sequencing the ceacal transcriptomes of *E. tenella*-infected chickens indicated that the Cytokines Interaction Pathway was considerably enriched with IL-2, IL-4, IFγ, IL-17, and Transforming Growth Factor Beta (TGFB) in comparison to non-infected chickens (Lee *et al.,* 2022). Gene expression analyses of *Eimeria*-infected tissues revealed a function for IL-8, IL-2 and IL-17 in the progression of infection at predilection sites. There is a significant enrichment of cytokine-cytokine receptor pathway interactions among differentially expressed genes, including IL-6, IL-8, IL-10, IL-17, TNF super family (TNFSF) and TGFβ. Resistance to *E. maxima* infection in chickens was found to increase in correlation with the presence of IL-6, IL-8 and CXCLi2, while resistance to *E. tenella* infection was found to be augmented by the presence of IL-6, IL-8 (CXCLi2) and CCLi. Given that the body's defence operates primarily as a unit, it is not surprising that the interplay between various immunological components is crucial during pathogen infection (Yu *et al.,* 2020).

Supplementing poultry-diet with different herbs in several researches was found to change the in vivo expression of a family of genes involved in the innate immune response, as compared to chickens fed a normal diet (Moraes *et al.,* 2019; Ruff *et al.,* 2021). At 3rd and 5th weeks of age, birds fed Rosemary at varying concentrations showed an immunostimulant effect, with boosts in blood levels of IgM, IgG, INF-γ, and IL-10 (Moraes *et al.,* 2019; Ruff *et al.,* 2021). Thousands of REO components improve cellular and humoral immunity. Studies have shown that EO supplementation in broiler chickens, regulates immune system gene expression, suppresses pro-inflammatory cytokines, lessens inflammatory cell recruitment, and minimizes oxidative damage. To the best of our knowledge, there has been no previous exploration of the impact of REO supplementation in feed on the expression of inflammatory, proinflammatory, and regulatory cytokines in broiler chickens challenged with *Eimeria*, notably in our geographical zone. The objective of the current investigation was to explore the impact of dietary supplementation of Rosemary essential oil on the expression of intestinal and ceacal cytokines in broiler chickens that were infected with *Eimeria maxima* and *E. tenella*. The hypothesis that supplementation of REO will modify the immunological response of broiler chickens when exposed to coccidiosis was thus tested using a diet supplemented with the REO.

Materials and methods

Institutional Review Board Statement: The stringent adherence to the animal ethical and welfare regulations, as well as the institutional requirements set forth by the Advanced Studies and Research Board (ASRB) at the University of Veterinary and Animal Sciences, was diligently

maintained throughout all stages of the aforementioned procedures under letter issued 609-A Dated 16-12-21.

Management and Housing of Birds

A total of 700 day-old broiler chicks were purchased from a hatchery in Lahore, Pakistan, and raised under standard managerial protocols at the experimental facility, Department of Pathology, University of Veterinary & Animal Sciences Lahore, Pakistan. Floor dividers were employed to provide individual housing for each treatment birds. The administration of the Newcastle disease vaccination commenced on the second day through nasal spray. Starter diets given to the animals between the age of 0 and 11 days, while finisher diets were given to them from age of 12 to 23 days. The diets were designed and used as a benchmark in accordance with NRC (1994) guidelines. All birds had free access to water and feed. After starting at 33°C for the first week, the room temperature was lowered to 22°C by day 14 and subsequently maintained at that level. The deployed illumination program was a continuous 23-hour light. On the 14^{th} day of age, the chickens were distributed at random into seven groups (n=10, 5 replicates) negative control (NC), Positive *Eimeria maxima* (PCM), Positive *Eimeria tenella* (PCT), *Eimeria maxima* treated with anticoccidial drug (SM), *Eimeria tenella* treated with anticoccidial drug (ST), Rosemary fed *E. maxima* challenged group (RM), Rosemary fed *E. tenella* challenged group (RT) (n=10 in each group, one replicate except the NC). The field samples underwent meticulous scrutiny, leading to the discernment and separation of profoundly virulent species, specifically *Eimeria tenella* and *Eimeria maxima*. It is of considerable significance to acknowledge that the manifestation of *Eimeriosis* in avian livestock was primarily ascribed to the existence of *Eimeria tenella* and *Eimeria maxima* (Amin *et al.*, 2014). Since day 0, 10 birds (5 replicates) in the RT and RM groups were fed a diet enriched with 100 parts per million (ppm) of Rosemary Essential Oil (REO). Previous studies in broilers helped establish the optimal dietary supplementation level of rosemary essential oil (Gumus and Gelen, 2023). On day 14 of age, birds in 4 treatment groups and 2 controls were given experimental *Eimeria* challenge through oral gavage: 1 ml of PBS solution containing 35,000 sporulated oocytes. PBS 1ml, was given to the NC group, which served as the negative control. The absence of coccidia in chickens was confirmed by coprological examination before challenge. On day 16, anticoccidial drug was administered to SM and ST birds (Amprolium HCl: 900 gm. 1g/16 liters water, administered orally for 5 days from 2dpi to 6dpi). The entire duration of the experiment was 23 days. Intestinal tissue specimens were collected at time point 0 (the day of infection, wherein the baseline condition of tissues in uninfected and untreated avian subjects is known), as well as at 5^{th}, 7^{th}, and 9^{th} dpi. At each designated time interval, two broiler chickens were subjected to a process of humane slaughter. Subsequently, the ceacal and intestinal tissues were meticulously gathered and placed in dealt tubes designed for cryopreservation. These tubes were promptly submerged in liquid nitrogen to ensure optimal preservation. Finally, the tubes containing the tissues were transferred to a freezer set at a temperature of -80°C, where they were securely stored.

Quantitative Polymerase Chain Reaction (qPCR)

Intestinal tissue specimens were collected on 0^{th}, 5^{th}, 7^{th}, and 9^{th} dpi for Quantitative Polymerase Chain Reaction (qPCR) to determine the expression of inflammatory cytokines Interlukine-17, Interlukine-2, Interlukine-4, Interlukine-10, Interferon-γ, tumor necrosis factor superfamily-15 and transforming growth factor-beta 4 (IL-17, IL2, IL-4, IL-10, IF-γ, TNFSF-15 and TGF-β4).

TRIzol methodology to extract the total RNA

The TRIzol methodology (Chomczynski and Sacchi. 1987) was employed to extract the complete RNA from ceacal and intestinal tissues according to the guidelines provided by the manufacturer (TRIzol® Plus RNA Purification Kit, Thermo Fisher Scientific, Waltham, USA).

RNA Quantification

Subsequently, the quantification of RNA was carried out using a NanoPhotometer spectrophotometer (Nano Drop 2000, Thermo Fisher Scientific, Waltham, USA) and the integrity of the RNA was expressed based on the optical density ratio of 260 to 280 nm. The RNA was preserved at a temperature of -80°C, ensuring its stability and integrity, until the commencement of the complementary DNA (cDNA) synthesis process.

Reverse Transcriptase PCR for cDNA

The primer sequences pertaining to IFN-γ, IL-2 (cytokines associated with Th1 response), IL-17 (cytokines associated with Th17 response), IL-4, IL-10, TGF-β4, and TNFSF-15 (cytokines associated with Treg response) along with the reference genes, have been meticulously compiled and are conveniently displayed in Table 1. The synthesis of all primers was conducted by Dingguo Biotech Co., China. The total RNA was subjected to reverse transcription using the RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, USA) using cDNA synthesis. The cDNA specimens were carefully preserved at a temperature of -20°C.

Table 1 Primer sequences used in Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) for relative expression of broiler chicken genes.

[1]Genes	Primers	Accession No.	References
IL-17	Forward:5′-CTCCTCTGTTCAGACCACTGC-3′ Reverse: 5′-ATCCAGCATCTGCTTTCTTGA-3′	NM-204460.1	Yu *et al.* (2021)
IL-2	Forward: 5′-TCTGGGACCACTGTATGCTCT-3′ Reverse: 5′-ACACCAGTGGGAAACAGTATCA-3’	AF-000631	Liu *et al.* (2018)
IFN-γ	Forward: 5′-AGCTGACGGTGGACCTATTATT3’ Reverse: 5′-GGCTTTGCGCTGGATTC-3′	Y07922	Liu *et al.* (2018)
IL-4	Forward: 5′-ACCCAGGGCATCCAGAAG-3’ Reverse: 5′-CAGTGCCGGCAAGAAGTT-3′	AJ-621735	Liu *et al.* (2018)
TNFSF-15	Forward: 5′-CCTGAGTTATTCCAGCAACGCA-3′ Reverse:5′-ATCCACCAGCTTGATGTCACTAAC-3′	NM-001024578	Liu *et al.* (2018)
IL-10	Forward:5′-CGCTGTCACCGCTTCTTCA-3′ Reverse:5′-TCCCGTTCTCATCCATCTTCTC-3′	NM-001004414.2	Yu *et al.* (2021)
TGF-β4	Forward: 5′-CGGGACGGATGAGAAGAAC-3′ Reverse: 5′-CGGCCCACGTAGTAAATGAT-3′	M-31160	Liu *et al.* (2018)
GAPDH	Forward: 5′-GGTGGTGCTAAGCGTGTTAT-3′ Reverse: 5′-ACCTCTGTCATCTCTCCACA-3′	K01458	Liu *et al.* (2018)

[1]Genes analyzed Interleukin-17 (IL-17), IL-2, IL-4, IL-10, Interferon gamma (IFN-γ), Transforming Growth Factor- beta4 (TGF-β4) and Tumor necrosis factor superfamily-15 (TNFSF-15), Glyceraladehyde-3-phosphate dehydrogenase (GAPDH).

Statistical Analysis

The data for all groups were analyzed by using recorded parameter values as variables for each sampling day. The results were expressed as mean values±standard deviation. The data collected from all groups were tested with Multivariate Analysis of Variance (ANOVA) and Duncan's test using SPSS 23.0 software (International Business Machines Corporation, Armonk, NY, USA). The significance of the difference in results was tested using Duncan's test, with a P-value<0.05.

Results

IL-17

Levels of IL-17 were shown in Figure 1. On 5th dpi, expressions of IL-17 in PCT and PCM groups were significantly lower than the ST and SM groups but higher than the expressions of CN, RT and

RM groups. On 7th and 9th dpi, IL-17 values of PCT and PCM were recorded highest than the values of all other groups (P<0.05).

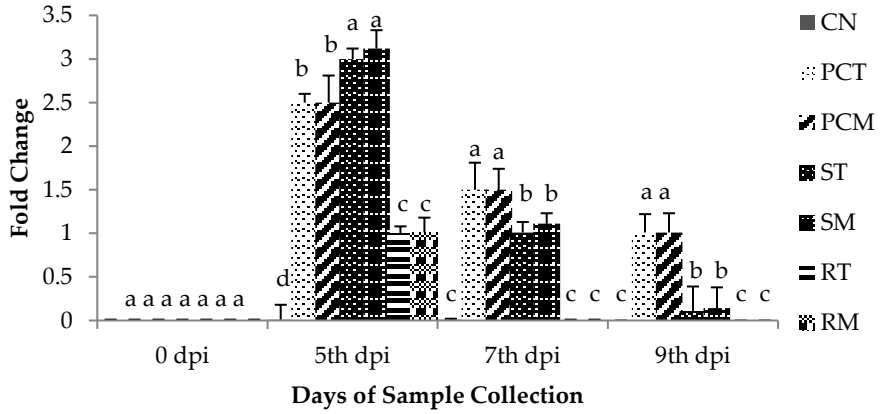

Figure1 Effects of REO and anticoccidial drug on Expressions of IL-17 of broiler chickens. a-c: different alphabets indicate significant difference (P<0.05) between treatments within the same day (dpi). CN: negative control; PCM: Positive *E. maxima*; PCT: Positive *E. tenella*; SM: *E. maxima* treated with anticoccidial drug; ST: *E. tenella* treated with anticoccidial drug; RM: Rosemary fed *E. maxima* challenged group; RT: Rosemary fed *E. tenella* challenged group. Values were expressed as mean±standard deviation (SD).

IL-2 and IFN-γ

Effects of dietary supplemented Rosemary essential oils and anticoccidial drug on expressions of IL-2 and IF-γ of broiler chickens were shown in Figure 2 and Figure 3 respectively. On 0th dpi, expressions of IL-2 and IFN-γ were similar among all groups (P>0.05). On 5th dpi, expressions of IL-2 and IFN-γ in PCT and PCM groups were lower than the expressions in RT and RM but higher than the expressions in CN, ST and SM groups. On 7th dpi, levels of IL-2 were highest in RT and RM groups; however, levels of IFN-γ were highest in PCT and PCM groups. On 9th dpi, expressions of IL-2 and IFN-γ were recorded lowest in CN, RT and RM groups than those of the PCT, PCM, ST and SM groups (P<0.05). Overall, it may be concluded that levels of both IL-2 and IFN-γ were kept higher in PCT and PCM groups on 5th, 7th and 9th dpi.

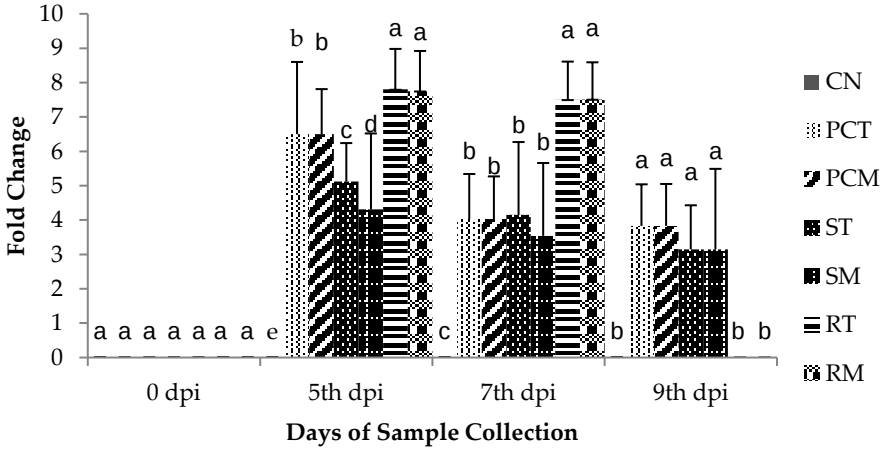

Figure 2 Effects of REO and anticoccidial drug on expressions of IL-2 of broiler chickens. a-c: different alphabets indicate significant difference (P<0.05) between treatments within the same day (dpi). CN: negative control; PCM: Positive *E. maxima*; PCT: Positive *E. tenella*; SM: *E. maxima* treated with anticoccidial drug; ST: *E. tenella* treated with anticoccidial drug;

RM: Rosemary fed *E. maxima* challenged group; RT: Rosemary fed *E. tenella* challenged group. Values were expressed as mean±standard deviation (SD).

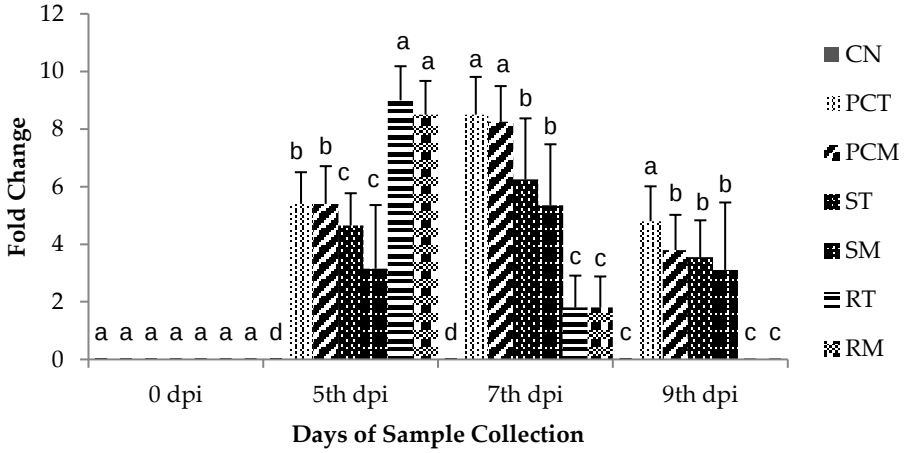

Figure 3 Effects of REO and anticoccidial drug on expressions of IFN-γ of broiler chickens. a-d: different alphabets indicate significant difference (P<0.05) between treatments within the same day (dpi). CN: negative control; PCM: Positive *E. maxima*; PCT: Positive *E. tenella*; SM: *E. maxima* treated with anticoccidial drug; ST: *E. tenella* treated with anticoccidial drug; RM: Rosemary fed *E. maxima* challenged group; RT: Rosemary fed *E. tenella* challenged group. Values were expressed as mean±standard deviation (SD).

IL-4 and TNFSF-15

Effects of dietary supplemented Rosemary essential oils and anticoccidial drug on expressions of IL-4 and TNFSF-15 of broiler chickens were shown in Figure 4 and Figure 5, respectively. On 5th dpi, expressions of IL-4 and TNFSF-15 in PCT and PCM were lower than the expressions in RT and RM, however, higher than those in the CN, ST and SM groups. On 7th and 9th dpi, expressions of IL-4 and TNFSF-15 were recorded highest in PCT and PCM as compared to the all other groups. Notably, levels of IL-4 and TNFSF-15 were recorded lowest in CN, RT and RM groups on 7th and 9th dpi.

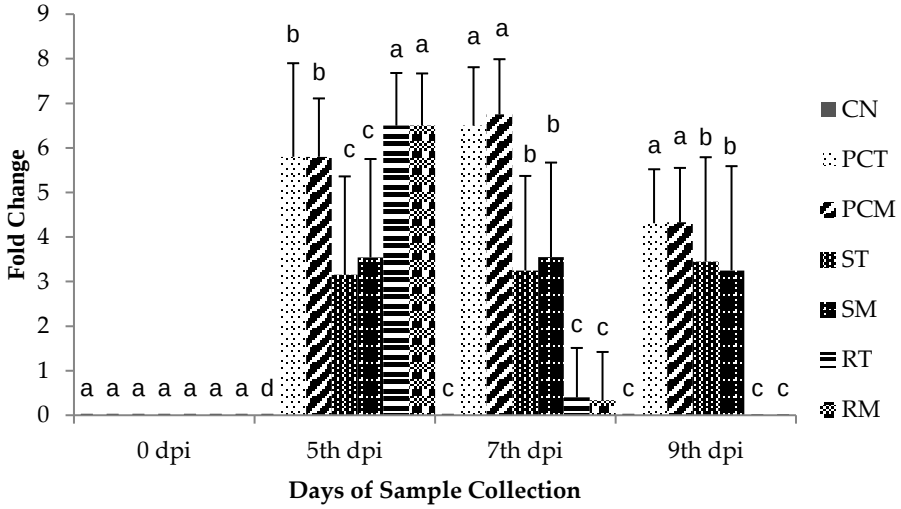

Figure 4 Effects of REO and anticoccidial drug on expressions of IL-4 of broiler chickens. a-c: different alphabets indicate significant difference (P<0.05) between treatments within the same day (dpi). CN: negative control; PCM: Positive *E. maxima*; PCT: Positive *E. tenella*; SM: *E. maxima* treated with anticoccidial drug; ST: *E. tenella* treated with anticoccidial drug;

RM: Rosemary fed *E. maxima* challenged group; RT: Rosemary fed *E. tenella* challenged group. Values were expressed as mean±standard deviation (SD).

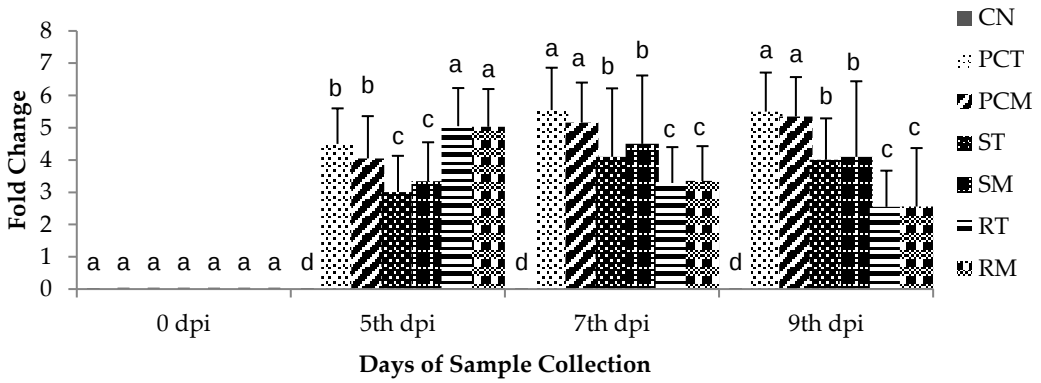

Figure 5 Effects of REO and anticoccidial drugs on expressions of TNFSF-15 of broiler chickens. a-d: different alphabets indicate significant difference (P<0.05) between treatments within the same day (dpi). CN: negative control; PCM: Positive *E. maxima*; PCT: Positive *E. tenella*; SM: *E. maxima* treated with anticoccidial drug; ST: *E. tenella*

IL-10 and TGF-β4

Effects of dietary supplemented Rosemary essential oils and anticoccidial drug on expressions of IL-10 and TGF-β4 of broiler chickens were shown in Figure 6 and Figure 7, respectively. On 5th, 7th and 9th dpi, birds from RT and RM groups presented significantly higher expressions of IL-10 and TGF-β4 as compared with the all other groups. Levels of both IL-10 and TGF-β4 were similar among PCT, PCM, ST and SM groups but higher than the levels of CN group. Levels of TGF-β4 were higher in ST and SM groups than those of the PCT and PCM groups on on 7th and 9th dpi. Overall, it can be seen from these findings that REO successfully stimulated the production of cytokines in chickens. Therefore, it can be concluded that REO helped the chickens to produce more cytokines and birds fed with REO produced significant immune response after infection.

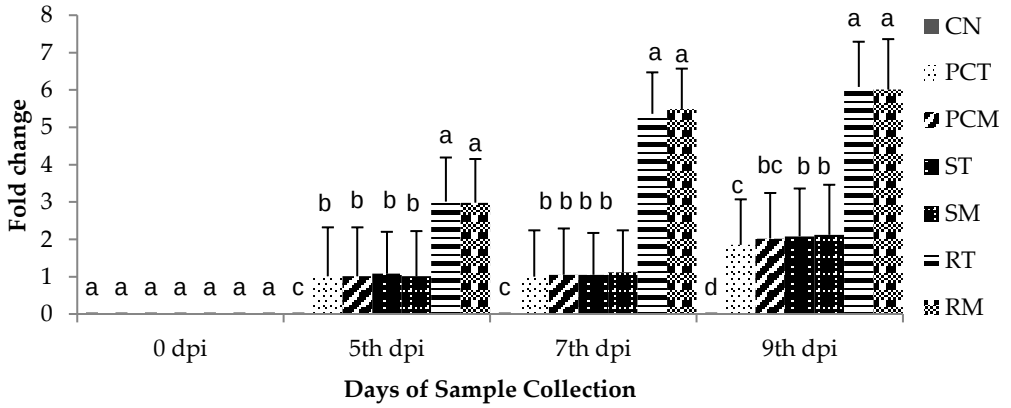

Figure 6 Effects of REO and anticoccidial drug on expressions of IL-10 of broiler chickens. a-d: different alphabets indicate significant difference (P<0.05) between treatments within the same day (dpi). CN: negative control; PCM: Positive *E. maxima*; PCT: Positive *E. tenella*; SM: *E. maxima* treated with anticoccidial drug; ST: *E. tenella* treated with anticoccidial drug; RM: Rosemary fed *E. maxima* challenged group; RT: Rosemary fed *E. tenella* challenged group. Values were expressed as mean±standard deviation (SD).

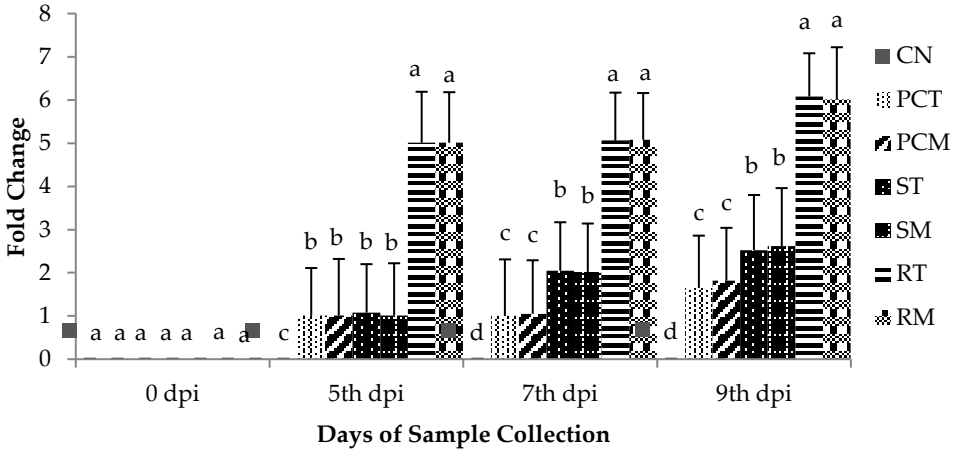

Figure 7 Effects of REO and anticoccidial drug on expressions of TGF-β4 of briler chickens. a-d: different alphabets indicate significant difference (P<0.05) between treatments within the same day (dpi). CN: negative control; PCM: Positive *E. maxima*; PCT: Positive *E. tenella*; SM: *E. maxima* treated with anticoccidial drug; ST: *E. tenella* treated with anticoccidial drug; RM: Rosemary fed *E. maxima* challenged group; RT: Rosemary fed *E. tenella* challenged group. Values were expressed as mean±standard deviation (SD).

Discussion

Coccidiosis immunity is primarily mediated by cellular immunological responses (Gabbani *et al.* 2023). Current investigation looked at the effect that organic essential oils of *Rosmarinus officinalis* had on the cellular immunological response of the body. Chicken coccidiosis is a significant enteric disease that has negative impact on growth and the immune system, ultimately leading to a high mortality rate. It has been estimated that this disease costs the poultry industry over US$3 billion each year (Blake & Tomley. 2014). The literature has reported the anti-parasitic activity of the herbal essential oils (Puvaca *et al.* 2022). Additionally, there is not plenty of documentation regarding the coccidiostat activity of terpenes found in other essential oils against *Eimeria* species in poultry (Gabbani *et al.* 2023). Due to their strong antibacterial and anti-inflammatory properties, essential oils (EOs) have been employed in the treatment of several ailments (Gharaibeh *et al.* 2021; Puvaca *et al.* 2022). Changes in immunological modulation through cytokine release are reflected in a reduction in illness incidences (Khatun *et al.* 2021). It has been demonstrated that immunological responses to *Eimeria* infection entail the participation of more than one cytokine (Nold *et al.* 2010. The detection and analysis of cytokine profiles can be used as a valuable tool to identify and characterize the specific type of immune response that is preferentially activated (Th1, Th2, T reg, Th 17) (Mucksov´a. *et al.* 2018). In terms of avian immunological response, IL-2, IL-15, IL-17 and IL-21 were shown to stimulate T-cell proliferation, IL-12 and IFN-γ improved cell-mediated immunity, IL-4 and IL-19 improved antibody-mediated immunity, and IFN-α, IFN-β, and IFN-γ showed antiviral activity (Kaiser and Stäheli. 2014). Along with the aforementioned, cytokines are crucial for the recovery of damaged tissues, especially injuries brought on by inflammation (Saleh and Zghoul. 2019).

We found considerably higher levels of IFN-γ, IL-2 and IL-4 in the RT and RM groups post infection, which suggests that the use of essential oils has had an impact on the immune system in an upbeat manner. Cytokines of the Th1 class, including IFN-γ, IL-2 and Th2 cytokine IL-4 (Giansanti *et al.* 2006) are primarily responsible for cellular immunity and predominate when an infection with *Eimeria* is evident (Shao *et al.* 2014; Saleh and Zghoul. 2019). Our findings corroborated those of Al Gabbani et al. (2023), who discovered an effective immune response and an increase in Th1 cytokines in broiler chickens that had been exposed to *E. tenella* and fed essential oil. They hypothesized that this could be due to the ability of essential oil to increase

productivity in broiler chickens and protect them from coccidiosis infection. Our results are supported by findings of Ghozlan et al. (2017) who found increased IFN-γ level after REO supplemented diet feeding to broilers.

The Th2-defining cytokine IL-4 is thought to protect birds from avian coccidiosis by stimulating a humoral immune response in the host (Liu *et al.* 2018). Our results agree with the previous findings of Gharaibeh *et al.* (2021) and Ohtsu *et al.* (2015) who found a significant increase in various cytokines including IL-4, in the birds fed with essential oils as compared to birds not fed essential oils. An elevated level of Th1-type (IFN-γ, IL-2) and Th2-type (IL-4) cytokines were released as a result of vaccine-induced immune responses, which showed to be efficient defenses against *Eimeria,* according to Liu et al. (2019). According to Annamalai and Selvaraj (2012) IL-4 possesses significant anticoccidial immune response capabilities in broiler chickens. Researchers found an increased level of anti-inflammatory cytokine IL-4 as result of plant essential oils (Zhang *et al.* 2022), agree with our results. Our findings are also supported by previous study (Liu *et al.* 2019) that demonstrated that vaccine-induced immune responses effectively protected against *Eimeria* by stimulating the production of high levels of Th1-type cytokines (IFN-γ, IL-2) (Figure 3 and 2) and Th2-type cytokine (IL-4) (Figure 4).

IL-17 is a prominent cytokine that is produced by Th17 cells, is associated with the production of cytokines, in particular IL-6, CXCL8, and GM-CSF, and it has an impact that is earlier and aggressive when there is an infection with *Eimeria* (Min *et al.,* 2013). In our study there was a slight rise in expression of IL-17 on 5^{th} dpi followed by very low values on 7^{th} and 9^{th} dpi (Figure 1). This corroborates with Yu et al. (2021) who found rise in IL-17A and F on 2^{nd}, 4^{th} dpi but negligible on 6^{th} and 8^{th} dpi. Also our study agree with the previous findings that IL-17D produced by Th17 cells is involved in the development of inflammation during protozoan infection since it was found to be much more expressed in lymphocytes of the spleen immediately following infection with *E. maxima* and *E. tenella* (Liu *et al*. 2018). Our results are also supported by the findings that *Eimeria*-infected tissues had higher levels of IL-17 implies that this cytokine may have a role in the regulation of the local immune response to coccidia (Giansanti *et al.* 2006; Khatun *et al.* 2020). Cloning of chicken IL-17 was accomplished by using an EST cDNA library that had been generated from the intestinal epithelial lining of chickens infected with *Eimeria* (Kim et al. 2014). According to the findings of the present study, feeding REO supplemented diet to broilers led to a significant increase in the expression of intestinal and ceacal IL-17 cytokine immediately after infection on day 14 of age but dropped down at 5^{th} dpi (Figure 1). After *E. maxima* and *E. tenella* infection, T-lymphocytes showed a strong rise in IL-17 expression. In the present study, IL-17 levels spiked in birds given REO supplementation ($P<0.05$) as compared to positive controls and anticoccidial treated infected birds. This agrees with the findings of previous study (Saleh and Zghoul. 2019). Our results also agree with the earlier studies that found that the upregulated IL-17 expression in chickens and mammals alike, while simultaneously downregulating IL-4 plasma levels in chickens (Giansanti *et al.* 2006; Khatun *et al.* 2020).

As shown here, IL-17 expressions was upregulated in the *Eimeria* challenged positive control birds and ST and SM birds on 5th dpi than birds fed REO ($P<0.05$). On 7^{th} and 9^{th} dpi, IL-4 and IL-17 expression in REO-fed chickens were significantly lower than in ST, SM and PCT and PCM. Pro-inflammatory cytokines such as IL-17 play important roles in innate immunity and the induction of the acute phase response (Liu *et al.* 2018). The present findings demonstrated that chickens fed REO had increased expression of pro-inflammatory cytokines in response to an *Eimeria* challenge; agree with earlier findings (Khatun *et al.* 2020).

TNF is a pro-inflammatory cytokine that regulates the inflammatory response in the early stages of *Eimeria* infection which is helpful in preventing coccidiosis (Liu *et al.* 2018). Some of these roles include protecting against parasitic invasion, blocking the formation of inflammation, and facilitating the natural repair and healing processes in the body. TNF promotes the expansion and differentiation of IL-2 and IFN-γ, which in turn results in an increase in antigen-induced B-cell activation (Liu *et al.* 2018; Khatun *et al.* 2021). In our study the birds fed with REO showed

significant increase in TNFSF-15 levels earliest on 5th dpi, it was higher than those treated with anticoccidial drug and positive control. The findings of our study (Figure 5) align with the research conducted by Miguel et al. (2021) that reported elevated levels of TNFSF-15 following the application of essential oils. The authors suggested that herbal essential oils have the potential to be effective in the prevention of inflammatory diseases. Consistent with earlier publications, our findings that REO-fed chickens showed significant increase in IL-4, IL-17, and TNFSF-15 suggest that these cytokines play crucial roles in protecting against emerging *Eimeria* infection (Mohamed *et al.* 2022).

The effects of pro- and anti-inflammatory cytokines on endothelial dysfunction have not been well studied (Shao *et al.* 2014). Additionally, prior studies shown that the pro-inflammatory signaling may be suppressed by the anti-inflammatory cytokines IL-10 and TGF-family members. The major anti-inflammatory cytokines that have been identified include IL-1Ra, IL-10, IL-11, IL-13, and TGF-β (Liu *et al.* 2018). These novel anti-inflammatory cytokines may have therapeutic benefits for treating endothelial dysfunction, although these effects are yet unknown (Frangogiannis. 2012). By dampening the inflammatory response, Treg cytokines like IL-10 and TGF- β4 may help reduce the severity of intestinal damage. The presence of elevated levels of TGF-β4 in the REO fed birds (Figure 7), may potentially contribute to the restoration of the mucous membrane that has been compromised by *Eimeria* parasites, corroborates with earlier findings (Khatun *et al.* 2020).

A variety of immune cells, including monocytes, macrophages, type 2 T helper cells (Th2), mast cells, natural killer (NK) cells, and CD4+, CD25+, Foxp3+, Regulatory T cells (Tregs), generate the anti-inflammatory cytokine IL-10. The fundamental physiological function of this is to restrict and conclude inflammatory responses, as well as control the development and growth of various immune cells (Shao *et al.* 2014). In this study, a robust rise in the transcript level of the Treg cytokines IL-10 (Figure 6) and TGF-β4 (Figure 7) was identified in REO fed birds that had been inoculated with *Eimeria maxima* and *E. tenella*, it is conceivable that this was the reason of the least intestinal damage on 5th dpi and hasted repair from of the lesions on 7th and 9th dpi, as compared to anticoccidial drug treated birds where the transcript level of these cytokines remained negligible on 5th and 7th dpi. During an infection with *Eimeria*, mice lacking the anti-inflammatory cytokine IL-10 showed an increased susceptibility to illness, which suggests that IL-10 may play a role in downregulating inflammatory responses to prevent host immunopathology (Shao *et al.* 2014). There is new evidence that suggests IL-10 is produced during coccidiosis; however, its function in the pathogenesis of the disease has not been studied (Khatun *et al.* 2020). These cytokines release from T reg cells to overcome release of pro-inflammatory cytokines and thus reduce the inflammation (Kaiser and Stäheli. 2014; Gharaibeh *et al.* 2021). Our results are supported by findings of Ghozlan et al. (2017) who found increased IL-10 level after REO supplemented diet feeding to broilers. Also Yao et al. (2023) found that REO feeding increased IL-10 levels in ducks.

The anti-inflammatory cytokine known as Transforming growth factor-sbeta4 (TGF-β4) is responsible for promoting the restoration of compromised mucosal epithelial integrity as well as downregulating inflammatory responses (Liu *et al.* 2018). After infection with *E. tenella* and *E. maxima*, the expression of TGF-β4 in birds that were fed REO supplemented feed, was significantly higher than the level of TGF-β4 in the birds that were not fed REO supplemented feed (Figure 7). This agrees with previous studies (Puvaca *et al.* 2022; Huang and Lee. 2018; Khatun *et al.* 2020). It is possible that having a high TGF-β4 level will help the mucous membrane to recover faster from damage caused by Eimeria parasites. Cytokines produced by Tregs, such as IL-10 (Figure 6) and TGF- β4 (Figure 7), may reduce intestinal damage by suppressing the inflammatory response during an *Eimeria* infection (Khatun *et al.* 2020).

According to the results of our research, the levels of cytokines were dramatically rose in the chickens fed REO supplemented feed. We can conclude that the increased level of cytokine protein caused by REO feeding may be responsible for the protection against *E. maxima* and *E. tenella* challenge. This protection may occur because the increased level of cytokine protein interferes with the invasion and damage caused by the parasite. Moreover, higher levels of anti-inflammatory and

healing and repair cytokines further reduced the damage and caused earlier repair as compared to anticoccidial drug treatment and positive controls, against both *Eimeria* species.

Conclusions

It is concluded that adding Rosemary essential oil to the diet fed to broiler chicken, had beneficial effect on immunity and the regulation of intestinal damage following infection with *Eimeria* species as it improved intestinal healing and restoration imperatively. The high levels of interferon gamma, interleukin-2, interleukin-4, and transforming growth factor-β4 shown by REO feeding all performed key roles in the immunological responses that fought off the *Eimeria* infection. In addition, REO supplementation in the diet reduced the upregulation of pro-inflammatory cytokines during *Eimeria*-induced inflammation. These findings lend support to the hypothesis that REO supplemented broiler diet improves the innate immunity. Broiler diets supplemented with REO may also be used to enhance the host immunological response of birds.

In order to gain a deeper understanding of the nature of protective immunity, it is recommended that further research may be conducted on the role that cytokines play in pre- and post- Eimeria infection. Further investigation is imperative to enhance our comprehension of T cell immunity, involved intricate molecular signaling mechanisms, the incipient stages of immune responses, and the precise mode of action exhibited by Rosemary essential oils in combatting chicken coccidiosis. This additional research will serve as a fundamental framework for the development of preventive measures against current incapacitating coccidiosis condition.

Author contributions: Conceptualization, S.B., G.S. plotted the basic idea of the work; methodology, software, validation, S.B. conducted research, compiled results and written the manuscript; formal analysis, G.S., A.A. supervised the research, reviewed the manuscript; investigation, S.B., G.S., A.A. and K.A. supervised the research work and guided through; resources, S.B., K.A.; writing original draft, S.B.; draft preparation, S.B.; writing review and editing, G.S.; supervision, G.S., A.A.

References

1. Bremner A, Kim S, Morris KM, Nolan MJ, Borowska D, Wu Z, Tomley F, Blake DP, Hawken R, Kaiser P and Vervelde L. 2021. Kinetics of the Cellular and Transcriptomic Response to Eimeriamaxima in Relatively Resistant and Susceptible Chicken Lines. Front. Immunol. 12:653085. doi: 10.3389/fimmu.2021.653085
2. Chengli Wang, Hailiang Yu, Xiaohui Wang, Wenbin Zou, Changhao Mi, Tingyan Hu, Guojun Dai. 2020. Effect of IL-8, IL-2 and IL-15 Gene Expression in Different Tissues of Jinghai Yellow Chicken Infected by Eimeria Tenella Journal of Physics: Conference Series. doi:10.1088/1742-6596/1629/1/012022 1.
3. Chen, J., Xu, Y., Han, Q., Yao, Y., Xing, H., and Teng, X. 2019. Immunosuppression, oxidative stress, and glycometabolism disorder caused by cadmium in common carp (Cyprinus carpio L.): Application of transcriptome analysis in risk assessment of environmental contaminant cadmium. J. Hazard. Mater. 366, 386–394. doi: 10.1016/j.jhazmat.2018.12.014
4. Dalloul, R.A. and Lillehoj, H., 2004. Poultry coccidiosis: Recent developments in control measures and vaccine development. Expert review of Vaccines. 5(1): 143-163.
5. Elazab, M. A., Khalifah, A. M., Elokil, A. A., Elkomy, A. E., Rabie, M. M., Mansour, A. T., et al. 2022. Effect of dietary rosemary and ginger essential oils on the growth performance, feed utilization, meat nutritive value, blood biochemicals, and redox status of growing NZW rabbits. Animals 12:375. doi: 10.3390/ani12030375
6. Habtamu, Y., and Gebre, T., 2019. Poultry Coccidiosis and its Economic Impact: Review Article. Br. Poult. Sci. 8 (3): 76-88. DOI: 10.5829/idosi.bjps.2019.76.88.
7. Iwona Skomorucha, Ewa Sosnówka-Czajka. 2021. The effect of adding herbal extracts to drinking water on body temperature, level of thyroid hormones and H:L ratio in the blood of

broiler chickens exposed to elevated ambient temperature. Ann. Anim. Sci., 21(4) 1511–1522 DOI: 10.2478/aoas-2021-0014.

8. Jonatas Rafael de Oliveira, Samira Esteves Afonso Camargo and Luciane Dias de Oliveira. 2019. *Rosmarinus officinalis L*. (rosemary) as therapeutic and prophylactic agent. J. Biomed. Sci. 26:5. https://doi.org/10.1186/s12929-019-0499-8.
9. Juárez-Estrada MA, Gayosso-Vázquez A, Tellez-Isaias G, Alonso-Morales RA. 2021. Protective Immunity Induced by an Eimeria tenella Whole Sporozoite Vaccine Elicits Specific B-Cell Antigens. Animals (Basel). 11(5):1344. doi: 10.3390/ani11051344. PMID: 34065041; PMCID: PMC8151427.
10. Ruff, J.; Tellez, G., Jr.; Forga, A.J.; Señas-Cuesta, R.; Vuong, C.N.; Greene, E.S.; Hernandez-Velasco, X.; Uribe, Á.J.; Martínez, B.C.; Angel-Isaza, J.A.; et al., 2021. Evaluation of Three Formulations of Essential Oils in Broiler Chickens under Cyclic Heat Stress. Animals. 11, 1084. https://doi.org/10.3390/ani11041084
11. Ruojun Bai, Chunlin Yuan, Tongzhuang Wang, Li Liu, Jinxiu Li, Ying Lai, Haifei Li, Zhiwei Chen, Chunli Li, Dazhi Ke, Johji Yamahara, Ling Yao, Jian-Wei Wang. 2020. Apple pomace and rosemary extract ameliorates hepatic steatosis in fructose-fed rats: Association with enhancing fatty acid oxidation and suppressing inflammation. 1975-1986https://doi.org/10.3892/etm.2020.8910.
12. Lee, Y.; Lu, M.; Lillehoj, H.S. 2022. Coccidiosis: Recent Progress in Host Immunity and Alternatives to Antibiotic Strategies. Vaccines. 10, 215. https://doi.org/10.3390/ vaccines10020215
13. Liu Y, Li C, Huang X, Zhang X, Deng P, Jiang G and Dai Q. 2022. Dietary rosemary extract modulated gut microbiota and influenced the growth, meat quality, serum biochemistry, antioxidant, and immune capacities of broilers. Front. Microbiol. 13:1024682. doi: 10.3389/fmicb.2022.1024682.
14. Liu, Y., Yu, M., Cui, J., Du, Y., Teng, X., and Zhang, Z. 2021. Heat shock proteins took part in oxidative stress-mediated inflammatory injury via NF-kB pathway in excess manganese-treated chicken livers. Ecotoxicol. Environ. Saf. 226:112833. doi: 10.1016/j.ecoenv.2021.112833
15. Lillehoj H S, Min W, Kang D C, et al. 2001. Molecular, cellular, and functional characterization of chicken cytokines homologous to mammalian IL-15 and IL-2. Vet. Immunol. Immunopathol. 82(3):229-244.
16. Mehtap Güney, Serhat Karaca, Sibel Erdogan, Askin Kor, Cagri Kale, Sukru Onalan, Murat Demirel & Nuriye Tugba Bingol. 2021. Effects of dietary supplementation with rosemary oil on methanogenic bacteria density, blood and rumen parameters and meat quality of fattening lambs, Italian Journal of Animal Science, 20:1, 794-805, DOI: 10.1080/1828051X.2021.1906165
17. Mici´c, D., Đurovi´c, S. Riabov, P., Tomi´c, A., Šovljanski, O., Filip, S., Tosti, T., Doj˘cinovi´c, B., Božovi´c, R., Jovanovi´c, D., et al. 2021. Rosemary Essential Oils as a Promising Source of Bioactive Compounds: Chemical Composition, Thermal Properties, Biological Activity, and Gastronomical Perspectives. Foods. 10, 2734. https://doi.org/10.3390/foods 10112734.
18. Jessy Moore, Michael Yousef and Evangelia Tsiani. 2016. Anticancer Effects of Rosemary (Rosmarinus officinalis L.) Extract and Rosemary Extract Polyphenols. Nutrients. 8, 731; doi:10.3390/nu8110731.
19. Khaled M. M. Saleh and Mohammad. 2019. Effect of Acute Heat Stress on the mRNA Levels of
20. Cytokines in Broiler Chickens Subjected to Embryonic Thermal Manipulation. Animals, 9, 499; doi:10.3390/ani9080499.
21. Moraes, P.O., Andretta, I., Cardinal, K.M., Ceron, M., Vilella, L., Borille, R., Frazzon, A.P., Frazzon, J., Santin, E., and Ribeiro, A.M.L., 2019. Effect of functional oils on the immune response of broilers challenged with Eimeria spp. Animal. 13-10. 2190-2198.

22. Saif, Y.M., 2003. Disease of poultry, 11 ed.
23. Shivaramaiah, C., Barta, J.R., Hernandez-Velasco, X., Téllez, G., Hargis, B.M., 2014. Coccidiosis: recent advancements in the immunobiology of Eimeria species, preventive measures, and the importance of vaccination as a control tool against these Apicomplexan parasites. Vet Med (Auckl). 5:23-34. doi: 10.2147/VMRR.S57839. PMID: 32670843; PMCID: PMC7337151.
24. Stojiljkovic J, Trajchev M, Nakov D, et al. 2018. Antibacterial activities of rosemary essential oils and their components against pathogenic bacteria. Adv Cytol Pathol. 3(4):93-96. DOI: 10.15406/acp.2018.03.00060.
25. Tovar Acero C, Ramı´rez-Montoya J, Velasco MC, Avile´s-Vergara PA, Ricardo-Caldera D,
26. Duran-Frigola M, *et al*. 2022. IL-4, IL-10, CCL2 and TGF-β as potential biomarkers for severity in Plasmodium vivax malaria. PLoS Negl Trop Dis 16(9): e0010798. https://doi.org/10.1371/journal. pntd.0010798
27. Xu Y., Lai X., Li Z., Zhang X., Luo Q. 2018. Effect of chronic heat stress on some physiological and immunological parameters in different breed of broilers. Poultry Sci., 97: 4073–4082.
28. Zhang, J., Cui, J., Wang, Y., Lin, X., Teng, X., and Tang, Y. 2022. Complex molecular mechanism of ammonia-induced apoptosis in chicken peripheral blood lymphocytes: MiR-27b-3p, heat shock proteins, immunosuppression, death receptor pathway, and mitochondrial pathway. Ecotoxicol. Environ. Saf. 246:113471. doi: 10.1016/j.ecoenv.2022.113471.