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DIETARY INCLUSION OF SCENT LEAF MEAL (*Ocimum Gratissimum*) AFFECTS IMMUNE GENES EXPRESSION IN CHICKEN SPLEEN AT 28 AND 56DAYS

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ABSTRACT

This study was conducted to examine the effects of scent leaf meal (Ocimum gratissimum) on expression of inflammatory cytokines in the spleen of two chicken strains. A total of 150birds (75 of each strain) were randomly allotted into five dietary treatments at fifteen birds per treatment. Birds were fed diet containing varying levels of Ocimum gratissimum leaf meal. Treatment one (T1) had 0% OG, while treatment two (T2), treatment three (T3), treatment four (T4) and treatment five (T5) had 0.5% OG, 1.00% OG, 1.5% OG and 2% OG respectively. Feed and water was provided adlibitum throughout the feeding trial. Three birds were slaughtered from each treatment at day 28 and day 56, spleen samples were collected and stored using RNALater in a -20°c freezer prior to RNA extraction. Real-time qPCR was performed in 40cycles using the PowerUp SYBR Green reagent and analyzed with the $2^{-\Delta\Delta Ct}$ method. Gene expression data were subjected to two-way analysis of variance. Strain effect was significantly different (P<0.05) at both time points. All the genes studied significantly differed (P<0.05) in their expression patterns at 28 and 56days of age. Increased inclusion rate of the test ingredients significantly (P<0.05) reduced IL1β and NF-KB1, while increasing IL10 and NF-KB2. *Ocimim gratissimum* leaf meal shows promise in the regulation of inflammation in chickens and can be used to efficiently replace antibiotics in broiler production.

Contribution/Originality: This work is one of very few studies that have unraveled expression patterns of inflammatory cytokines in chickens subjected to dietary inclusion of scent leaf meal as replacement for in-feed antibiotic, and contributes to existing literature on the role of phytochemical containing plants in immunomodulation in chickens.

1. INTRODUCTION

Any direct or indirect damage of intestinal epithelial cells may cause a breakdown in gut barrier and consequentially disruption of normal mucosal immune balance that can potentially lead to uncontrolled chronic intestinal and systemic inflammation [1]. Inflammation necessitates cells to hire more cells to the centre of activity by the way of secreting inflammatory cytokines and chemokines, however, delayed inflammation can cause the

animals to lose weight due to high energy expended in the process [2]. Phytochemicals have been reported to reduce cytokine gene expression IL1β, IL6 and TNF (Pro-inflammatory cytokines) and increase IL-10 (anti-inflammatory cytokines) in LPS-activated cells [3]. Phytochemicals also induced molecular changes in NF-Kb pathways [4], therefore, applying materials possessing the ability to suppress NF-kB signaling would be promising diet supplements in poultry husbandry since NF-Kb supports the expression of different inflammatory cytokines and chemokines, inclusive of genes related to cellular transformation, inflammation, invasion, and metatastasis [5]. The expression of genes related to tissue repair and inflammation is dependent on the time, strength and the site of stimulation, hence when birds are infected, it generates pro-inflammatory cytokines and mitogen that activate the expression of NFKB which is quickly followed by inflammatory cytokines [6].The NF-kb mediated pathways involves many physical reactions and is extremely important in the modulation of immunity, inflammation and apoptosis [7]. Interlukin1 (IL1β), Interleukin 6 (IL6) and tumor necreotic factor alpha are three known pro-inflammatory cytokines expressed in monocytes and macrophages after invasion of pathogens are identified [2, 3]. These cytokines mediates metabolic changes that end up boosting immune response and resistance to diseases, thereby stunting growth and performance [2, 3]. Reports indicated a strong relationship between the levels of IL1β and the amount of intestinal inflammation in chickens, making IL1β the chief cytokine responsible for the initiation and multiplication of the inflammatory responses [8]. On the other hand, IL-10 helps in promoting the development of T2 responses which makes it inhibits the synthesis of pro-inflammatory cytokines inclusive of IL1β both at the level of transcription and post-transcriptionally, thereby down regulating inflammatory T1 responses and boosting erythrocyte immunity capacity [9]. IL-10 is been regarded as the signature member of the cytokines because it modifies immune response through direct effects on many cell types, hence low level of IL10 increases resistance in chickens while high levels increases susceptibility to intracellular pathogens since the level of pathogen correlates with the magnitude of Tcell response [10, 11]. In chickens, the spleen act as both reservoir and activation site for leukocytes and therefore an analysis of the mRNA levels in the spleen should be reliable in ascertaining systemic immune function [12]. There have been great breakthroughs in the past using various techniques and feed additives to achieve more cost effective and higher genetic potentials in poultry production. Antibiotics have been in livestock feed including poultry diets for a long period of time to prevent diseases, promote growth and improve feed conversion efficiency [13]. Antibiotics have also been known to have antibacterial effect and improved digestibility and performance of birds [14, 15]. The continuous and prolonged use of antibiotics has resulted to deposition of residues in animal products including milk and meat [16] and the resistance among enteric bacteria as well as the transference of antibiotic resistance from livestock to man [17, 18]. These health hazards [19] have led to discontinued use of antibiotics and in some countries, and Europe total ban, and this has resulted to a new search for natural alternatives [16]. Plant derived products have been reported to contain less toxic substances than antibiotics, and scent leaf (*Ocimum gratissimum L*) is a good example of Herbs and spices that possess antimicrobial, antioxidative, anti-inflammatory, as well as immuno-modulatory properties [20]. Scent leaf is a leafy vegetable, and good source of dietary fibre, carotenoids, vitamin C, foliate, phytochemicals and certain minerals [21]. Phytochemical analysis of the leaf extract shows the availability of steroids, flavonoids, alkaloids, saponins, tannins, terpenoids, cardiac glycosides and anthraquinone [22-24]. There is however, inadequate research information on the effect of *Ocimum gratissimum* on the immunomodulation in the chicken. This study was therefore designed to examine the role of scent leaf meal in the expression of immune genes in chicken spleen.

2. MATERIALS AND METHODS

2.1. Experimental site, Animals and Diets

The study was carried out at the Poultry Unit of the Teaching and Research Farms of Delta State College of Education Mosogar. One Hundred and Fifty (150) day old unsexed and healthy commercial broiler chicks, including 75 Cobb 500 and 75 Arbor Acre strains were sourced from Zartech Farms, Ibadan, Nigeria. The birds

were raised on deep-litter system, and fed for a period of eight weeks (56 days). Feed and water were provided ad-libitum throughout the experimental period. Scent leaves were harvested from a farm at Oki, Agbor, in Ika South Local government Area, Delta State, Nigeria. The leaves were air dried while still showing greenish coloration. The dried Scent leaves were hammer milled to obtain a final leaf meal. Five (5) Experimental broiler starter and finisher diets were formulated, and scent leaf meal was incorporated at the rate of 0.00%, 0.50%, 1.00%, 1.50% and 2.00% dietary levels in five (5) treatments as Treatment 1, Treatment 2, Treatment 3, Treatment 4 and Treatment 5 respectively.

2.2. Experimental Design

The 150birds were randomly allotted into five dietary treatment groups of 75birds per strain. Each strain had 15 birds per treatment. The birds in group I (T1) were given normal medications for broilers [25], while birds in groups II (T2), III (T3), IV (T4) and V (T5) had Scent leaf meal included in their feed at the rate of 0.50%, 1.00%, 1.50% and 2.00% respectively.

2.3. Sample Collection

Three birds from each of the treatment groups were slaughtered at 4weeks (28days) and at the end of the feeding trial 8weeks (56 days) to examine the mRNA expression patterns of genes regulating immune responses in Chicken. The spleen was collected from the experimental birds and stored in eppendorf tubes with the aid of RNALater solution and stored in a freezer prior to RNA extraction.

2.4. RNA Concentration and Reverse Transcription

RNA was extracted from a 25mg tissue in a mixture of 400ul RB buffer and β-mertacaptoethanol. The total RNA extraction protocol as contained in Geneaid was then followed. The concentration and purity of the isolated RNA were assessed by a spectrophotometer (Nanodrop) followed by reverse transcription. The 20μL reverse transcription reaction system was comprised of the following: 10 μl of RNA, 0.5μL of Oligo dT, 0.5μL of random primers, 5 μL of nuclease-free water, 1μl of FIREScript Reverse Transcriptase, 0.5μl RiboGrip RNAs inhibotor, 2μl 10x RT reaction buffer with DTT and 0.5μL of DNTP mix. The reaction procedure was performed under the following conditions: denaturation for 5 min at 70°C, Primer annealing for 5min at 25°C, reverse transcription at 40°C for 20mins followed by enzyme inactivation at 85°C for 5mins and then storage at 4°C.

Table-1. Primer sequence for real-time PCR.

S/N	Genes	F/R	Primer sequences for Chicken	NCBI accession number	%GC
1	IL1B	F	5'GTCAACATCGCCACCTACAA3'	NM_204524.1	50
		R	5'CGGTACATACGAGATGGAAACC3'		50
2	IL10	F	5'AGCTGAGGGTGAAGTTTGAG3'	NM_001004414.2	48
		R	5'AACTCATCCAGCAGTTCAGAG3'		50
3	NFkB1	F	5'CCTCAACCTCACTTCCTTACT C3'	NM_205134.1	48
		R	5'CTTCAGTGTCCAGTCCTTTGT3'		50
4	NFkB2	F	5'GACATTGAGGTGCGGTTCTAT3'	NM_204413.1	50
		R	5'GATGGCGTACTGCTTGTGTA3'		50
5	GAPDH	F	5'CCTCTCTGGCAAAGTCCAAG3'	NM_204305.1	45
		R	5'CATCTGCCCATTTGATGTTG3'		55

Note: IL1B: Interleukin1beta; IL10: Interleukin 10; NF-kB1: Nuclear Factor Kappa B1; NF-kB2: Nuclear Factor Kappa B2; F: Forward, R: Reverse; GC: Guanine-Cytosine content

2.5. Real-Time Polymerase Chain Reaction

Table 1 shows the genes, sequences (forward and reverse), guanine-cytocine content, and gene bank accession numbers of the primers used for the study. The mRNA expression level of Interleukin 1-beta, Interleukin 10, NF-kB1, and NF-kB2 were measured using a 7500 Real-Time PCR System with a 20 μL reaction system containing the

following: 1 µL of cDNA, 10 µL of 2× SYBR Premix Dimer Eraser, 0.6 µL of each gene specific primer (100 nM) and 7.8µl nuclease free water. The reaction procedure were performed as follows: 1 cycle of 95°C for 30secs followed by 39 cycles of 95°C for 5secs, 60°C for 30secs, and 72°C for 60secs; and 1 cycle of 95°C for 15secs, 60°C for 60secs, 95°C for 30secs, and 60°C for 15secs.

2.6. Statistical Analysis

All data collected were subjected to ordinary two-way analysis of variance (ANOVA) with the Prism 7.0 software (Graphpad Software Inc, San Diego, California). Multiple comparisons were corrected using the Bonferroni's method.

3. RESULTS AND DISCUSSION

Figure 1 and Figure 2, shows the expression patterns of interleukin1β in the spleen of chickens fed varying levels of *Ocimim gratissimum* leaf meal at 28days and 56days in two chicken strains. Interleukin1β expression patterns decreased with increased inclusion rate of the test ingredients at 28 and 56 days in both chicken strains. The expression of IL1β in the Arbor-acre strain was significantly different ($P<0.05$) for the control and treated groups, but treated groups did not differ significantly ($P<0.05$) at 56days, while T1 and T2 significantly differed ($P<0.05$) from T5; T3, T4 and T5 did not significantly differ ($P>0.05$) from each other at 28days. However, in Cobb500, T1 significantly differed ($P<0.05$) from T1, but not T3, T4 and T5 at 28days and 56days respectively. At 56days, IL1β was expressed in 1.5folds and 2.19folds at 0.5% inclusion rate compared to 2% and down-regulated by 0.67folds and 0.46 folds at 2% inclusion rate compared to 0.5% inclusion in the Abor-acre and Cobb 500 strain. In Figures 3, and 4, the relative expression of interleukin10 shows significant differences ($P<0.05$) for the two strains at both time point, however, expression patterns were inconsistent at all ages in the two strains, while increased inclusion rate increased fold change in Abor-acre at 28days and 56days, in Cobb500, increased inclusion reduced expression of IL10 at 56days, but increased IL10 expression at 28days. IL10 was expressed in 0.05folds and 5.71folds at 0.5% inclusion rate compared to 2% and up-regulated by 19.43folds and 0.17 folds at 2% inclusion rate compared to 0.5% inclusion in the Abor-acre and Cobb 500 strain at 56days respectively. Figures 5, and 6, shows the expression patterns of Nuclear Factor Kappa B (NF-KB1) in two chicken strains at different time point. The control (T1) was significantly different ($P<0.05$) from other groups at 28days and 56days in the Arbor-acre, while T1 and T2 differed significantly from other groups at 56days in Cobb500; T1 and T4, as well as T2 and T3, were not significantly different ($P>0.05$), but other groups showed significant differences ($P<0.05$) at 28days. NF-KB1 was expressed in 58.41folds and 2.24folds at 0.5% inclusion rate compared to 2% and down-regulated by 0.02 folds and 0.45 folds at 2% inclusion rate compared to 0.5% inclusion in the Abor-acre and Cobb 500 strain at 56days. The effect of dietary inclusion of Ocimum graticimum leaf meal on expression of NF-KB1 and IL10 was not consistent in the experimental birds at both ages, since results shows that increased inclusion did not constantly reduce or increase expression patterns of the gene for the two strains studied. Figures 7, and 8, shows the expression patterns of NF-KB2 in two chicken strains at 28 and 56days of age. NF-KB2 was differentially expressed in both time points in the strains studied. Non-significant difference ($P>0.05$) was recorded for T1, T3 and T4, while significant differences ($P<0.05$) were obtained at 56days, while T1 was not significantly different ($P>0.05$) from T2 and T3. T3 and T4 followed the same trend, while significant differences ($P<0.05$) were recorded for other groups at 28days. NF-KB2 expression was not significant for T2 and T3, while other groups reported significant differences ($P<0.05$) at 56days in the Cobb500 strain, but at 28days, T1 and T4, T2 and T3 were not significant ($P>0.05$), while significant differences ($P<0.05$) were obtained for other groups. NF-KB2 was expressed in 0.12 folds and 2.44 folds at 0.5% inclusion rate compared to 2% and up-regulated by 8.31folds and 0.14 folds at 2% inclusion rate compared to 0.5% inclusion in the Abor-acre and Cobb 500 strain respectively.
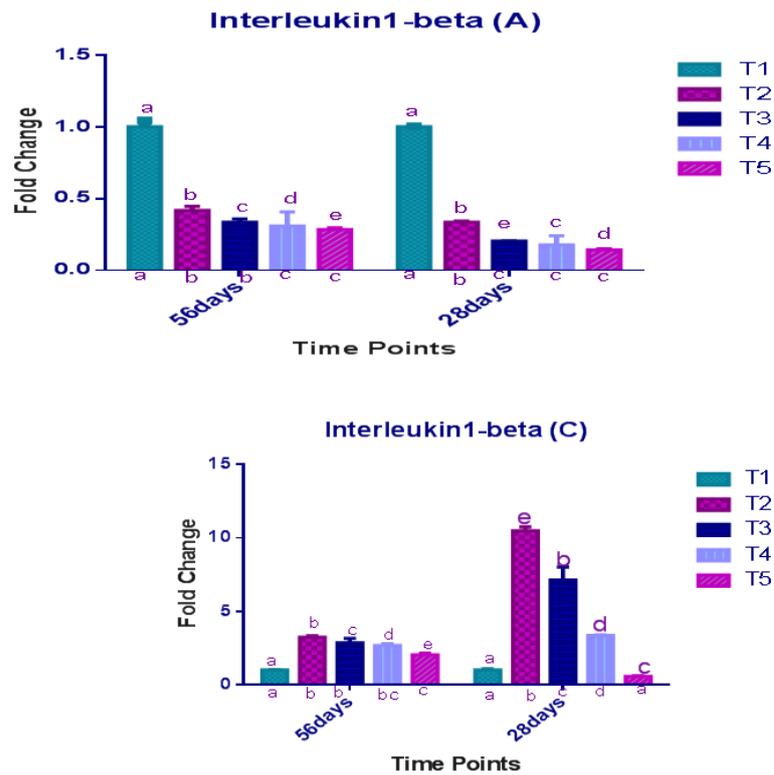
Figure-1 & Figure-2. Relative expression pattern of interleukin 1-beta (IL 1B) in the spleen of Arbor-acre (A) and Cobb500 (C) strains at 28 days and 56 days. abcde- bars with different superscript between time points were significantly different (P<0.05), while bars carrying different subscript within same time points were significantly different (P<0.05). 2-way ANOVA test was done with multiple comparisons corrected using Bonferroni's method.
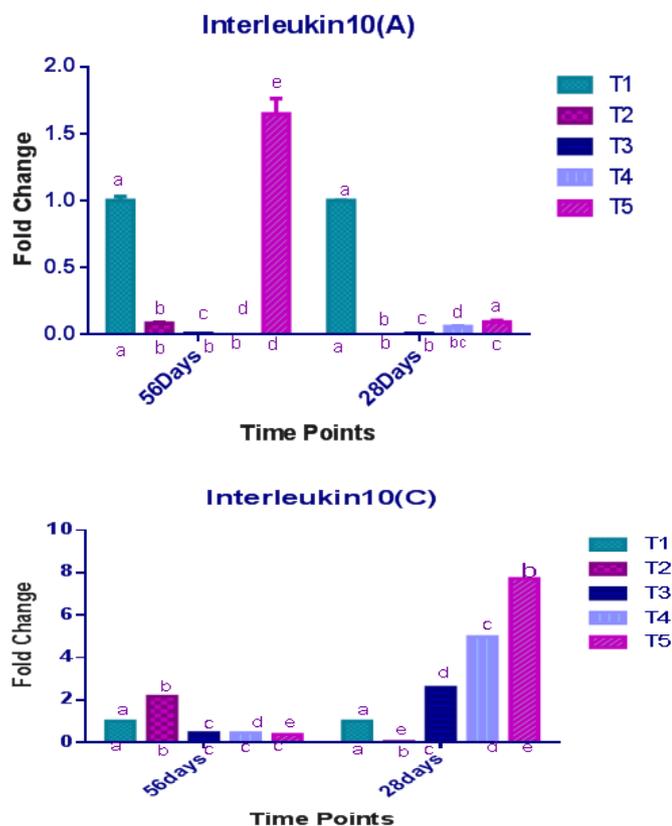

Figure-3 & Figure-4. Relative expression pattern of interleukin10 (IL 10) in the spleen of Arbor-acre (A) and Cobb500 (C) strains at 28 days and 56 days. abcde- bars with different superscript between time points were significantly different (P<0.05), while bars carrying different subscript within same time points were significantly different (P<0.05). 2-way ANOVA test was done with multiple comparisons corrected using Bonferroni's method.
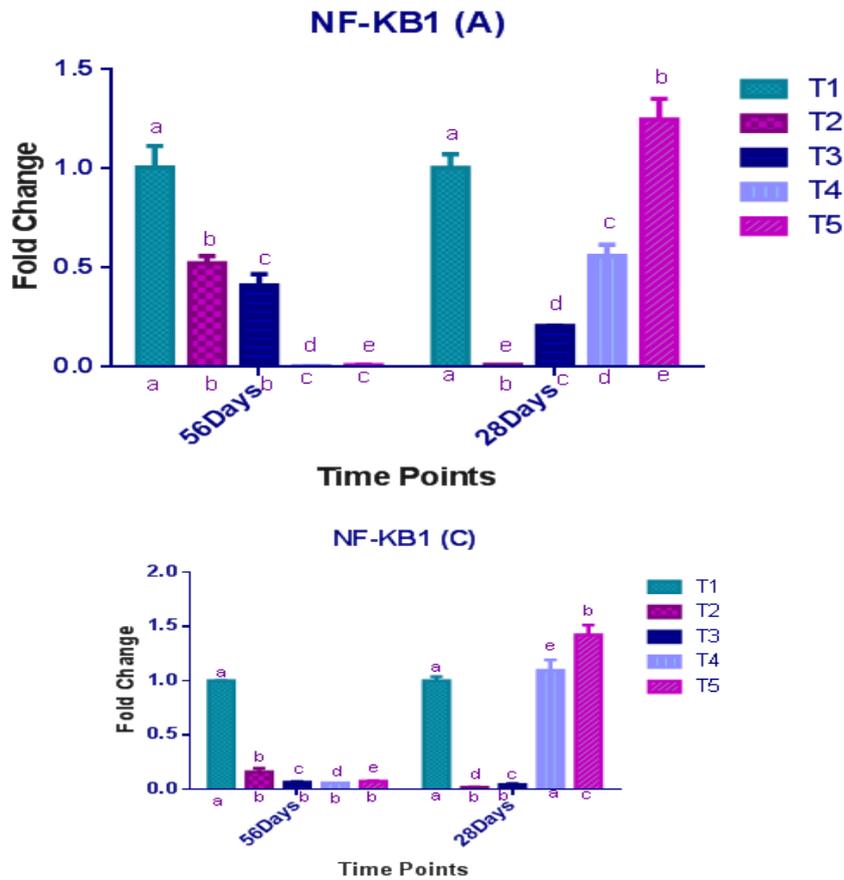
NF-KB1 (A)

NF-KB1 (C)

Figure-5 & Figure-6. Relative expression pattern of Nuclear factor kappa B1 (NF-KB1) in the spleen of Arbor-acre (A) and Cobb500 (C) strains at 28 days and 56 days. abcde- bars with different superscript between time points were significantly different (P<0.05), while bars carrying different subscript within same time points were significantly different (P<0.05). 2-way ANOVA test was done with multiple comparisons corrected using Bonferroni's method.
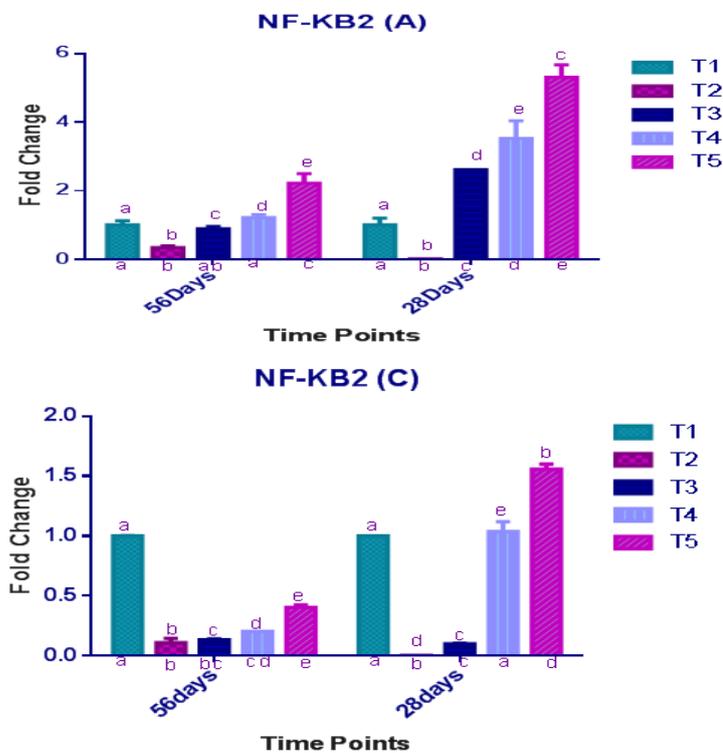
NF-KB2 (A)

NF-KB2 (C)

Figure-7 & Figure-8. Relative expression pattern of Nuclear factor kappa B2 (NF-KB2) in the spleen of Arbor-acre (A) and Cobb500 (C) strains at 28 days and 56 days. abcde- bars with different superscript between time points were significantly different (P<0.05), while bars carrying different subscript within same time points were significantly different (P<0.05). 2-way ANOVA test was done with multiple comparisons corrected using Bonferroni's method.

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4. DISCUSSIONS

Knowing full well that birds can ingest feeds that may contain both nutrients and non-nutrient that can pose positive or negative effects, the intestinal wall becomes the first barrier to entertaining such effects. These consumed feeds can cause stress to birds that will trigger immune responses that are caused by the release of pro and anti-inflammatory cytokines. Inflammation necessitates cells to hire more cells to the centre of activity by the way of secreting inflammatory cytokines and chemokines, however, delayed inflammation can cause the animals to lose weight due to high energy expended in the process. Therefore, to ease and obstruct too much inflammation while bringing the immune system to standard state is vital to livestock production (2). Phytochemicals have been reported to decrease the expression of pro-inflammatory cytokines IL1β, IL6 and TNF and increase anti-inflammatory cytokines IL-10 in LPS-activated cells (3), which is in tandem with the present study as increased inclusion of dietary Ocimum gratissimum consistently reduced expression of interleukin1β in the spleen of the two chicken strains studied. The effect of strains in response to dietary levels were significant ($P<0.05$) at both time points. This is in line with previous studies that cytokine expression differs with type of bird as reported for layers and broilers [26, 27]. This could be attributed to the different responses in the immune systems of the chicken strains to the test ingredients. Although lack of sufficient antioxidants to eliminate reactive oxygen species can also trigger inflammation due to oxidative damage [2, 9, 28], hence, while including ingredients that improves anti-inflammatory responses in chicken diets, the antioxidant capacity needs to be put into consideration in other to avoid oxidative damages. As a compliment and justification that the test ingredients covers this aspects, earlier reports indicated that compounds from medicinal plants reduced small intestine legions, reduced oxidative stress and improved body weight in chickens challenged with coccidial infection [20, 29]. This study is also in agreement with reports that Scent leaf (Ocimum gratissimum L) possesses antimicrobial, antioxidative, anti-inflammatory, as well as immuno-modulatory properties [20]. The anti-inflammatory role of Scent leaf was evident, as increased inclusion rate consistently down regulated IL1β, which is known to be the chief pro-inflammatory cytokine in cells. Lactobacillus species was also found to differentially induced IL-10 and IL1β in spleen cells (8). Since one of the biological effects of IL-1β is to stimulate inflammation by activating the immune system in an acute phase response [30], dietary inclusion of scent leaf can be used in place of antibiotics to reduce inflammation in chickens thereby curbing stunted growth, excessive energy expenditure, and weight loss in birds. IL-10 together with 1L4 helps in promoting the development of T2 responses which makes it inhibits the synthesis of pro-inflammatory cytokines inclusive of IL1β, TNF and IL12 both at the level of transcription and post-transcriptionally, thereby down regulating inflammatory T1 responses and boosting erythrocyte immunity capacity (9). IL-10 is been regarded as the signature member of the cytokines because it modifies immune response through direct effects on many cell types; this corresponds with the present study as results indicated a decrease in the expression of IL1β, as IL10 levels increased in chicken spleen at 28 and 56days in the experimental birds in both strains studied. *Ocimum gratissimum* increased IL10 expression while simultaneously decreasing IL1β as observed in the current study. The anti-inflammatory response of the test ingredient is vital for averting weight loss and stabilizing growth rate in chickens if they are eventually faced with pathogen invasion. Bearing in mind that IL10 is the signature member of anti-inflammatory cytokines, higher amount of inclusion of the test ingredients in the diets of chickens may further strengthen the fight against inflammation in chicken, which invariably will result in body weight and growth stability. The differential expression of cytokines is not new as non antibiotic alternatives have previously been reported to significantly affect cytokine gene expression in coccidia challenged chickens [31]. While NF-kB2 consistently increased with increased inclusion rate of the test ingredient, NF-kB1 expression was affected in no specific direction. Reports indicate that phytochemicals have induced molecular changes in Nf-Kb pathways (4), but the directions and dimensions were unstable in this study. Carvacoal, cinnamaldehyde, curcumin and thymol were reported to surpress NF-kB expression [32-35], while our study shows NF-kB1 and NF-kB2 did not progress in the same manner with respect to the test ingredient. Earlier studies indicates that NF-kB can be induced and

activated by pro-inflammatory cytokines, it has also been shown that NF-Kb activation increased production of pro-inflammatory cytokines that eventually resulted to systemic inflammation during stress period among birds [36]. Eimeria Tenella challenged birds increased expression of NF-kB together with other pro-inflammatory cytokines in chickens [37]. Therefore, reduced IL1β and NF-kB1 expression with increased levels of Ocimum gratissimum in the diet of the experimental birds is expected to down-regulate the expression of NF-kB2 in this study, but the reverse was the case in the expression of NF-kB2 at 28 and 56 days. More so, applying materials possessing the ability to suppress NF-kB signaling would be promising diet supplements in poultry husbandry since NF-Kb supports the expression of different inflammatory cytokines and chemokines, inclusive of genes related to cellular transformation, inflammation, invasion, and metatastasis (5). Since the expression of genes related to tissue repair and inflammation is dependent on the time, strength and the site of stimulation (6), the expression of NF-kB1 and NF-kB2 in the current study could have been modulated by the test ingredient in different directions with respect to the NF-kB pathways.

5. CONCLUSION

This study further provides information on the anti-inflammatory roles of phytochemical containing plants in the immune system of chickens. It reveals that Ocimum graticimum can be used in place of antibiotics in broiler diet, which can reduce the negative effects occasioned by the use synthetic antibiotics.

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