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THE IMMUNE MODULATING POTENTIAL OF SUPPLEMENTING COBB500 BROILER CHICKENS WITH CRICKET AND SHRIMP CHITIN AND CHITOSAN

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ABSTRACT

This study explored the potential impacts of cricket-derived chitin and chitosan on the immune systems of Cobb500 broilers. One hundred and fifty broiler chicks of the Cobb500 strain were randomly assigned to any one of the five dietary groups in order to accomplish this. While the first set of birds (group 1) were only served the basal diet with no supplementation, the second to fifth sets of birds (groups 2 to 5) were served a diet supplemented with 500 mg/kg of the following: cricket-chitin, cricket-chitosan, shrimp-chitin, and shrimp-chitosan. The bursa and spleen were weighed relative to the body weight, and qPCR was used to determine the spleen's relative expression of toll-like receptor 4 (TLR4), toll-like receptor 15 (TLR15), interleukin-1β (IL-1β), and inducible nitric oxide synthase (iNOS) genes. After 42 days of dietary cricket-chitin, the bulk of the index immunological organs increased ($P<0.05$). At day 21, TLR4, TLR15, IL-1β, and iNOS expression were unaffected by chitin and chitosan, but at day 42, they were down-regulated ($P<0.05$). However, during day 21, dietary shrimp-derived chitosan enhanced ($P<0.05$) the relative expression of TLR4, TLR15, and IL-1β, whereas the expression of TLR15 was lowered ($P<0.05$) but that of TLR4 was increased by cricket-chitin and shrimp-chitin. According to our findings, feeding broiler chicks with 500 mg/kg of shrimp chitosan and cricket-derived chitin can positively boost their immunity.

Keywords: Broiler chicken, cricket- and shrimp-chitosan, cricket- and shrimp-chitin, immunity, and gene expression.

INTRODUCTION

The chicken business is the fastest-growing agricultural sector in the world and is important to human nutrition and the economy (Zaid and Ali, 2016). In order to satisfy the growing need for global animal protein, there is a need for a greater supply of poultry products, which has been partially met by increasing poultry production. Low doses of antibiotics are given to broiler chickens in order to promote rapid growth (Costa *et al.*, 2017). As a result, feed additives containing antibiotics have been employed proactively to increase poultry output (Ayalew *et al.*, 2022). However, over time, the use of antibiotics in food has resulted in drug-resistant strains of microorganisms and drug residue issues (Bostami *et al.*, 2015). This has produced an increase in the assessment of substitute additives for feed (European Union, 2018). Research has indicated that chitosan (CHS), a (1-4)-linked d-glucosamine, and chitin (CHT), a (1-4)-N-acetyl-d-glucosamine, can enhance the immune responses of broiler chickens (Menconi *et al.*, 2014). There have been findings that CHT enhances macrophage activation and the production of inflammatory cytokines (Behera *et al.*, 2022; Komi *et al.*, 2018). Additional investigations found that CHS increased the nitric oxide (NO) manufacturing process, activated macrophages, increased iNOS expression and activity, and secreted more TNF, IL-1, and IL-2 in broiler chicks (Li *et al.*, 2014).

Moreover, the lymphoid tissue plays a vital role in the defensive process (Zaid and Ali, 2016). Organ size and function are related, according to Shingleton (2010) and Gokhale and Shingleton (2015), and immunosuppression has been associated with a drop in body weight, particularly in lymphoid organs (Halouzka *et al.*, 1991). In research by Shi-bin and Hong (2012), the weight of lymphoid organs increased in all treatment groups given dietary CHS at a maximum dose of 1200 mg/kg. Regarding CHT and

CHS's ability to modulate the immune system, there are disagreements. Furthermore, the majority of studies on the effects of CHT and CHS on broiler chicken immune systems that are currently accessible had focused on CHT and CHS derived from crustaceans; little to nothing is known about the effects of CHT and CHS derived from insects on broiler chicken immune systems. This knowledge will reveal a fresh strategy for raising chickens that are healthier and more productive. Additionally, information is needed about the nutrigenomic aspect of dietary CHT and CHS derived from shrimp and crickets. Consequently, the immunological responses of Cobb500 chickens to dietary cricket-derived and shrimp-derived CHT and CHS are compared in this study.

MATERIALS AND METHODS

Handling experimental bird populations

The study's methodology was authorized by the Universiti Putra Malaysia Animal Care and Use Committee (UPM/IACUC/AUP-R025/2017). The Poultry House, Department of Animal Science, Faculty of Agriculture, Universiti Putra Malaysia was the study's location. In a completely randomized design, 150 male Cobb500 broilers were divided into five dietary groups (n = 30), each with three replicates of ten birds. A locally available antibiotic-free commercial chicken feed was remixed for the purpose of this study (Table 1: starter diets and Table 2: grower diets). Shrimp-CHT and -CHS were supplied by Sigma-Aldrich in the USA, whereas cricket-CHT and -CHS were obtained from house crickets (*Brachytrupes portentosus*) according to Ibitoye *et al.* (2018). As shown in Tables 1 and 2, the animals in groups 2 to 5 respectively had their diets enriched with 500 mg/kg of cricket-CHT, cricket-CHS, shrimp-CHT and shrimp-CHS. Investigations in this study followed the broiler chicken production phases of starter (days 0-21) and grower (days 22-42). A battery cage system with 2.3 × 9.1 × 3.8 m cage dimensions was used to raise the birds in an open-sided pen.

Immune organ index

Following each feeding phase, the birds were allowed to starve for 12 hours overnight. Then, three experimental birds were selected at random per replicate, they were weighed and sacrificed (Malaysian Standard 1500:2009). After that, the spleen and bursa of Fabricius (BF) were quickly separated, collected, and weighed on a digital scale. The weight of BF and spleen were estimated relative to the body weight (Aguihe *et al.*, 2017).

Table 1. Diet composition of broiler chicken starter feed supplemented with CHT and CHS derived from cricket and shrimp

Feed resources (kg)	Experimental groups				
	T1	T2	T3	T4	T5
Corn	50.32	50.32	50.32	50.32	50.32
Soybean meal	39.12	39.12	39.12	39.12	39.12
Palm oil	6.67	6.67	6.67	6.67	6.67
Limestone	1.22	1.22	1.22	1.22	1.22
Dicalcium phosphate	1.62	1.62	1.62	1.62	1.62
Sodium chloride	0.50	0.50	0.50	0.50	0.50
Mineral premix	0.10	0.10	0.10	0.10	0.10
Vitamin premix	0.10	0.10	0.10	0.10	0.10
L-Lysine, hydrochloric acid	0.15	0.15	0.15	0.15	0.15
DL-Methionine	0.20	0.20	0.20	0.20	0.20
Analyzed nutrient composition					
Metabolizable energy (kcal/kg)	3129.90	3129.90	3129.90	3129.90	3129.90
Crude protein (%)	22.24	22.24	22.24	22.24	22.24
Available phosphorus (%)	0.47	0.47	0.47	0.47	0.47

Methionine + Cysteine (%)	1.00	1.00	1.00	1.00	1.00
Lysine (%)	1.30	1.30	1.30	1.30	1.30

Key: T1: only basal diet; T2: 500 mg/kg cricket-chitin; T3: 500 mg/kg cricket-chitosan; T4: 500 mg/kg shrimp-chitin; T5: 500 mg/kg shrimp-chitosan

Table 2. Diet composition of broiler chicken grower feed supplemented with CHT and CHS derived from cricket and shrimp

Feed resources (kg)	Experimental groups				
	T1	T2	T3	T4	T5
Corn	59.20	59.20	59.20	59.20	59.20
Soybean meal	30.68	30.68	30.68	30.68	30.68
Palm oil	6.25	6.25	6.25	6.25	6.25
Limestone	1.41	1.41	1.41	1.41	1.41
Dicalcium phosphate	1.28	1.28	1.28	1.28	1.28
Vitamin premix	0.05	0.05	0.05	0.05	0.05
Sodium chloride	0.52	0.52	0.52	0.52	0.52
Mineral premix	0.25	0.25	0.25	0.25	0.25
L-Lysine, hydrochloric acid	0.21	0.21	0.21	0.21	0.21
DL-Methionine	0.15	0.15	0.15	0.15	0.15
Analyzed nutrient composition					
Metabolizable energy (kcal/kg)	3210	3210	3210	3210	3210
Crude protein (%)	18.75	18.75	18.75	18.75	18.75
Available phosphorus (%)	0.38	0.38	0.38	0.38	0.38
Methionine + Cysteine (%)	0.80	0.80	0.80	0.80	0.80
Lysine (%)	1.23	1.23	1.23	1.23	1.23

Key: T1: only basal diet; T2: 500 mg/kg cricket-chitin; T3: 500 mg/kg cricket-chitosan; T4: 500 mg/kg shrimp-chitin; T5: 500 mg/kg shrimp-chitosan

Investigation of relative gene expression

This was carried out using the spleen (Kannaki *et al.*, 2010). At days 21 and 42, total RNA was isolated from about 25 mg of spleen samples previously preserved with the RNAlater solution (Invitrogen) using the protocol of the Total RNA Mini Kit (Tissue) (Geneaid Biotech Ltd.). A spectrophotometer (Infinite M200PRO TECAN) at a wavelength of 450 nm ± 2 nm was used to determine RNA purity and concentration, and then the RNA was stored at -80℃ until further use. ReverTra Ace® qPCR RT Master Mix with gDNA remover kit (TOYOBO Bio-Technology, CO., LTD) was used for reverse transcription, followed by a quantitative real-time PCR using a PCR machine (Bio-Rad CFX96). In this study, pre-denaturation was at 98℃ for 2 minutes, 45 cycles of denaturation were done at 98℃ for 10 seconds, while annealing and extension were respectively achieved at 60℃ for 10 seconds and 68℃ for 30 seconds. Genes of interest in this investigation were Toll-like receptor-15 (TLR-15), Toll-like receptor-4 (TLR-4), interleukin-1 beta (IL-1), and inducible nitric oxide synthase (iNOS) (Table 3). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-actin (β-actin) were utilized to attain endogenous controls (Table 3). Using the Auto mode, the Bio-Rad CFX96 qPCR machine's software (Bio-Rad CFX Manager 3.1) was used to independently check each plate's quality. The relative gene expression level was then ascertained with the ΔΔCT technique as prescribed by Livak and Schmittgen (2001), where ΔCT is obtained by subtracting the CT values of reference genes (GAPDH and β-actin) from the CT value of gene of interest, and the mean values were then computed. CT is produced by relating the CT of treated groups with the CT of the control. The information was presented as fold changes in the target gene expression of the treated sample relative to the control samples.

Statistical evaluation

One-way ANOVA was applied on the data using SPSS and thereafter displayed as mean ± standard error (SE) in tables and charts. The difference between means was examined using the least significant difference (LSD) technique. Additionally, a student t-

test was employed in SPSS to search for noteworthy variations in the information gathered from the gene expression study (IBM, 2020). A P-value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Immune organ index of experimental broiler chickens

Table 4 shows that on day 21, in broiler chicks, the weights of spleen and BF relative to the body weights were not affected (P>0.05) by 500 mg/kg CHT and CHS dietary treatments. Shi-bin and Hong (2012) concurred with this, which may suggest that a sufficient amount of time is required for CHT and CHS supplementation to have a noticeable impact on these organs. According to Shi-bin and Hong (2012) investigation into the impact of dietary CHS on duck immune organs, the immune organ index significantly rose at 2400 mg/kg. However, Shi-bin and Hong (2012) research did demonstrate that broilers given a little amount of CHS may lead to a rise in the body-weight-adjusted lymphoid organs and immunological organs. Conversely, the relative weights of the spleen and BF of Cobb500 broiler chickens at day 42 were significantly (P<0.05) impacted by the addition of CHT and CHS. This correlated with the findings of Chi *et al*. (2017), who noted that at grower phase (day 42), the relative weights of the BF, spleen, and thymus of hens supplemented with 350 mg/kg of CHS were significantly larger than those of the control unit. Despite this, compared to the shrimp-CHT (T4) treatment, the cricket-CHT (T2) group produced noticeably (P<0.05) weightier spleen and BF. The spleen weights of the birds treated with shrimp-CHT and shrimp-CHS were considerably lesser (P<0.05) compare to those of the controls. While the relative weights of spleen and BF in CHT and CHS derived from cricket (T2 and T3) differed non-significantly when compared to the control. This was anticipated because, in contrast to the cricket-CHT group, the broilers' body weight and body weight gain had been insignificantly reduced by cricket-CHS supplementation (Table 4). This result might have been affected by the earlier publication by Ibitoye *et al.* (2018) that the quality and purity of cricket-CHT were higher than shrimp-CHT. It is noteworthy to emphasize that variations in the ages, species, molecular weights, dosages, and durations of CHS feeding could have contributed to disparate study outcomes for the immunological organs index, according to Chi *et al.* (2017).

The immune systems of experimental broiler chickens

The immune system's response to 500 mg/kg of CHT and CHS generated from both shrimp and crickets is demonstrated in Figure 1 by looking at the gene fold expression of a few toll-like receptors, cytokines, and iNOS.

Table 3. Primer sequences (5′ → 3′) for immune genes used for real-time PCR

Name	Forward primer	Reverse primer	Product size (bp)	Annealing Temp (℃)
**TLR15	GTTCTCTCTCCCAGTTTTGTAAATAGC	GTGGTTCATTGGTTGTTTTTAGGAC	262	56
**TLR4	GTCTCTCCTTCCTTACCTGCTGTTC	AGGAGGAGAAAGACAGGGTAGGTG	187	56
*GAPDH	GCCGTCCTCTCTGGCAAAG	TGTAAACCATGTAGTTCAGATCGATGA	128	60
*β-actin	CAACACAGTGCTGTCTGGTGG	ATCGTACTCCTGCTTGCTGAT	205	55
#IL-1β	ACAGAGATGGCGTTCGTTCCCGA	TCAGCTCGACGCTGTCGATGT	635	
#iNOS	AATGCTGTGCCCATGGCAGTTTGCA	CACCTCAAGGAGCATGTTGGCAACA	1072	

** Higgs *et al.* (2006); Lu *et al.* (2009); # Cheng *et al.* (2006); Xing & Schat (2000)* Faseleh Jahromi *et al.* (2017).

Table 4. Body weight and index immune organs response of experimental birds (Mean ± SE)

Parameters (g)	Experimental groups				
	T1	T2	T3	T4	T5
			Day 21		
Body weight	734.66 ± 14.25	733.00 ± 7.81	724.33 ± 6.39	748.67 ± 9.39	749.00 ± 16.09
Body weight gain	363.33 ± 7.84	367.33 ± 3.84	354.33 ± 8.69	370.67 ± 7.22	377.67 ± 15.62
Bursa of Fabricius	0.16 ± 0.02	0.18 ± 0.01	0.18 ± 0.03	0.20 ± 0.04	0.26 ± 0.03
Spleen	0.12 ± 0.02	0.18 ± 0.03	0.11 ± 0.02	0.09 ± 0.09	0.14 ± 0.03
			Day 42		
Body weight	2670.14± 60.89^{a}	2565.4 ± 47.0$^{a, b}$	2359.36 ± 16.6^{c}	2518.99 ± 15.6^{b}	2514.35 ± 21.77^{b}
Body weight gain	2620.31± 60.88^{a}	2515.8 ± 46.8$^{a, b}$	2310.2 ± 16.2^{c}	2470.30±14.96^{b}	2465.42 ± 21.56^{b}
Bursa of Fabricius	0.18 ± 0.02^{b}	0.28 ± 0.03 ^{a}	0.18 ± 0.03 ^{b}	0.13 ± 0.01^{c}	0.17 ± 0.01^{b}
Spleen	0.16 ± 0.14^{a}	0.11 ± 0.02$^{a, b}$	0.10 ± 0.01$^{a, b, c}$	0.06 ± 0.0^{c}	0.10 ± 01$^{b, c}$

Data on the same row with different superscripts a, b, and c are significantly different (*P<0.05*).

Key: T1: basal diet alone; T2: 500 mg/kg cricket-chitin; T3: 500 mg/kg cricket-chitosan; T4: 500 mg/kg shrimp-chitin; T5: 500 mg/kg shrimp-chitosan

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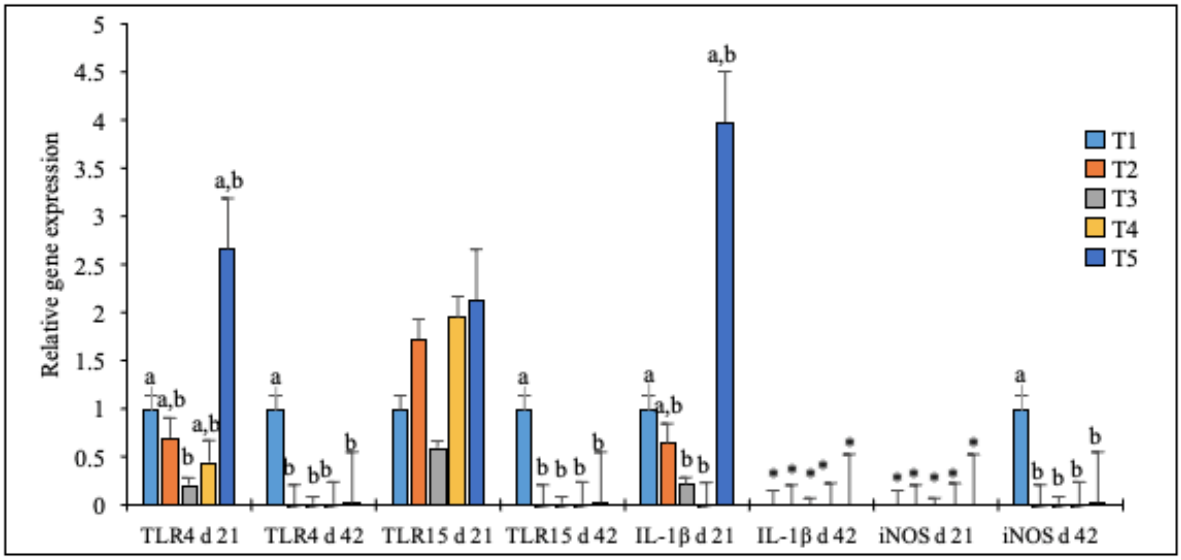

Figure 1. *Effect of dietary cricket and shrimp, CHT and CHS on IL-1, iNOS, TLR4, and TLR15 expression. While bars with a * are not available, those with the letters a and b are statistically different (P<0.05).*
Key: T1: only basal diet; T2: 500 mg/kg cricket-chitin; T3: 500 mg/kg cricket-chitosan; T4: 500 mg/kg shrimp-chitin; T5: 500 mg/kg shrimp-chitosan.

In this assessment, CHT and CHS treatment groups differed non-significantly (P>0.05) at either of the two production stages. On day 21, the relative gene expression of TLR4 showed a significant down-regulation (P<0.05) by cricket-CHS (T3) compared to the untreated group, while it was not significantly impacted by the supplementations of shrimp-CHT (T4) (P>0.05) and cricket-CHT (T2) (P>0.05). On the other hand, broiler chicks' expression of TLR4 was upregulated (P>0.05) in response to 0.5 g of shrimp-CHS in 1 kg of feed (T5) as opposed to T1. Furthermore, the gene expression of TLR15 was quadratically up-regulated (P>0.05) in cricket-CHT, shrimp-CHT, and shrimp-CHS treated groups, whereas it was down-regulated (P>0.05) in cricket-CHS group. This could be the result of dietary CHT and CHS having no discernible effect on the spleen's weight on day 21. Furthermore, as the battery-cage management system could have avoided the experimental birds ingesting oocysts and eggs, therefore, TLR15 overexpression could not have resulted from helminth or coccidial infections (Burakova *et al.*, 2018). Nonetheless, it is possible that shrimp-CHS's capacity to attach to mannose receptors on macrophages is what causes the enhanced immunological responses seen in birds given supplemented shrimp-CHS (T5) (Shibata *et al.*, 1997). This contact starts the complement pathway, activates inflammasomes, and produces antibodies and pro-inflammatory cytokines (Bueter *et al.*, 2011). According to Li *et al.* (2004), dietary supplementation of CHS derived from shrimp at 500 mg/kg diet (T5) in this investigation quadratically (P>0.05) up-regulated the relative expression of IL-1β in the spleen. This suggests that red pulp macrophages' secretory function may have been positively impacted by shrimp-CHS supplementation, which is connected to the dietary supplementation of shrimp-CHS. The way that CHS enhanced the secretory capabilities of red pulp macrophages may have been caused by the presence of the structural amino groups, which the receptor on macrophage surfaces may be able to detect and somewhat activate (Yeramian *et al.*, 2006). Upon activation, macrophages emit a multitude of bioactive chemicals such as reactive oxygen, NO, IL-1, IFN-γ, TNF-α, and many more that are connected to inflammation and the immune system (Duque and Descoteaux, 2014). In contrast to shrimp-CHS (T5), this study found that supplementing with cricket-CHS (T3) led to the down-regulation (P<0.05) of IL-1β. This might be because the particles employed in this study, shrimp-CHS, and cricket-CHS, have different particle sizes. Additionally, because smaller particles are simpler to phagocytose, IL-1β is expressed more in the smaller particle CS (Bueter *et al.*, 2013). Likewise, variations in the purification processes and material sources may also have played a role in these discrepancies. However, when it comes to the expression of the IL-1β gene, there is no change (P>0.05) comparing CHT with CHS. This may be explained by the fact that Chia's action (Tabata *et al.*, 2017) breaks down big CHT particles, exposing their core CHS, which in turn activates IL-1β.

The relative gene expression of TLR4, TLR15, and iNOS were significantly down-regulated (P<0.05) in chickens supplemented with CHT and CHS; while no change

(P>0.05) was observed between the CHT and CHS groups at day 42. This could be explained by the fact that the spleen weight of birds receiving CHT and CHS supplements decreased. This implies that function may be impacted by splenic size and that certain cytokine secretions may be inhibited by supplementing with CHT and CHS at a 500 mg/kg diet. Furthermore, this study showed that, while CHT and CHS supplementations may be beneficial for the immune system until day 21, they were not able to improve the immune response in broiler chickens at age 42 days. This may be explained by the effects of aging in chickens, which is corroborated by research done in mice by Kovacs *et al.* (2009), who discovered that aging can affect all innate immune cells, changing both the quantity and type of cells. Moreover, Kogut *et al.* (2005) state that one of the two important variables found for assessing the immune response in poultry is the age of the birds. However, this study was unable to explain the lack of data for the relative expression of IL-1β at day 42 and iNOS at day 21.

CONCLUSION

This study indicated that broiler chicken's immunological responses might be enhanced by adding 500 mg/kg of chitosan and chitin derived from shrimp and cricket respectively to their feed. Consequently, it is advised to conduct additional research on the impact of varying concentrations of chitin and chitosan on the expression and control of immune gene substrates in broiler chickens.

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