Universidade de São Paulo
Escola Superior de Agricultura "Luiz de Queiroz"

Farinhas de larvas de insetos (*Tenebrio molitor* e *Hermetia illuscens*) melhoram o desempenho e modulam o sistema imune inato de frangos de corte

José Matheus de Moura Andrade

Dissertação apresentada para obtenção do título de Mestre em Ciências. Área de concentração: Ciência Animal e Pastagens

Piracicaba
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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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À Escola Superior de Agricultura “Luiz de Queiroz”

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SUMÁRIO

RESUMO

Farinhas de larvas de insetos (*Tenebrio molitor* e *Hermetia illuscens*) melhoram o desempenho e modulam o sistema imune inato de frangos de corte

Cada vez mais o uso de insetos na alimentação animal ganha espaço; as inúmeras pesquisas realizadas utilizando farinha de insetos apontam reais benefícios sobre seu uso, dentre eles melhoria no desempenho animal e em respostas imunológicas, revelando propriedades nutracêuticas que se somam às nutricionais. Os insetos mais pesquisados atualmente são o tenébrio (*Tenebrio molitor*) e a black soldier fly - BSF (*Hermetia illucens*). Ambos os insetos produzem peptídeos antimicrobianos que são responsáveis por sua defesa e são efetivos contra bactérias Gram-negativas e positivas, fungos e, recentemente, foi mostrado que têm efeito modulador da resposta imune de aves. Diversos estudos avaliaram farinhas de insetos com foco em seu valor nutricional como fonte de energia e proteína. Devido a suas propriedades nutracêuticas, o uso do ingrediente como alimento funcional foi avaliado neste estudo, com foco em modulação do sistema imune de frangos de corte e possibilitando a produção sem uso de antibióticos promotores do crescimento. O presente projeto é composto por 3 capítulos: (1) avaliação do desempenho de frangos de corte alimentados na fase inicial com 0,5 ou 1,0% de farinha de larvas de tenébrio ou black soldier fly e possível efeito residual dos ingredientes até a fase final; (2) dois experimentos independentes, o primeiro para avaliar o desempenho de frangos de corte até os 35 dias alimentados com 0,5 ou 2,0% de farinha de larvas de tenébrio e a contagem bactérias no ceco; o segundo, usando as mesmas dietas experimentais, para avaliar o desempenho e o sistema imune inato de frangos de corte desafiados aos 21 dias com LPS (lipopolissacarideo) de *E. coli*,; (3) avaliação do desempenho de frangos de corte alimentados com 0,5 ou 2,0% de farinha de larvas de black soldier fly desengordurada e o sistema imune das aves desafiadas aos 35 dias com LPS de *E. coli*. Ração contendo 10 mg/kg de enramicina e 66 mg/kg de salinomicina foi o controle positivo (CP) e ração isenta de suplementação foi o controle negativo (CN). No capítulo 1 não foi observada diferença estatística significativa entre os tratamentos dietéticos. No capítulo 2, as aves alimentadas com 2,0% de farinha de tenébrio tiveram ganho de peso intermediário ao CN e CP, aumento no consumo de ração (CR) e a conversão alimentar (CA) não foi influenciada. Não foi observada diferença na contagem de bactérias no ceco. No exp.2B os tratamentos não diferiram estatisticamente no ganho de peso (GP) e CR, no entanto as aves alimentadas com 2,0% de farinha de tenébrio tiveram uma melhor CA. Os tratamentos não alteraram os parâmetros do sistema imune das aves desafiadas; os frangos não desafiados e alimentados com 2% de farinha de tenébrio tiveram maior atividade do sistema imune complemento e da mieloperoxidase. No capítulo 3, as aves alimentadas com 2,0% de farinha de BSF também tiveram desempenho intermediário ao CN e CP, aumento no CR e a CA não foi influenciada. As aves não desafiadas com LPS e alimentadas com 2,0% farinha de BSF tiveram maior atividade da lisozima e do sistema complemento em relação ao CN e após o desafio com LPS tiveram maior atividade bactericida contra *Escherichia coli* que as aves CP. Não foi observada melhora no desempenho das aves alimentadas com 0,5% de farinha de insetos em nenhum dos experimentos realizados. Os resultados deste projeto comprovam que as farinhas de insetos avaliadas usadas em baixo nível de inclusão (2,0%) podem melhorar o desempenho das aves (com valor intermediário ao uso de antibiótico + coccidiostático) e modular o sistema imune inato das aves.

Palavras-chave: Alternativa aos antibióticos, Black soldier fly, Ingrediente alternativo, Insetos comestíveis, Tenebrio

ABSTRACT

Insect larvae meals (*Tenebrio molitor* and *Hermetia illuscens*) enhance performance and modulate the innate immune system of broiler chickens

The use of insects in animal feed is increasingly gaining ground; the numerous studies carried out using insect meal point to real benefits on its use, among them improvement in animal performance and in immune responses, revealing nutraceutical properties that are added to nutritional ones. The insects most researched today are the mealworm (Tenebrio molitor) and the black soldier fly - BSF (Hermetia illucens). Both insects produce antimicrobial peptides that are responsible for their defense and are effective against Gram-negative and positive bacteria, fungi and, recently, they have been shown to have a modulating effect on the immune response of birds. Several studies have evaluated insect meals focusing on their nutritional value as a source of energy and protein. Due to its nutraceutical properties, the use of the ingredient as a functional food was evaluated in this study, focusing on modulating the immune system of broilers and enabling production without the use of growth-promoting antibiotics. The present project is composed of 3 chapters: (1) performance evaluation of broilers fed in the initial phase with 0.5 or 1.0% of meal of larvae of larvae of larvae or black soldier fly and possible residual effect of the ingredients until the final phase; (2) two independent experiments, the first to evaluate the performance of broilers up to 35 days old fed with 0.5 or 2.0% Tenebrio molitor larvae meal and the bacterial count in the cecum; the second, using the same experimental diets, to evaluate the performance and the innate immune system of broilers challenged at 21 days with LPS (lipopolysaccharide) from *E. coli*; (3) performance evaluation of broilers fed with 0.5 or 2.0% defatted black soldier fly larvae meal and the immune system of birds challenged at 35 days with E. coli LPS. Feed containing 10 mg/kg of enramycin and 66 mg/kg of salinomycin was the positive control (PC) and feed without supplementation was the negative control (NC). In chapter 1, no statistically significant difference was observed between dietary treatments. In chapter 2, birds fed with 2.0% of tenebrio meal had an intermediate weight gain at CN and CP, increase in feed intake (CR) and feed conversion (FC) was not influenced. No difference was observed in the bacterial count in the cecum. In exp.2B the treatments did not differ statistically in weight gain (WG) and CR, however the birds fed with 2.0% of mealworm meal had a better CA. The treatments did not change the parameters of the immune system of the challenged birds; non-challenged chickens fed with 2% turkey meal had higher immune system complement and myeloperoxidase activity. In chapter 3, birds fed with 2.0% BSF flour also had an intermediate performance to CN and CP, increase in CR and CA was not influenced. Birds not challenged with LPS and fed with 2.0% BSF meal had higher activity of lysozyme and complement system in relation to NC and after challenge with LPS they had higher bactericidal activity against *Escherichia coli* than CP birds. No improvement in the performance of birds fed with 0.5% insect meal was observed in any of the experiments performed. The results of this project prove that the evaluated insect meals used in low inclusion level (2.0%) can improve the performance of the birds (with intermediate value to the use of antibiotic + coccidiostat) and modulate the innate immune system of the birds.

Keywords: Alternative to antibiotics, Black soldier fly, Alternative ingredient, Edible insects, Tenebrio

1. INTRODUÇÃO

O uso de insetos como ingrediente ou aditivo vem ganhando rapidamente o interesse dos pesquisadores e da indústria avícola no Brasil e no mundo. O valor nutritivo da farinha de insetos tem variações de acordo com sua espécie, podendo ter entre 35 e 69% de proteína, apresentando um bom balanceamento de aminoácidos, dentre eles os aminoácidos essenciais (Hwangbo et al., 2009; Veldkamp et al., 2012).

A black soldier fly - BSF (*Hermetia illucens*) e o tenébrio (*Tenebrio molitor*) são capazes de utilizar resíduos orgânicos como substrato para seu crescimento, funcionando assim com recicladores de resíduos orgânicos muito eficientes. O uso de farinhas de larvas de insetos para a alimentação animal além de ter uma grande importância nutricional também engloba a reutilização de alimentos que não seriam mais aproveitados, sendo esses utilizados na alimentação dos insetos. Dessa forma, a criação de insetos, está inserida no conceito de economia circular onde é objetivado a redução, reutilização e reciclagem de materiais e energia, melhorando assim o aproveitamento dos alimentos. Em estudo realizado por Nguyen *et al.* (2015), foi observado que a BSF foi capaz de utilizar resíduos (fígado suíno, dejeto de suíno, resíduos domésticos, resíduos de pescado, frutas e vegetais) para realizar seu desenvolvimento. Sendo assim, é possível utilizar os insetos como forma de auxílio na redução de resíduos.

Além de possuírem um alto valor nutritivo, insetos são notáveis quanto a suas propriedades nutracêuticas por produzirem uma gama diversa de peptídeos antimicrobianos (AMPs) que funcionam como ‘‘antibióticos naturais’’. De acordo com uma revisão de literatura elaborada por Józefiak e Engberg (2017), os peptídeos antimicrobianos são pequenos, catiônicos, compõem o sistema imune dos insetos e exibem atividade antitumoral (Sun *et al.*, 2014), bactericida, antifúngica, antiparasitária e antiviral (Jenseen et al., 2006; Yi et al., 2014; Mylonakis et al., 2016; Józefiak e Engberg, 2017). Com base nisso, pode-se propor a classificação dos produtos de insetos como “alimentos funcionais”; Hasler (2002) define alimentos funcionais como *alimentos que, devido a presença de compostos fisiologicamente ativos, proporcionam benefícios à saúde além de fornecer nutrientes”*.

As características dos insetos despertam interesse na utilização dos mesmos para o desenvolvimento de um alimento funcional/aditivo nutricional com propriedades nutracêuticas que pode minimizar os efeitos da exclusão dos antibióticos promotores do crescimento dos programas nutricionais de frangos de corte e outros animais (Ratclifee *et al*., 2011; Van Huis, 2015).

1.1 Peptídeos antimicrobianos e propriedades nutracêuticas dos insetos

Enquanto vertebrados superiores possuem uma imunidade inata e outra adaptativa baseada na expansão de populações de células T e B com especificidade particular para antígenos, os insetos e a maioria dos outros animais contam com um sistema imune inato evolutivamente mais antigo. Quando o sistema imune inato dos insetos é ativado, este produz um amplo espectro de moléculas efetoras incluindo os AMPs (Bulet *et al*., 2004).

Os AMPs estão presentes em diversas espécies animais, mas têm sido muito mais intensivamente estudados em espécies de insetos devido ao amplo repertório de substâncias que esse grupo taxonômico possui principalmente em termos de número e variedade de AMPs produzidos (Yi et al., 2014; Mylonakis et al., 2016). Além disso, a quantidade de AMPs produzida pelas espécies de insetos varia consideravelmente e, para efeito de escala, enquanto o inseto *Harmonia axyridis* é conhecido por produzir mais de 50 tipos de AMPs, a *Acyrthosiphon pisum* não produz nenhum (Vilcinskas, 2013). O repertório de AMPs produzido pelos insetos está intimamente associado à natureza das ameaças enfrentadas durante a evolução; assim, é esperado que aqueles insetos expostos a mais diversos patógenos apresentem um repertório também mais amplo (Vilcinskas *et al*., 2013)

Bioquimicamente, AMPs são proteínas com sequência aminoacídica curta (< 100 resíduos) com atividade microbicida contra bactérias, fungos, parasitas e até mesmo vírus (Jenseen et al., 2006; Mylonakis et al., 2016). Em termos gerais, AMPs de cadeia curta (< 20 aminoácidos) têm como alvo principal bactérias Gram –, enquanto os AMPs de cadeia longa (> 20 aminoácidos) são mais efetivos contra bactérias Gram + (Otvos, 2002; Rahnamaeian e Vilcinskas, 2012).

Os AMPs atuam por diversos modos de ação incluindo a) alteração dos gradientes eletroquímicos transmembrana necessários à homeostase microbiana; b) indução da permeabilidade e consequente formação de poros e ruptura da membrana; c) indução da síntese de espécies reativas de oxigênio e/ou d) neutralização de endotoxinas que culminam na morte do invasor (Thevissen *et al*., 2004; Rahnamaeian, 2015). Adicionalmente, os AMPs modulam a resposta imune inata dos insetos pelo aumento de suas concentrações na hemolinfa de forma mais prolongada que respostas iniciais celulares levando a uma possível capacidade de "back up'' contra infecções persistentes (Makarova *et al*., 2016).

Quatro diferentes AMPs foram identificados no tenébrio. A tenecina 1 é um peptídeo de defesa que atua contra bactérias Gram + e fungos (Moon *et al.,* 1994). A tenecina 2 é um peptídeo semelhante a coleoptericina e a diptericina que tem como alvo bactérias Gram – (Roh *et al.,* 2009). A tenecina 3 é um peptídeo rico em glicina ativo contra fungos (Lee *et al.,*

1996). A tenecina 4 foi descrita sendo uma proteína de 120 aminoácidos com 14% de glicina que se assemelha com atacinas e gloverinas (AMPs produzidos por outras espécies de insetos) com atividade bactericida contra *E. coli*, uma bactéria Gram – (Chae et al., 2012).

Assim como para o tenébrio, também foram realizados estudos buscando saber os reais efeitos dos peptídeos antimicrobianos produzidos pelas larvas de BSF. Park *et al.* (2014) demonstraram efeito positivo da larva em combate a bactérias Gram + e – e observaram que um peptídeo isolado das larvas de BSF é eficaz no combate ao *S. aureus* resistente a meticilina. Em seguida, esse peptídeo foi sequenciado e descrito (Park *et al.*, 2015) e chamado DLP4. Alvarez *et al*. (2019) observaram que os peptídeos antimicrobianos produzidos pelas larvas de BSF possuem atividade contra *Helicobacter pylori*. Para esse ensaio as larvas de BSF foram desafiadas com *E.coli*, incubadas por 36 h após a inoculação e, em seguida foi realizada extração de pequenos peptídeos da hemolinfa e purificação dos mesmos.

Pesquisas dos últimos 10 anos têm evidenciado as propriedades nutracêutcas dos insetos, especialmente via AMPs, e seu potencial uso como bactericida e modulador da resposta imune (Jenseen *et al*., 2006; Ratclifee *et al*., 2014; Yi *et al*., 2014; Rahnamaeian et al., 2015; Mylonakis et al., 2016), especialmente pelos cada vez mais alarmantes números sobre resistência a antibióticos tanto para humanos quanto animais (Ventola, 2015). Apesar das inúmeras vantagens, o progresso no estudo dos insetos e suas propriedades nutracêuticas tem sido lento e tem explicação principalmente na falta de interesse da indústria farmacêutica (entre 1998 e 2004, 290 novas drogas antibacterianas foram desenvolvidas e apenas 4 das maiores indústrias farmacêuticas apoiaram) e altos custos de produção devido à baixa concentração dos AMPs nos insetos (Ratclifee *et al*., 2014).

O uso de insetos tanto como ingrediente funcional e/ou aditivo recentemente ganhou muita notoriedade e sinaliza positivamente para as vantagens dos insetos e como esse grupo animal representa uma solução real e possível à indústria animal (Józefiak e Engberg, 2015; Józefiak et al., 2016) e farmacêutica (Mylonakis et al., 2016). Neste sentido, está integrado ao conceito *One Health*, que une o cuidado humano, animal e do meio-ambiente, de forma conjunta, como estratégia bem-sucedida de esforços em saúde pública e bem-estar das populações. Os peptídeos antimicrobianos exercem diversas atividades antimicrobianas que podem prevenir o desenvolvimento de resistência pelas bactérias (Peschel e Sahl, 2006).

1.2 *Tenebrio molitor* e *Hermetia illuscens*: Características

Tenébrio (*Tenebrio molitor*) e BSF (*Hermetia illucens*) são espécies de insetos candidatos para uso na nutrição de frangos de corte. O tenébrio é um inseto coleóptero que pode ser utilizado como fonte proteica para a nutrição animal. Seu ciclo de vida apresenta cerca de 12 instars larvais e é variável de acordo com a temperatura de criação, entre 5 e 18 meses. O último estágio larval tem variação de coloração entre amarelo e marrom claro, pesa aproximadamente 120-160 mg e tem tamanho máximo de 20-32 mm. A farinha de tenébrio pode apresentar composição bromatológica diferente dependendo da sua forma de criação, mas apresenta valores de 47-60% de proteína bruta (PB) e 31-43% de lipídeos (Makkar et al., 2014).

A BSF é um inseto da ordem Diptera, sua fase larval pode atingir até 27 mm de comprimento e pesar até 220 mg. As larvas são cinza opacas (Diclaro e Kaufman, 2009). Podem ser alimentadas com diversos resíduos orgânicos, como frutas e legumes, resíduo de ração e dejetos (Diener et al., 2011; Van Huis et al., 2013). O ciclo de vida é menor que o do tenébrio; em 2 meses as larvas atingem a maturidade, porém em situação de pouco alimento pode levar até 4 meses (Hardoiune Mahoux, 2003).

As larvas de BSF possuem valor nutricional de grande interesse para a nutrição animal. Possuem 40-44% de PB, mas de acordo com Arango Gutierrez *et al. (*2004), sua composição pode se alterar de acordo com a sua alimentação. Com relação à composição de ácidos graxos, está diretamente relacionada à dieta.

1.3 Insetos como aditivo nutricional em dietas para frangos de corte

Em uma revisão recente sobre o uso de insetos na alimentação de frangos, Gasco *et al.* (2019) coletaram e analisaram dados de publicações de diversas revistas e observaram que o número de pesquisas utilizando insetos na alimentação animal teve um aumento considerável nos últimos 5 anos. Porém, mais estudos são necessários para a comprovação do real efeito das farinhas de insetos na alimentação animal. Apesar do aumento no número de estudos com o uso de insetos na alimentação de aves, a maioria das pesquisas realizadas abordam o uso como ingrediente, sendo necessários resultados que comprovem o uso como aditivo nutricional e modulador do sistema imune.

Józefiak *et al.* (2018) adicionaram 0,2% de farinha de tenébrio ou de BSF em rações para frangos de corte até 35 dias de idade e observaram que o uso em baixas dosagens pode modular a microbiota do trato digestório das aves. Eles avaliaram a microbiota da digesta ileal e cecal e obervaram que o uso de tenébrio resultou em contagens mais baixas de *Bacteroides*-

Prevotella enquanto a dieta com BSF diminuiu a contagem de subgrupos de *Clostridium leptum* e resultou em um aumento da contagem de L*actobacillus spp./Enterococcus spp.*, quando comparados ao tenébrio.

Islam e Yang (2016), ao adicionarem 0,4% de farinha de tenébrio na dieta de frangos de corte desafiados com *Salmonella* Enteritidis e *Escherichia coli* inoculadas via oral, obtiveram aumento no ganho de peso utilizando a farinha de tenébrio. Quando analisadas as respostas imunológicas das aves, o uso de farinha de tenébrio resultou em um aumento na produção de IgG e IgA e reduziu *Salmonella* Enteritidis e *E. coli* no conteúdo cecal.

Benzertiha *et al.* (2020), ao realizarem experimento comparando o uso de farinha de tenébrio (0,2 e 0,3% de inclusão) com salinomicina (coccidiostático) na ração de frangos, observaram que a utilização desses níveis resultou em aumento no ganho de peso das aves e no consumo de ração sem alterar a conversão alimentar. Quando comparado o uso de tenébrio com a dieta controle (sem aditivos), foi verificada uma redução de IgY e IgM; , também observaram uma redução linear de IgM com o aumento do ganho de peso, o que resultou em uma correlação negativa significativa entre esses dois parâmetros.

Resultados semelhantes a respeito dos benefícios da baixa inclusão de tenébrio na alimentação de frangos de corte foram observados por Benzertiha *et al.* (2019), ao realizarem experimento comparando o uso de farinha de tenébrio (0,2 e 0,3% de inclusão) com salinomicina (coccidiosático) e verificaram um aumento de atividade de α e β-glucosidase e α-galactosidase e uma diminuição da contagem cecal de *Prevotella* quando comparada ao controle positivo e negativo.

Apesar de pesquisas estarem em andamento a respeito da utilização de farinhas de insetos na nutrição animal, ainda são necessárias muitas outras para que níveis confiáveis e o modo de ação sejam estabelecidos. Além disso, apesar dos inúmeros benefícios para a nutrição, é preciso estabelecer os níveis adequados para a inclusão e quais são os reais efeitos do aditivo no sistema imunológico das aves para que se possam obter dados suficientes para possível recomendação como aditivo e potencial uso na substituição dos antibióticos promotores do crescimento (APCs).

1.4 Justificativa e principais objetivos

Nos últimos anos muitas pesquisas vêm sendo realizadas buscando resultados sobre a inclusão de insetos na alimentação de frangos de corte. As farinhas de tenébrio e de BSF têm mostrado reais benefícios de sua inclusão nas dietas. Porém, ainda é necessário que mais

estudos sejam realizados para que se possam definir os níveis mais adequados de inclusão nas dietas e o efeito sob o sistema imune de aves.

Devido aos grandes avanços em pesquisas e as descobertas dos AMPs em BSF e tenébrio, esses produtos foram avaliados envolvendo o desempenho e sistema imunológico de frangos de corte alimentados com ambos os insetos. O fornecimento de farinhas de insetos na alimentação de frangos de corte busca elucidar benefícios dos insetos na nutrição das aves, pelo seu valor nutritivo, ao fornecer nutrientes, e reforçar as defesas naturais, melhorando sua saúde. Esta estratégia pode, em consequência, resultar em redução do uso de APCs.

O experimento (exp.) 1 foi realizado inicialmente para definir o melhor nível de inclusão de farinha de larvas de tenébrio e de BSF (0,5 ou 1,0%) como alimento funcional na ração. O desempenho das aves, representado pelo ganho de peso, consumo de ração e conversão alimentar foi avaliado durante o período de criação de 42 dias. O nível de inclusão a ser definido seria aplicado nos experimentos subsequentes. Entretanto, ao final do período experimental não foram observadas respostas positivas no desempenho das aves com nenhum dos níveis de farinha de insetos escolhidos inicialmente, sendo necessária a alteração nos dois experimentos seguintes.

O exp. 2 foi realizado para avaliar o desempenho de frangos de corte alimentados com 0,5 ou 2,0% de farinha de tenébrio considerando as mesmas variáveis do exp. 1 e a contagem de *E. coli* e *Enterococcus spp*. no ceco das aves. Adicionalmente foi realizado um experimento em gaiolas metálicas com os mesmos tratamentos do exp. 2 para avaliar o desempenho e o sistema imune inato das aves 6 horas após desafio com LPS de *E. coli*.

O exp. 3 foi realizado para avaliar o desempenho de frangos de corte alimentados com 0,5 ou 2,0% de farinha de larvas de black soldier fly desengordurada considerando as mesmas variáveis de desempenho dos experimentos anteriores e para avaliar o sistema imune inato das aves 12 horas após desafio com LPS de *E. coli.*

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2. SHORT COMMUNICATION: EFFECT OF LOW DIETARY LEVEL OF TENEBRIO OR BLACK SOLDIER FLY FULL-FAT MEALS ON BROILER PERFORMANCE

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Abstract

The objective of this study was to evaluate tenebrio and black soldier fly larvae full-fat meals added at 0.5 or 1.0% in the starter diet (1-21 d) on broilers growth performance and the possible residual effects during grower and finisher phases. 840 d-old male broiler chicks were used. Birds were assigned to 6 treatments, 7 replicate pens per treatments, and 20 birds per pen in an open-sided poultry house, during 42 d, to evaluate performance. The experimental treatments used were as following: NC (negative control) – no additives; PC (positive control) – NC + enramycin (10 ppm); TM0.5 – NC + 0.5% tenebrio meal (TM); TM1.0 – NC + 1.0% TM; BSF0.5 – NC + 0.5% black soldier fly meal (BSF) and BSF1.0 – NC + 1.0% BSF. Birds were weighed to evaluate body weight gain, feed intake and feed conversion ratio. There were no effects of the antibiotic or the insect meals added to the feed on body weight gain in the starter (average 958 g) or the overall period (average 3,362 g). Feed conversion in the 1-42 d was not changed by the insect meals (average 1.511) but it was improved by enramycin (1.475, $P<0.01$). In conclusion the use of low levels of inclusion (0.5 or 1.0%) of tenebrio and black soldier fly full-fat meals in the starter diet did not improve broilers performance and did not have carryover effects in the grower and finisher phases.

Key-words: functional ingredient, feed additive, antibiotic, insects.

2.1 Introduction

Use of insect meals in animal production has been gaining prominence in research around the world. Production of insect larvae makes use of by-products as substrates and requires less land and water to produce high quality animal origin protein feed ingredients. Lower cabon emission brings even more interest to the feed chain. The European Union has recently approved the use of insect meals for poultry and swine feeds.

Insect meals have good nutritive value and amino acid profile (Veldkamp et al., 2012). Yellow mealworm (*Tenebrio molitor;* TM) is an insect of the order Coleoptera and

black soldier fly (*Hermetia illuscens;* BSF) belongs to the order Diptera. Their larvae contain high amount of protein and fat (Makkar et al., 2014). In addition to its nutritional value, insects produce antimicrobial peptides (AMPs) for their defense, which play an important role in immune response. These AMPs can be effective against bacteria, virus, fungi and other pathogens (Yi et al., 2014; Elhag et al., 2017), and can modulate the immune response and enhance the performance of broiler chickens (Benzertiha et al.,2019).

In a previous study utilizing high inclusion levels of TM (4, 8 or 12%) in the diet of broilers we observed that body weight gain was improved by up to 140 g at 35 days of age compared to the basal diet and favored modulation of the innate immune response (unpublished data). Several studies have been conducted in the past years aiming partial or total replacement soybean meal and fish meal in chicken diets and concluded that insect meals can be used as alternative protein sources in broiler nutrition (Bovera et al., 2015; Biasato et al., 2017; Sedgh-Gooya et al., 2020; Tippayadara et al., 2021). High cost and available volumes of insect products make unviable the use in complete substitution to conventional protein sources for poultry production (Veldkamp et al., 2021). Benzertiha et al. (2020), using low inclusion levels (0.2 or 0.3%) *Tenebrio molitor* and *Zophobas morio* meals, reported increased broiler growth performance compared to treatment with salynomicin. They also found improved immune response traits, expressed as lower serum IgM and IgY. The positive response obtained with low levels of insect meals observed in the latter study constitutes an indication that functional properties are involved, as opposed to their nutritional contribution. Therefore, the classification of insect meals as 'functional food' is proposed. This evidence aroused our interest in studying low inclusion levels and the possibility to reduce the need for antibiotic growth promoters (AGPs) in the starter phase for broiler production.

So, we hypothesized that the use of low inclusion levels of selected insect meals can enhance broiler performance. The present study was conducted to evaluate TM and BSF added at 0.5 and 1.0% in the starter diet on broilers growth performance and the possible residual effects during grower and finisher phases.

2.2 Material and Methods

This experiment was conducted in Department of Animal Science, University of São Paulo, Piracicaba, SP, Brazil (Lat: 22o 42' 30'' south - Long: 47o 38' 00'' west - Alt: 546

m). All procedures were approved by the Institutional Animal Care and Use of Committee (protocol number: 8400081020).

A total of 840 one-day-old male broiler chicks (Ross) were purchased from a local hatchery, weighed individually upon arrival, distributed to 42 homogeneous groups (20 chicks, 45.5 g initial body weight) and randomly assigned to floor pens (3 m^2). Experimental design was randomized complete blocks. Chicks were divided in 6 treatments with 7 replicates. The birds were housed in pens with rice hulls as bedding material, with water and feed *ad libitum,* using nipple waterers and tubular feeders, respectively. The maximum and minimum temperature in the experimental facility was recorded daily and ranged from 24.5 to 31.5 °C in the starter phase, 24.6 to 31.6 °C in the grower phase and 25.0 to 29.3 °C in the finisher phase. Heating lamps were used in each pen during the ten initial days. The lighting schedule adopted was 18 h light: 6 h dark after 10 days from the start of the experiment.

TM was obtained from Vida Proteína Cia. Ltda, Neirópolis, Goiás, Brazil and BSF was obtained from BSF Nutrição e Biotecnologia Ltda., Piracicaba, São Paulo, Brazil.

TM was analyzed for dry matter, crude protein, ether extract, ash, crude fiber, gross energy, calcium, phosphorus and amino acid profile. The methodology can be found in the publication of Nascimento Filho et al. (2021). For BSF, components analyzed were dry matter, crude protein, ether extract, ash, crude fiber and gross energy. Amino acid profile and digestibility coefficients of amino acids and metabolizable energy of BSF were based on Mwaniki et al. (2018); digestible amino acid levels and metabolizable energy value were adjusted according to the crude protein and gross energy in the product utilized in the present study. Nutrient composition of the TM and BSF used in this experiment are summarized in Table 1.

Diets were formulated according to Brazilian tables for poultry and pigs (Rostagno et al., 2017), based on corn and soybean meal, being isonutritive and isoenergetic, prepared in mash form. Starter diets were offered to all birds from 1 to 21 day of age, grower diets from 21 to 35 day of age, and finisher diets from 35 to 42 day of age. The experimental treatments used were as following: NC (negative control) – no additives; PC (positive control) – NC + enramycin (10 ppm); TM0.5 – NC + 0.5% TM; TM1.0 – NC + 1.0% TM; BSF0.5 – NC + 0.5% BSF and BSF1.0 – NC + 1.0% BSF. After 21 days, all chickens receiving the treatments containing insect meals were fed the same grower and finisher diets (NC); only the PC chickens remained in the treatment with

enramycin. The composition of the diets is shown in Table 2. TM and BSF were removed from the diets after 21 days to check if the effects obtained in the first weeks were maintained throughout the growth period due to the health and immunity benefits conferred by the insect meals. On days 21, 35 and 42 of the experiment, birds were weighed on a pen basis for determination of weight gain and feed intake. Adjusted feed conversion ratio was calculated considering the losses occurred during the experiment. The variables analyzed were body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) for each phase and for the cumulative period.

Performance data were submitted to PROC MIXED (Linear Mixed Models) of SAS (Statistical Analysis System, version9.2). All data were tested for normality of residuals through Shapiro-Wilk test. When a significant effect was verified, the variables were submitted to mean comparison by Tukey test considering the level of 5% of significance.

2.3 Results

The effects of TM and BSF supplementation on the growth performance of broiler chickens are shown in Table 3. No significant effect was observed on BWG and FI in the starter phase (1 to 21 day). However, it was observed that chicks of PC had better FCR ($P<0.05$). During the grower and finisher phases (21 to 42 day), no difference in BWG was observed between treatments. In the grower phase, treatment PC had better FI and FCR compared to the other treatments ($P<0.05$). In the overall period (1 to 42 day), no significant effects of dietary treatments were observed in BWG; however, the broilers on the antibiotic treatment (PC) presented lower FI and improved FCR ($P<0.05$).

2.4 Discussion

Insect meals are being used in animal feed in substitution of soybean (Bovera et al., 2015, 2016) and fish meal (Tippayadara et al., 2021) as an alternative protein source. There are some reports of increased BWG and FI when insect meals are fed (Ballitoc and Sun, 2013; Hussain et al., 2017). However, in the present study we did not observe any improvement in broiler performance fed tenebrio meal or black soldier fly meal. Moreover, antibiotic supplementation in the feed also did not result in greater weight gain; the additive resulted in lower FI and better FCR possibly because the antibiotic improved energy utilization (Harms et al., 1986).

Biasato et al. (2017) observed increased average daily gain during the ten initial days of growth when broilers were fed 5, 10 or 15% TM, but not after that. They observed greater FI without affecting FCR, and no effects on hematochemical parameters. Similar finding was reported by Sedgh-Gooya et al. (2020) when 2.5% TM was fed in the first 10 days. BSF can also improve broiler performance (Dabbou et al., 2018; Popova et al., 2021). Benzertiha et al. (2020), using low inclusion (0.2 or 0.3%) of *Tenebrio molitor* and *Zophobas morio* full-fat meals, obtained an increase in broiler performance and improvement in some parameters of the immune system of the birds. However, in another report of the same research group (Józefiak et al., 2018) low inclusion levels (0.05 or 0.2%) did not affect the performance of the chickens. The results obtained in the present study using 0.5 or 1.0% insect meals in the feed are in agreement with the latter reports in which insect meals did not improve broiler performance.

Ban of AGPs makes necessary alternatives to substitute them without harming performance. AGPs are used in animal production as a way to fight pathogenic microorganisms that can attack the animals in addition to favor bacteria that are beneficial to the environment (Sunde et al., 1990), allowing the animals to express their productive potential, improve fat digestibility and energy uptake (Harms et al., 1986). Nevertheless, use of AGP in broiler feed may not result in increase on BWG, FI and better FCR of the birds (Broderick et al., 2021). This fact that can be related to non-contact with pathogens or challenging situations (Cakır et al., 2008). In this experiment, the use of AGP did not improve broiler performance, which may have occurred because the experimental facilities have not been used for almost one year before this trial; as a consequence, no challenging environment was presented to the birds. The final liveweight of 3,407 g at 42 d with a FCR of 1.511 constitute an additional evidence that environmental conditions were near optimal, because according to the Performance Objectives of Ross 308 (Aviagen, 2019) the weight at 42 d should be 3,136 g with a FCR of 1.596. Under these conditions, any response to management strategies is not likely to be achieved. An additional topic that remains to be better known is about the actual content of antimicrobial compounds in the insect larvae. According to Veldkamp et al. (2021), the insect haemolymph acquires antimicrobial properties after the insect has been injured, so the conditions in which the larvae are raised may affect the AMPs.

2.5 Conclusion

The use of low levels of inclusion (0.5 or 1.0%) of yellow mealworm and black soldier fly full-fat meals in starter diet did not improve broilers performance and did not have effects in the grower and finisher phases under the conditions of this study.

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Table 1: Nutrient composition of yellow mealworm (TM) and black soldier fly full-fat meals (BSF) used in the experiment (as fed basis).

Item	*TM*	*BSF*
Dry matter (%)	97,02	97.10
Crude protein (%)	50,00	50.46
Ether extract (%)	29.73	28.16
Crude ash (%)	4.12	6.30
Crude fiber (%)	4.50	6.77
Calcium (%)	0.125	0.121
Phosphorus (%)	0.570	0.095
Gross energy (kcal/kg)	6366	6408
AMEn (kcal/kg)	4854	4853
Amino acids composition (%)		
Digestible indispensable amino acids, %		
Arginine	2,68	2.17
Histidine	1.48	2,93
Isoleucine	1,91	1,86
Leucine	3,31	2,95
Lysine	2,90	2,42
Methionine	0,68	0.70
Phenylalanine	2,12	1,65
Threonine	1,71	1.68
Valine	2,76	2.61
Diegestible dispensable amino acids, %		
Alanine	3.16	2,94
Aspartic acid	3,89	3,62
Cysteine	0.54	0.25
Glycine	2,25	2.05
Glutamic acid	5,58	5.15
Proline	2,85	2.62
Serine	1,72	1.90
Tyrosine	4,07	2,14

Table 2: Composition of the experimental diets

Ingredients (g/kg)	Starter (1 to 21d)						Grower (22 to 35d)		Finisher (36 to 42d)	
	NC [1]	PC [2]	TM0.5 [3]	TM1.0 [4]	BSF0.5 [5]	BSF1.0 [6]	NC	PC	NC	PC
Corn	490.3	490.0	494.0	497.8	494.6	498.9	563.9	563.7	626.3	626.2
Soybean meal	434.0	434.1	427.8	421.7	427.6	421.2	353.9	353.9	300.9	300.9
Soybean oil	36.8	36.9	34.2	31.6	34.0	31.2	49.3	49.3	44.9	44.9
Dicalcium phosphate	18.0	18.0	17.9	17.9	17.8	17.6	14.7	14.7	10.9	10.9
Limestone	8.9	8.9	9.0	9.0	8.9	8.9	7.0	7.0	6.7	6.7
Salt	5.3	5.2	5.2	5.2	5.2	5.2	4.9	4.9	4.6	4.6
Vitamin premix	0.5*	0.5	0.5	0.5	0.5	0.5	0.4**	0.4	0.4	0.4
Mineral premix ***	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
DL-Methionine	3.24	3.24	3.24	3.24	3.25	3.26	2.74	2.74	2.33	2.33
L-Lysine.HCl 77%	1.22	1.22	1.23	1.22	1.26	1.30	1.52	1.52	1.67	1.67
L-Threonine	0.45	0.45	0.45	0.45	0.45	0.45	0.38	0.38	0.28	0.28
Choline chloride 60%	0.8	0.8	0.8	0.8	0.8	0.8	0.6	0.6	0.4	0.4
Yellow mealworm full-fat meal	0	0	5.0	10.0	0	0	0	0	0	0
Black soldier fly full-fat meal	0	0	0	0	5.0	10.0	0	0	0	0
Enramycin 8%	0	0.125	0	0	0	0	0	0.125	0	0.06
Calculated nutritive value (g/kg)										
Crude protein	239.0	239.0	239.0	239.0	239.0	239.0	208.4	208.4	189.1	189.1
Total phosphorus (P)	6.90	6.90	6.89	6.88	6.88	6.86	6.03	6.03	5.17	5.17
Calcium (Ca)	9.35	9.35	9.35	9.35	9.35	9.35	7.58	7.58	6.34	6.34
Sodium (Na)	2.22	2.22	2.22	2.22	2.22	2.22	2.08	2.08	1.97	1.97
Methionine	6.34	6.34	6.34	6.34	6.36	6.38	5.51	5.51	4.91	4.91
Lysine	12.90	12.90	12.90	12.90	12.90	12.90	11.24	11.24	10.14	10.14
Methionine + Cysteine	9.50	9.50	9.5	9.50	9.50	9.50	8.32	8.32	7.50	7.50
Threonine	8.50	8.50	8.50	8.50	8.50	8.50	7.42	7.42	6.69	6.69
AME (kcal/kg)	2975	2975	2975	2975	2975	2975	3150	3150	3200	3200

* Salus Vitamin Products, provided the following per kilogram of diet: vitamin A, 8,500 IU; vitamin D_3, 3,000 IU; vitamin E, 18 IU; vitamin K_3, 2,5 mg; Vitamin B_1, 2 mg; vitamin B_2, 6 mg; vitamin B_6, 3 mg; vitamin B_{12}, 14 µg; vitamin B_5, 14 mg; folic acid, 1,2 mg; biotin, 0,08 mg and selenium, 0,5 mg.

**Salus Vitamin Products, provided the following per kilogram of diet: vitamin A, 6,800 IU; vitamin D3, 2,400 IU; vitamin E, 14 IU; vitamin K_3, 2,0 mg; Vitamin B_1, 1,6 mg; vitamin B_2, 4,8 g; vitamin B_6, 2,4 mg; vitamin B_{12} , 11 µg ; vitamin B_5, 14 mg ; folic acid, 1,0 mg; biotin, 0.06 mg and selenium, 0,4 mg.

***Salus Mineral Products, provided the following per kilogram of diet: manganese, 80 mg; zinc, 70 mg; Iron, 50 mg; copper, 10 mg and iodine, 1 mg.

[1] NC- Negative control.

[2] PC – Positive control – NC + Enramycin (10 ppm), AGP.

[3] TM05 – NC + 0.5% TM.

[4] TM10 – NC + 1.0% TM.

[5] BSF05- NC + 0.5% BSF.

[6] BSF10- NC + 1.0% BSF.

Table 3: Growth performance of broiler chickens fed two different full-fat insect meals: tenebrio and BSF

	Treatments[1]							
Item[2]	NC	PC	TM0.5	TM1.0	BSF0.5	BSF1.0	SEM[3]	P-value
1 to 21 d								
BWG	967	958	953	942	953	978	13.56	0.4541
FI	1121	1101	1115	1116	1129	1151	12.45	0.1240
FCR	1.161[ab]	1.149[a]	1.171[ab]	1.177[ab]	1.187[b]	1.177[ab]	0.0076	0.0109
21 to 35 d								
BWG	1552	1567	1536	1530	1571	1545	17.21	0.3495
FI	2375	2334	1343	2306	2375	2367	20.84	0.0600
FCR	1.531[b]	1.490[a]	1.525[b]	1.507[b]	1.516[b]	1.532	.0097	0.0178
35 to 42 d								
BWG	866	857	864	847	870	851	16.86	0.8626
FI	1596	1557	1602	1576	1642	1605	22.30	0.1074
FCR	1.844	1.818	1.856	1.860	1.892	1.887	0.0018	0.2177
1 to 42 d								
BWG	3385	3347	3353	3319	3394	3374	29.12	0.4270
FI	5092[a]	4964[b]	5021[ab]	5028[ab]	5147[a]	5121[a]	31.71	0.0004
FCR	1.505[b]	1.475[a]	1.510[b]	1.506[b]	1.517[b]	1.518[b]	0.0071	0.0019

[1] NC= negative control; PC= NC + enramycin addition (AGP), 10 ppm; TM0.5= CN + 0.5% TM; TM1.0= CN + 1.0% TM; BSF0.5= CN + 0.5% BSF; BSF1.0= CN + 1.0% BSF

[2] BWG= body weight gain; FI= feed intake; FCR= feed conversion ratio.

[3] SEM: standard error of the mean.

[a, b] Means values within a row having different superscripts are statistically different by Tukey test ($p<0.05$).

3. TENEBRIO LARVAE MEAL AS A FUNCTIONAL FOOD MODULATES IMMUNITY AND IMPROVES GROWTH PERFORMANCE OF BROILER CHICKENS.

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ABSTRACT

This study aimed to determine the effects of full-fat tenebrio larvae meal (TM) in the diet of broilers on growth performance and to study serum innate immunity in birds challenged with *Escherichia coli* lipopolysaccharide (LPS). In two independent experiments, 992 d-old Cobb male broiler chicks were used. In the first trial, birds were assigned to 4 treatments, 10 replicates of 20 chicks per treatment, in a randomized block design, in an open-sided poultry house, during 35 d, to evaluate performance and count of selected bacteria in ceca. Dietary treatments were as follows: **NC** (negative control); **PC** (positive control) – NC + 10 mg/kg enramycin and 66 mg/kg salinomycin; **TM0.5** – NC + 0.5% TM; and **TM2.0** – NC + 2.0% TM. In the second experiment, the same treatments were used but birds were raised in battery cages, during 21 d; at the end of the experiment birds was inject with LPS or with sterile saline solution. Blood was collected and lysozyme activity (**LYZ**), hemolytic activity of the alternative complement system (**HACS**), myeloperoxidase activity (**MPO**) and bactericidal activity (**BA**) were measured in serum. In both trials the supplementation with 0.5% TM did not improve performance. 2.0% TM resulted in an increase in body weight gain (**BWG**) and feed intake (**FI**) of 3.8% and 4.2% ($P<0.01$ and $P<0.01$, experiment 1, respectively) and 5.7% and 2.1% ($P<0.01$ and $P<0.03$, experiment 2, respectively) but did not affect the feed conversion ratio (**FCR**) in experiment 1 ($P>0.05$). In the second trial TM2.0 had better FCR compared to NC ($P<0.05$). Birds non-challenged with LPS (NCLPS) and fed 2,0% TM had HACS and MPO favored compared to CN and even CP ($P<0.05$). In conclusion the use of 2% TM in broilers feed can increase BWG and FI of birds and modulates the innate immune response of birds prior to LPS challenge.

Key Words: antibiotic growth promoter, feed additives, ionophores, mealworm, poultry, *Tenebrio molitor*.

3.1 Introduction

The use of insect larvae meal as a nutritional component of broilers feed or as a functional food has been gaining attention for food-producing animals. In recent years, there has been an increase in published research on insect feeding, but studies to define its application in nutritional programs, such as inclusion levels, are still necessary.

Tenebrio molitor, a species of the order Coleoptera, family Tenebrionidae, is a beetle known as mealworm. Its larvae meal can have 44-69% crude protein and 23-47% fat on dry matter basis (Veldkamp et al., 2012; Makkar et al., 2014). The protein has a good amino acid profile, camparable to soybean or fish meal protein (De Marco et al., 2015). We determined that the standardized ileal digestibility coefficient of amino acids in TM is greater then 0.81 (Nascimento Filho et al., 2021).

Insects such as tenebrio and others are be able to synthesize antimicrobial peptides (**AMPs**) as a protective mechanism (Bulet et al., 2004; Józefiak and Engberg, 2017). AMPs are proteins with low amino acid sequence (<100) and have shown microbicidal effect against bacteria, virus, fungi and other pathogens (Jenssen et al., 2006; Mylonakis et al., 2016; Józefiak and Engberg, 2017). Four AMPs are described for tenebrio, *tenecin 1, 2, 3 and 4*, which have spectrum against Gram-negative and positive bacteria and against fungi (Moon et al., 1994; Lee et al., 1996; Roh et al., 2009; Chae et al., 2012). Insects are also rich in chitin and chitosan which possess microbicidal activity (Vartiainer et al., 2004) and can thrive an immune response and modulation after recognition by *toll-like receptors* (**TLR**) (Lee et al., 2008; Islam and Yang, 2017; Komi et al., 2018). According to Fuchs et al. (2018), TLR2 is a mammalian immune cell pattern recognition receptor that directly binds chitin with high affinity. In this way, after the recognition of the immune system, immune responses can be triggered.

Most nutritional studies with insect meals have focused on their nutritive value as a high protein and lipid animal ingredient (Veldkamp et al., 2021); however, considering cost of the inclusion in practical feeds, the availability of the products and the effects of insect meals as a functional food, evaluation of low inclusion level is necessary. In a previous study of our research group, diets formulated with 0, 4, 8 or 12% full-fat tenebrio larvae meal were fed to broilers from 1 to 35 d of age in floor pens. It has been shown that the feed containing 4% TM resulted in significant increase of 140 g in body weight gain (**BWG**) and modulation effect of the innate immune response of broilers challenged with LPS “R. T. Pereira (University of São Paulo, Piracicaba, São Paulo, personal communication).” Recently, Benzertiha et al. (2020) demonstrated that the inclusion of small amounts (0.2 or 0.3%) of *Tenebrio molitor* full-fat meals in broiler diets improved growth performance and modulated the immune response, reducing serum IgM and increasing serum IL-2 and TNF-α. In this case, improvement in performance cannot be attributed to the nutrient contribution of the insect meals, and some nutraceutical properties might be involved. Recent research have also evaluated the immune response with other insect meals and have reported their ability to modulate the microbiota,

immune response and to increase antioxidant capacity (Józefiak et al., 2018; Biasato et al., 2019; Kozlowski et al., 2021).

To the best of our knowledge this is the first study to evaluate the effects of full-fat TM meal in the innate immune response of broiler chickens under an LPS-induced inflammation. Therefore, we hypothesized that low inclusion levels of 0.5 or 2.0% of TM in broiler diets may improve growth performance and modulate the innate immune response of chickens. The effects of full-fat tenebrio larvae meal (TM) on growth performance and serum innate immunity in broilers challenged with *Escherichia coli* lipopolysaccharide (LPS) was investigated

3.2 Material and Methods

The study was conducted at Department of Animal Science, University of São Paulo (Piracicaba, SP, Brazil). All procedures were approved by the Institutional Animal Care and Use of Committee (protocol number: 8400081020).

Two experiments were carried out with the use of TM meal in the feed for broiler chickens. The first trial assessed bird performance and bacterial counts in the gastrointestinal tract and the second trial studied the innate immune response under an LPS-induced inflammation.

In both experiments, diets met the nutritional specifications of the Brazilian tables for poultry (Rostagno et al., 2017), based on corn and soybean meal, being isonutritive and isoenergetic. Diets were prepared in mash form. In experiment 1, starter diets were offered from 1 to 21 d of age and grower diets from 22 to 35 d of age; in experiment 2, only the starter diet was utilized. The experimental treatments were as follows: **NC** (negative control) – no additives; **PC** (positive control) – NC + 10 mg/kg enramycin and 66 mg/kg salinomycin; **TM0.5** – NC + 0.5% TM; and **TM2.0** – NC + 2.0% TM. TM was analyzed for dry matter, crude protein, ether extract, ash, crude fiber, gross energy, calcium, phosphorus and amino acid profile. The methodology of bromatological analysis can be found in the publication of Nascimento Filho et al. (2021). TM was obtained from Vida Proteína Cia. Ltda., Neirópolis, Goiás, Brazil and its analyzed composition is depicted in Table 1. Diet composition is shown in Table 2.

Randomized complete block design was used in both experiments. We adopted this design because the poultry house used for experiment 1 has trees on only one side and an even distribution of treatments was preferred.

3.2.1 Experiment 1

Chickens were raised in an open-sided poultry house equipped with curtains, fans, spraying nozzles, heating lamps, nipple drinkers and tube feeders, in 3.0 m^2 floor pens covered with reused rice hulls litter. Environmental conditions were maintained close to the chickens needs at each stage of growth. Minimum and maximum temperatures were recorded daily. The heaters provided adequate temperatures for chick development during the first three weeks; in the grower phase, the averages for low and high temperatures were 18.5 ºC and 26.4 ºC, respectively.

A total of 800 one-day-old male broiler chicks (Cobb 500) were obtained from a local hatchery, weighed individually upon arrival, uniformly distributed to groups and randomly assigned to 40 pens. Chicks, with average weight of 45.3 g, were divided in 4 treatments with 10 replicates (20 birds/pen).

At the age of 7, 14, 21, 28 and 35 d, the birds were weighed on a pen basis and feed consumption was recorded. Weekly data and cumulative values for BWG and feed intake (**FI**) were determined, and feed conversion ratio (**FCR**) was calculated. Mortality was considered in calculations.

At the end of the trial (35 d of age), eight birds per treatment, randomly taken from eight pens in each treatment, were sacrificed and the ceca was quickly removed. The left ceca were collected and sent to a private laboratory for bacterial counting analysis of *Escherichia coli* and *Enterococcus spp*. according to APHA 2001 methodology (MKBLAB, Jundiaí, São Paulo, Brazil).

3.2.2 Experiment 2

Experiment 2 was designed to assess bird health, not performance, conducted in a ventilated poultry house, in battery cages with heating control, wired floors and stainless steel trough feeders and drinkers. During the entire experiments, chickens had *ad libitum* access to water and feed.

A total of 192 one-day-old male broiler chicks (Cobb 500) were obtained from a local hatchery, weighed individually upon arrival and randomly divided into 24 metallic cages. Chicks, with 42.5 g initial body weight, were divided into 4 treatments, with 6 replicates (8 birds/cage). The cages were equipped with stainless steel trough feeders and waterers, with mesh floor. On 7, 14 and 21 d of the experiment, the birds were weighed and the same variables of the experiment 1 were obtained. Weekly data and cumulative values for BWG and FI were determined and FCR was calculated. Mortality was considered in calculations.

On 21 d of the experiment, one bird/pen (n=6) was inoculated intra-abdominally with 2 mL (1,48 mg/kg of body weight) *Escherichia coli* LPS (serotype O55:B5, LPS L2880; Sigma-Aldrich) and one bird/pen (n=6) of the same pen was inoculated with 2mL of 0,9% (w/v) sterile saline solution as negative control. After 6 hours, blood was collected from the brachial vein into 4 mL clot accelerator tubes to obtain serum for the analysis of the innate immune system. Then, blood samples were centrifuged at 2000 × g for 10 min at 4 ºC, aliquoted in Eppendorf vials and stored at -80 ºC.

After serum collection, analyses were performed to assess the response of the innate immune system: lysozyme activity (**LYZ**), hemolytic activity of the complement (**HACS**), myeloperoxidase activity (**MPO**) and bactericidal activity (**BA**) against *E. coli* and BA *Salmonella* Gallinarum. All these analyses were carried out in serum of the birds challenged with LPS (**CLPS**) and non-challenged LPS (**NCLPS**).

3.2.3 Lysozyme activity

The LYZ of birds was determined by a turbidimetric assay as described by Jorgensen et al. (1993). Briefly, a suspension (200 µL) of *Micrococcus lysodeikticus* (M3770, Sigma-Aldrich) in PBS (0.2 g/L) at pH 6.2 was mixed with serum (10 µL) in a flat-bottomed 96-well plate. Plates were read with a microplate reader (Synergy H1 Multi-Mode Reader, Winooski, VT, USA). Lysozyme activity (units/mL) was calculated using the following formula: [(Δabsorbance (4 min–1 min)/3)/0.001] x 100. Quantification of lysozyme activity was done as per the standard definition of one unit of lysozyme activity from chicken egg hen lysozyme (L6876, Sigma Aldrich) corresponding to the linear decrease in optical density (OD) at 450 nm of 0.001 per minute.

3.2.4 Hemolytic activity of the alternative complement system

The HACS was measured as described by Sutili et al. (2016). An 80% diluted sample (80 µL of serum + 20 µL PBS) was mixed with 100 µL of 2% washed sheep red blood cells (SRBC) and incubated at 25 ºC for 45 min. Following incubation, the mixture was centrifuged at 2500 × g for 5 min. Then, 100 µL of supernatant were transferred to a 96-well plate and measured at 450 nm in a plate reader (Synergy H1 Multi-Mode Reader, Winooski, VT, USA). The percent of hemolysis was calculated by comparing between total hemolysis (100%: SRBCs + DI water) and no-hemolysis (0%: SRBCs + PBS + heat-inactivated serum) controls as follows: % hemolysis = [(A_{450} sample – A_{450} no-hemolysis)/ (A_{450} total hemolysis – A_{450} no-hemolysis)] × 100.

3.2.5 Myeloperoxidase activity

The MPO of serum was quantified following the protocol described by Kreutz et al. (2011), with modifications. Ten microliters of serum were diluted in 40 µL of PBS in flat-bottomed 96-well plates. Then, 100 µL of a solution containing o-3,3′, 5,5′-tetramethylbenzidine (TMB, T0440, Sigma-Aldrich) and hydrogen peroxide prepared in citrate (0.2 M); a phosphate buffer (0.01 M) at pH 5.3 was added to each well. The peroxidase reaction was stopped after 5 min by adding 100 µL of HCl (3 M). Plates were read with a microplate reader (Synergy H1 Multi-Mode Reader, Winooski, VT, USA), and the myeloperoxidase activity reported in absorbance at 450 nm. Wells without samples were added in the microplate as negative controls.

3.2.6 Bactericidal activity

The serum BA was determined by evaluating its effect on growth of *Salmonella* Gallinarum and *Escherichia coli*. The bacterial strains were provided by Laboratory of Veterinary Microbiology and Immunology - Farroupilha Federal Institute of Education, Science and Technology. The bacterial solution for each strain was prepared in tryptone soy broth medium (TSB) from cultures grown in tryptone soy agar medium (TSA) (Himedia Laboratories) {($1x10^8$ colony forming units (CFU)/mL; 0.15 optical density (OD) at 600 nm) (30 °C/24 h)}. Then, 20 µL of serum and 20 µL of bacterial dilution at a concentration of 10^6 bacteria/mL were added to each well of 96-well plates. The samples were incubated for 4h at 37 ºC. After incubation, 25 µL/well of 2,3,5-triphenyltetrazolium chloride (TTC), 0.5 mg/mL, (Sigma) were added and the samples were incubated for another 10 min (37 ºC), before being centrifuged (2000 × g, 10 min). The supernatant was removed, and the precipitate dissolved with 200 µL/well of dimethylsulfoxide (DMSO). Aliquots of 100 µL from each well were transferred to a new plate of 96 flat-bottomed wells. Absorbance was then measured at 450 nm (Synergy H1 Multi-Mode Reader, Winooski, VT, USA). The percent of BA was calculated by comparing between controls: 0% BA (TSB + bacterial solution) and 100% BA (PBS) (Albaladejo-Riad et al., 2020).

All these analysis (LYZ, HACS, MPO, BA *E. coli* and BA *Salmonella* Gallinarum) were carried out in serum of the birds challenged with LPS (**CLPS**) and non-challenged LPS (**NCLPS**).

3.2.7 Statistical analysis

Performance and bacterial count data were submitted to PROC MIXED (Linear Mixed Models) of SAS software. All data was tested for normality of residuals through Shapiro-Wilk test. When a significant effect was verified, the variables were submitted to mean comparison by Tukey test considering the level of 5% of significance. Serum immune parameters (LYZ, HACS, MPO and BA) were were subjected of Tukey-Kramer test to compare estimated means among treatment CLPS and NCLPS groups separately. Results were reported as least square means with standard error of the mean (S.E.M).

3.3 Results

3.3.1 Experiment 1

There was no response in performance of the broilers receiving the diet containing 0.5% TM in any phase of the trial (Table 3). However, the feed containing 2% TM resulted in an improvement in broiler performance compared with NC group. Weight gain (**WG**) was significantly higher for the chickens fed 2% TM on week 1 (1-7d, +7.1 g, P=0.01), week 2 (7-14d, +44 g, P<0.001) and week 4 (21-28d, +9.0 g, P<0.001). Although the advantage of TM2.0 compared to NC was not significant (P>0.05) in the remaining weeks, numerical differences were observed, resulting in higher BWG in the starter period, 1-21d (+76 g, P<0.001) and a final BWG 102 g higher at 35d (2808 g vs. 2706 g, P<0.001). This represents an advantage of 7.2% in the BWG in the starter phase and 3.8% in the total period for the broilers fed TM2.0 over the controls.

The feed intake of the birds consuming the diet with 2% TM was significantly higher than the NC on weeks 2 (P=0.005), 4 (P=0.001) and 5 (P=0.022). During the starter period, FI was 76 g greater for TM2.0 than NC (P=0.08). In the overall period, FI was 160 g higher for TM2.0 (3958 g vs. 3798 g, P<0.001), a difference of 4.2%. Feeding TM2.0 resulted in significantly (P<0.001) improved FCR on week 2 compared to NC, but it did not result in significant difference (1.410 vs. 1.403, P>0.05) in the total period.

The broilers fed the diet supplemented with enramycin + salinomycin (PC) had a greater response in BWG in relation to the NC than TM2.0. The response in BWG was 13.4% in the starter phase (1187 g vs. 1047 g, P<0.001) and 6.7% in the overall period (2890 g vs. 2706 g, P<0.001). PC broilers had higher BWG than TM2.0 at the end of the trial (+82 g, 2.9% higher, P<0.001). FI was very similar for PC and TM2.0; at the end of the trial the broilers fed the antimicrobial additives had a better FCR (1.374 vs. 1.410, P<0.001). Dietary

treatments did not affect (P>0.05) bacterial counts of *Escherichia coli* and *Enterococcus spp*. in the ceca (Table 4).

3.3.2 Experiment 2

There was no response in performance of the broilers receiving the diet containing 0.5% TM (Table 5). TM2.0 resulted in higher BWG than the NC on week 3 (+53 g, P=0.020), but the advantage of 60 g at 21 d was not significant (1113 g vs. 1053 g, P>0.05). The difference in FI between TM2.0 and NC was not significant (P>0.05), but FCR was improved for broilers fed TM2.0 (1.108 vs. 1.158, P=0.003).

Feeding the antimicrobial additives improved BWG, FI and FCR relative to NC at the end of the trial (P<0.01). The results for TM2.0 did not differ of PC for growth variables (P>0.05).

3.3.3 Innate immune response

The results of the immunity parameters for the birds receiving the dietary treatments and challenged or non-challenged with LPS are presented graphically in Figure 1 for visualization of the comparisons. Within each challenge condition, dietary treatments were compared using Tukey-Kramer test. After LPS challenge, no treatment differences were detected (P>0.05) for the immune parameters studied. However, in NCLPS broilers, TM2.0 resulted in greater HACS (P<0.001) and MPO activity (P=0.005) than the control (NC). Compared to the PC, the TM2.0 birds had higher HACS. The chickens of PC had activity of MPO similar to TM2.0 (P>0.05). The broilers fed TM2.0 had BA *E. coli* similar to the other treatments (P>0.05), but the BA *Salmonella* Gallinarum was inferior to CN (P=0.004).

3.4 Discussion

Two experiments were conducted to evaluate the performance of broilers fed diets containing low levels (0.5 and 2.0%) of tenebrio larvae meal, the effect on cecal counts of bacteria and on the innate immune response of birds challenged or not with LPS. Feeding the diet containing 2.0% TM resulted in an increase in WG of 3.8% (significant effect, floor pens, experiment 1) and 5.7% (non-significant, metallic cages, experiment 2) compared to the control diet, but inclusion of 0.5% in the diet did not improve performance. The difference in significance of results in both experiments may be attributed to the smaller number of birds per experimental unit and to the smaller number of replicates in experiment 2, which increases

the experimental error. However, experiment 2 was designed to assess the health of the birds, not the performance.

Comparing TM2.0 to NC in experiment 1, we obtained the same percentage increase, 3.8%, in bird WG observed by Cardinal et al. (2019) in a meta-analysis in which the response to AGPs was studied. Thus, the use of tenebrio meal can be a way to minimize the use of AGPs.

In experiment 1, the improvement in BWG of the chickens on diet TM2.0 was evident on the first week and it continued for the entire growth period of 35 d. At 21 d of age, the difference in WG was 76 g; in experiment 2, which was terminated at 21 d, the difference in WG at this age was 60 g. In other studies, broilers fed tenebrio meal had better WG only in the starter phase. This was reported by Biasato et al. (2017), with diets containing 0, 5, 10 or 15% tenebrio meal, and Sedgh-Gooya et al. (2020), feeding a 2.5% tenebrio meal diet; the authors indicated that this was due to a better efficiency of utilization of nutrients in the initial period. Ballitoc and Sun (2013) also obtained an increase in broiler WG when fed 2.0% tenebrio meal, but not 0.5%.

A series of studies have been published recently using elevated inclusion levels of tenebrio meal in the diet of broiler chickens, considering its nutritional value as an alternative protein ingredient. The insect meal was used in partial or total substitution to soybean meal (Bovera et al., 2016; Biasato et al., 2017; Elahi et al., 2020) or fish meal (Zadeh et al., 2020). The results are variable probably due to differences in the composition of the insect meal, which is affected by the stage of the larvae, raising conditions and differences in substrate (Veldkamp et al., 2012; Makkar et al., 2014).

In the present study, there was no growth performance improvement due to the TM0.5 diet. A recent report of Benzertiha et al. (2020) was successful in obtaining improved performance of broilers receiving diets containing levels as low as 0.2 or 0.3% tenebrio meal full-fat larvae meal in comparison to a salinomycin supplemented diet. In another study (Islam and Yang, 2017), chicks innoculated with *Salmonella* Enteritidis and *Escherichia coli* also showed improved performance when receiving diets containing the insect meals reported above. Contrarily, Jósefiak et al. (2018) did not obtain any growth improvement of broilers fed 0.05 to 0.2% of several insect meals, including tenebrio.

Utilizing the same feed formulation and the same batch of insect meal, it was found an increased feed intake for the diet TM2.0 in experiment 1, but not in experiment 2 reported here. The greater FI in the first trial (3958 vs. 3798 g, $P<0.01$) at 35 d promoted the improved BWG, because FCR was not affected by the TM2.0 diet compared to control. Even

considering the low inclusion of tenebrio meal in the diet, the demonstrated preference for the insect meal (Nascimento Filho et al., 2020) may explain the higher FI. This effect on FI has been observed in other reports (Biasato et al., 2017; Islam and Yang, 2017; Benzertiha et al., 2020); in the case of Biasato et al. (2017) this occurred in some phases of growth but not in the overall period. When the efficiency of feed utilization is improved by feeding insect meal, as indicated by a better FCR, the FI may not be affected, as observed in experiment 2; this finding corroborates the reports by Ballitoc and Sun (2013), Bovera et al. (2016) and Islam and Yang (2017). In experiment 2, TM2.0 resulted in a significantly better FCR (0.039, or 3.4%, $P<0.05$). Nutraceutical properties of insect meal may be responsible for a variety of effects, including the reduction of intense inflammatory processes. This reduction may maintain feed intake and lead to economy of nutrients. In both experiments, the positive control diet resulted in the best performance among all treatments, an indication that the experimental conditions allowed for the expression of the effects of the AGP + ionophore utilized.

As previously mentioned, insects only have an innate immune system, which is responsible for the synthesis of AMPs for their defense (Bulet et al., 2004). AMPs exhibit action against bacteria, fungi, parasites and other pathogens (Yi et al., 2014; Mylonakis et al., 2016; Jósefiak and Engberg, 2017). The microbicidal action exerted by these AMPs may be directly related to the improvement in the performance of the broilers fed tenebrio meal.

Despite the possible antimicrobial effect of insect meal, in this study there was no reduction in the counts of *E. coli* and *Enterococcus* spp. in the cecal contents of the chickens. Different results were found by Islam and Yang (2017) feeding diet containing 0.4% TM; after challenging birds with *E. coli* and *Salmonella* Enteritidis, they observed lower cecal counts of the bacteria, and attributed this effect to the chitin/chitosan present in the exoskeleton of the larvae of tenebrio. More recently, Sedgh-Gooya et al. (2021) also observed a reduction in the counting of *E. coli* in the cecum of broilers, but only with 5.0% of tenebrio meal inclusion, and not with 2.5%. They also attributed this result to chitin/chitosan.

In addition to the possible effect of TM in reducing pathogenic bacteria in the intestinal tract due to its nutraceutical properties, it may also modulate the intestinal microbiota. Biasato et al. (2019) evaluated the microbiota of broilers fed diets containing 0, 5, 10 or 15% of tenebrio meal and observed an increase in bacteria of the genera *Clostridium*, *Alistipes* and *Sutterella*, indicating a positive influence on the cecal microbiota; however, they observed a reduction of *Ruminococcus*, which is directly related to the production of butyrate. In another study, Józefiak et al. (2020) added 0.3% of TM in the feed and observed an

increase in the abundance of the family Ruminococcacea in the ceca. They concluded that the insect meals may stimulate the colonization with probiotic and commensal bacteria and develop barriers against infection with pathogenic bacteria.

The innate immune system is the first defense barrier. It is responsible for triggering responses by cellular and chemical mechanisms (Tizzard, 2014), with the purpose of returning to homeostasis. In situations of intense microbial challenge, this response mechanism can divert the nutrients used for maintenance and growth, impairing the animals' zootechnical performance (Roura et al., 1992). To date, there are few studies on the innate immune system and insect feeding, thus many of the comparisons about immune response presented below are related to the use of probiotics and prebiotics in broilers feed.

Lysozyme is an important bactericidal agent secreted by macrophages and polymorphonuclear leukocytes (Melnick et al., 1985). It exhibits bactericidal activity by hydrolyzing the b-1,4-glycosidic linkage between N-acetylmuraminic acid and N-acetylglucosamine of bacterial cell wall peptidoglycan and is most effective against many Gram-positive bacteria (Phillips, 1966). The increased activity of cells and enzymes of the innate immune system such as macrophages, MPO and LYS after challenge with LPS can be considered an indicator of an inflammatory response (Jang et al., 2003).

In this experiment, no statistically significant difference among treatments was observed for lysozyme activity, before and after the challenge with LPS, but after the challenge with LPS it was observed a higher enzyme activity, showing that the innate immune system of birds was active due to the challenge after 6 hours. However, the response of innate immune system will mostly be active after 12 hours of the injury suffered (Tizzard, 2009). Wang et al. (2016) supplemented the diet of broilers with *Saccharomyces cerevisiae* and observed an increase in lysozyme activity after 8 hours of challenge with *Escherichia coli* LPS (serotype O55:B5); they attributed this increase to the inflammation caused by LPS, which led to activation of the innate immune system. Earlier, Gao et al. (2009) concluded that feed with *S. cerevisiae* increased the innate immune system of chickens after verifying higher lysozyme activity before and after *Eimeria tenella* challenge. Increased lysozyme activity is associated with increased destructive activity of phagocytic cells (Kreukniet et. al., 1995).

One of the most important responses of the innate immune system is the complement system, formed by about 35 proteins that are activated in a cascade (Dunkelberger and Song, 2010) and are responsible for opsonization, attracting phagocytic cells and activating cells to release cytokines and chemokines, inducing an inflammatory response, acting as an antimicrobial agent (Hawlisch and Kohl, 2006). In this experiment, it was determined an

increase in the HACS in birds NCLPS and fed with 2.0% TM; this result provides evidence that tenebrio meal can help modulate the innate immune response of broilers. However, after the challenge with LPS, no statistically significant difference of treatments was found, possibly due to the collection time, which was only 6 hours. Supplementing the diet with the probiotic *Clostridium butyricum*, it was observed an increase in complement components, C3, at 21 d, in addition to a higher concentration of IgA and IgM in serum of broilers (Yang et al., 2012) and layers (Zhan et al., 2018).

Benzertiha et al. (2020) reported that feeding broilers with 0.2 or 0.3% of TM resulted in improved performance and also a reduction in the level of IgM and a significant increase in IL-2 and TNF-α; they attributed this effect to a possible relationship with AMPs produced by insects.

According to Hajati et al. (2014), mannan and glucan that are present in fungal cells can activate the innate immune system of birds as they are recognized by pattern recognition receptors. The same activation potential is attributed to insect meals because the exoskeleton is formed by chitin which can trigger an immune response after recognition by the TLR (Komi et al., 2018).

Myeloperoxidase also plays an important role in the immune system; it is a bactericidal enzyme that produces reactive oxygen species, destroying intracellular pathogens (Khan et al., 2018). MPO is produced by cells responsible for the individual's first line of defense after an injury suffered, innate immunity, such as monocytes, macrophages (Nicholls and Hazen, 2005) and neutrophils (Khan et al., 2018). In this experiment, the MPO activity was increased in NCLPS birds fed 2.0% TM, but after the challenge with LPS, the MPO activity was reduced in all treatments, with no statistically significant difference ($P>0.05$), a result different from that reported by Wang et al. (2016).

In addition to the effects on cells and enzymes related to the immune system, birds fed with tenebrio meal can have their hematological profile altered with no effect on the health of the birds, as observed by Biasato et al. (2017). Kozlowski et al. (2021) fed 3% tenebrio meal to young turkeys and observed that the inclusion, despite not improving the performance of birds, exerted anti-inflammatory, immunomodulator and antioxidant function with an effect similar to the addition of monensin.

The bactericidal activity of the serum is an important indicator of the immunity of chickens. However, although broilers fed 2.0% TM in this study showed higher activity of HACS and MPO in NCLPS ($P<0.05$), BA of *E. coli* and *Salmonella* Gallinarum was lower than for the birds from NC and PC ($P<0.05$). After the challenge, all treatments increased

their potential for bactericidal activity, but there were no statistically significant differences between treatments.

Inclusion of 2.0% TM to broiler diets in two independent experiments showed a similar response in the WG of the birds. There was an improvement in the WG of birds in experiment 1 compared to CN, but in experiment 2 this difference was only numerical possibly due to the experimental conditions.

3.5 Conclusions

Feeding TM at 2.0% in broiler diet appears to be effective as a functional food, promoting significant increase in feed intake and weight gain of the chickens and modulates the innate immune system. Feeding 0.5% tenebrio meal did not express this nutraceutical effect.

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Table 1: Analyzed nutrient composition of the *Tenebrio molitor* full-fat larvae meal (TM) used in the experiments, as fed basis. Values in % unless otherwise stated

Item	*Tenebrio molitor*
Dry matter	97.02
Crude protein	50.00
Ether extract	29.73
Crude ash	4.12
Crude fiber	4.50
Calcium	0.125
Phosphorus	0.570
Gross energy (kcal/kg)	6366
AMEn (kcal/kg)	4854
Indispensable amino acids	
Arginine	2.68
Histidine	1.48
Isoleucine	1.91
Leucine	3.31
Lysine	2.90
Methionine	0.68
Phenylalanine	2.12
Threonine	1.71
Valine	2.76
Dispensable amino acids	
Alanine	3.16
Aspartic acid	3.89
Cysteine	0.54
Glycine	2.25
Glutamic acid	5.58
Proline	2.85
Serine	1.72
Tyrosine	4.07

Table 2: Ingredient and nutrient composition of the experimental diets[1]. Experiments 1 and 2.

Ingredients (g/kg)	Starter (1 to 21d)				Grower (21 to 35d)			
Corn	490.3	489.5	494.0	505.1	564.0	563.2	654.6	587.1
Soybean meal	434.0	434.1	427.8	409.4	353.9	354.0	353.1	322.9
Soybean oil	36.8	37.0	34.2	26.4	49.2	49.5	48.9	37.3
Dicalcium phosphate	18.0	18.0	18.1	18.2	14.7	14.7	14.7	15.0
Limestone	8.9	8.9	8.9	8.9	7.0	7.0	7.0	7.0
Salt	5.3	5.2	5.2	5.2	4.9	4.9	4.9	4.9
Mineral premix [a]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin premix	0.5 [b]	0.5	0.5	0.5	0.4 [c]	0.4	0.4	0.4
DL-Methionine	3.24	3.24	3.24	3.23	2.74	2.74	2.74	2.74
L-Lysine.HCl 77%	1.22	1.22	1.23	1.24	1.52	1.52	1.53	1.57
L-Threonine	0.48	0.48	0.48	0.48	0.41	0.41	0.41	0.44
Choline chloride	0.8	0.8	0.8	0.8	0.6	0.6	0.6	0.6
Tenebrio molitor full-fat meal	0	0	5.0	20.0	0	0	0	20.0
Enramycin 8%	0	0.125	0	0	0	0.125	0	0
Salinomycin 24%	0	0.275	0	0	0	0.275	0	0
Calculated nutritive value (g/kg)								
Crude protein	239.00	239.00	239.00	239.00	208.38	208.37	208.32	208.26
Available phosphorus	4.45	4.45	4.45	4.45	3.74	3.74	3.74	3.74
Calcium	9.35	9.35	9.35	9.35	7.58	7.58	7.58	7.58
Sodium	2.22	2.22	2.22	2.22	2.08	2.08	2.08	2.08
Dig. Methionine	6.34	6.34	6.34	7.58	5.51	5.51	5.51	5.52
Dig. Lysine	12.90	12.90	12.90	12.90	11.24	11.24	11.24	11.24
Dig. Methionine + Cysteine	9.50	9.50	9.5	9.50	8.32	8.32	8.32	8.32
Dig.Threonine	8.50	8.50	8.50	8.50	7.42	7.42	7.42	7.42
AME (kcal/kg)	2975	2975	2975	2975	3150	3150	3150	3150

[a] Salus Mineral Products, provided the following per kilogram of diet: Manganese, 80 mg; Zinc, 70 mg; Iron, 50 mg; Copper, 10 mg and Iodine, 1 mg.
[b] Salus Vitamin Products, provided the following per kilogram of diet: vitamin A, 8,500 IU; vitamin D_3, 3,000 IU; vitamin E, 18 IU; vitamin K_3, 2,5 mg; Vitamin B_1, 2 mg; vitamin B_2, 6 mg; vitamin B_6, 3 mg; vitamin B_{12}, 14 µg; vitamin B_5, 14 mg; folic acid, 1,2 mg; biotin, 0,008 mg and selenium, 0,5 mg.

[c] Salus Vitamin Products, provided the following per kilogram of diet: vitamin A, 6,800 IU; vitamin D3, 2,400 IU; vitamin E, 14 IU; vitamin K_3, 2,0 mg; Vitamin B_1, 1,6 mg; vitamin B_2, 4,8 g; vitamin B_6, 2,4 mg; vitamin B_{12}, 11 µg; vitamin B_5, 11mg; folic acid, 1,0 mg; biotin, 0,006 mg and selenium, 0,4 mg.
[1]NC- Negative control; PC – Positive control – NC + 10 mg/kg enramycin + 66 mg/kg salinomycin; TM0.5 – NC + 0.5% tenebrio molitor full-fat meal; TM2.0 – NC + 2.0% tenebrio molitor full-fat meal. All diets had no phytase.

Table 3: Performance characteristics of broilers receiving diets supplemented with antimicrobial additives or containing full-fat tenebrio larvae meal. (Experiment 1, 1-35d)

	Treatments[1]					
Item[2]	NC	PC	TM0.5	TM2.0	SEM[3]	P-value
1 to 7d						
BWG, g	160.8[b]	168.4[a]	162.8[ab]	167.9[a]	1.86	0.010
FI, g	169	175	174	175	2.14	0.161
FCR, g:g	1.052	1.037	1.066	1.043	0.013	0.208
7 to 14d						
BWG, g	320[c]	383[a]	328[bc]	364[ab]	11.11	<0.001
FI, g	414[c]	461[a]	420[bc]	452[ab]	10.33	0.005
FCR, g:g	1.298[c]	1.206[a]	1.290[bc]	1.253[ab]	0.015	<0.001
14 to 21d						
BWG, g	566[b]	636[a]	561[b]	591[b]	9.48	<0.001
FI, g	731[b]	784[a]	727[b]	763[ab]	10.08	<0.001
FCR, g:g	1.292[b]	1.233[a]	1.295[b]	1.291[b]	0.010	<0.001
1 to 21d						
BWG, g	1047[b]	1187[a]	1052[b]	1123[a]	18.72	<0.001
FI, g	1314[c]	1420[a]	1321[bc]	1390[ab]	19.70	0.008
FCR, g:g	1.256[b]	1.196[a]	1.256[b]	1.239[b]	0.007	<0.001
21 to 28d						
BWG, g	750[b]	786[a]	746[b]	759[ab]	9.58	0.015
FI, g	1092[b]	1135[a]	1088[b]	1136[a]	10.18	<0.001
FCR, g:g	1.458	1.446	1.460	1.498	0.014	0.055
28 to 35d						
BWG, g	910	918	920	927	11.18	0.683
FI, g	1392[b]	1415[a]	1404[ab]	1432[a]	10.72	0.022
FCR, g:g	1.531	1.544	1.526	1.548	0.014	0.640
21 to 35d						
BWG, g	1659	1703	1666	1685	15.68	0.135
FI, g	2484[c]	2550[ab]	2492[bc]	2568[a]	16.45	<0.001
FCR, g:g	1.498	1.498	1.496	1.525	0.012	0.272
1 to 35d						
BWG, g	2706[c]	2890[a]	2718[c]	2808[b]	22.95	<0.001
FI, g	3798[b]	3970[a]	3813[b]	3958[a]	30.53	<0.001
FCR, g:g	1.403[b]	1.374[a]	1.403[b]	1.410[b]	0.006	0.002

[1]NC= negative control; PC= NC + 10 mg/kg enramycin + 66 mg/kg salinomycin; TM0.5= NC + 0.5% tenebrio molitor full-fat meals and TM2.0= NC + 2.0% tenebrio molitor full-fat meals.

[2] BWG= body weight gain; FI= feed intake; FCR= feed conversion ratio (g of FI/g of BWG, g/g)

[3] SEM: standard error of the mean.

[a,b,c] Means values within a row having different superscripts are statistically different by Tukey's test ($p<0.05$).

Table 4: Bacteria counts in ceca contents of broiler chickens fed 0.5 or 2% of tenebrio full-fat meal or antimicrobial additive.

	Treatments[1]					
Itens[2]	NC	PC	TM0.5	TM2.0	SEM[3]	P-value
E. coli	8.31	8.44	8.54	8.80	0.185	0.315
Enterococcus spp.	4.15	4.07	4.48	4.62	0.213	0.222

[1]NC= negative control; PC= NC + 10 mg/kg enramycin + 66 mg/kg salinomycin; TM0.5= NC + 0.5% tenebrio molitor full-fat meal and TM2.0= NC + 2.0% tenebrio molitor full-fat meal.

[2]$\log_{10}$ count/g cecal contents.

[3] SEM: standard error of the mean.

Table 5: Growth performance of broiler chickens fed 0.5 and 2% of tenebrio full-fat meal or antimicrobial additives or AGP Experiment 2: 1-21 d in cages)

	Treatments[1]					
Item[2]	NC	PC	TM.05	TM2.0	SEM[3]	P-value
1 to 7d						
BWG, g	166.4	174.7	170.5	165.7	4.99	0.286
FI, g	158	168	164	158	4.41	0.106
FCR, g:g	0.949	0.961	0.959	0.954	0.011	0.892
7 to 14d						
BWG, g	356 ^{b}	385 ^{a}	361 ^{ab}	363 ^{ab}	8.46	0.020
FI, g	397	418	404	408	7.21	0.061
FCR, g:g	1.110 ^{ab}	1.087 ^{a}	1.136 ^{b}	1.125 ^{ab}	0.013	0.045
14 to 21d						
BWG, g	531 ^{c}	605 ^{a}	545 ^{bc}	584 ^{ab}	12.65	0.002
FI, g	656	701	662	667	12.02	0.062
FCR, g:g	1.233 ^{c}	1.159 ^{ab}	1.215 ^{bc}	1.142 ^{a}	0.016	0.002
1 to 21d						
BWG, g	1053 ^{b}	1165 ^{a}	1067 ^{b}	1113 ^{ab}	18.66	<0.001
FI, g	1208 ^{b}	1287 ^{a}	1230 ^{ab}	1233 ^{ab}	17.72	0.030
FCR, g:g	1.147 ^{b}	1.105 ^{a}	1.153 ^{b}	1.108 ^{a}	0.097	0.003

[1]NC= negative control; PC= NC + 10 mg/kg enramycin + 66 mg/kg salinomycin; TM0.5= NC + 0.5% tenebrio molitor full-fat meal and TM2.0= NC + 2.0% tenebrio molitor full-fat meal.

[2] BWG= body weight gain; FI= feed intake; FCR= feed conversion ratio (g of FI/g of BWG, g/g)

[3] SEM: standard error of the mean.

[a,b,c]Means values within a row having different superscripts are statistically different by the Tukey test ($p \leq 0.05$).

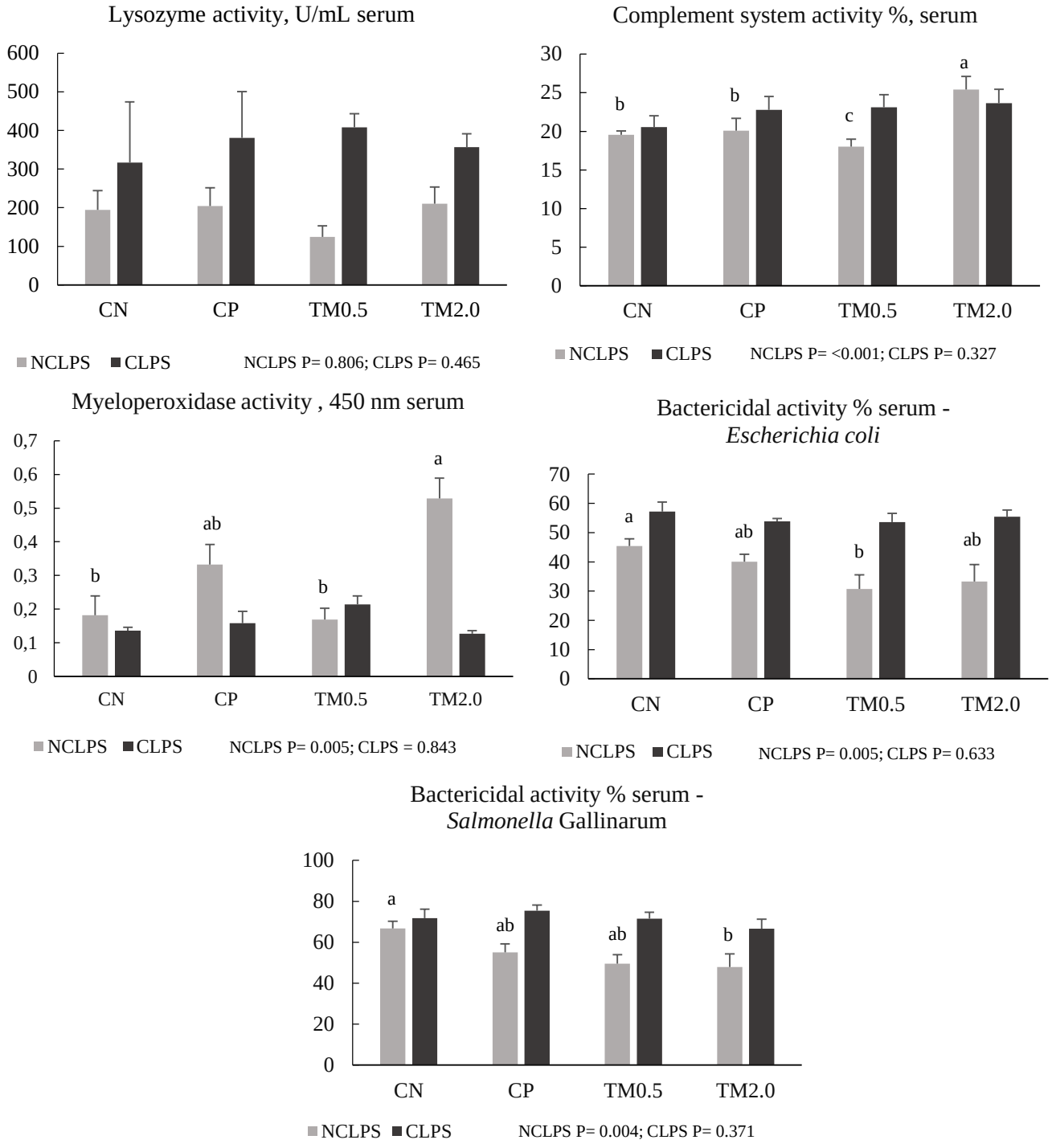

Figure 1. Effect of dietary supplementation with antimicrobial additives or containing full-fat tenebrio larvae meal in innate immune response in broiler serum, NCLPS and CLPS**.**

CLPS: chickens were injected with LPS (1.43 mg/kg BW); NCLPS: chickens were injected with sterile saline solution 0.9%. Samples were collected 6 h after injection. Results from two-way ANOVA and Tukey-Kramer test among treatments into NCLPS and CLPS groups separately, P<0.05. a – c: means among NCLPS chickens' group with different letters are statistically different

4. INSECT BLACK SOLDIER FLY LARVAE MEAL DEFFATED AS A FUNCTIONAL INREDIENT MODULATES IMMUNITY AND IMPROVES GROWTH PERFORMANCE OF BROILER CHICKENS.

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Abstract

The effects of defatted black soldier fly larvae meal (**BSF**) on growth performance and serum innate immunity in broilers challenged with *Escherichia coli* lipopolysaccharide (LPS) was investigated. One experiment was conducted to evaluate this, 800 d-old male broiler chicks were used. Birds were assigned to 4 treatments, 10 replicate pens per treatment in a randomized block, and 20 birds per pen in an open-sided poultry house, during 35 d, to evaluate performance. Dietary treatments used were as follows: **NC** (negative control); **PC** (positive control) – NC + 10 mg/kg enramycin and 66 mg/kg salinomycin; **BSF0.5** – NC + 0.5% BSF; and **BSF2.0** – NC + 2.0% BSF. BSF were calculated in the diets. At the end of the experiment birds was inject with LPS or with sterile saline solution. Blood was collected and lysozyme activity (**LYZ**), hemolytic activity of the alternative complement system (**HACS**), myeloperoxidase activity (**MPO**) and bactericidal activity (**BA**) was measured on serum. Supplementation with 0.5% BSF did not improve performance, but 2.0% BSF resulted in an increase in body weight gain (**BWG**) and feed intake (**FI**) of 3.8% and 3.8% ($P<0.01$, respectively) but did not affect the feed conversion ratio (**FCR**). Birds of BSF2.0 non-challenged with LPS (**NCLPS)** have greater LYZ than the controls, NC and PC ($P<0.01$) and greater HACS compared to NC ($P<0.05$). However, birds BSF2.0 challenge with LPS (**CLPS**) have BA *Salmonella* Gallinarum greater than NC. In conclusion the use of 2% BSF broilers feed increase BWG and FI of birds, and not have an effect on FCR, and modulates the innate immune system of birds before and prior LPS challenge.

Key Words: antibiotic growth promoter, feed additives, *Hermetia illuscens,* ionophores, poultry.

4.1 Introduction

The use of insect larvae meal as a nutritional or functional ingredient in feed has been gaining attention as a non-conventional alternative of animal origin for food-producing animals. In recent years, there has been an increase in published research on insect feeding, but studies to define its application in nutritional programs, including inclusion levels, are still necessary.

The nutrient composition of insect meals varies among species, ranging between 35 to 69% crude protein, with good amino acid profile, and 4 to 47% crude fat (Hwangbo et al., 2009; Veldkamp et al., 2012). *Hermetia illuscens* is a species of the order Diptera, family

Stratiomyidae, is a fly also known as black soldier fly. Its larvae meal can have 35- 57% crude protein an 15-49 % fat on dry matter basis (Veldkamp et al., 2012; Makkar et al., 2014). Black soldier fly meals can be whole or defatted and black soldier fly oil also can be used for animal feed (Kim et al., 2020; Kim et al., 2021).

Insects such as black soldier fly and others are be able to synthetize antimicrobial peptides (**AMPs**) as a protective mechanism (Bulet et al., 2004; Józefiak and Engberg, 2017). AMPs are proteins with low amino acid sequence (<100) and have shown microbicidal effect against bacteria, virus, fungi and other pathogens (Jenssen et al., 2006; Mylonakis et al., 2016; Józefiak and Engberg, 2017). Larvae black soldier fly have an AMP described, DLP4, which have spectrum against methicillin-resistant *Staphylococcus aureus* (Park et al., 2015). In addition to AMPs, black soldier fly larvae meal has high concentrations of lauric acid (C12:0), up to 60¨%, in the fat (Spranghers et al., 2017) and it is known for its antiviral and antibacterial activities (Lieberman et al., 2006). Insects are also rich in chitin/chitosan which has microbicidal activity (Vartiainer et al., 2004) and trigger an immune response and modulation after recognition by *toll-like receptors* (Lee et al., 2008; Islam and Yang, 2017; Komi et al., 2018).

Currently insect meals have a low supply on the market leading to a high cost to purchase for use in animal feed (Veldkamp et al., 2021) and most nutritional studies using them have focused on their nutritive value with and ingredient providing proteins and lipids. However, due to its immunomodulatory properties, it is necessary to evaluate it at a low inclusion level as a functional ingredient. Lee et al. (2018) demonstrated that the inclusion of 2 and 3% of black soldier fly meal in broiler diets after challenge with *Salmonella* Gallinarum, enhance survive rate and modulate the innate immune system of birds. So are true that the insect meals have microbicidal properties and then can modulate the immune response of broiler chickens.

To the best of our knowledge this is the first study to evaluate the effects of of defatted black soldier fly larvae meal (**BSF**) in the innate immune response of broiler chickens under an LPS-induced inflammation. Therefore, we hypothesized that low inclusion levels of 0.5 or 2.0% of BSF in broiler diets may improve growth performance and modulate the innate immune response of chickens. The present study was conducted to evaluate the effects of BSF (0.5 or 2.0% in the diet) on broiler growth performance and innate immune response when challenged with *Escherichia coli* LPS.

4.2 Material and Methods

The study was conducted at Department of Animal Science, University of São Paulo, Piracicaba, SP, Brazil. All procedures were approved by the Institutional Animal Care and Use of Committee (protocol number: 8400081020).

Defatted BSF larvae meal used in the feeding trial was analyzed for the content of dry matter, crude protein, ether extract, ash, crude fiber, gross energy and amino acid profile. The methodology of bromatological analysis can be found in the publication of Nascimento Filho et al. (2021). Digestibility coefficients of amino acids and metabolizable energy of BSF were based on Matin et al. (2021); digestible amino acid levels and metabolizable energy value were adjusted according to the amino acid profile and gross energy in the product utilized in the present study. Analyzed composition is depicted in Table 1.

Diets met the nutritional specifications of Brazilian tables for poultry and pigs (Rostagno et al., 2017), based on corn and soybean meal, being isonutritive and isoenergetic and prepared in mash form. Starter diets were offered from 1 to 21 d of age and grower diets 22 to 35 d of age. The experimental treatments used were as follows: NC (negative control) – no additives; PC (positive control) – NC + 10 mg/kg enramycin addition and 66 mg/kg salinomycin; BSF0.5 – NC + 0,5% BSF and BSF2.0 – NC + 2,0% BSF. BSF was obtained from BSF Animal Nutrition. Ltda, Piracicaba, São Paulo, Brazil. Diet composition is shown in Table 2.

The experiment design were randomized blocks. We adopted this design because the poultry house used 1 has trees on only one side, so no treatment could be harmed during the draw. Chickens were raised in an open-sided poultry house equipped with curtains, fans, spraying nozzles, heating lamps, nipple drinkers and tube feeders, in 3 m^2 floor pens covered with reused rice hulls litter. The birds were housed eight d after the batched was removed, so the period of sanitary vacuum recommended to poultry house was not given. Environmental conditions were maintained close to the chickens needs at each stage of growth. Minimum and maximum temperatures were recorded daily. The average for low and high temperatures were 19.4 ºC and 27.5 ºC in the starter phase and 15.3 ºC and 24.8 ºC in the grower phase, respectively. During the entire experiments, chickens had *ad libitum* access to water and feed. The lighting schedule adopted in both experiments was 18 h light: 6 h dark after 10 d from start experiment.

A total of 800 one-day-old male broiler chicks (Cobb) were purchased from a local hatchery, weighed individually upon arrival, uniformly distributed to 40 groups and randomly

assigned to 40 pens. Chicks, with average weight of 41.6 g, were divided in 4 treatments with 10 replicates (3 m^2, 20 birds/pen).

At the ages of 7, 14, 21, 28 and 35 d, the birds were weighed on a pen basis and feed consumption recorded. Weekly data and the totals for phase and cumulative values for body weight gain (**BWG**) and feed intake (**FI**) were determined and feed conversion ratio (**FCR**) was calculated. Mortality and culling were considered in calculations.

On 35 d of the experiment, one bird/pen (n=6), treatment CN, CP and BSF2.0, was injected intra-abdominally with 2 mL (1,78 mg/kg of body weight) *Escherichia coli* LPS (serotype O55:B5, LPS L2880; Sigma-Aldrich) and one bird/pen (n=6) of the same pen was inoculated with 2 mL of 0,9% (w/v) sterile saline solution as negative control. After 12 hours, blood was collected from the brachial vein into 4 mL clot accelerator tubes to obtain serum for the analysis of the innate immune system. Then, blood samples were centrifuged at 2000x g for 10 min at 4ºC, aliquoted in Eppendorf vials and stored at -80ºC ultrafreezer.

After serum collection, analyzes were performed to assess the response of the innate immune system. For that were analyzed Lysozyme activity (**LYZ**), hemolytic activity of the complement (**HACS**), myeloperoxidase activity (**MPO**) and bactericidal activity (**BA**) *E. coli* and BA *Salmonella* Gallinarum. All these analysis were carried out in serum of the birds challenged with LPS (**CLPS**) and non-challenged LPS (**NCLPS**).

4.2.1 Lysozyme activity

The LYZ of birds was determined by a turbidimetric assay as described by Jorgensen et al. 1993. Briefly, a suspension (200 μL) of *Micrococcus lysodeikticus* (M3770, Sigma-Aldrich) in PBS (0.2 g/L) at pH 6.2 was mixed with serum (10 μL) in a flat-bottomed 96-well plate. Plates were read with a microplate reader (Synergy H1 Multi-Mode Reader, Winooski, VT, USA). Lysozyme activity (units/mL) was calculated using the following formula: [(Δabsorbance (4 min–1 min)/3)/0.001]x100. Quantification of lysozyme activity was done as per the standard definition of one unit of lysozyme activity from chicken egg hen lysozyme (L6876, Sigma Aldrich) corresponding to the linear decrease in optical density (OD) at 450 nm of 0.001 per minute.

4.2.2 Hemolytic activity of the alternative complement system

The HACS was measured as described by Sutili et al. 2016. An 80% diluted sample (80 μL of serum + 20 μL PBS) was mixed with 100 μL of 2% washed sheep red blood cells (SRBC) and incubated at 25 ◦C for 45 min. Following incubation, the mixture was centrifuged

at 2500 × g for 5 min. Then, 100 µL of supernatant was transferred to a 96-well plate and measured at 450 nm in a plate reader (Synergy H1 Multi-Mode Reader, Winooski, VT, USA). The percent of hemolysis was calculated by comparing between total hemolysis (100%: SRBCs + DI water) and no-hemolysis (0%: SRBCs + PBS + heat-inactivated serum) controls as follows: % hemolysis = [(A_{450} sample – A_{450} no-hemolysis)/(A_{450} total hemolysis – A_{450} no-hemolysis)] × 100.

4.2.3 Myeloperoxidase activity

The MPO of serum was quantified following the protocol described by Kreutz et al. 2011, with modifications. Ten microliters of serum were diluted in 40 µL of PBS in flat-bottomed 96-well plates. Then, 100 µL of a solution containing o-3,3′, 5,5′-tetramethylbenzidine (TMB, T0440, Sigma-Aldrich) and hydrogen peroxide prepared in citrate (0.2 M) and a phosphate buffer (0.01 M) at pH 5.3 was added to each well. The peroxidase reaction was stopped after 5 min by adding 100 µL of HCl (3 M). Plates were read with a microplate reader (Synergy H1 Multi-Mode Reader, Winooski, VT, USA), and the myeloperoxidase activity reported in absorbance at 450 nm. Wells without samples were added in the microplate as negative controls.

4.2.4 Bactericidal activity

The serum BA was determined by evaluating its effect on growth of *Salmonella* Gallinarum and *Escherichia coli*. The bacterial strains were provided by Laboratory of Veterinary Microbiology and Immunology - Farroupilha Federal Institute of Education, Science and Technology. The bacterial solution for each strain was prepared in tryptone soy broth medium (TSB) from cultures grown in tryptone soy agar medium (TSA) (Himedia Laboratories) {($1x10^8$ colony forming units (CFU)/mL; 0.15 optical density (OD) at 600 nm) (30 °C/24 h)}. Then, 20 µL of serum and 20 µL of bacterial dilution at a concentration of 10^6 bacteria/mL were added to each well of 96-well plates. The samples were incubated for 4h at 37ºC. After incubation, 25 µL/well of 2,3,5-triphenyltetrazolium chloride (TTC), 0.5 mg/mL, (Sigma) were added and the samples were incubated for another 10 min (37ºC), before being centrifuged (2000 x g, 10 min). The supernatant was removed and the precipitate dissolved with 200 µL/well of dimethylsulfoxide (DMSO). Aliquots of 100 µL from each well were transferred to a new plate of 96 flat-bottomed wells. Absorbance was then measured at 450 nm (Synergy H1 Multi-Mode Reader, Winooski, VT, USA). The percent of BA was

calculated by comparing between controls: 0% BA (TSB + bacterial solution) and 100% BA (PBS) (Albaladejo-Riad et al., 2020).

All these analysis (LYZ, HACS, MPO, BA *E. coli* and BA *Salmonella* Gallinarum) were carried out in serum of the birds challenged with LPS (**CLPS**) and non-challenged LPS (**NCLPS**).

4.2.5 Statistical analysis

Performance data were submitted to PROC MIXED (Linear Mixed Models) of SAS software. All data was tested for normality of residuals through Shapiro-Wilk test. When a significant effect was verified, the variables were submitted to mean comparison by Tukey test considering the level of 5% of significance. Serum immune parameters (LYZ, HACS, MPO and BA) were subjected of Tukey-Kramer test to compare estimated means among treatment CLPS and NCLPS groups separately. Results were reported as least square means with standard error of the mean (S.E.M).

4.3 Results

4.3.1 Performance

The growth performance of the broilers fed experimental diets containing TM are shown in Table 3. No differences were found in performance of the broilers receiving the diet containing 0.5% BSF in any phase of the trial. However, the feed containing 2% BSF resulted in an improvement in broiler performance compared to those receiving the negative control diet, NC. Weight gain (**WG**) was significantly higher for the chickens fed 2% BSF on week 2 (7-14d, +19 g, $P<0.01$), week 3 (14-21d, +20 g, $P<0.01$) and week 4 (21-28d, +26 g, $P<0.01$). Although the advantage of BSF2.0 compared to NC was not significant ($P>0.05$) in the remaining weeks, numerical differences were observed, resulting in higher BWG in the starter period (+44 g) and a final BWG 99 g higher at 35d (2714 g vs. 2615 g, $P<0.01$). This represents an advantage of 4.7% in the BWG in the starter phase and 3.8% in the total period for the broilers fed BSF2.0 over the controls.

The feed intake of the birds consuming the diet with 2% BSF was significantly higher ($P<0.05$) than the control on weeks 1, 2, 3 and 4. During the starter period, FI was 71 g greater for BSF2.0 than NC ($P<0.05$). In the overall period, FI was 140 higher for BSF2.0 (3827 g vs. 3687 g, $P<0.01$), a difference of 3.8%. Feeding BSF2.0 resulted in significantly ($P<0.05$) worse FCR in week 1 compared to NC, but in the total period resulting in non-significant difference (1.410 vs 1.410).

The broilers fed the diet supplemented with enramycin and salinomycin (PC) had a greater response in BWG in relation to NC, compared to the response obtained with BSF2.0. The response in BWG was 20.3% in the starter phase (1137 g vs. 945 g, P<0.01) and 9.4% in overall period (2.860 g vs. 2.615 g, P<0.01). The magnitude of the growth response obtained with BSF2.0 was intermediate. This advantage was consistent, and significant, during the starter phase (1137 g vs. 989 g, 15% higher, P<0.01) and at the end of trial (2860 g vs. 2714 g, 5.38% higher, P<0,01) favoring PC in relation to BSF2.0. FI was higher for PC compared to BSF2.0 (3937 g vs. 3827 g, 2.9% higher, P<0.01). At the end of the trial the broilers fed the antimicrobial additives had a better FCR (1.377 vs. 1.410, P<0.05).

4.3.2 Innate immune response

The results of the immunity parameters for the birds receiving the dietary treatments and challenged or non-challenged with LPS are presented graphically in Figure 1 for visualization of the comparisons. Within each challenge condition, dietary treatments were compared using Tukey-Kramer test. In the non-challenged birds, there was no difference among treatments for MPO, BA *E. coli* and BA *Salmonella* Gallinarum, (P<0.05). However, broilers BSF2.0 resulted in greater LYZ than the controls, NC and PC (P<0.01), and greater HACS compared to NC (P<0.05). No difference was observed enter among treatments after CLPS for LYZ, HACS and BA *Salmonella* Gallinarum (P>0.05). Birds BSF2.0 have greater BA *E coli* compared to NC (P<0.05); however, broilers PC have greater MPO activity compared to BSF2.0 and NC (P<0.05).

4.4 Discussion

The experiment was conducted to evaluate the performance of broilers fed diets containing low levels (0.5 and 2.0%) of defatted black soldier fly larvae meal and the innate immune system of birds challenged or not with LPS. Feeding the diet containing 2.0% BSF resulted in an increase in WG of 3.8% (P<0.01) compared to the NC, but inclusion 0.5% did not improve performance. The birds fed with antimicrobial additives possibly presented a superior performance to the other treatments, possibly due to the fact that the poultry house did not remain in a sanitary vacuum for 14 d as recommended. Although studies using black soldier fly meals in poultry feed are found in the literature, but the effect on performance and the best level of insect meal is not yet defined. Popova et al. (2020) observed that broilers fed 5% black soldier fly had higher WG in the growth phase result different from that observed by Dobbou et al. (2018), who found no improvement in performance with 5% but observed an

improvement in the starter phase using 10% black soldier fly meal. The same authors reported decrease in WG and feed conversion ratio when used 15% black soldier fly meal. Contrarily, Souza Vilela et al. (2021) observed an improvement in performance when used 15 and 20% black soldier fly meal.

Thus, studies with different levels of inclusion and supply periods are needed to elucidate possible variations in the performance and health results of birds, which may be related to the stage of rearing the larvae, larvae nutrition and even the process of removing fat from the meals.

Due to its high protein value, good amino acid profile and nutraceutical properties, researchers aimed at partial and even total replacement of soybean meal in broiler feed, however they concluded that 50% replacement is more adequate so that there is no loss in the performance of the birds (Murawska et al., 2021). In addition to the black soldier fly meal, the oil extracted from the larvae has potential for feeding birds. Studies are evaluating its use as a replacement for soybean oil and as verified by Kim et al. (2020) the use of 50 g/kg during the first thirty d of rearing broilers did not affect the performance of the birds, but improved feed conversion. They concluded that the use of black soldier fly oil can be used as an ingredient to enrich meat with medium chain fatty acids. Other researchers have also concluded that black soldier fly oil can totally or partially replace soybean oil without impairing growth and yield performance for broilers, laying hens (Schiavone et al., 2017; Schiavone et al., 2018; Patterson et al., 2021; Kim et al., 2021) and turkeys (Sypniewski et al., 2020).

An important factor for birds to express their growth potential is the feed intake; in our experiment we observed an increase in the FI of birds fed 2% BSF, a result that is partially justified due to the birds' food preference for insect meals (Nascimento Filho et al. 2020). Like us, other researchers also observed an increase in CR when using insect meal (Benzertiha et al., 2020; Dobbou et al., 2020) however other researchers such as de Souza Vilela et al. (2021) did not observe difference in FI. In this experiment, FCR feed with 2% BSF was not affected, similar to that found by Dobbou et al. (2020), but the same author observed that the use of 15% black soldier fly meals worsened the FCR of birds, differently from Souza Vilela et al. (2021) who throughout the experimental period (1 to 42d) observed an improvement in the FCR of birds fed with 15 and 20% black soldier fly meal.

Insect meal has properties that possible can help modulate the immune system of animals that consume them, as insects produce antimicrobial peptides (AMPs) that are responsible for their defense against pathogens (BULET et al., 2004). These AMPs can exhibit bactericidal, fungicidal and anti-pathogen action (Yi et al., 2014; Mylonakis et al.,

2016; Jósefiak e Engberg, 2017). The modulation of the immune response caused by AMPs may be one of the reasons for the improvement in the performance of birds fed with 2% BSF.

Birds have an innate and adaptive immune system, innate being the first responsible for the inflammatory process after contamination with pathogens or injury suffered and the second for the defense memory (Tizzard, 2009). The cells of the immune system, like other cells in the body, use nutrients to develop their functions. Animals that need to trigger an intense immune response may have their performance reduced due to a shift in nutrients for its activation (Roura et al., 1992).

To date, there are few studies evaluating the immune system of broiler chickens fed with black soldier fly, however Lee et al. (2018) when feeding broilers with 1, 2 and 3% black soldier fly meals and challenging them with *Salmonella* Gallinarum observed a higher survival rate in the birds. In addition to larger subpopulations of CD3+ and CD4+ T lymphocytes. They also observed greater serum lysozyme activity and concluded that black soldier fly meal has nutraceutical properties that stimulate the innate immune system of birds. Pasotto et al. (2020) also observed and enhance in lysozyme activity in broiler quails feed 10% black soldier fly larvae meal. In this experiment, we also evaluated the activity of lysozyme in serum, as this is an important bactericidal agent secreted by defense cells (Melnick et al., 1985) that works by hydrolyzing the binding between peptidoglycans in the bacterial cell wall, being more effective against Gram-positive bacteria (Phillips, 1966) and we observed that NCLPS birds fed with 2% BSF had a higher lysozyme activity compared to the other treatments, however after the challenge, CLPS, no difference was observed between treatments. A possible justification for the reduction in lysozyme activity after challenge in BSF2.0 and PC birds is the consumption of lysozyme circulating in the bird's body to combat the infection triggered by LPS. While in NC birds due to low lysozyme activity before the challenge needed to increase their production to fight the infection caused by LPS, but without statistically differing ($P>0.05$). An effect similar to that observed by Li et al. (2015) who observed an increase in lysozyme activity in the plasma of broiler chickens fed with *Bacillus amyloliquefaciens* and when challenging with LPS observed a reduction in its activity after 2 hours. They also observed an activation of the *toll-like receptor* 4 after the challenge, an effect that possibly happened in our experiment due to the observed immune responses. Results show that the use of probiotics in broiler feed increases survival after challenge with *Salmonella* Gallinarum, an effect partially attributed to increased lysozyme enzyme activity after stimulation of phagocytic cell activation by the probiotic (Jung et al., 2010). Other authors have also observed an increase in lysozyme activity in probiotic-fed

broilers over a lifetime (Wu et al., 2019; Khattab et al., 2021). Despite this significant increase in LYZ (517%) compared to NC ($P<0.01$), we did not observe any loss in the performance of the birds, as they had higher BWG ($P<0.01$).

The complement system is one of the most important responses triggered by the innate immune system, formed by approximately 35 proteins that are activated in a cascade (Dunkelberger & Song, 2010). Together or individually, they are responsible for opsonization to accelerate the phagocytosis process and activate cells to release cytokines and chemokines, inducing an inflammatory response, acting as an antimicrobial agent (Hawlisch & Kohl, 2006). In the present study we observed a higher complement system activity in birds fed 2% BSF in relation to NC ($P<0.05$) and a numerical increase in relation to PC. However, after the challenge, no difference was observed between the dietary treatments evaluated ($P>0.05$). As previously mentioned, the use of probiotics can help modulate the immune system and Khattab et al. (2021) when supplementing the broilers diet with a blend of *Lactobacillus acidophilus* and *Lactobacillus casei* verified a greater activation of the complement system and lysozyme, as mentioned above. In addition to modulating the immune system, they also observed a higher status of the antioxidant enzymes superoxide dismutase and catalase.

Reactive oxygen species are important allies in the elimination of intracellular pathogens and can be produced by the MPO enzyme, having great importance for the immune system (Khan et al., 2018). Defense cells such as macrophages, monocytes and neutrophils are responsible for the production and release of MPO. The use of BSF did not result in increased MPO activity before and after the LPS challenge ($P>0.05$), however the use of antibiotic + coccidiostatic did ($P<0.05$), indicating that possibly the CP animals had a greater release of reactive oxygen species. Different from what we found Wang et al. (2016) observed an increase in MPO activity in the serum of birds supplemented with *Saccharomyces cerevisiae* after challenge with LPS, but without differing from the control treatment, 8 hours after inoculation.

One of the ways to measure the effectiveness of the defense of the innate immune system of birds is to evaluate the bactericidal activity in the serum against a pathogen. However, no difference was observed in BA E. coli and *Salmonella* Gallinarum from birds fed 2% BSF in relation to NC, before and after the LPS challenge ($P>0.05$), meanwhile the reduction of BA E. coli from PC birds ($P>0.05$) indicate a lower defense capacity of the animal against a possible infection.

Inclusion 2% of defatted black soldier fly larvae meal to broiler diets showed response in WG of the birds. There was an improvement in the WG of birds BSF2.0 compared to CN but an intermediate response compared to CP.

4.5 Conclusion

It was determined that birds fed 2% BSF had a higher activity of some immunological parameters such LYZ and HACS in relation to CN and LYZ in relation to CP. So, we concluded that the use of 2% BSF broilers feed can increase WG and FI of birds and not affect feed efficiency, and can modulates the innate immune response of birds prior to LPS challenge.

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Table 1: Analyzed nutrient composition of the defatted black soldier fly larvae meal (BSF) used in the experiment, as fed basis. Values in % unless otherwise stated.

Item	*Hermetia illuscens*
Dry matter	95,61
Crude protein	62,55
Ether extract	8,71
Crude ash	8.33
Crude fiber	7,56
Calcium	1,71
Phospthorus	1,21
Gross energy (kcal/kg)	5268
AMEn (kcal/kg)	3840
Digestible indispensable amino acids	
Arginine	2,66
Histidine	1.62
Isoleucine	2,77
Leucine	4,02
Lysine	3,74
Methionine	1,00
Phenylalanine	2,29
Threonine	1,91
Valine	2,09
Digestible ispensable amino acids	
Alanine	2,66
Cysteine	0.35
Glycine	2,46
Glutamic acid	5,30
Proline	2,69
Serine	1,80

Table 2: Ingredient and nutrient composition of the experimental diets[1].

	Starter (1 to 21d)				Grower (22 to 35d)			
Ingredients (g/kg)	NC	PC	BSF0.5	BSF2.0	NC	PC	BSF0.5	BSF2.0
Corn	490.3	489.5	495.5	510.3	564.0	563.2	567.0	575.9
Soybean meal	434.0	434.1	426.2	403.6	353.9	354.0	348.1	330.6
Soybean oil	36.7	37.0	34.5	28.2	49.2	49.5	47.4	42.0
Dicalcium phosphate	18.0	18.0	17.7	16.9	14.7	14.7	14.5	13.6
Limestone	8.9	8.9	8.9	8.9	7.0	7.0	7.0	7.0
Salt	5.3	5.2	5.2	5.2	4.9	4.9	4.9	4.9
Mineral premix [a]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin premix	0.5 [b]	0.5	0.5	0.5	0.4 [c]	0.4	0.4	0.4
DL-Methionine	3.24	3.24	3.24	3.25	2.74	2.74	2.73	2.70
L-Lysine.HCl 77%	1.22	1.22	1.23	1.20	1.52	1.52	1.46	1.29
L-Threonine	0.48	0.48	0.49	0.51	0.41	0.41	0.39	0.50
Choline chloride	0.8	0.8	0.8	0.8	0.6	0.6	0.6	0.6
Black soldier fly meal	0	0	5.0	20.0	0	0	5.0	20.0
Enramycin 8%	0	0.125	0	0	0	0.125	0	0
Salinomycin 24%	0	0.275	0	0	0	0.275	0	0
Calculated nutritive value								
Crude protein	239.00	239.00	239.00	239.29	208.38	208.37	209.02	210.9
Available phosphorus (P)	4.45	4.45	4.45	4.45	3.74	3.74	3.74	3.74
Calcium (Ca)	9.35	9.35	9.35	9.35	7.58	7.58	7.58	7.58
Sodium (Na)	2.22	2.22	2.22	2.22	2.08	2.08	2.08	2.08
Methionine dig.	6.34	6.34	6.36	6.42	5.51	5.51	5.52	5.59
Lysine dig.	12.90	12.90	12.90	12.90	11.24	11.24	11.24	11.24
Methionine + Cysteine dig.	9.50	9.50	9.50	9.50	8.32	8.32	8.32	8.32
Threonine dig.	8.50	8.50	8.50	8.50	7.42	7.42	7.42	7.42
AME (kcal/kg)	2975	2975	2975	2975	3150	3150	3150	3150

[a] Salus Mineral Products, provided the following per kilogram of diet: Manganese, 80 mg; zinc, 70 mg; Iron, 50 mg; copper, 1 mg and iodine, 1 mg.

[b] Salus Vitamin Products, provided the following per kilogram of diet: vitamin A, 8,500 IU (retinyl acetate); (); vitamin D_3, 3,000 IU (cholecalciferol); vitamin E, 18 IU (DL-a-tocopherol acetate); vitamin K_3, 2,5 mg; Vitamin B_1, 2 mg; vitamin B_2, 6 mg; vitamin B_6, 3 mg; vitamin B_{12}, 14 µg; vitamin B_5, 14 mg; folic acid, 1,2 mg; biotin, 0,008 mg and selenium, 0,5 mg.

[c] Salus Vitamin Products, provided the following per kilogram of diet: vitamin A, 6,800 IU (retinyl acetate); vitamin D3, 2,400 IU (cholecalciferol); vitamin E, 14 IU (DL-a-tocopherol acetate); vitamin K3, 2,0 mg; Vitamin B1, 1,6 mg; vitamin B2, 4,8 g; vitamin B6, 2,4 mg; vitamin B12, 11 mg; vitamin B5, 14 g; folic acid, 1,0 mg; biotin, 0,006 mg and selenium, 0,4 mg.
[1]NC- Negative control; PC – Positive control – NC + 10 mg/kg enramycin + 66 mg/kg salinomycin; BSF0.5 – NC + 0.5% defatted black soldier fly larvae meal; BSF2.0 – NC + 2.0% defatted black soldier fly larvae meal. All diets had no phytase.

Table 3: Growth performance of broiler chickens fed 0.5 and 2% of defatted BSF meal or antimicrobial additives or AGP.

	Treatments[1]					
Item[2]	NC	PC	BSF.05	BSF2.0	SEM[3]	P-value
1 to 7 D						
BWG	139.5^{ab}	142.8^{ab}	136.2^{b}	143.8^{ab}	1.61	0.003
FI	142^{b}	146^{ab}	144^{b}	152^{a}	1.88	0.002
FCR	1.016^{a}	1.021^{a}	1.056^{b}	1.054^{b}	0.009	0.003
7 to 14 D						
BWG	273^{c}	359^{a}	263^{c}	292^{b}	5.74	<0.001
FI	366^{c}	431^{a}	373^{bc}	399^{b}	7.05	<0.001
FCR	1.324^{b}	1.210^{a}	1.419^{c}	1.371^{bc}	0.015	<0.001
14 to 21 D						
BWG	533^{c}	636^{a}	529^{c}	553^{b}	5.36	<0.001
FI	792^{b}	905^{a}	805^{b}	821^{a}	13.83	<0.001
FCR	1.487^{b}	1.424^{a}	1.521^{b}	1.506^{b}	0.011	<0.001
1 to 21 D						
BWG	945^{c}	1137^{a}	928^{c}	989^{b}	11.22	<0.001
FI	1300^{c}	1482^{a}	1322^{c}	1371^{b}	19.27	<0.001
FCR	1.375^{b}	1.303^{a}	1.424^{c}	1.387^{bc}	0.012	<0.001
21 to 28 D						
BWG	747^{c}	799^{a}	745^{c}	773^{b}	7.22	<0.001
FI	1048^{b}	1105^{a}	1044^{b}	1084^{a}	10.22	<0.001
FCR	1.402	1.383	1.401	1.395	0.008	0.120
28 to 35 D						
BWG	923	924	929	953	9.55	0.097
FI	1339^{ab}	1351^{ab}	1332^{b}	1373^{a}	9.94	0.027
FCR	1.452	1.462	1.434	1.441	0.011	0.236
21 to 35 D						
BWG	1671^{b}	1723^{a}	1674^{b}	1725^{a}	13.62	0.002
FI	2387^{b}	2456^{a}	2376^{b}	2457^{a}	17.56	<0.001
FCR	1.430	1.425	1.419	1.424	0.008	0.800
1 to 35 D						
BWG	2615^{c}	2860^{a}	2602^{c}	2714^{b}	21.60	<0.001
FI	3687^{c}	3937^{a}	3697^{c}	3827^{b}	32.10	<0.001
FCR	1.410^{b}	1.377^{a}	1.421^{b}	1.410^{b}	0.008	<0.001

[1]NC= negative control; PC= NC + 10 mg/kg enramycin + 66 mg/kg salinomycin; BSF0.5= NC + 0.5% defatted black soldier fly larvae meal and BSF2.0= NC + 2.0% defatted black soldier fly larvae meal.

[2] BWG= body weight gain; FI= feed intake; FCR= feed conversion ratio (g of FI/g of BWG, g/g)

[3] SEM: standard error of the mean.

[a,b,c] Means values within a row having different superscripts are statistically different by the Tukey test ($p<0,05$).

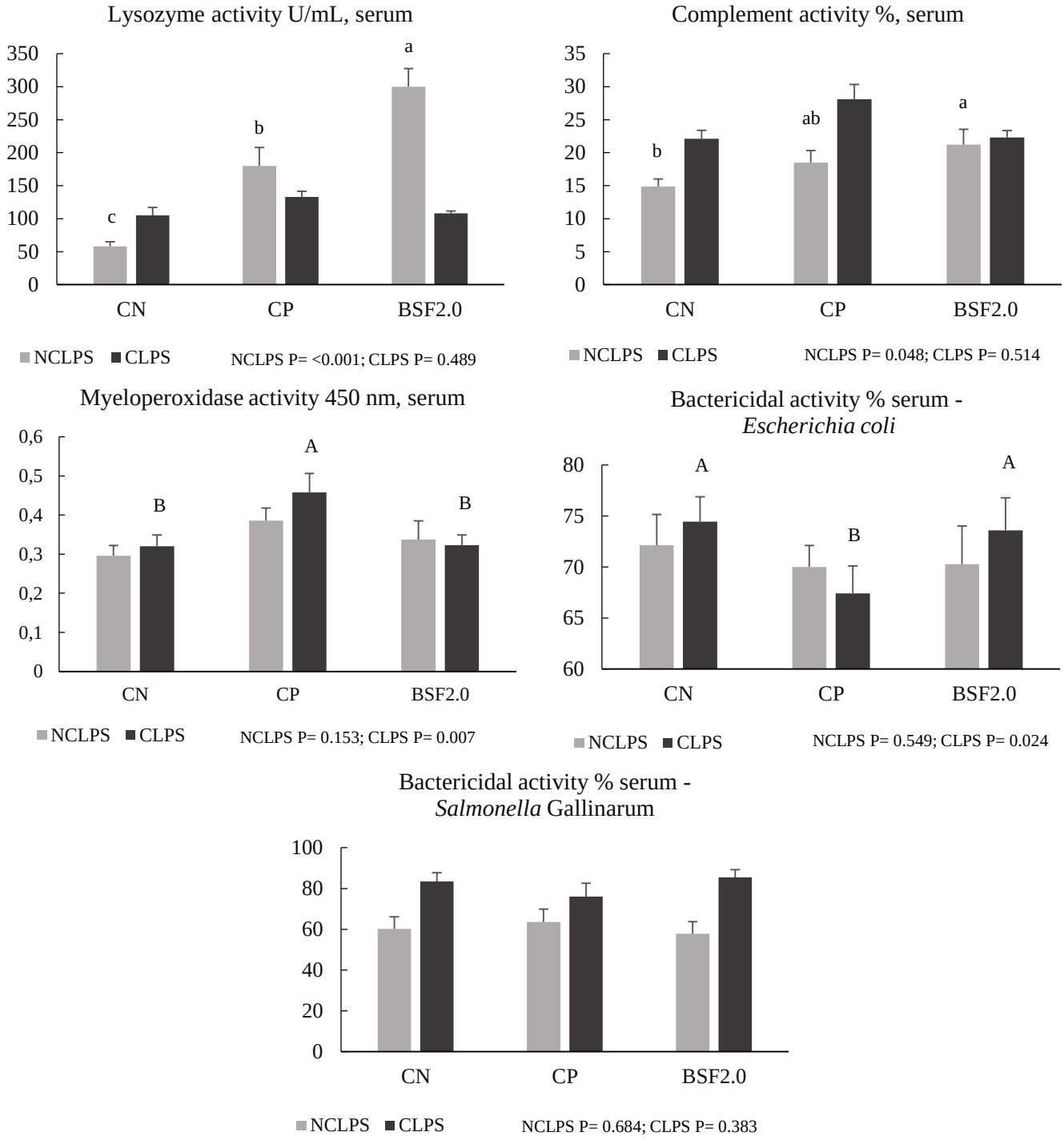

Figure 1. Effect of dietary supplementation with antimicrobial additives or containing defatted black soldier fly larvae meal in innate immune response in broiler serum, NCLPS and CLPS**.** CLPS: chickens were injected with LPS (1.78 mg/kg BW); NCLPS: chickens were injected with sterile saline solution 0.9%. Samples were collected 12 h after injection. Results from two-way ANOVA and Tukey-Kramer test among treatments into NCLPS and CLPS groups separately, $p<0.05$. a – c: means among NCLPS chickens' group with different letters are statistically different. A – C: means among CLPS chickens' group with different letters are statistically different.

5. CONSIDERAÇÕES FINAIS

O uso de farinhas de insetos na alimentação animal cada vez mais ganha destaque no Brasil e no mundo. A recente liberação pela UE a respeito do uso de farinhas de insetos para a alimentação de aves e suínos tende a alavancar a produção e o interesse de empresas por esse setor.

Além do ótimo valor nutricional já conhecido das farinhas de insetos como proteína, gordura, minerais e vitaminas, as farinhas de larvas de insetos como a de *Tenebrio molitor* e *Hermetia illuscens* podem possuir peptídeos antimicrobianos, usados como defesa dos insetos, que podem exibir ação contra bactérias, fungos e outros patógenos. Esse efeito antimicrobiano das farinhas de larvas de insetos pode auxiliar na modulação do sistema imune de frangos de corte.

Os resultados encontrados demonstram que frangos de corte alimentados com níveis de inclusão como 0,5 e 1,0% não tem seu desempenho melhorado. Entretanto, o uso de 2,0% de farinha de tenébrio e BSF melhoram o desempenho em cerca de 100 g, aos 35 dias de idade, e aumenta o consumo de ração das aves. Tendo efeito de melhora parcial no desempenho quando comparado ao com uso de antibióticos melhoradores de desempenho e coccidiostáticos.

Além da melhoria no desempenho, aves alimentadas com 2,0% de farinha de tenébrio ou BSF são capazes de modular o sistema imune inato este efeito pode estar diretamente relacionado aos peptídeos antimicrobianos e quitina/quitosana produzidos pelos insetos.

Desta forma, a partir dos resultados encontrados, conclui-se que as farinhas de insetos quando usadas em baixo nível de inclusão, 2,0%, além de fornecerem nutrientes essenciais, melhoram o desempenho e modulam o sistema imune inato de frangos de corte. Sendo assim as farinhas de insetos podem ser uma possível alternativa, de ingrediente funcional, aos antibióticos melhoradores de desempenho, tendo desempenho parcialmente compensado devido a melhora na saúde e defesa natural dos animais.