UNIVERSIDADE ESTADUAL PAULISTA – UNESP
CAMPUS JABOTICABAL

IMPACTO DO DESAFIO POR COCCIDIOSE E DE NÍVEIS DE PROTEÍNA BALANCEADA NAS RESPOSTAS DE FRANGOS DE CORTE

Luís Filipe Villas Boas de Freitas
Zootecnista

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Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – UNESP, Campus de Jaboticabal, como parte das exigências para a obtenção do título de Doutorado em Zootecnia.

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REGISTRO DE IMPACTO

O impacto esperado da tese é que a sociedade tenha o conhecimento das alterações nutricionais de aves criadas em desafio por coccidiose. A doença é responsável por causar redução no aproveitamento de nutrientes e no desempenho das aves, modificando as exigências nutricionais das mesmas, causando impacto ambiental e econômico.

IMPACT RECORD

The expected impact of the thesis is that society has knowledge of the nutritional alterations of birds raised in challenge by coccidiosis, which is responsible for causing economic and environmental impact, promoting a reduction in the use of nutrients and in birds performance, modifying the birds nutritional requirements.

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Câmpus de Jaboticabal

CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: IMPACTO DO DESAFIO POR COCCIDIOSE E DE NÍVEIS DE PROTEÍNA BALANCEADA NAS RESPOSTAS DE FRANGOS DE CORTE

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Jaboticabal, 09 de março de 2023

DADOS CURRICULARES DO AUTOR

Luís Filipe Villas Boas de Freitas, filho de João Mario Mendes de Freitas e Lucimara Villas-Bôas de Freitas, nascido no dia 31 de maio de 1995 em Varginha, Minas Gerais. Ingressou no curso de graduação em Zootecnia em 2013 na Universidade Federal de Lavras (UFLA), Minas Gerais, finalizando em 2017. Neste período foi bolsista de iniciação científica pela CAPES pelo Programa Jovens Talentos para a Ciência no período de 01 de agosto de 2013 a 01 de julho de 2014, sob orientação da Profª. Drª. Sarah Laguna Conceição Meirelles. Em sequência, durante o período de 01 de agosto de 2016 a 01 de dezembro de 2016, foi participante de iniciação científica voluntária e no período de 01 de dezembro de 2016 a 01 de junho de 2017 foi bolsista CNPq de iniciação científica ambas sob orientação do Prof. Dr. Antônio Gilberto Bertechini. Iniciou o curso de Mestrado em Zootecnia em julho de 2017 na mesma instituição e sob orientação do Prof. Dr. Antônio Gilberto Bertechini, defendendo sua dissertação no dia 12 de julho de 2019. No mês de agosto de 2019, teve início ao curso de Doutorado em Zootecnia pela Faculdade de Ciências Agrárias e Veterinárias da Universidade Estadual Paulista, campus Jaboticabal sob orientação da Profa. Dra. Nilva Kazua Sakomura, obtendo bolsa CNPq. No período de dezembro de 2021 a maio de 2022 realizou doutorado sanduíche financiado pelo projeto CAPES Print na Université Laval sob orientação da Profa. Marie-Pierre Letourneau Montminy. Finalizou o curso de Doutorado em Zootecnia com a defesa de sua tese no dia 9 de março de 2023.

“*A grande tragédia da ciência: o massacre de uma bela hipótese por parte de um horrível fato”* (Thomas Huxley)

Dedicatória

A todos que estão em busca do conhecimento. Que façam bom proveito.

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SUMÁRIO

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"JÚLIO DE MESQUITA FILHO"
Câmpus de Jaboticabal

CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **"Resposta de frangos de corte a níveis crescentes de proteína balanceada e submetidos a desafio sanitário"**, protocolo nº 014470/19, sob a responsabilidade da Profª Drª Nilva Kazue Sakomura, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 14 de novembro de 2019.

Vigência do Projeto	01/12/2019 a 05/05/2023
Espécie / Linhagem	*Gallus gallus domesticus* / Cobb 500
Nº de animais	3600
Peso / Idade	40 g / 1 dia
Sexo	Machos
Origem	Incubatório Pluma agroavícola – Descalvado - SP

Jaboticabal, 14 de novembro de 2019.

Fabiana Pilarski
Prof.ª Dr.ª Fabiana Pilarski
Coordenadora – CEUA

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IMPACTO DO DESAFIO POR COCCIDIOSE E DE NÍVEIS DE PROTEÍNA BALANCEADA NAS RESPOSTAS DE FRANGOS DE CORTE

RESUMO – A coccidiose é responsável por promover lesões intestinais nos animais podendo alterar o aproveitamento de nutrientes, como a da proteína, e promover dispêndios nutricionais para reparação do tecido danificado e promoção das respostas imune para defesa do organismo, o que reduz o desempenho das aves. Determinar como os nutrientes são utilizados por frangos de corte em desafio sanitário auxilia na tomada de decisões dos formuladores quanto a dieta de frangos desafiados. Assim, teve-se como objetivo na presente tese [1] determinar o impacto do desafio sanitário promovido por *Eimeria maxima* sobre a mantença e eficiência de utilização de proteína balanceada, além de, [2] determinar as modificações nas respostas de saúde intestinal, digestibilidade ileal aparente dos aminoácidos e proteína bruta. E por último [3] determinar os efeitos da coccidiose no desempenho de frangos de corte determinando os fatores influentes ao mesmo. Com intuito de responder os dois primeiros objetivos, um experimento foi realizado com 2400 frangos de corte machos da linhagem Cobb500, de 14 a 28 dias de idade, distribuídos em delineamento inteiramente casualizado, alimentados com cinco níveis de proteína balanceada e aves desafiadas ou não por *Eimeria maxima*. Variáveis de desempenho, deposição de tecidos, saúde intestinal e digestibilidade ileal aparente foram mensuradas para obtenção da resposta. Os fatores principais e suas interações foram analisadas pelo teste de análise de variância seguidas pelo teste de comparação de média de Tukey a 5% de probabilidade. Para responder o terceiro e último objetivo, uma meta-análise foi realizada avaliando os fatores que influenciam na reposta das aves desafiadas comparadas ao grupo não desafiado, determinando o impacto do desafio por coccidiose no desempenho avaliados pelo modelo linear misto a 5% de probabilidade. Foi observado que a mantença de proteína balanceada não foi alterada ($P>0.05$) quando as aves foram desafiadas por *Eimeria maxima*, contudo a eficiência da utilização de proteína foi reduzida significativamente. Em geral, o desafio reduziu ($P<0.05$) o desempenho das aves, a saúde intestinal, a digestibilidade ileal aparente de alguns aminoácidos. Níveis alto de proteína resultaram em efeito associativo com o desafio reduzindo o desempenho e a saúde intestinal. A partir da meta-análise foi possível identificar que os dias pós-infecção é o principal fator que atua na piora do desempenho das aves ($P<0.05$), e que a coccidiose em si, impacta mais o ganho de peso que o consumo de ração das mesmas, fatores que podem ser ligados há um menor aproveitamento de nutrientes. Conclui-se que a coccidiose impacta negativamente no desempenho e na eficiência de utilização de proteína balanceada em frangos de corte.

Palavras-chave: Eficiência de utilização, *Eimeria maxima*, mantença, meta-análise, saúde intestinal

IMPACT OF COCCIDIOSIS CHALLENGE AND BALANCED PROTEIN LEVELS ON THE RESPONSES OF BROILER CHICKENS

ABSTRACT – Coccidiosis is responsible for promoting intestinal lesions in animals, which can alter the use of nutrients, such as protein, and promote nutritional expenditure to repair damaged tissue and promote immune responses, which reduces the performance of birds. Determining how nutrients are used by broiler chickens in health challenges helps to make decisions regarding the diet of challenged chickens. Thus, the objective of this thesis [1] was to determine the impact of the health challenge promoted by *Eimeria maxima* on the maintenance and efficiency of utilization of balanced protein, in addition to [2] determining changes in intestinal health responses, amino acids, and crude protein ileal apparent digestibility. And finally [3] to determine the effects of coccidiosis on the performance of broiler chickens by determining the influence factors. To answer the first two objectives, an experiment was carried out with 2,400 male broilers, Cobb500, from 14 to 28 days old, distributed in a completely randomized design, fed with five levels of balanced protein, and birds challenged or not by *Eimeria maxima*. Performance variables, tissue deposition, gut health, and apparent ileal digestibility were measured to obtain the response. The main factors and their interactions were analyzed by the analysis variance test followed by the Tukey test at 5% probability. To answer the last objective, a meta-analysis was performed evaluating the factors that influence the response of the challenged birds compared to the unchallenged group, determining the impact of the coccidiosis challenge on the performance evaluated by the mixed linear model at a 5% probability. It was observed that the maintenance of balanced protein was not altered ($P>0.05$) when birds were challenged by *Eimeria maxima*, however, the efficiency of protein utilization was significantly reduced. In general, the challenge reduced ($P<0.05$) bird performance, gut health, and apparent ileal digestibility of some amino acids. High protein levels resulted in an associative effect with *E. maxima* challenge impairing performance and gut health. From the meta-analysis, it was possible to identify that the days post-infection was the main factor that acts in the performance of the birds ($P<0.05$), and coccidiosis impacts more the weight gain than the feed intake on birds, response that can be linked to reduced use of nutrients. It is concluded that coccidiosis harms the performance and efficiency of using balanced protein in broiler chickens.

Keywords: Efficiency of utilization, *Eimeria maxima*, gut health, maintenance, meta-analysis.

CAPÍTULO 1 - Considerações gerais

1. INTRODUÇÃO

A cadeia avícola brasileira possuí destaque mundial, de forma que no ano de 2021, o país foi responsável pela produção de 14,3 milhões de toneladas de carne de frango, posicionando-se como o terceiro maior produtor e o maior exportador a nível mundial (ABPA, 2022). Resultados que só foram atingidos devido a organização e tecnificação dos elos da cadeia avícola (ambiência, nutrição, saúde e bem-estar). Com a intensificação para uma maior produtividade, a produção de frangos de corte no Brasil se baseia em sua grande maioria no sistema de confinamento, ou seja, a partir do uso de pequenas áreas com uma maior densidade de indivíduos, onde é atingido índices produtivos maiores. Contudo, maior densidade de animais em um mesmo local possibilita maior propagação de doenças, o que faz com que lotes quando acometidos por desafios sanitários resultem em grandes prejuízos ao setor.

Ao longo dos anos a doença coccidiose tem despertado a atenção do setor produtivo avícola, por ser uma doença recorrente na produção dos frangos. Causada pelo protozoário do gênero *Eimeria,* possui como especificidade o desenvolvimento de parte do seu ciclo de vida no intestino das aves, atuando de forma intracelular, o que causa o rompimento das mesmas resultando em menor aproveitamento dos nutrientes (Chapman, 2014). Em sua forma subclínica é responsável pela redução do desempenho das aves, sem apresentar sintomatologias clínicas, levando a perdas econômicas advindas da queda do desempenho e também associada aos gastos profiláticos (Kipper et al., 2013). A coccidiose pode ser ocasionada por diferentes espécies em frangos de corte, de forma que cada uma possuí suas especificidades de atuação e consequências, com destaque as espécies *acervulina, maxima* e *tenella* (Williams, 2005).

Animais em crescimento acometidos por doenças apresentam retardamento do desenvolvimento, o que gera prejuízos ao setor. De forma que, o crescimento diminuído pode ser atribuído a diversos fatores, desde gastos nutricionais para promoção do sistema imune e de sintomalogias ocasionadas pelo próprio organismo, até a própria ação do patógeno que impede o aproveitamento de nutrientes pelo hospedeiro e causa dispêndios para reparo dos tecidos danificados (Sandberg et al., 2007). Além do mais, fatores associados ao ambiente x nutrição x doença podem a vir a influenciar a resposta da ave frente ao desafio. Dessa forma, determinar como um nutriente, em exemplo a proteína, é utilizado pela ave no momento em que a mesma é desafiada contribui para a tomada de decisões técnicas como forma de intervenção nutricional de lotes recorrentes da infecção.

Com isso, estudos foram realizados com intuito de [1] determinar a utilização de proteína balanceada pelas aves em situação de desafio sanitário por coccidiose (*Eimeria maxima*), além de, [2] determinar as respostas das aves frente a diferentes níveis de proteína sobre desempenho, saúde intestinal e digestibilidade, e se diferem quando as aves são desafiadas por coccidiose. E mais, [3] determinar os efeitos da coccidiose no desempenho relativo de frangos de corte determinando os fatores influentes ao mesmo.

2. REVISÃO DE LITERATURA

2.1. Coccidiose

A coccidiose é causada pela infecção de uma ou mais espécies do protozoário *Eimeria* ssp., pertencente ao filo apicomplexa, de tal forma que existem sete espécies descritas que promovem doença em aves domésticas (*Gallus gallus domesticus*), que são: *E. acervulina, E. maxima, E. praecox, E. mitis, E. brunetti, E. necatrix, E. tenella*, as quais, as quatro primeiras são reconhecidas como o grupo que causam má absorção e as três últimas causam doença hemorrágica (Blake et al., 2020). Todas possuem como característica invadir e desenvolver dentro das células epiteliais do trato intestinal, mas cada uma possuí suas especificidades, em exemplo, o local de atuação no trato, a aparência das lesões ocasionadas, a morfologia do oocisto, o tempo mínimo de esporulação, o tempo mínimo do período pré-patente, o tamanho e a forma do oocisto, o local do desenvolvimento e outros (Conway and McKenzie, 2007).

O ciclo de vida da *Eimeria* spp. basicamente possui duas fases, a fase externa (esporogônia), no ambiente, e a fase interna ao hospedeiro (esquizogonia e gametogonia) (figura 1). Para início da infecção, a ave ingere os oocistos esporulados do ambiente e, a partir da ação mecânica da moela e química do proventrículo, o oocisto se rompe liberando quatro esporocistos. Os esporocistos vão ao intestino onde sofrem ação de atividade enzimáticas (tripsina) e da bile, e associado as condições favoráveis (CO_2 e temperatura) do local, os mesmos são rompidos liberando dois esporozoítos (Lopez-Osorio et al., 2020). Os esporozoítos livres infectam as células epiteliais do intestino e desenvolvem a primeira fase assexuada, a esquizogonia ou merogonia, onde os esporozoítos se tornam em trofozoítos que se prolongam e dividem seu núcleo, dando a origem aos merozoítos. Os merozoítos repetem esse processo de replicação assexuada por mais uma ou duas vezes, dependendo da espécie, e em seguida, inicia-se o processo de gametogonia, dando origem aos microgametas (masculinos) e aos macrogametas (feminino) (Conway and McKenzie, 2007). Após a formação do gameta dos machos, ocorre a maturação e liberação dos mesmos que irão adentrar as células onde se localiza os macrogametas, os fertilizando. A fertilização gera a formação do oocisto imaturo, que após o processo de maturação irá romper a célula intestinal e será liberado no lúmen do intestino, sendo excretado juntamente as fezes. No ambiente, o oocisto é esporulado quando submetido a condições de temperatura e umidade ideal, normalmente as encontradas na cama do aviário, e novamente se inicia o ciclo de reprodução do parasita (Conway and McKenzie, 2007).

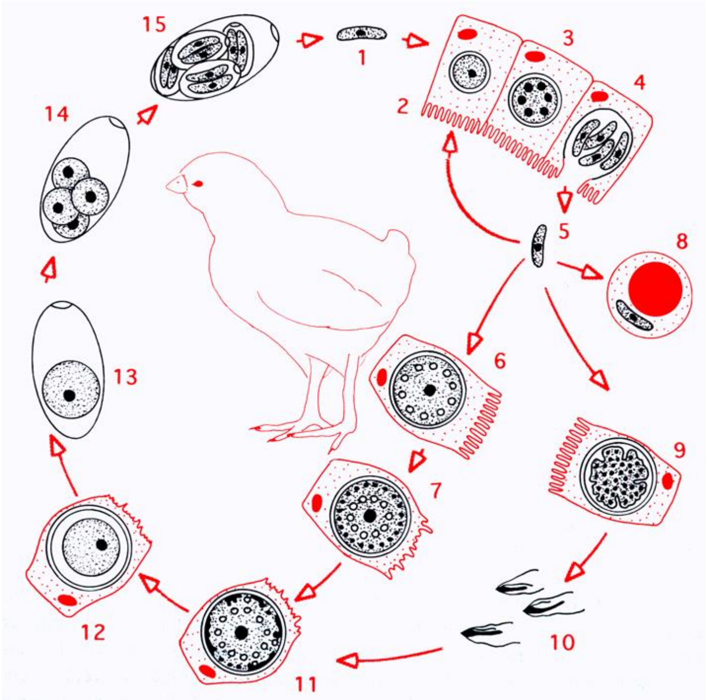

Figura 1. Ciclo de vida *Eimeria* spp. 1 – Esporozoíto; 2- Trofozoíta em célula epitelial; 3- esquizogonia; 4- merogonia; 5-merozoíto; 6- Macrogametócito; 7- Macrogameta; 8-Estágio de repouso dentro de linfócito; 9-microgametócito; 10- microgametas; 11- zigoto; 12- oocisto intracelular; 13- oocisto imaturo livre; 14-emporoblastos dentro do oocisto- 15- oocistos esporulado. Fonte: Adaptado de Lucius e Loos-Frank (1997).

A coccidiose é reconhecida na avicultura industrial como uma das principais doenças causadora de prejuízos a produção avícola, de tal forma que no ano de 2016 foi responsável por um prejuízo de 10,4 bilhões de libras ao setor á nível global, o equivalente a 0,16 libras por ave, sendo esse custo associado a redução de desempenho e gastos profiláticos a doença (Blake et al., 2020). O nível subclínico da infecção é considerado o mais preocupante, pois dificilmente é diagnosticado na prática, e quando evidenciado e tratado, as maiores perdas já ocorreram em consequência da redução do consumo de ração pelas aves (anorexia) e da redução do aproveitamento dos nutrientes (Kipper et al., 2013). Além disso, o parasita atua dentro das células do hospedeiro, gerando danos ao tecido, alterando o metabolismo do animal, desviando nutrientes destinados ao ganho de peso para produção do sistema imune e reposição dos tecidos danificados (Allen et al., 1998, Sandberg et al., 2007).

Uma das principais características da doença coccidiose é seu impacto no aproveitamento de nutrientes e na alteração das características intestinais e metabólicas, pois, a contaminação de um simples oocisto esporulado nas condições ideais, resulta em destruição de aproximadamente 2048 células epiteliais, que são responsáveis pela absorção de nutrientes (Kawazoe, 2009). De acordo com revisão realizada por Chapman (2014) já foram evidenciadas redução na absorção de glicose, ácido oleico, vitamina A, carotenoides, cálcio e minerais traço de aves infectadas por E. *acervulina*. Também foram relatadas alteração na permeabilidade do intestino ocasionando extravasamento de proteínas plasmáticas no lúmen intestinal, aumento de pH e redução da motilidade intestinal. O mesmo autor ainda relata sobre os achados das consequências da doença na redução de níveis hormonais de tiroxina e triiodotironina e aumento plasmático de corticosterona e prolactina. Outra consequência determinada dos efeitos do desafio por coccidiose em frangos de corte é a redução da digestibilidade aparente de aminoácidos. Em meta-análise realizada com um conjunto de dados de 13 estudos, Kim et al. (2022) identificaram que na ordem cronológica decrescente os aminoácidos mais impactados, são cisteína, arginina, metionina, alanina, tirosina e treonina.

A infecção por *Eimeria* spp. pode agir como um cofator a novas infecções, principalmente para o desenvolvimento de microbiota indesejável devido as suas características de atuação e lesões. Por exemplo, a primeira resposta do sistema imune inato a infecção por *Eimeria* spp. é o aumento da produção de muco, em resposta a indução local das células T inflamatórias. O muco é substrato para bactérias mucolíticas como *Clostridium perfringens*, aumentando a proliferação da mesma, e nesse sentido, uma possível predisposição a enterite necrótica pode se iniciar (Collier et al., 2008). Além disso, o rompimento de células epiteliais ocasionadas pela *Eimeria* spp. responsáveis por maior espaçamento entre as junções oclusivas o que torna o intestino mais permeável (como já comentado), e desse modo, mais susceptível a entrada de patógenos na circulação sistêmica, podendo desencadear outras contaminações em outros lugares no organismo (Santos et al., 2020).

Na prática, a tentativa da redução dos prejuízos ocasionados pela doença se faz com a utilização do fornecimento de produtos químicos em dosagem baixa na ração, como os ionóforos, ou a utilização de vacinas vivas de baixa atenuação como forma de prevenção a infecção da doença (Parent et al., 2018). Contudo, ainda assim existe a prevalência de contaminação da doença no campo. Nesse intuito, entender e quantificar a relação entre patógeno e hospedeiro e relaciona-lo com a nutrição pode nos apresentar conhecimentos que ajudariam a reduzir as perdas do setor e nos demonstraria o mecanismo de ação da doença no hospedeiro (Sandberg et al., 2007).

2.2. Consequências do desafio sanitário em animais

Animais quando desafiados tem como principal resposta ao desafio a redução do consumo de ração, por consequência, redução na ingestão de nutrientes e energia (Le Floc'H-Burban, 2020). Dessa forma, alguns nutrientes podem se tornar escassos, e sua quantidade ingerida pode não atender as exigências dos animais para o determinado período. Assim, de acordo com Coop e Kyriazakis (1999), animais em situações de recursos alimentares escassos possuem uma possível ordem de prioridades para realizar suas várias funções corporais por meio da utilização dos nutrientes (Tabela 1). Segundo esses autores, quando o animal sem imunidade a uma determinada doença é submetido a um desafio imunológico, a prioridade desse animal é primeiro manter a proteína corporal, em seguida, produzir respostas imunológicas, deixando o ganho de proteína como terceira prioridade. No entanto, em animais que já adquiriram a imunidade adaptativa e só precisam expressa-la, sua prioridade será após o ganho de proteína. Segundo os autores, o lipídeo sempre será o último componente priorizado, por ser um constituinte de reserva.

Tabela 1. Prioridades (1 maior a 4 menor) por um animal em crescimento para suas várias funções corporais ao dividir um recurso alimentar escasso[1].

Animal em crescimento	
Fase de aquisição de imunidade	Expressão de imunidade
1.Matença da proteína corporal[2]	1. Mantença da proteína corporal
2.Aquisição de imunidade	2. Ganho de proteína
3.Ganho de proteína	3. Expressão da imunidade
4.Mantença e ganho de lipídeo	4. Mantença e ganho de lipídeo

[1]Para *naive*, animal em crescimento que não teve nenhuma experiencia anterior ao desafio, a fase de aquisição da imunidade é considerada separada da expressão.
[2]Inclui reparação, substituição e reação de tecidos danificados ou perdidos.
Adaptado de Coop e Kyriazakis (1999).

Ao ser submetido a um desafio sanitário, o sistema imunológico inicia a fase aguda de resposta, que se inicia pelo recrutamento de macrófagos (linfócitos de defesa) para o combate ao patógeno e produção do sistema imune (figura 2) (Johnson, 1997).

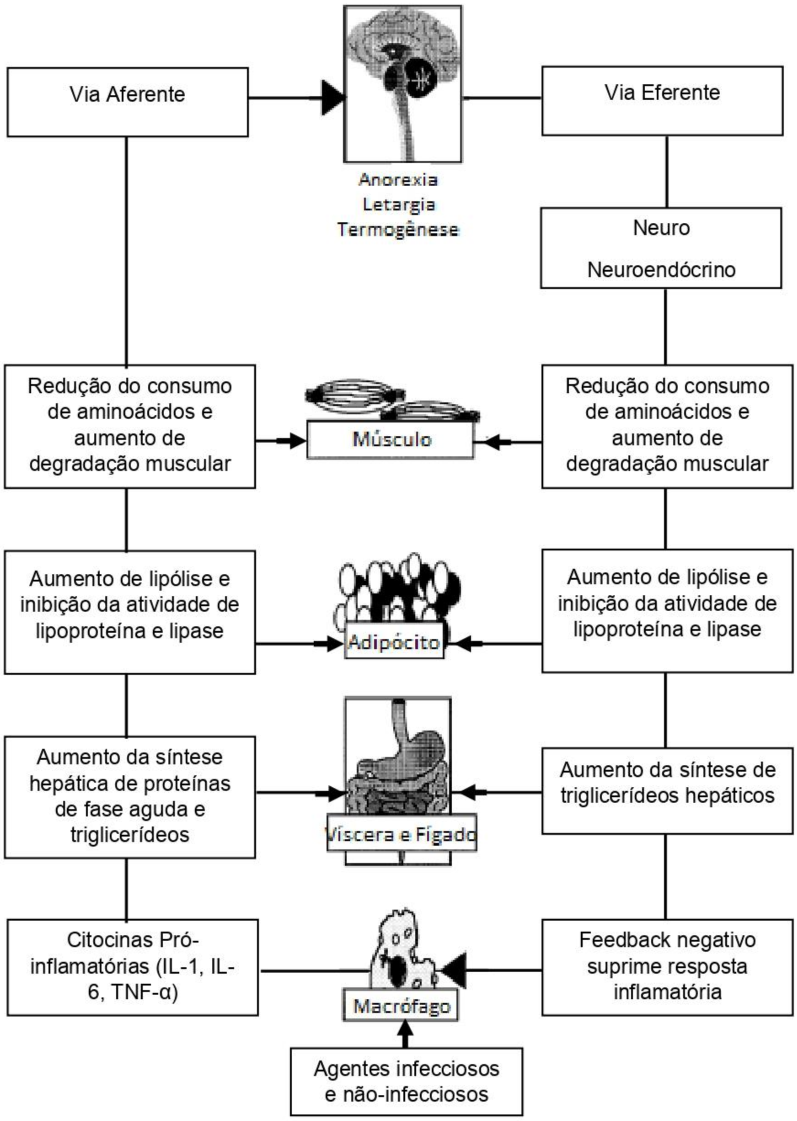

Figura 2. Representação esquemática dos possíveis mecanismos que as citocinas de respostas pró-inflamatórias inibem o crescimento. Fonte: Adaptado de Johnson (1997).

Os macrófagos promovem o desencadeamento de cascata de citocinas pró-inflamatórias (em exemplo, Interleucina (IL)-1, IL-6, fator de necrose tumoral) que são responsáveis pela alteração do metabolismo dos animais e redução no consumo de ração voluntário, que pode variar entre 30 a 60%, denominada anorexia, (Poppi et al., 1990). A alteração no metabolismo induz a degradação muscular e a lipólise, liberando aminoácidos que serão utilizados no fígado para síntese de proteína de fase aguda e aumento da concentração de triglicerídeos no sangue, respectivamente (Johnson, 1997).

A alteração no metabolismo de energia e proteína, ocorre em função da tentativa de adquirir o sistema imunológico, de forma que Li et al. (2007) descreveram a função dos aminoácidos na resposta imune (tabela 2), demonstrando a necessidade de determinados aminoácidos para promoção da resposta, dessa forma o organismo cataboliza o tecido muscular para ter as quantidades necessárias de aminoácidos para promoção do sistema imunológico.

Tabela 2. Papeis dos aminoácidos nas repostas imunes.

Aminoácidos	Produtos	Principais funções
Aminoácidos	Proteínas	Fatores e enzimas do sistema humoral e celular
Alanina	Diretamente	Inibição de apoptose, estimulo de proliferação de linfócitos
Arginina	Óxido Nítrico	Molécula sinalizadora, Mata patógenos, regula produção de citocinas, mediador de doenças autoimunes
Cisteína	Taurina	Antioxidante, regulação do estado redox celular
Metionina	Homocisteína	Oxidante, inibidor de síntese de óxido nítrico;
	Betaina	Metilação da homocisteína para metionina
	Colina	Síntese de Betaina
	Cisteína	Síntese de glutationa
Glutamato	GABA	Neurotransmissor, inibição da resposta da célula T e inflamação
Lisina	Diretamente	Regulação da síntese de óxido nítrico, atividade antiviral
Prolina	H_2O_2	Mata patógenos, integridade intestinal, imunidade
Serina	Diretamente	Inibição de apoptose, estimulo de proliferação de linfócitos
Treonina	Diretamente	Síntese de proteína de mucina que é requerida para manter o sistema imune intestinal, inibição de apoptose, estimulo de proliferação de linfócitos
Triptofano	Serotonina	Neurotransmissor, inibe a produção de citocinas inflamatórias e superóxido
Tirosina	Dopamina	Neurotransmissor, regula resposta imune
	Melanina	Antioxidante, inibe a produção de citocinas inflamatórias e superóxido

Adaptado de Li et al., (2007)

A energia também é requerida em maiores quantidades em desafio imunológico, uma vez que com o desafio, ocasiona maior produção de linfócitos T e B, cujo são as células que apresentam a maior velocidade de divisão no corpo, sendo requerente de grande quantidade de nutrientes e energia para sua produção, no entanto, essas células frequentemente morrem por apoptose, dessa forma, existe uma ineficiência no sistema, perdendo consideráveis quantidade de energia e nutrientes (Klasing, 2007). Além disso, a energia é necessária para a produção de febre, e reparação de tecidos (turnover) (Klasing, 2007). Assim, a redução no consumo de ração e maior utilização de energia para produção e expressão do sistema imune pode atuar na deposição de lipídeo em animais desafiados.

O impacto do desafio sanitário, promove o estresse imunológico que desencadeia um conjunto de resposta metabólicas, neuroendócrinas e comportamentais, essas repostas terão como consequência o comprometimento do crescimento dos indivíduos, ou seja, redução na taxa de crescimento dos mesmos. Além disso, o tipo do desafio influencia na taxa de crescimento dos indivíduos, pois desafios que acometem o trato gastrointestinal são responsáveis, na maioria das vezes, por redução na digestão e absorção dos nutrientes, impactando ao final ao digestibilidade dos mesmos, de forma que os nutrientes consumidos de forma escassa, devido a redução do consumo em consequência ao estresse imunológico, podem ser menos aproveitados, e dessa forma, impactando mais ainda no crescimento e produção dos animais, principalmente em desafios crônicos, gerando retardo no crescimento do animal, não permitindo atingir o máximo potencial dos mesmos.

2.3. Proteína balanceada

Os frangos de corte, possuem altas exigências de proteína em sua dieta para atender suas necessidades de mantença, crescimento, produção e reprodução, sendo que a mesma é o segundo maior constituinte corporal desses animais (11 a 18%), menor somente que a água, variando de acordo com a idade e o sexo, e dependendo da idade e alimentação pode ser menor que a gordura (Vargas et al., 2020). Para alcançar o máximo potencial de crescimento de forma adequada, ou máximo potencial de deposição de proteína, valores mínimos de proteína devem ser fornecidos na dieta. Contudo, a exigência por proteína bruta (PB) pelos monogástricos foi substituído pelo conceito de proteína balanceada, uma vez que esses animais não possuem exigência para o nitrogênio (N) não-proteico e o fornecimento de um mínimo de PB resultaria em excesso de fornecimento de aminoácidos, que devem ser digeridos e metabolizados, promovendo aumento no incremento calórico, além de gastos de energia para excreção de N em excesso, e ainda aumento no custo da dieta, visto que esse nutriente é o mais oneroso da ração (Bertechini, 2021).

Dessa forma, o conceito de proteína balanceada refere-se à exigência dos animais monogástricos, por uma quantidade de proteína da dieta que leva em consideração a inclusão de um aminoácido referência (lisina digestível) e os demais aminoácidos são mantidos constante de acordo com um perfil de aminoácidos ideal determinado (em exemplo o proposto por Rostagno et al., 2017). Já o perfil ideal de aminoácidos seria o balanço exato de aminoácidos que é capaz de promover sem excesso ou deficiência os requerimentos de todos os aminoácidos necessários para a manutenção animal e máxima deposição proteica (Mitchel, 1964).

Os aminoácidos podem ser classificados de acordo com sua síntese endógena e utilização pelo organismo, classificados como essenciais, parcialmente essenciais e não-essenciais. Os aminoácidos essenciais são aqueles que os animais não conseguem produzir endogenamente ou produzem em baixas quantidades, e assim, devem ser fornecidos via dieta. Os aminoácidos parcialmente essenciais são aqueles que as aves produzem em condições suficientes, mas em determinado período de criação, o mesmo pode se tornar essencial. E os aminoácidos não-essenciais são aqueles que as aves produzem endogenamente e conseguem suprir suas exigências (Bertechini, 2021).

A lisina é tomada como aminoácido referência devido a ser estritamente essencial; seu metabolismo é principalmente para deposição corporal; as análises para tal nutriente são bem mais desenvolvidas e mais precisas; sua suplementação é economicamente viável; é o primeiro limitante para suínos e o segundo para aves (em dietas de milho e farelo de soja); e há bastante estudos na literatura para determinação das exigências (Batterham, 1990).

Outro fator que a utilização de proteína balanceada permitiu foi a redução da excreção do componente N em excesso no ambiente, o que reduz o impacto ambiental e também pode melhorar a qualidade do ambiente de criação em que as aves se encontram, uma vez que o excesso de N se torna em amônia, que é prejudicial à saúde das aves (Van Harn et al., 2019). O excesso de N nas rações também está associado com a inclusão de altos teores de ingredientes proteicos nas rações, como o farelo de soja. O farelo de soja possui grande quantidade de potássio em sua constituição, que pode contribuir para o maior consumo de água pelos animais e consequentemente, maior teor de umidade nas excretas, aumentando o teor de umidade na cama (Youssef et al., 2011). A associação da alta umidade da cama, com temperaturas altas no ambiente e o mal manejo da cama, podem ser cofatores para o aparecimento de lesões de calos de peito e de patas nas aves, o que está relacionado ao bem-estar das aves e prejuízos econômicos com a condenação de um produto com alto valor agregado (Abd El-Wahab et al., 2013).

Vale ressaltar ainda, que na prática o nível de proteína balanceada utilizada pode não ser aquele que irá atingir o potencial de crescimento da ave e sim aquele que trará maior retorno econômico, como já dito, pois é o nutriente mais oneroso da ração.

2.4. Alteração nas exigências nutricionais de animais em desafio

Para aves domésticas, a determinação das exigências nutricionais preconizadas pelo NRC (1994) e pelas Tabelas Brasileiras para Aves e Suínos (2017), usualmente, foram baseadas na avaliação do crescimento de aves em condições ideais, ou seja, em locais onde as mesmas possam expressar o seu máximo potencial. No entanto, em situações práticas as aves são expostas a diferentes tipos de estresse, doenças e combinações de diferentes condições ambientais, o que podem vir a alterar a utilização dos nutrientes fornecidos as mesmas, sendo necessário levar tais situações em consideração (Taghinejad-Roudbaneh et al., 2013). Os desafios sanitários responsáveis por ocasionar o estresse imunológico, promovem alterações no metabolismo, e em consequência, podem aumentar o custo de nutrientes para produção e expressão de imunidade, reparação dos tecidos e inflamação (Sandberg et al., 2007), além também de reduzir a capacidade dos animais em digerir e absorver nutrientes (Kim et al., 2022) e quando submetidos a estresse imunológico crônico ocasionam redução na taxa de crescimento, assim, o somatório dessas consequências irão atuar sobre o crescimento da ave, possivelmente alterando as exigências nutricionais das mesmas no período de infecção.

Trabalhos com intuito de entender e descrever os custos do desafio imunológico sobre os nutrientes já foram realizados, no entanto, existem contradições sobre tal temática, de tal forma que alguns autores assumem que as necessidades nutricionais para o desenvolvimento da resposta imunológica e reparo dos danos teciduais são pequenas, assim o fornecimento dos níveis dietéticos estimados para a máxima resposta de desempenho atendem as exigências pelos animais, e a redução no crescimento dos mesmo sempre irá ocorrer devido a redução do consumo de ração “inevitável” (Kidd et al., 2001; Taghinejad-Roudbaneh et al. 2011). Por outro lado, há autores que evidenciaram em seus estudos que a redução do ganho de peso médio diário não está relacionada somente com a redução no consumo diário, e sim também ao aumento da necessidade de nutrientes e energia para melhor resposta imune pelo organismo, muitas vezes sendo os mesmos associados ao aumento da mantença e/ou diminuição da eficiência de utilização (Pastorelli et al., 2012; Van der Meer et al., 2020), o que ao final resultará em menor quantidades dos nutrientes e energia para produção líquida. Assim, maior quantidade de nutrientes e energia seriam desejáveis para atender todas as exigências dos animais.

Entender e quantificar a interação entre patógeno (doença), hospedeiro (resposta) e nutrição, é uma boa alternativa para tomada de decisões sobre os programas alimentares de lotes infectados. Para tanto o formato de estudos com alimentação controlada (*pair* feeding) e avaliação de respostas marginais em função de determinados nutrientes ou planos alimentares, seriam ideais, de maneira que a associação das duas metodologias obteria resultados mais conclusivos (Sandberg et al., 2007).

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CAPÍTULO 2 – Coccidiosis infection and growth performance of broilers: insights from a meta-analysis including modulating factors

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Coccidiosis infection and growth performance of broilers: insights from a meta-analysis including modulating factors

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ABSTRACT

An infection by protozoa *Eimeria* spp. can cause coccidiosis, which negatively affects broiler chicken performance and causes economic and production losses. To understand the effect of coccidiosis on broilers' performance, we evaluated the independent variables and their interactions on the severity of coccidiosis in broilers that cause variation (**Δ)** of average daily feed intake (**ADFI**), average daily gain (**ADG**), and gain per feed (**G:F**) of broiler chicks using a meta-analysis approach. A database of 55 papers describing 63 experiments was gathered; broilers were challenged by *Eimeria* species (*E. acervulina, E. maxima, E. tenella,* and mixed) and at least two variables among ADFI, ADG, and feed conversion ratio (**FCR**) were studied. The variation induced by the challenge was calculated relative to the control group of each experiment. The indirect factors evaluated were days post-infection (**DPI**), *Eimeria* type and dose, infection age (**IA**), bird's mean age in the analyzed period, genetic line, gender, and whether they were raised in a cage or a pen. Graphical, correlation and variance analyses were performed to evaluate the form of the responses. Then, a linear plateau model was adjusted for each response variable as a function of DPI to determine the consequences of the disease on the variation of performance over time after infection. The impact of the infection challenge on the variation of performance vs non-challenge broilers was only impacted by DPI ($p < 0.05$). The adjustment of the data with the linear plateau model allows us to determine the host response to the coccidiosis disease at different stages. At 5 DPI (acute phase), ΔADFI, ΔADG, ΔG:F were of –19.0; –39.8, and –25.5, respectively. After almost 13 DPI birds achieved the recovery phase for all variables with Δ varying from –19% to –3.75% for ADFI, from –39.8% to –10.5% for ADG, and from –25.5% to –7.24% for G:F. The *Eimeria* impact was higher in ADG than ADFI in all periods due to *Eimeria* aggressive action form causing lesions in gut epithelial reducing the use of nutrients and energy. The results can be used as a quantitative approach to determine the consequences of *Eimeria* spp*.* on broiler performance.

Keywords: average daily feed intake, average daily gain, *Eimeria* spp., disease, meta-analysis.

Abbreviations: ADFI, average daily feed intake; ADG, average daily gain; DPI, days post-infection; G:F, gain to feed; IA, infection age; FCR, feed conversion ratio.

INTRODUCTION

Coccidiosis is caused by a protozoan of the genus Eimeria that is a global broiler chicken production concern. Its annual cost is estimated to be about $11.93 billion, due to a reduction in the broiler performance (as measured by the feed intake, weight gain and the feed conversion ratio), increased mortality, and the expense of medication and vaccines (Blake et al., 2020). Seven species of Eimeria are recognized as responsible for causing pathogenicity in domestic birds (Gallus gallus domesticus) including E. acervulina, E. maxima, E. tenella, E. necatrix, E. mitis, E. praecox, and E. brunetti. Each species had its specificity regarding the location of actionin the gut, oocyst morphometrics, and immunogenicity (Chapman, 2014). According to Reid et al. (2014), Eimeria species can be classified by the consequences on the host as hemorrhagic (E. tenella, E. necatrix and E. brunneti), malabsorption (E. maxima and E. acervulina), and lesser degree infection (E. mitis and E. praecox). Of all species, four are of concern in terms of global disease and economic impact: E. acervulina, E. maxima, E. tenella, and E. necatrix (Williams, 2005).

According to Willians (2005), coccidiosis disease severity can be classified into three levels: coccidiasis (a mild infection, causing no adverse effect), subclinical coccidiosis, and clinical coccidiosis. In a subclinical infection, coccidiosis is responsible for reducing bird growth performance as a result of reduced voluntary feed intake (anorexia), reduced nutrient digestibility, reduced efficiency of nutrient use, and expenditure of nutrients to repair damaged tissues (higher maintenance;

Moraes et al., 2019, and Kim et al., 2022). Furthermore, to activate and maintain the immune system, some specific nutrients and energy were required to produce immune responses cells diverting them from the anabolic process and modifying the metabolism (Allen et al., 1998; Sandberg et al., 2007).

The effects of the Eimeria on bird growth performance can be influenced by various factors inherent to the pathogen, host, and the environment and their interactions (Kipper et al., 2013). Factors such as age, gender, genetic line (factors inherent to the host), and Eimeria species (factor inherent to the pathogen) were able to influence the impact of Eimeria infection. Also, a significant factor that could influence the infection and modify the response on performance is the different periods of response to disease (incubation, acute phase, recovery phase). Therefore, trials that report only measurements at the acute phase normally would show a higher impact on the performance than trials that report only the recovery phase.

The aim of this study was to use meta-analysis to evaluate the consequences according to days post-infection, which was calculated as the age when we measured the growth performance minus the infection age (IA), in addition to all the factors reported in the publications that may affect the response of birds to an Eimeria challenge in terms of the variation (Δ) of average daily gain (ΔADG), average daily feed intake (ΔADFI), and gain per feed relationship (ΔG:F).

MATERIAL AND METHODS

Database collection and coding

An exhaustive literature search was performed using Pubmed, ScienceDirect, and the Web of Science database with the keywords Eimeria, coccidiosis, E. acervulina, E. maxima, E. tenella, broilers, and growth performance (i.e., daily gain, feed intake, body weight, feed efficiency). Only data reported in articles published in peer-review journals were used as quality criteria. Coccidia species selected were E. acervulina, E. maxima, E. tenella, and mixed (simultaneous infection with two or more Eimeria spp.). All references obtained in each database were reviewed independently by two researchers to select the papers.

Selection criteria for the final database were papers that: 1) reported on at least two of these variables: average daily gain, average feed intake, or feed conversion ratio (FCR), 2) the experiments should include a control group of birds that were not challenged, 3) controlled and treated birds received the same diet, 4) the experiments should challenge the broilers only using sporulated oocysts (vaccines were not accepted), and 5) reported the dose.

Articles were excluded if they: 1) contained an additive that affects the Eimeria life cycle, 2) did not show a reduction in ADFI during the acute phase period (5 to 10 DPI), and 3) reported reinfection (reduced performance after 12 DPI). With those exclusions, we were left with 55 publications reporting about 63 trials from 2000 to march of 2022 (Figure 1).

For each article selected, we made an Excel database with general information (i.e., author names, journal of publication, year of publication), the information describing the animals (e.g., gender, lineage, mean age, number of birds per treatment), the information about the challenge (i.e., Eimeria species, dose, IA, days post-infection), experimental conditions (e.g., type of housing, diet composition), and animal performance (ADFI, ADG, FCR). Each publication, trial, and treatments was assigned a code to categorize the data (Sauvant et al., 2008). An overall database description was made with data used in the statistical proceedings (Table 2 and 3).

Data examination and statistical analyses

To determine the impact of Eimeria spp. on the growth performance, we compared the reductions caused by the disease in the challenged vs. unchallenged groups, as suggested by Pastorelli et al. (2012). This procedure also accounted for heterogeneity between experiments. To better understand the factors that affect the response of birds to coccidiosis, the following independent variables were tested: effects of challenge (types of Eimeria, DPI, IA, and doses of oocysts), experimental conditions (diet composition, cage vs. pen, gender, and mean age). Before starting the analysis, all studies that report the diets were verified for standard ileal digestibility of lysine, methionine, methionine + cystine, arginine, and threonine requirements (mg/day) and for apparent metabolizable energy (kcal/kg), in accordance with genetic guidelines of the corresponding genetic line and year to avoid the confounding effects of nutrient deficiency. The first analysis performed was graphical (not shown) to visualize the relationships between the dependent and independent variables, identify outliers, and observe the biological coherence of data. The second step used was a correlation between the independent variables to identify any collinearity. Then, the General Linear Mixed model analysis was performed using the code of each trial as a random effect with Minitab 19 software (State College PA) with a significant effect at $P < 0.05$.

The analysis aimed to study the dynamics of the response of broilers after coccidiosis infection by evaluating the evolution of ΔADFI, ΔADG, and ΔG:F as a function of the days per infection. According to the meta-design graphical examination, a linear-plateau (Eq. 1) model with the trial as a random effect was fitted:

$\Delta Y = (X > T1) \times L + (X \leq T1) \times (R \times (X - T1) + L) + RE$ Eq. 1

Where Y is the dependent variable; X is the variable on the X-axis (DPI); L is the plateau value; R is the inclination of the line segment (slope), T1 is the time necessary to reach the plateau value, or in other words, the time for performance recovery after an infection, and RE is the randomized effect (study effect). To evaluate a full recovery of performance, the value estimated for L was tested for significance with 0 (no variation) by an F-test. The fit of linear plateau regression and the comparison of the parameter was performed using the procedure PROC NLMIXED on SAS (2004). The maximum variation (L0) was calculated with Equation 2:

$L_0 = L/(R \times (T1 - T_0))$ Eq. 2.

Where T0 is the first DPI where there is a change in ADFI, ADG, and G:F.

RESULTS

The effects of IA, dose, genetic line, gender, and housing type were never significant. The variables mean age and DPI had a significant effect when evaluated independently, however, they were colinear, thus only DPI was maintained in the final model. As DPI increases, the variation on ADFI, ADG, and G:F fitted a linear-plateau model (Figure 2; Table 3). The time necessary for recovery (T1) was 11.7 ± 1.28 DPI for ΔADFI, 12.9 DPI for ΔADG, and 13.0. The R parameter was –2.27 for ΔADFI, –3.73 for ΔADG and –2.29 for ΔG:F. The maximum value attainable (plateau value, L) was –3.75 for ΔADFI, –10.50 for ΔADG, and –7.23. The L for all variables was different ($P < 0.05$) from 0. The L0 calculated with Eq. 2 was equal to –19.0, –39.8, and –25.5 for ΔADFI, ΔADG, ΔG:F, respectively.

By evaluating the dynamic between the ΔADFI and ΔADG as DPI increases, we can distinguish the percentage of growth reduction when voluntary feed intake was reduced (Figure 3). The anorexia induced by the coccidia infection was responsible for 39% of the reduction of ADG (by the absence of intake), so we can say it was mostly explained by the direct Eimeria impact. In the latent phase of the challenge (5 to 7 DPI) the reduction in feed intake represents about 46% of the reduction of ADG.

DISCUSSION

Identifying the main factors that modulate the interactions in the host-disease-environment gives us a perspective on how coccidiosis reduces broilers' performance, and allows us to measure the impact of the disease on contaminated flocks. Modelling the relative response of challenged birds over time with DPI gives a holistic and dynamic view of the effects of the disease on the growth of the host broiler chickens, and of the average impact of the disease in a general rearing period.

Different species of Eimeria can promote coccidiosis in broilers, but each species has a specific mode of action. According to López-Osorio et al. (2020), the species E. acervulina and E. maxima were classified as species with moderate pathogenicity while E. tenella is highly pathogenic. Kipper et al (2013) reported that Eimeria maxima induces a greater impact on the feed conversion ratio than E. acervulina, E. tenella, and mixed pathogens. However, no significant effect was observed in our database, assuming that regardless of the Eimeria species, performance was reduced similarly compared to the control and to the combined result.

None of the independent factors (IA, dose, genetics, gender, and type of installation) seemed to have any significant effect. Literature regarding the age at infection is scarce. It is generally considered that young broilers have less developed immune systems than older birds, and therefore young birds would be more susceptible to diseases and more readily display signs of disease (Yun et al., 2000). However, in older studies (e.g., Rose, 1967; Kouwenhoven, 1972), it has been shown that older birds suffer more from the consequences of coccidiosis than younger birds, because the older ones shed more oocysts (Rose, 1967) and have their growth reduced more and in the longer term (Kouwenhoven, 1972). The authors related these responses to the high capacity of oocyst excitation in older birds promoted by a well-developed gastrointestinal tract and associated with a high quantity of cells prone to infection. However, reinfections are less common in older birds than younger birds because of the older birds' well-developed immune system (Rose, 1967). The lesser impact on young birds was explained by an inefficient oocyst wall break by the gizzard and suboptimal tryptic enzyme production (enzymes that act in the wall break). In addition, Rose (1967) affirmed that naive birds do not have immunity to coccidiosis, independent of age, because they had no passive immunity from the breeders for coccidiosis (Rose and Long, 1962). Thus, further study of the effects of the coccidiosis infection age is important.

Another factor that had already been reported to make a difference was the inoculum dose (Kouwenhoven, 1972; Teng et al., 2020; Teng et al., 2021). All papers showed that the reduction in growth caused by the disease was worse with higher doses of sporulated oocysts. In our database, only four studies aimed to evaluate the effect of inoculum doses on the broiler growth performance

(Rochell et al., 2016; Sakkas et al., 2018; Teng et al., 2020; Teng et al., 2021); all show the same trend, a significant reduction in performance with increased dose (Figure 4). Increasing the amount of inoculum probably results in higher numbers of cells infected, and a greater impact on gut health. Lastly, some authors reported that female broilers were more sensitive than male broilers to coccidiosis (Kipper et al., 2013), but 96% of studies in our database used only male broilers.

The protozoa Eimeria spp. has necessary stages for development (exogenous and endogenous), the durations of which are well known, with small differences between species. For example, for the pre-patent phase we expected that Eimeria acervulina would have a range of 4 days, Eimeria maxima 5 to 6 days, and tenella a range of 6 to 7 days (Bangoura and Daugschies, 2018). The endogenous phases of the parasite cycle (schizogony and gametogony) disrupt the host intestinal cells to form parasite “zoites”. However, the pathological changes and clinical signs only occur during the gamont phase, and this is when broiler performance suffers. The disease destroys the mucous membranes in all segments of the gut, causing imbalances in absorption that result in diarrhea and a failure to thrive (Lópes-Osorio et al., 2020).

This sequence of the Eimeria cycle development leads to the host response sequence (incubation period, acute phase, and recovery) and in the present study was approached as DPI. The effect of DPI on the variation in broiler performance shows the importance of considering the disease stages when comparing the impact of pathogens on performance. Here we analyzed the effect by fitting the variation responses (ΔADFI, ΔADG, ΔG:F) to the linear plateau model with a good fit.

During the incubation period, considered as the first day of infection (inoculation day) to 4 days post-infection, pathological changes and clinical signs were not expected. In our model, this period was not considered, because we did not expect differences in performance between challenged and unchallenged birds in this period (Lópes-Osorio et al., 2020), and because few studies measure the broilers during this time.

Based on the meta-design, the acute phase period, which showed a worse reduction in performance, was between 5 to 12 DPI, with the highest impact occurring at 5 days. The "recovery" phase was from 13 to 30 DPI, corresponding to the days where birds recover from the impact of the disease compared to the control, probably by an effective immune response (Figure 2). Nevertheless, some limitations had to be assumed when considering the fitted model. First, the first days per infection is always 5 days in the database, probably because before that (incubation phase) a reduction of performance was not expected by the authors considering the intern cycle of Eimeria infection. The other limitation is that the model should be used to determine only the variation in performance on the range between 5 to 30 DPI, the maximum range found in our database, and values estimated before or after this range could be wrong.

The coccidiosis disease reduced the daily food intake, as was evident at the acute phase, with the worst reduction, ~19%, estimated to be at 5 days post-infection, calculated by Eq. 2. The reduction in food consumption is associated with the production of inflammatory cytokines that produce negative feedback in the central nervous system, reducing the animals' appetite (Johnson, 1997). In the same way, the worst reduction in daily weight gain, ~39%, was 5 days post-infection. The Eimeria infection induces a worse reduction in ADG due to the lesions in epithelial cells in the gut, which reduces digestibility of nutrients and energy (Amerah and Ravindran, 2014; Teng et al., 2021, Kim et al., 2021) and the efficiency of nutrient transport from the intestinal lumen to epithelial cells (Teng et al., 2021) resulting in a higher feed conversion rate and an increase in maintenance to repair tissue damage (Moraes et al., 2019b).

To better understand why daily gain was affected more than the daily food intake, we separated the consequences of the Eimeria challenge to explain the reduction in daily gain relative to the number of days post-infection (Figure 4). We separated the consequences in response to the reduced food intake (promoted by anorexia symptoms) and due to an Eimeria consequence (consequences related directly *Eimeria* damage). Results showed that on the first day, the impact of anorexia and *Eimeria* cost was almost the same, and as the days post-infection increased, anorexia had less impact. In other words, in the acute phase anorexia caused the most growth reduction, and then it was more related to gut damage and the energy requirements of the immune response.

It is interesting to see that as the days post-infection increase, the daily food intake increased as well as the daily gain and gain to feed ratio (L parameter) which, although the recovery percentage differed, occurred practically on the same day (12 to 13 DPI). Even describing a recovery phase, a full recovery (when the infected and non-infected did differ in the variables) was not observed for any of the growth performance parameters. This indicates that the Eimeria infection impact on growth performance is not resolved even at 30 DPI (maximum range evaluated). This absence of full recovery may be due to reduced feed intake due to reduced weight and size of birds in challenged birds.

In conclusion, using a meta-analytic approach, we observed that the number of days since infection was the principal modulating factor of the impact of Eimeria spp. on the growth performance of broilers, and therefore had to be considered when developing strategies to reduce coccidiosis. The variation in ADFI, ADG, and G:F is most significant in the acute phase of the disease, and the impact in the G:F and ADG are more pronounced than ADFI. The recovery phase after infection of Eimeria spp. occurs near 13 DPI, however, full recovery was in general not possible to achieve.

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Table 1. Descriptive characteristics of used database

Authors	Sex	Genetics	Installation	Species
Matthews and Southern (2000)	Male	Hubbard	Pen	*E. acervulina*
Yadav and Gupta (2001)	-	Ross 308	Cage	*E. tenella*
Klasing et al. (2002)	Mixed	Cobb500	Pen	*E. acervulina*
Allen and Fetterer (2002)	Male	Ross 308	Cage	*E. maxima*
Kidd et al. (2003)	Male	Ross 308	Pen	*E. acervulina*
Christaki (2004 a, b and c)	Mixed	Cobb 500	Cage	*E. tenella*
Koinarski et al. (2005)	-	Cobb 500	Cage	*E. acervulina*
Watson et al. (2005)	Male	Ross x Ross	-	*E. acervulina*
Oviedo-Rondón et al. (2006)	Male	Cobb 500	Pen	*E. acervulina*
Persia et al. (2006)	Male	Ross x Ross	-	*E. acervulina*
Elmusharaf et al. (2007)	Female	Ross 308	Pen	*E. tenella*
Nollet et al. (2007)	Male	Ross 308	Cage	Mixed
Bafundo et al. (2008)	Male	Cobb x Cobb	Pen	Mixed
Hassan et al. (2008)	-	Ross x Ross	Cage	*E. tenella*
Abbas et al. (2011)	-	Hubbard	-	*E. tenella*
Bun et al. (2011)	Male	Arbor Acress	Cage	*E. tenella*
Küçükyilmaz et al. (2012)	Mixed	Ross 308	Pen	Mixed
Bozkurt et al. (2012)	Mixed	Ross 308	Pen	Mixed
Faber et al. (2012)	Male	Ross x Ross	Pen	*E. acervulina*
Abdelrahman et al. (2014)	Male	Cobb 500	Pen	Mixed
Bozkurt et al. (2014)	Mixed	Ross 308	Pen	Mixed
Ritzi et al. (2014)	Male	Cobb 500	Pen	Mixed
Bortoluzzi et al. (2015)	Male	Ross 308	Pen	Mixed
	-	Ross 308	Cage	*E. acervulina*
Pop et al. (2015 a, b and d)	-	Ross 308	Cage	*E. maxima*
	-	Ross 308	Cage	Mixed
Singh et al. (2015)	Male	Ross 308	Pen	Mixed
Aziza et al. (2016)	-	Cobb 500	Cage	*E. tenella*
Rochell et al. (2016)	Male	Ross 308	Cage	*E. acervulina*
Laika and Jahanian (2017)	-	Ross 308	-	Mixed
Rochell et al. (2017b)	Male	Ross 308	-	*E. acervulina*
Rochell et al. (2017a)	Male	Ross 308	-	*E. acervulina*
Aziza and Awadin (2018)	-	Cobb 500	Cage	*E. tenella*
Sakkas et al. (2018)	Female Male	Ross ranger Classic Ross 308	Pen	*E. maxima*
Tonda et al. (2018)	Male	Cobb 500	Cage	Mixed
Moraes et al. (2019)	Male	Cobb 500	Pen	Mixed
Sakkas et al. (2019)	Male	Ross 308	Pen	*E. maxima*
De Souza Khatlab et al. (2019)	Male	Cobb 500	Pen	Mixed
Oelschlager et al. (2019)	Male	Ross 308	Cage	Mixed
Pop et al. (2019)	-	Ross 308	-	Mixed
Park et al. (2020)	Male	Ross 308	Cage	*E. maxima*
Santos et al. (2020)	Male	Cobb 500	Cage	Mixed
Teng et al. (2020a)	Male	Cobb 500	Cage	Mixed
Sadeghi et al. (2020)	-	Ross 308	Cage	Mixed
Teng et al. (2020c)	Male	Cobb 500	Cage	Mixed
Yazdanabadi et al. (2020)	Mixed	Ross 308	Pen	Mixed

Talghari et al. (2020)	-	Ross 308	Pen	Mixed
Yadav et al. (2020)	Male	Cobb 500	Cage	Mixed
Teng et al. (2020b)	Male	Cobb 500	Cage	Mixed
Wang et al. (2021)	Male	Arbor Acres	Cage	Mixed
Lin and Olukosi (2021a)	Male	Cobb 500	Cage	Mixed
Teng et al. (2021)	Male	Cobb 500	Cage	*E. maxima*
Qaid et al. (2021b)	Mixed	Ross 308	Cage	*E. tenella*
Youssef et al. (2021)	-	Cobb 500	Pen	*E. tenella*
Qaid et al., (2021a)	Mixed	Ross 308	Cage	*E. tenella*
Qaid et al. (2022)	Mixed	Ross 308	Cage	*E. tenella*
Yadav et al. (2022)	Male	Cobb 500	Cage	*E. maxima*

Table 2. Overall descriptive database

Species	Mixed				*Eimeria acervulina*				*Eimeria maxima*				*Eimeria tenella*			
Nº of papers	27				12				7				11			
Nº of trials	28				14				8				13			
N° of NCH	75				59				32				34			
N° of challenge	45				30				18				17			
Ind. Var.[1]	n	Mean	Min	Max	n	Mean	Min	Max	n	Mean	Min	Max	n	Mean	Min	Max
Initial age	111	18.8 (±6.48)	12	36	67	11.5 (±6.88)	1	22	34	17.1 (±4.23)	11	22	40	16.4 (±8.62)	1	29
Final age	111	30.0 (±9.03)	19	42	67	19.6 (±5.17)	10	28	34	24.9 (±3.28)	20	28	40	30.5 (±8.85)	17	42
Infection age*	57	15.4 (±2.50)	13	21	34	10.3 (±5.94)	1	19	19	13.8 (±1.98)	11	21	21	16.6 (±4.89)	10	21
DPI*	57	14.4 (±8.38)	6	28	34	9.35 (±3.02)	5	13	19	10.8 (±3.39)	6	14	21	13.2 (±6.50)	7	28
Dosage*	57	1.8×10^5	5×10^3	7.6×10^5	34	6.4×10^5	3×10^4	1×10^7	19	6.2×10^4	2.5×10^3	1.8×10^5	21	4.3×10^4	5×10^3	1×10^5
Dependent variable																
ADFI (g)																
Unchallenged	54	119 (±37.7)	56.9	211	33	65.4 (±27.49)	29.5	119	15	109 (±14.9)	81.5	134	19	97.8 (±29.8)	42.1	135
Challenged	57	110 (±40.7)	49.9	217	34	60.2 (±27.1)	28.5	119	19	100 (±18.4)	68.3	133	21	88.0 (±24.9)	40.8	130
ADG (g)																
Unchallenged	54	67.5 (±16.7)	24.0	102	33	40.3 (±14.5)	14.5	68.7	15	67.9 (±19.3)	37.7	102	19	58.4 (±17.5)	31.9	85.2
Challenged	57	53.9 (±20.9)	19.2	103	34	33.8 (±14.3)	15.7	67.4	19	53.9 (±20.4)	22.0	88.1	21	45.9 (±14.1)	24.7	75.6
FCR (g/g)																
Unchallenged	54	1.77 (±0.341)	1.14	3.18	33	1.63 (±0.421)	1.17	2.88	15	1.70 (±0.421)	1.29	2.62	19	1.69 (±0.383)	1.12	2.32
Challenged	57	2.10 (±0.412)	1.18	3.85	34	1.80 (±0.397)	1.23	2.76	19	2.12 (±0.792)	1.34	3.75	21	1.99 (±0.468)	1.21	2.92

*Data only for the challenged group; means are followed by the standard deviation in parenthesis. Abreviations: DPI, days post-infection; NCH, unchallenged

Table 3. Parameters estimated (R, T1 and L) and standard deviations (in parentheses) by adjusting the data to the linear plateau model for ΔADFI, ΔADG, and ΔGain:Feed and the maximum variation calculated at 5 days post-infection (L0)

Parameters	ΔADFI	ΔADG	ΔGain:Feed
R	-2.27 (± 0.57)	-3.727 (± 0.57)	-2.293 (± 0.53)
T1[a]	11.71(± 1.28)	12.85 (± 0.93)	12.99 (± 1.42)
L (Ymax)	-3.75 (± 1.03)	-10.50 (± 1.90)	-7.24 (± 1.65)
P value (L=0)[b]	<0.001	<0.001	<0.001
L_0	-19.00	-39.77	-25.55

[a] Number of days post-infection (DPI) to the variation achieve the plateau value; [b] P-value obtained by the T-test, comparing parameter L from zero; $L_0 = L/R \times (T-T_0)$ where T0 is the first day post-infection that occurs variation in response.

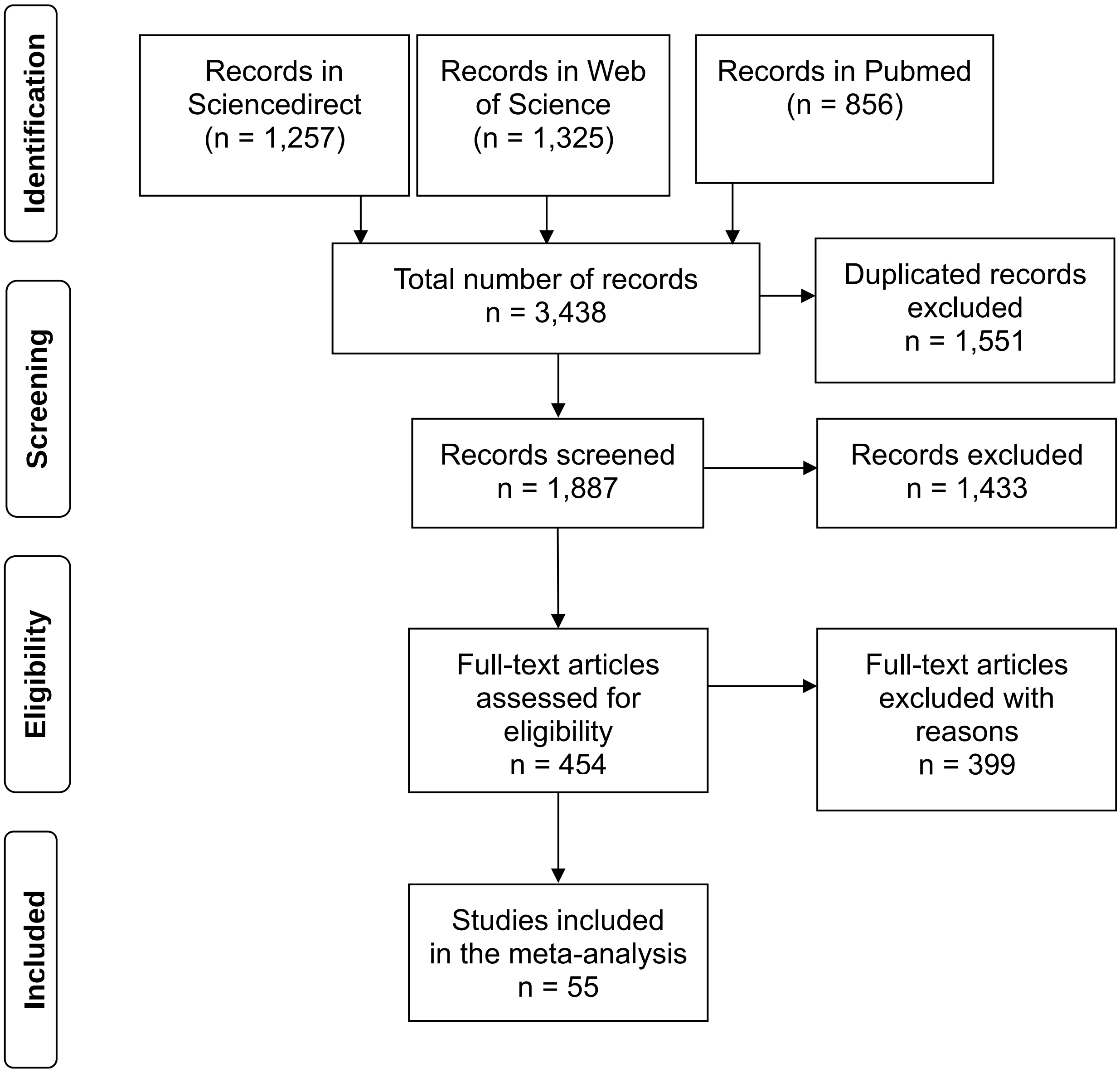

Figure 1. Prisma flow diagram for the meta-analysis

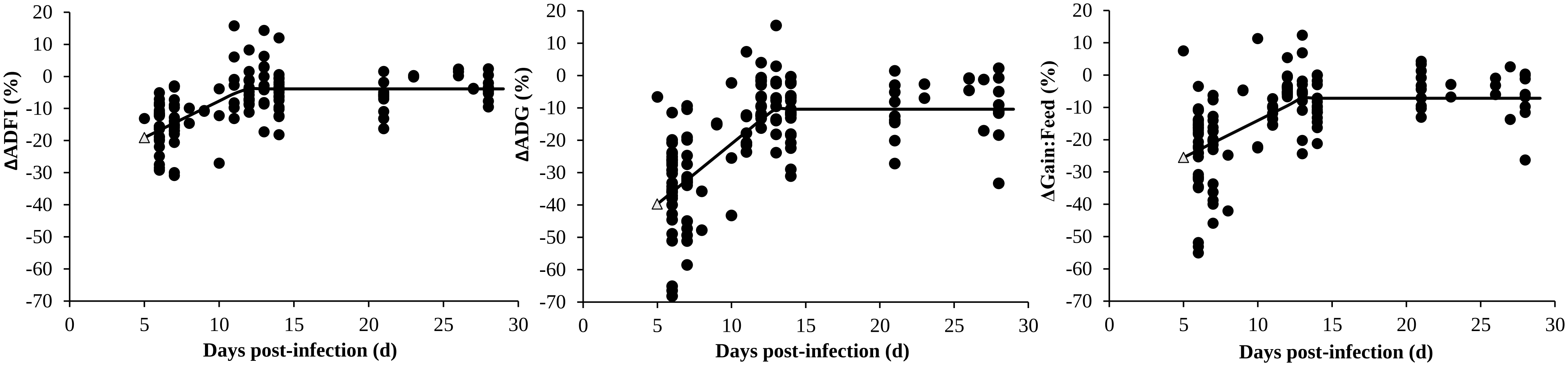

Figure 2. Dynamics between the variation of ADFI, ADG and Gain:Feed versus days post-infection in broilers challenged by coccidiosis compared to control treatments (unchallenged broilers). The points represent the data observed and the line shows the adjustment with a linear plateau in each case. The triangle represents the estimated maximum variation.

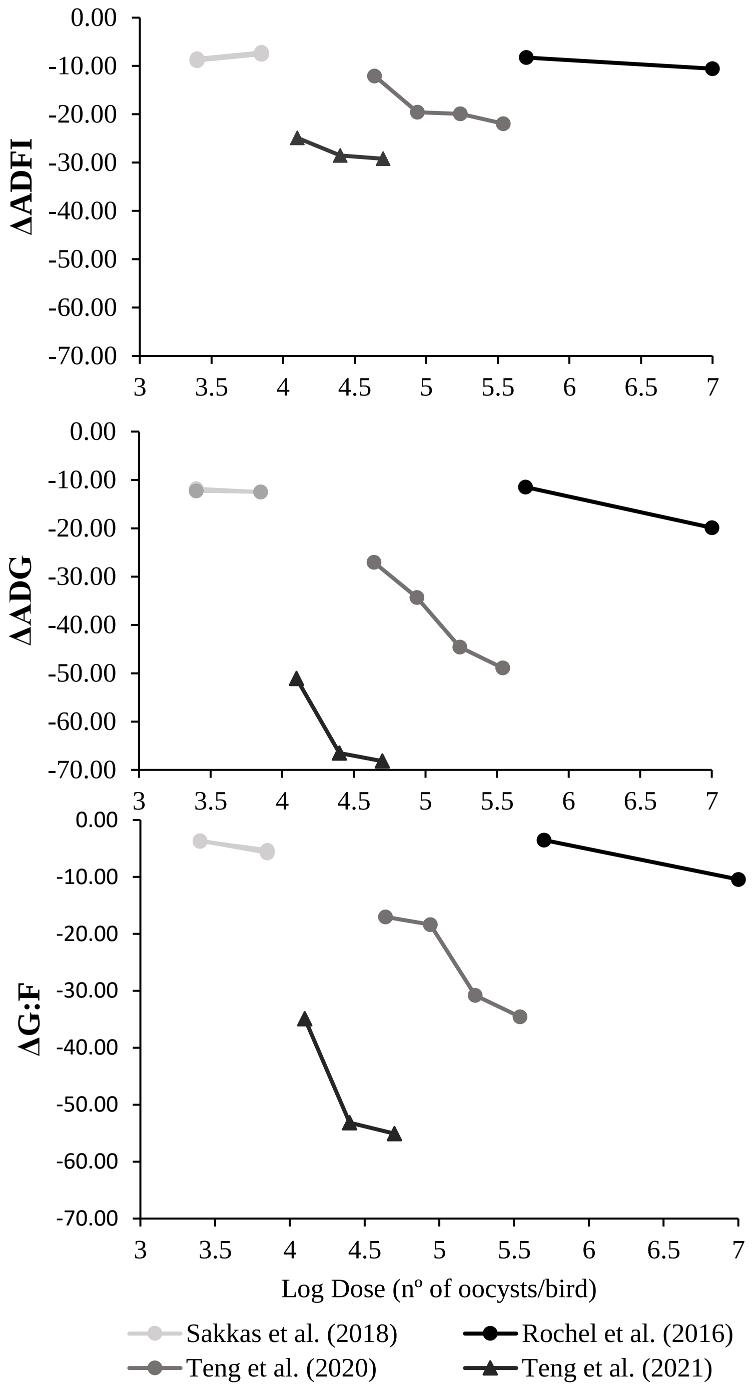

Figure 3. Effects of inoculum dose (log dose) of *Eimeria* spp. in the variation (Δ) of average daily feed intake (ADFI), average daily gain (ADG) and gain per feed (G:F).

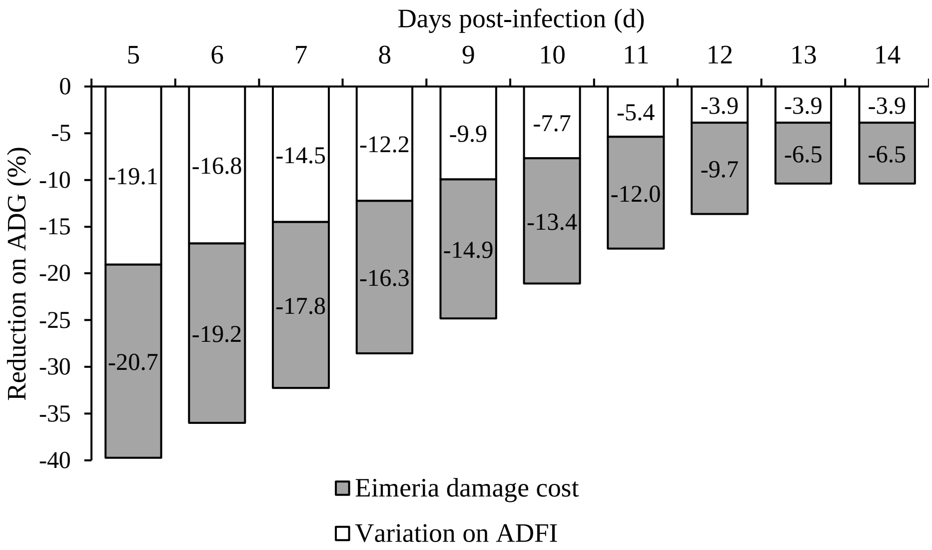

Figure 4. Division of the consequences caused by an *Eimeria* challenge in broiler chickens, which reduced the average daily weight gain (ADG). The variation is due to reduced average daily feed intake (ADFI) related to anorexia and to extra nutrition expenditure for broilers to repair damage done by *Eimeria* protozoa.

CAPÍTULO 3 – Eimeria maxima infection impacted the protein utilization of broiler chicks from 14 to 28 days of age

Este capítulo é apresentado segundo as normas da revista Animal

Eimeria maxima infection impacts the protein utilization of broiler chicks from 14 to 28 days of age

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Abstract

In floor-raised broilers, coccidiosis is responsible for reducing the use of nutrients, mainly by impairing intestinal tissue function and activating the immune system. Understanding and quantifying how balanced dietary protein (**BP**) is used when birds are challenged will allow nutritionists to make decisions regarding challenged flocks. This study aimed to determine the effects of *Eimeria maxima* on broiler performance and body composition, and to calculate changes in the maintenance and efficiency of protein utilisation (**Ep**). A total of 2,400 male 14-day-old Cobb500 broiler chickens were randomly allotted to ten groups with six replications of 40 birds each, with a 5 × 2 factorial arrangement of treatments. Five levels of BP in reference to digestible lysine (3.6, 7.2, 10.8,14.4, and 18.0 g/kg) were fed to unchallenged **(NCH**) and challenged (**CH**) broilers with 7x10^3 *E. maxima* sporulated oocysts from 14 to 28 days of age. Performance and body deposition were measured using a comparative slaughter technique to compare BP maintenance requirements and Ep. ANOVA followed by a posthoc test was performed to compare the effects of BP levels, challenge, and their interactions. A monomolecular model describing the responses of NCH and CH broilers to BP intake, maintenance, and maximum protein deposition was compared. There were significant interactions between body weight gain and digestible lysine intake among the factors studied. Infection had a negative impact on all variables analysed, proving the efficacy of the challenge. The maintenance did not differ between the CH and NCH groups. Increased levels of dietary BP did not recover the maximum protein deposition in CH broilers. *Eimeria maxima* significantly reduced Ep by a factor of 0.09 times on Ep compared to the control group. The Eimeria maxima challenge was responsible to modify the use of BP altering the body composition and impairing broilers performance.

Keywords: coccidiosis, health challenge, nutrient use, performance, protein partitioning

Implications

This study demonstrates how challenge with *Eimeria maxima* affects the performance of growing broilers, resulting in protein use, not by a change in protein maintenance, but by a reduction in the efficiency of protein utilisation and the maximum response to protein deposition. These results indicate that increasing the dietary balanced protein density in broilers challenged with *E. maxima* will not recover protein deposition compared to healthy broilers. We demonstrated that a reduction in protein utilisation is a consequence of the *E. maxima* infection, likely since the protozoa causes tissue damage and immune cost.

Introduction

One of the most common health challenges in broiler chicken production is coccidiosis, which is caused by *Eimeria* spp., particularly *E. acervulina, E. maxima,* and *E. tenella*. This infection is responsible for annual global losses of over £10.4 billion in the poultry industry, equivalent to £0.16/bird (Blake et al., 2020). These protozoa can cause lesions in gastrointestinal tissue, resulting in impaired nutrient utilisation, health, and compromised animal performance (Allen et al., 1998). Therefore, understanding how broilers infected with coccidiosis respond to nutrient intake may be helpful in understanding how the pathogen affects performance and how broilers incorporate these nutrients into the body. Conducting trials to investigate marginal nutrient responses allows for the quantification of changes in maintenance, net efficiency utiliation, and maximum response of the birds (Sandberg et al., 2007). Amino acids, particularly essential amino acids, are necessary for bird development because they cannot be produced via *de novo* synthesis. As poultry do not have a specific protein requirement, but rather a definite requirement for essential amino acids, feeds are formulated based on the ideal protein concept. Formulations based on the ideal protein concept reduce feed costs and nitrogen excretion due to the more efficient use of amino acids. Recent studies on protein nutrition have focused on balanced proteins (**BP**) which refers to the protein content of a diet with lysine as a reference amino acid. Other essential amino acids are kept constant with lysine

(Eits et al., 2005), according to ideal amino acid profiles, as proposed by Rostagno et al. (2017). Evaluating the use of BP by modern broilers and elucidating how they respond to different dietary BP levels comprise important pathways for optimising production systems (Azevedo et al., 2021). However, in poultry farms, several factors influence the response of broilers to a given feed, such as sex, age, environment, and health challenges (Gous, 2018). Additionally, the different statistical models applied to describe the obtained responses to various feeds may lead to different results (Siqueira et al., 2011).

Modifications in the utilisation and response of birds to proteins and amino acids may occur after a minor immune system challenge (Sandberg et al., 2007); however, contradictory results are available in the literature. Certain authors have reported that the nutritional requirements of challenged animals are higher either due to increased maintenance requirements or by reducing the efficiency of nutrient utilisation (Pastorelli et al., 2012; Remus et al., 2014; Moraes et al., 2019), which was observed in our preliminary results published in abstract form (Freitas et al., 2022). Other authors claim that a reduction in the maintenance and efficiency of nutrient use does not occur, reporting that challenged animals only reduced the maximum response, likely due to a decrease in feed intake (Webel et al., 1998). The main difference between these two concepts is the possibility of maintaining regular growth in challenged broilers by altering the nutrient density of the feed. Uncertainty regarding the effect of disease challenges on broiler metabolism does not help nutritionists make decisions regarding feed changes for infected flocks, highlighting the necessity for further study in this field.

Describing and interpreting the use of nutrients and energy and measuring their biological occurrence in birds can often be performed using mathematical models.

Therefore, this study aimed to determine the effects of a health challenge caused by *Eimeria maxima* on the performance and body composition of broiler chickens from 14 to 28 days of age and to determine the maintenance and efficiency of protein utilisation (**Ep**).

Material and methods

Experimental design and management

A total of 2,400 14-day-old male Cobb500 chicks were used from 14 to 28 days of age. Day-old chicks were purchased from a commercial hatchery and vaccinated against Marek 'sand Gumboro's disease. At 7 days of age, the birds were vaccinated against Newcastle disease using drinking water. Before the trial started (1 to 13 days of age), birds were housed in groups of 40 birds across 60 pens (dimensions 1.8 x 1.0 m), located in a conventional facility equipped with a nipple drinker, semi-automatic tubular feeder (25 kg), and a floor covered with 8 cm of fresh wood shavings. All birds had free access to water and feed, formulated to meet or exceed Rostagno et al. (2017) recommendations (12.77 MJ/kg ME and 228.9 g/kg of CP). The temperature was kept at 32 ºC during the first two days of ageDOA, using infrared lamps, and gradually reduced according to the recommendations of Cobb (2018) guidelines. The light program used was 24 h of light in the first week, and 18 h of light, and 6 h of dark in the following weeks.

At 14 days of age, birds were weighed and randomly distributed with similar initial weights (478 g ± 8 g) into ten treatments with six replicates of 40 birds each in a factorial design of 5 (dietary BP levels) x 2 (challenged or not), totalling 60 experimental units, with the respective pens comprising the experimental unit. Five experimental diets were formulated to contain increasing levels of dietary BP, using digestible lysine as the reference amino acid, and were provided to all birds. Water and food were provided *ad libitum* throughout the experiments. The temperature and humidity were measured daily using four thermo-hygrometers located at different points of the poultry house, obtaining the averages of maximum temperature (31.37 ± 2.19 °C), minimum temperature (22.39 ± 1.56 °C), maximum relative humidity (89.66 ± 5.71%), and minimum relative humidity (59.44 ± 8.92%). Mortality was checked daily and the weight of the deceased birds was recorded.

Dietary treatments

Experimental diets with different levels of dietary BP were prepared using the dilution technique (Fisher and Morris, 1973), mixing a low-BP diet with a high-BP diet (Table 1) in the proportions 100:0, 75:25, 50:50, 25:75, and 0:100, obtaining the values of 66.6, 133.2, 199.8, 266.8 and 333.0 g/kg of CP, in reference to digestible lysine (3.6; 7.2; 10.8; 14.4 and 18.0 g/kg), respectively. All diets were isoenergetic and maintained the ideal protein ratio proposed by Rostagno et al. (2017). After mixing, the feed samples were collected and analysed for CP (Kjeldahl, AOAC method 2001.11) and total amino acid content by HPLC. The digestible amino acids were calculated using the digestibility method proposed by AMINODat 5.0® (Wiltafsky et al., 2010).

Challenge model

To induce health challenges, birds were individually inoculated with *Eimeria maxima* (*E. maxima* BVD 4). The inoculum, acquired from Biovet Vaxxinova®, was diluted in phosphate-buffered saline at 7×10^3 sporulated oocysts/ml concentration and stored between 1-7 °C until use. Each broiler from the challenged (**CH**) treatment received 1 mL of the solution via gavage, using 5 ml syringes to aspirate the previously stirred inoculum coupled to probe N_o. 8, which was inserted directly into the crop of the birds at 14 days of age.

Data collection

Performance variables included initial BW at 14 days of age, final BW at 28 days of age, feed intake, digestible lysine intake (**iLys**), body weight gain (**BWG**), and feed conversion rate (**FCR**). Feed intake was corrected for mortality and calculated by subtracting the feed offered from the leftovers. iLys was calculated by multiplying the Feed intake with the analysed digestible lysine value. BWG was obtained by subtracting BW at 28 days of age from BW at 14 days of age, and FCR was obtained by dividing feed intake (adjusted for mortality) by BWG according to the methodology proposed by Sakomura and Rostagno (2016).

The protein retention efficiency rate (**PER**) was calculated by dividing BWG (g) by protein consumption (g), and the energy efficiency rate (**EER**) was calculated by multiplying BWG (g) by 100 and dividing the value by the intake of metabolizable energy. PER and EER were used as indicators of the efficiency of dietary protein and energy utilisation.

A comparative slaughter technique was used to determine the N and fat deposition in birds. On the first day of the experiment, 10 birds were fasted for 12 h, weighed, and euthanised. Samples of their feathers were manually removed and stored in paper bags for the determination of DM and N according to the AOAC methodology (methods 934.01 and 2001.11, respectively). The birds were then completely defeathered and weighed. The total weight of the feathers was calculated by subtraction. Carcasses were stored in plastic bags and frozen in a cold chamber (-10 ºC) for further processing.

All frozen carcasses were cut using a bandsaw and ground in an industrial mill (CAF 8 Inox; C.A.F. Maquinas, Rio Claro, SP, BR) to obtain homogeneous samples. Carcase subsamples (60 to 80 g) were collected from each bird, placed in disposable Petri dishes, and frozen in an ultra-freezer (-80 °C). The samples were analysed for DM, ether extract, and N content. DM was determined by freeze-drying for 72 h at -80 ºC and 10 atm (Edwards SuperModulo, Thermo Fisher Scientific, Waltham, MA). The nitrogen content of the carcase was quantified using the same methodology as feathers and then multiplied by a factor of 6.25 to obtain CP. The ether extract content was measured using an AnkonXT15 Extractor (ANKOM Technology, Macedon, NY, USA) with petroleum ether (AOAC method 920.39). At the end of the experiment (28 days of age), two birds per replicate with a weight close to the average (± 5%) were selected and analysed under the same processes mentioned above to measure the body components.

To obtain a balanced protein intake, feed samples from each treatment were analysed for nitrogen content using the Kjeldahl method (AOAC 2001.11) and multiplied by a factor of 6.25, thus obtaining the percentage of BP in the diet. Feed intake was then multiplied by dietary BP to estimate protein consumption, expressed as metabolic body weight (g/BW$^{0.75}$/d). The whole-body protein from 28 d-old broilers was subtracted from the whole-body protein of reference birds to calculate the total body protein deposition (g/BW$^{0.75}$/day). Body fat deposition (g/BW$^{0.75}$/day) was calculated using a similar process.

Estimation of maintenance requirement

The maintenance requirement for BP was estimated using a reparametrized monomolecular model, as suggested by Kuhi et al. (2019):

$$Y = Ymax * \left[1 - e^{-K*(X-Xm)}\right];\ \ X \geq 0 \qquad (1)$$

where Y is the deposition of protein (g/kg $BW^{0.75}$/d), Ymax is the maximum attainable value for Y (g/kg $BW^{0.75}$/d), K is the fractional rate parameter, X is the intake of BP (g/kg $BW^{0.75}$/d), and Xm is the protein intake when Y is equal to zero, that is, the amount of protein needed to meet the maintenance requirements.

Calculation of protein utilisation efficiency

To determine the Ep values, the calculations proposed by Kyriazakis and Emmans (1992a) and Emmans (1986) are as follows (Supplementary Material S1):

$$Ep = PR/[(FI * FCP * DCP * v) - 0.008 * Pm^{-0.27} * P\,](g/g) \qquad (2)$$

where PR is the protein deposition (g/d), FI is the feed intake (g/d), FCP is the protein content on feed (g/g), DCP is the crude protein digestibility (g/g), v is the ratio of ingested lysine to the concentration of lysine in the reference protein deposited, e.g., poultry body protein (Sandberg et al., 2007), 0.008 is the amount of ideal protein required per unit of maintenance (Emmans, 1986), Pm is the protein weight at maturity (kg) (considered as 1.56 kg for Cobb500 males, according to Vargas et al. (2020)), P is the average protein weight of the birds used in the experiment.
To determine the DCP coefficient, protein digestibility values for each ingredient (Rostagno et al., 2017) were considered. The total DCP value (%) was obtained by multiplying the digestible value with the ingredient concentration in the feed.

Statistical procedures

All data were evaluated for normality and homoscedasticity of errors using the Cramer–von Mises and Brown–Forsythe tests at the 5% significance level. Observed data that did not show normality in the errors and were log-linearised. Data were analysed using a Two-Way ANOVA test in the statistical software SAS version 9.4 (SAS Institute Inc., Cary, NC, USA), considering the effects of BP levels, the challenge by *E. maxima*, and their interactions, assuming significant effects when $P < 0.05$. Tukey's test was used to compare the means of interaction and levels at 5% probability (Supplementary Material S2).

Adjustments to the monomolecular model were performed using PROC NLMIXED from SAS, and the estimated parameters were compared using a t-test ($P < 0.05$; Supplementary material S2).

Results

Bird responses as a function of balanced protein levels and health challenge

There were significant interactions ($P < 0.05$) between dietary BP and CH for BW at 28 days of age, iLys, and BWG (Table 2). Birds in the unchallenged **(NCH)** and CH groups had the same response to the 66.6 g/kg dietary BP for all variables. However, for other dietary BP levels, the *E. maxima* challenge promotes a negative impact ($P < 0.05$). The level of 266.4 g/kg of dietary BP enables a higher BW at 28 days of age and BWG but was non significantly different ($P > 0.05$) from 333 g/kg of BP levels, followed by the levels of 199.8,133.2, and 66.6 g/kg of BP, respectively in both groups (NCH and CH). The increment of BP on the diet of CH birds of 266.4 g/kg of dietary BP resulted in a BWG and BW at 28 days of age of similar values ($P > 0.05$) to the birds in the NCH group receiving 199.8 g/kg of dietary BP. As the levels of dietary BP increased, a high amount of iLys was observed in both groups, with a higher intake at the lowest level.

There was no significant interaction ($P > 0.05$) between dietary BP levels and challenge for feed intake, FCR, PER, or EER; therefore, only the main effects were evaluated (Table 3). Broilers had the higher feed intake at 266.4 g/kg of BP followed by 333, 199.8, 133.2, and 66.6 g/kg of dietary BP, respectively. CH reduced ($P < 0.05$) the amount of feed intake. CH negatively impacted the FCR and EER, and the dietary BP levels of 266.4 and 333 g/kg promoted the best results. Birds receiving 133.2 g/kg of dietary BP levels had a higher ($P < 0.05$) value for PER, followed by 199.8 and 66.6 g/kg of BP. The increase in dietary BP levels of 266.4 to 333 g/kg of BP reduces the PER, and the CH reduces the PER of birds.

Protein intake, protein and fat deposition, and carcass composition at 28 days

There were no significant interactions ($P > 0.05$) between BP levels and CH for BP intake or protein and fat deposition (Table 3). As dietary BP levels increased, a high BP intake (g/kg$^{0.75}$/day) was observed in the treatments, promoting a difference ($P < 0.05$) between the levels showing the highest intake at 333 g/kg of dietary BP. However, protein deposition (g/kg$^{0.75}$/day) did not follow the same pattern as that of BP intake. The highest protein deposition occurred at the level of 266.4 g/kg of dietary BP, followed by levels 199.8 and 333 g/kg of dietary BP, and a reduced ($P < 0.05$) protein deposition was observed at 133.2, and 66.6 g/kg of dietary BP levels, respectively. For lipid deposition (g/kg$^{0.75}$/day), the first two levels (66.6 and 133.2 g/kg of dietary BP) had a higher deposition. As dietary BP levels increased, a significant reduction was observed at dietary BP levels of 199.8, 266.4, and 333 g/kg. The CH produced by *E. maxima* had a negative impact on all these variables.

Regarding carcass composition at 28 days of age, fat content showed an interaction between BP levels and CH (Table 2). The NCH group fed the lowest level of dietary BP (66.6 g/kg) showed a higher ($P < 0.05$) carcass lipid content, followed by the CH group fed the same level. In sequence, at 133.2 g/kg of BP, a reduction in lipid content was observed but was not different between groups. The increase in dietary BP level to 199.8 g/kg reduces the lipid at the carcass, and when compared to the groups (fed with 199.8 g/kg of BP), the CH reduces ($P < 0.05$) the lipid content compared to the NCH. With the same trade increasing the dietary BP level to 266.4

and 333 g/kg, a reduction was observed in lipid deposition; however, no differences were observed in each level when comparing groups. No interactions were observed between the treatments for water or protein content in the carcasses (Table 3). Body water content (g/kg) increased ($P < 0.05$) as the BP level in the feed increased, resulting in the highest value at 333 g/kg of dietary BP. The NCH had less water in the body than the CH group. For protein content (g/kg), birds fed with 199.8 and 266.4 g/kg of dietary BP levels showed a significatively higher value comparatively, followed by 333 g/kg, which did not differ from 266.4 g/kg ($P > 0.05$), and was followed by 133.2 and 66.6, in sequence.

Comparison of maintenance and maximum value of protein deposition of unchallenged and challenged groups

The coefficients estimated using the monomolecular equations for the CH and NCH groups are listed in Table 4. By comparing the estimates between groups, a difference ($P<0.05$) was observed only for the Ymax parameter, where the NCH group showed a higher value to Ymax (15.04 g of protein/$BW^{0.75}$/day) compared to the CH group (13.50 g of protein/$BW^{0.75}$/day). There were no significant differences ($P > 0.05$) between challenged groups for the parameters Xm and K. The estimated maintenance of BP was 7.53 g of protein/$BW^{0.75}$/day for broilers from 14 to 28 days of age.

Efficiency of balanced protein utilisation

No interactions ($P > 0.05$) were observed between BP levels and CH for Ep (Table 3). The Ep shows a better utilization ($P < 0.05$) at 133.2 and 199.8 g/kg of dietary BP, but no differences were observed for Ep between 266.4 and 133.2 g/kg. Increasing the dietary BP to 266.4 reduces the Ep compared to a dietary BP of 199.8 g/kg, and under a further increase to 333 g/kg, a lower Ep was observed when compared to 266.4, 199.8, and 133.2 g/kg of BP. No differences were observed in the Ep between the 66.6 and 333 g/kg dietary BP groups. The Ep estimated for CH broilers (0.56) was lower ($P < 0.05$) in comparison to the value calculated for the NCH group (0.61).

Discussion

Challenge by coccidiosis can be caused by one or more species of *Eimeria*, such as *E. maxima* which acts on the upper part of the small intestine and modifies the response of birds when challenged, mainly characterised by reduced performance. The reduction in voluntary feed intake is one of the most evident responses to infection caused by anorexia; however, *E. maxima* has a greater impact on BWG than other *Eimeria* species, resulting in a 10% drop in weight gain when their feed intake is kept constant (Kipper et al., 2013). Understanding and quantifying the relationship between this reduction in BWG, which is no longer responsive only to voluntary feed intake but also to the cost of the immune system (Sandberg et al., 2007), which may be attributed to the increase in maintenance costs and decrease in nutrient efficiency. Therefore, a wide range of dietary BP levels (a range of 66.6 g/kg of BP between each level) is necessary to observe the dose-response curve and to extrapolate the maintenance and maximum responses to dietary BP (Sakomura and Rostagno, 2016).

Bird's responses as a function of balanced protein levels and health challenge

Challenge with *E. maxima* at the dosage used negatively affected the feed intake, BW at 28 days of age, and FCR of the CH birds. These results were likely a consequence of damage to the middle of the small intestine (jejunum and ileum) (Kipper et al., 2013), which may have increased cytokine production. Consequently, cytokines may directly affect the central nervous system, increase leptin production by adipose tissue, cause anorexia, and increase energy expenditure (Johnson, 1998). These effects may explain the reduction in carcase fat deposition and lipid content observed in the CH group. A decrease in the voluntary feed intake and an increase in the mobilisation of nutrients and energy to activate the immune system and restore tissue damage can lead to a reduction in nutrient availability, thereby affecting broiler performance and body composition. According to our results, the reduction in voluntary feed intake was responsible for only a fraction of the decrease in BWG (2.5% of 7.6%), suggesting that other factors impair the growth of healthy CH broilers such as a reduction in nutrient digestibility (Kim et al., 2021), Ep, and an increase in maintenance requirements.

The interaction between health challenge and dietary BP on broilers responses may be due to the effect of *E. maxima* since it has been shown that the presence of this microorganism in the intestine contributes to the development of other pathogenic microorganisms, mainly *Clostridium perfringens* (Hauck, 2017). Therefore, the association between the health challenge and the increase in dietary protein may increase the rate of protein degradation, producing substrates such as amines and ammonia and increasing intestinal pH, which is favourable for the propagation of *C. perfringens*, negatively affecting the BWG and FCR of broilers (Hilliar et al., 2020; Adhikari et al., 2020). Comparing the daily weight gain between NCH and CH birds at different levels of CP using a meta-analytic approach, Remus et al. (2014) found that the reduction in daily weight gain was more pronounced when broilers were fed higher levels of dietary CP.

In the highest level of BP for both groups, BWG was reduced due to the high intake of protein and constancy of dietary energy, resulting in a lower dietary energy:protein ratio, altering the “protein-dependent” phase to an “energy-dependent” phase, resulting in poor performance (Gous et al., 2018). The increase in dietary BP was responsible for the reduction in body lipid content, which was related to the decrease in PER and the constancy of the EER. The energy:protein ratio may affect the efficiency of protein utilisation since increased protein requires more energy to be deposited, which may affect the amount of energy available for lipid deposition, thereby reducing BWG (Gous et al., 2018). A reduction in body fat and protein content in birds challenged with *Eimeria* spp. was also reported by Ott et al. (2018). The latter authors suggested that expenditure by the immune system is a likely reason for this reduction. However, some of this decrease is also linked to a lower voluntary feed intake, as suggested by Sandberg et al. (2007), reducing the intake of nutrients and energy.

The maintenance requirement, maximum response to balanced protein, and efficiency of protein utilisation

Previous studies have reported changes in maintenance values due to sanitation challenges. Pastorelli et al. (2012) performed a meta-analysis on pigs challenged with several microorganisms and reported that gastric parasites caused a slight increase in animal maintenance which was obtained by adjusting the response of the literature data to a first-degree linear regression. The latter study showed that challenged animals, with the same average daily intake as the unchallenged group, showed a reduction in average daily weight gain of 5.4% ± 1.2%, representing an increase in maintenance. Moraes et al. (2019) fractionated the variation in the growth of birds challenged with *Eimeria* spp. using the methodology reported by Pastorelli et al. (2012) by evaluating the effects of challenge on the variation in feed intake and weight gain. This change was found to be 86% responsive to an increase in animal maintenance; however, they assumed an increase in overall maintenance and did not focus directly on which nutrients or energy expenditure routes were predominant. In the literature, there is a lack of investigations which compare the change in the maintenance response to BP in challenged birds, the present study is one of the first studies which carried out this investigation. Thus, we can verify that the increase in maintenance reported in the literature is not caused by protein. Sandberg et al. (2007) reported that the cost of producing an immune response from challenged birds is between 0.57-1.2 g CP/kg BW/day. Nevertheless, in the present study, the maintenance value for CH birds (7.92 g CP/kg $BW^{0.75}$/day) (Table 4) had a slight increase of 0.78 g CP/kg $BW^{0.75}$/day or 0.94 g CP/kg BW/day, in relation to the maintenance estimated for the NCH group. Although not significant, these values were consistent with those reported by Sandberg et al. (2007).

The challenged birds showed a significant reduction in the maximum potential for protein deposition (Figure 1). Two factors can explain this reduction: the first is the reduction in voluntary feed intake compared to NCH birds, which is inherent to anorexia promoted by diseases, mainly parasitic diseases (Kyriazakis et al., 1998). Increasing the nutrient density of feed can overcome this issue. However, our study demonstrated that birds fed with higher levels of dietary BP achieved lower body weight gain than healthy broilers, leading to the second factor. We found that the Ep levels were reduced in health-challenged broilers. In this sense, even when providing higher levels of dietary BP, the CH birds never achieved the same maximum capacity to deposit protein because the Ep was reduced by an average of 9 percentage points, reducing performance and protein deposition.

Differences in maximum protein retention potential have been previously reported by other previous studies (Williams et al., 1997; Webel et al., 1998). This suggests that concentrating feed nutrients as a solution for reducing voluntary feed intake in CH birds is not viable because of their lower responsiveness. Thus, it is essential to note that concentrating feed to a certain level can increase bird performance, but the maximum response is expected to decrease. In this study, we verified that the addition of BP to the diet from 199.8-266.4 in CH birds resulted in protein deposition similar to the 199.8 g/kg level observed among NCH birds (Table 3). However, because the range of levels provided to the birds was too broad, this result was not investigated in detail. Predominantly, at a higher level of BP, birds reduce the intake and deposition of protein in the body, possibly due to a lack of energy to deposit tissue (Gous et al., 2018). According to these authors, a decrease in the response of birds consuming high levels of BP (or amino acids) occurs because the deposition of BP depends on the minimum amount of energy, and Ep is reduced with a reduction in the AMEn/kg DCP ratio. Similarly, Sandberg et al. (2007) suggested that a decrease in the plateau responses of challenged animals might occur because of an insufficient amount of energy in relation to protein.

The reduction in Ep in challenged birds explains the reduction in BWG and protein deposition in the body, being unresponsive to voluntary feed intake in such a way that it may be linked to a reduction in nutrient digestibility by coccidiosis, the use of proteins to repair damaged tissues, and exacerbated endogenous losses, thereby reducing the use of N. Few studies have been carried out to compare the nutrient partitions of protein in broiler chickens challenged by *Eimeria* spp., and further studies are needed to verify possible changes in the use of N and to determine its cost because it may differ according to the dosage used, the age of the birds, or due to the response of individuals, strains, and sexes (Sandberg et al., 2007).

Furthermore, this methodological approach can be extended to measure the impact of other nutrient, energy, and sanitary challenges on animal production.

Challenge with *E. maxima* ($7x10^3$ sporulated oocysts/ml) reduced broiler performance from 14-28 days of age. It showed different responses to dietary BP levels, modifying protein utilisation and body composition. This difference cannot be attributed to the change in maintenance because the comparison between the model parameters showed no difference between the groups, indicating that the reduction in consumption and in Ep were responsible for the decrease in the maximum responsiveness of the birds to balanced protein.

Ethics approval

This study was approved by the Animal Use Ethics Committee of the Faculty of Agricultural and Veterinary Sciences, São Paulo State University, Campus Jaboticabal (Process 14470/19).

Data and model availability statement

These data have not been deposited in the official repository. All data supporting the research findings were requested from the corresponding author.

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Declaration of interest

None.

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Table 1

Ingredients and nutrients composition, as fed basis, in the low and high balanced protein feeds used to mix and obtain the balanced protein levels offered to broilers at 14 to 28 days of age.

Ingredients	Low balanced protein[1] (g/kg)	High balanced protein (g/kg)
Corn, (79 g/kg CP)	57.8	289
Soybean meal, (450 g/kg CP)	87.7	438
Corn gluten (600 g/kg CP)	33.0	165
Soybean oil	63.5	55.6
Dicalcium phosphate	21.6	16.3
Limestone	7.86	8.83
Salt	4.95	3.57
Sodium bicarbonate	0.39	1.93
BioLys®	1.17	5.85
DL – Methionine (990 g/kg)	0.550	2.76
Premix Vitamin[2]	1.00	1.00
Premix Mineral[3]	1.00	1.00
Choline Chloride (600 g/kg)	0.700	0.700
Potassium Carbonate	13.7	0.000
Corn Starch	447	0.000
Inert[4]	40.0	0.000
Celite®	10.0	10.0
Rice Husk	88.4	0.000
Sugar	120	0.000
Nutritional composition		
Dry matter[5] (g/kg)	914	905
Metabolizable Energy, (MJ/kg)	13.1	13.1
Crude Protein, (g/kg)	66.6 (72.6)	333 (348)
Digestible Methionine, (g/kg)	1.6 (1.5)	8.1 (8.0)
Digestible Met + Cys, (g/kg)	2.7 (2.7)	13.3 (13.4)
Digestible Lysine, (g/kg)	3.6 (3.5)	18.0 (18.0)
Digestible Threonine, (g/kg)	2.4 (2.5)	12.0 (11.9)
Digestible Arginine, (g/kg)	3.9 (3.7)	19.3 (19.5)
Calcium, (g/kg)	8.5	8.5
Available phosphorus, (g/kg)	4.3	4.3

[1] Values within parentheses, total amino acids were analysed by HPLC and multiplied by digestibility factor provided by AMINOdat5.0®, the CP was evaluated by the AOAC method (2001.11).

[2] Content/kg of premix: Vitamin A = 11 000 000 IU; Vitamin D = 4 000 000 IU; Vitamin C = 55 000 IU; Vitamin K3 = 3 000 mg; Vitamin B1 = 2 300 mg; Vitamin B2 = 700 mg; Pantothenic acid = 12g; Vitamin B6 = 4 000 mg; Vitamin B12 = 25 000 µg; Nicotinic acid = 60 g; Folic acid = 2 000 mg; Biotin = 250 mg and Selenium = 300 mg.

[3] Content/kg of premix: Iron = 100 g; Cupper = 20 g; Manganese = 130 g; Zinc = 130 g and Iodine = 2 000 mg.

[4] Inert material comprised washed sand.

[5] Values analysed.

Table 2

Interaction effect of balanced protein levels and challenged by *E. maxima* (7×10^{3} oocyst/ml) on performance variables and fat composition of broilers at 14 to 28 days

	Factors											
BP levels (g/kg)	66.6		133.2		199.8		266.4		333.0		SEM	*P*-value
Challenge	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No		
Performance variables												
BW at 28 days of age (kg)	0.658^{g}	0.698^{g}	0.962^{f}	1.07^{e}	1.19^{d}	1.37^{bc}	1.33^{c}	1.54^{a}	1.31^{c}	1.47^{ab}	0.021	0.003
iLys (g)	3.77^{h}	4.07^{h}	8.82^{g}	9.60^{f}	14.2^{e}	16.1^{d}	19.2^{c}	22.4^{b}	23.2^{b}	26.6^{a}	0.161	<0.001
BWG (kg)	0.184^{f}	0.225^{f}	0.480^{e}	0.618^{d}	0.702^{d}	0.895^{bc}	0.842^{c}	1.07^{a}	0.822^{c}	0.996^{ab}	0.021	<0.001
Carcass composition												
Fat (g/kg, as is)	179^{b}	211^{a}	142^{c}	153^{c}	103^{e}	123^{d}	87.9^{ef}	99.1^{ef}	69.4^{g}	84.9^{fg}	3.55	0.043

Abbreviations: BP = balanced protein; iLys = intake of digestible lysine intake; BWG = body weight gain.

$^{a-b}$ Values within a row with different superscripts differ significantly at $P < 0.05$, as determined using Tukey's test.

Table 3

Performance variables, tissues deposition, carcass composition and protein efficiency utilization of broilers at 14 to 28 days in response to the main effects of balanced protein levels in reference to lysine digestible and challenged by *Eimeria maxima* (7×10^3 oocyst/ml)

	Factors											
	Balanced protein levels (g/kg)						Challenge			*P*-value		
	66.6	133.2	199.8	266.4	333.0	SEM	Yes	No	SEM	BP levels	CH	Interaction
Perfomance variables												
BW at 14 days of age (kg)	0.470	0.478	0.474	0.472	0.480	0.017	0.477	0.473	0.003	0.788	0.320	0.920
Feed intake (kg, as fed basis)	1.07^{d}	1.28^{c}	1.39^{ab}	1.44^{a}	1.35^{b}	0.015	1.24^{b}	1.38^{a}	0.010	<0.001	<0.001	0.213
FCR (g/g)	5.43^{d}	2.37^{c}	1.82^{b}	1.49^{a}	1.50^{a}	0.094	2.68^{a}	2.36^{b}	0.057	<0.001	<0.001	0.802
PER (%)	50.7^{bc}	59.1^{a}	51.4^{b}	46.9^{c}	36.3^{d}	1.17	46.1^{b}	51.6^{a}	0.703	<0.001	<0.001	0.344
EER (%)	5.99^{d}	13.6^{c}	17.8^{b}	21.7^{a}	21.4^{a}	0.345	15.1^{b}	17.1^{a}	0.208	<0.001	<0.001	0.208
BP intake (g/kg $BW^{0.75}$/day)	9.60^{e}	16.5^{d}	25.1^{c}	31.3^{b}	38.0^{a}	0.200	23.8^{b}	24.4^{a}	0.129	<0.001	<0.001	0.075
Tissues deposition												
Protein deposition (g/kg $BW^{0.75}$/day)	2.77^{d}	8.03^{c}	12.5^{b}	14.0^{a}	12.9^{b}	0.258	9.42^{b}	10.6^{a}	0.167	<0.001	<0.001	0.923
Fat deposition (g/kg $BW^{0.75}$/day)	10.1^{a}	9.68^{a}	8.06^{b}	6.97^{c}	5.05^{d}	0.288	6.78^{b}	9.16^{a}	0.182	<0.001	<0.001	0.434
Carcass composition (as is)												
Water (g/kg)	582^{d}	614^{c}	635^{b}	647^{b}	668^{a}	2.90	635^{a}	625^{b}	1.83	<0.001	0.001	0.764
Protein (g/kg)	151^{d}	162^{c}	183^{a}	180^{ab}	174^{b}	1.89	171	169	1.19	<0.001	0.235	0.772
Protein efficiency												
Ep	0.490^{c}	0.648^{ab}	0.705^{a}	0.628^{b}	0.460^{c}	0.014	0.558^{b}	0.613^{a}	0.010	<0.001	<0.001	0.130

Abbreviations: BP = balanced protein; FCR = feed conversion rate; PER = protein efficiency ratio; EER = energy efficiency rate; Ep = efficiency of protein utilisation; CH = challenge.

$^{a-b}$ Values within a row with different superscripts differ significantly at $P < 0.05$, as determined using Tukey's test.

Table 4

Parameters of the monomolecular model[1] estimated (±SE) to describe the response of broilers to balanced protein challenged or not by *E. maxima*

	Groups		
	Unchallenged	Challenged	*P*-value
Monomolecular parameters[2]			
Ymax	15.33[a] (±0.513)	13.50[b] (±0.513)	0.033
Xm	7.14 (±0.558)	7.92 (±0.458)	0.282
K	0.092 (±0.014)	0.111 (±0.015)	0.367
Monomolecular reparametrized[3]			
Ymax	15.04[a] (±0.462)	13.50[b] (±0.513)	0.001
Xm	7.53 (±0.362)		NA
K	0.101 (±0.011)		NA

Abbreviations: NA= not applicable.

[1] Monomolecular model: Y=Ymax*[1-e^(-K*(X-Xm))], where Y is protein accretion (g/BW$^{0.75}$/day), Ymax is the maximum value for Y, K is the fractional rate parameter, X is the balanced protein intake (g/BW$^{0.75}$/day) and Xm is the intake of protein when Y is equal to zero (maintenance) (g/BW$^{0.75}$/day).

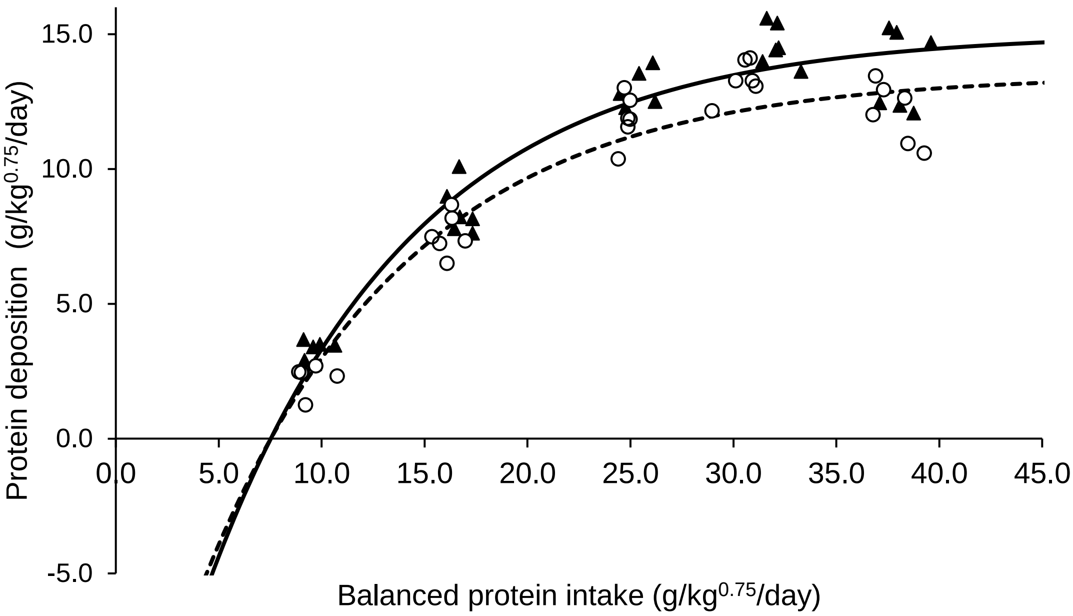
[2] Parameters estimated for each group were adjusted separately.

[3] Parameters were estimated fixing the parameters that did not have a difference between groups.

[a-b] Values within a row with different superscripts differ significantly at $P < 0.05$ by T test.

Figure captions

Fig. 1. Best-fit Monomolecular plots of protein accretion (g/kg BW$^{0.75}$/day) as a function of balanced protein intake (g/kg BW$^{0.75}$/day) for broilers unchallenged and challenged by *Eimeria maxima* at 14 to 28 days of age. Observed response of unchallenged (▲) and challenged (○) birds. Estimated response of no challenge (solid line) and challenge (dotted line).



CAPÍTULO 4 – Responses of broilers challenged by *Eimeria maxima* fed with different levels of dietary balanced protein

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Responses of broilers challenged by *Eimeria maxima* fed with different levels of dietary balanced protein

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Highlights

Eimeria maxima impair gut health and apparent ileal digestibility of some amino acids

High-balanced protein associated with *Eimeria maxima* worsened gut health and litter

High values of balanced protein worsened the litter quality and footpad dermatitis

Abstract

The study aimed to evaluate the responses of broiler chicks fed with different dietary BP levels infected or not by *Eimeria maxima* on gut health, amino acids and CP apparent ileal digestibility (**AID**), footpad dermatitis lesions, and litter quality. A total of 2,400 male 14-day-old Cobb500 broilers were randomly allotted into 10 treatments with six replication and 40 birds each, in a factorial design 5x2. The treatments consisted of five levels of dietary balanced protein (**BP**) in reference to digestible lysine (0.36%, 0.72%, 1.08%, 1.44%, and 1.80%) and broilers unchallenged (**NCH**) and challenged (**CH**) by *Eimeria maxima*. To perform the challenge, broilers in the CH group received 1 ml of *Eimeria maxima* inoculum in a concentration of $7x10^3$ sporulated oocysts/ml. Oocyst count in excreta (at six days post-infection (**DPI**)), presence of intestinal abnormalities score (at six DPI), morphology, and morphometrics of ileum (at six and 13 DPI) were determined to give a gut health status. In addition, amino acids and CP AID (at 14 DPI), litter quality (at 42 days old), and footpad dermatitis (at 14 DPI) were evaluated. An ANOVA and Kruskal-Wallis followed by a post-hoc test were performed to compare the effects of BP levels and challenge. Oocyst count was observed only in the CH group and increased with a higher amount of dietary BP level. The incidence of intestinal abnormalities was most observed in the CH group and increased with dietary BP level, but even NCH group shows abnormalities in the intestine in the two highest levels of BP. In general, the morphometrics (villus height, crypt depth, villus:crypt ratio) was impaired by the challenge, and the two highest levels of BP in the NCH group impact negatively these variables. Amino acids (Methionine, Methionine + Cystine, Arginine, and Serine) were reduced by *Eimeria maxima* challenged, but for CP was not different. The lowest dietary BP level reduces AID of all amino acids and CP. An increase in dietary BP levels results in poor litter quality and a high incidence of footpad dermatitis. The challenged and the increase in dietary BP worsened gut health, litter quality, and footpad dermatitis.

Keywords: apparent ileal digestibility, footpad dermatitis, immune parameters, gut health, litter quality.

Introduction

Coccidiosis is caused by protozoans of the genus *Eimeria* spp. and is one of the main diseases that affect poultry production, causing significant economic losses to the sector (Blake et al., 2020). Subclinical infection is considered the most problematic, as it often goes unnoticed resulting in impaired performance (Kipper et al., 2013). Due to its parasitic characteristic of intracellular action, *Eimeria spp.* can damage the intestinal epithelial cells, promotes lesions, reduces nutrient utilization, and can be a gateway to new infections, therefore reducing the intestinal health of birds (Santos et al., 2020). The protozoan is responsible to act directly on the enterocytes promoting cell disruption, reducing the capacity of the tissue to absorb nutrients and water and reducing the expression of amino acids (**AA**) transporters located at the brush edge worsening the feed conversion rate (Adedokun et al., 2016). For broilers, the most known Eimeria species are *E. acervulina*, *E. maxima*, *E. tenella*, *E. necatrix*, *E. mavatti*, *E. mitis*, *E. praecox, E. brunetti* (Collier et al., 2008). *Eimeria maxima* are generally known to cause a severe impact on broilers' performance compared to the others (Kipper et al., 2013) because this protozoan is mainly located in the jejunum and ileum, where most dietary nutrients are digested and absorbed. In addition, infected broilers will present a reduced feed intake (anorexic state) while part of the nutrients will be allocated for activation of the immune system to prevent disease propagation and ultimately dropping performance (Sandberg et a., 2007).

Dietary protein is an expensive nutrient in broiler feeds. To optimize the utilization of dietary protein and reduce its inclusion in the feed, nutritionists have to formulate diets based on the ideal protein concept. In this concept, the dietary protein has an amino acid profile to meet the broiler's requirement precisely and each of the AA in the feed is related to the requirement of lysine. However, not all of them can be produced via *de novo* synthesis and must be supplied to the diet (Mitchell, 1964). In this way, the concept of balanced protein (BP) has been used when formulating feeds and meeting nutritional requirements.

The nutritional requirements for broilers have been studied and updated for many years (Rostagno et al., 2017; Cobb 2022). However, some stress factors can modify the use of nutrients and expenditure; thus limiting the capacity of broilers to achieve their maximum growth potential. The health challenge is one of those factors, which increases the costs of nutrients and energy to produce response proteins, such as cytokines and acute phases proteins (Li et al., 2007). There are some concerns about increasing BP in diets of broilers challenged by coccidiosis that need to be evaluated not only by performance responses but also by evaluating the consequences at the intestinal level, considering the aspects to the animal health and welfare. Increasing protein levels could be associated with a great source of nutrients for the development of pathogenic bacterias, such as *Clostridium perfringens* (Hilliar et al., 2020). Feeding diets with a high crude protein content are associated with excess N excretion, which can lead to increased ammonia production in the environment and worsened litter quality (Van Harn et al., 2019). In addition, the challenge by coccidiosis can be associated to the reduction of litter quality, due to the lower use of nutrients, excreting them to the environment during the infection phase.

In this context, the present study was designed to evaluate the response of broilers challenged by *Eimeria maxima* fed with different levels of dietary balanced protein on gut health, apparent ileal digestibility, footpad dermatitis lesions, and litter quality.

Material and Methods

All activities were approved by the Ethics Committee on the Use of Animals, located at the Faculty of Agricultural and Veterinary Sciences, São Paulo State University – Campus Jaboticabal, under process number 14470/19.

Experimental design and dietary treatments

A total of 2,400 14-day-old male Cobb500 broilers were used from 14 to 28 days of age. Day-old chicks were purchased from a commercial hatchery vaccinated against Marek and Gumboro diseases. At seven days of age, birds were vaccinated against Newcastle disease *via* water. The chicks were housed in a conventional facility and raised in similar conditions, distributed in 60 pens (size 1.0 x 1.8 m) in groups of 40 birds each on the first day. Each pen was equipped with a semi-automatic tubular feeder (25kg), nipple drinker, and wood shavings used as litter material. Before the experimental period, the birds were fed a basal diet (3,050 Kcal EM/kg and 22.89% of CP) formulated to meet or exceed the requirements for the period as proposed by Rostagno et al. (2017). The temperature was maintained at 32ºC during the first two days, using infra-red lamps, and then reduced following the recommendations of Cobb guidelines (2022). The light program adopted was 24 hours of light in the first week and reduced to 18 hours of light and six hours of dark until the end of the experiment.

At 14 days of age, birds were weighed and randomly distributed with similar initial weight (478g ±8g) into ten groups with six replicates of 40 birds each in a factorial design 5 x 2. The treatments consisted of five levels of BP offered to birds unchallenged (NCH) or challenged (CH) by *Eimeria maxima*. CH birds were situated on the opposite side of the house to avoid contact with NCH birds, sanitary measures were taken to avoid cross-contamination, and daily management was performed from the NCH group to the CH group. The temperature and humidity were recorded daily throughout the experimental period using four thermo-hygrometers located at different points in the facility, obtaining the maximum temperature (31.37 ± 2.19 ºC), minimum (22.39 ± 1.56 ºC), and the maximum (89.66 ± 5.71 %), and minimum (59.44 ± 8.92 %) relative humidity.

Experimental diets were formulated using dilution technique (Fisher and Morris, 1973) by mixing a low BP diet (6.66% of BP) and a high protein BP diet (33.3% of BP) (Table 1) in the following proportions, 100:0; 75:25; 50:50; 25:75; 0:100, obtaining the values of 6.66%, 13.32%, 19.98%, 26.68% and 33.30% of dietary BP, in reference to digestible lysine (0.36%, 0.72%, 1.08%, 1.44% and 1.80%, respectively). The diets were isoenergetic and maintained the ideal ratio, as proposed by Rostagno et al. (2017). Representative samples of each diet were taken and analyzed for CP content (Kjeldahl, AOAC method 2001.11) and the amounts of total AA were determined by high-density liquid chromatography, and corrected for digestible values by multiplying the digestibility coefficients in AMINODat5.0®. After the experimental period, the birds received a common basal feed (3,170 Kcal EM/kg and 19% of CP) until 42-old-days.

Eimeria maxima challenge model

To induce the challenge, each bird situated in the CH group was individually inoculated with 1 ml of inoculum (*Eimeria maxima* BVD 4 in a concentration of $7x10^3$ sporulated oocysts/ml diluted in phosphate-buffered-saline solution). The inoculation was performed by oral gavage using a 5ml syringe attached to the probe that was introduced into the birds' crop at 14 days of age.

Oocyst count and presence of modifications in the small intestine score

At six days post-infection (DPI), the excreta were sampled on different points on top of the litter of each pen. The samples were stored in plastic bags for further oocyst count analysis in the Laboratory of Parasitic Diseases (LabEPar, São Paulo State University). The samples were weighed and homogenized, then a subsample (2g) was taken to quantify oocysts per gram of feces (OPG) according to the methodology of Gordon and Withlock (1939). On the same day, two birds per replication were randomly selected and euthanized to verify and determine intestinal health modifications. The middle intestine was evaluated using scores varying of from 0 to 3 by visual measurement according to the degree of severity, being 0 = normal, 1 = slight redness of the intestine, 2 = small petechiaes, red intestine, 3 = incidence of visible gross lesions.

Histology and morphometric analysis of the ileum

Three birds with a weight close to the mean (±5%) were selected and euthanized to evaluate ileum villus height, crypt depth, villus crypt ratio (V:C), and goblet cell (GC) count at 20 and 27 days of age. A proximal ileal sample of 5 cm (from Meckel’s diverticulum to the ileocecal junction) of each pen was taken and treated according to the procedure of Luquetti et al. (2012). Tissues sample of 5 µm were prepared and stained with hematoxylin-eosin according to Behmer (1976). Six sections from distinct regions of the same segment were arranged on each slide, enabling the observation of large amounts of villi suitable for analysis and providing the evaluation of a large part of the extension of the segment. For each intestinal portion, 30 villi and crypts were randomly evaluated. To calculate the V:C the values obtained for villus were divided by the crypt values. The sections were photographed at 50 to 100x zoom by a digital camera system coupled to a binocular imaging microscope and analyzed using Image-J software (Abràmoff et al., 2004).

For GC count, PAS staining was obtained from the dewaxing and rehydration of the sections, then they were incubated in 0.5% periodic acid for 15 minutes, washed, and incubated with Schiff's reagent for 30 minutes. The number of GC (PAS+) was determined by pictures with 50x objective lens along 200 µm in the middle part of the villus, in a total of 10 villi per sample (Torres et al., 2013).

Immunity parameters and FITC-dextran

T lymphocyte counts (CD4+ and CD8+) and FITC-dextran were analyzed by collecting blood from the jugular vein of a bird per pen with a weight close to average (±5%) at 20 days of age. Samples were kept in an insulated foam container with an ice pack until processing. T lymphocyte count (CD4+ helper T lymphocyte and CD8+ cytotoxic T lymphocyte) was performed by flow cytometry according to the methodology proposed by Stabel et al. (2000). FITC-dextran amount in the serum was measured according to the methodology proposed by Vicuña et al. (2015).

Apparent ileal digestibility (AID) of AA and crude protein

To measure the apparent ileal digestibility of AA and crude protein, 1% of Celite® (silica) was added to the diet of the birds (Sakomura and Rostagno, 2018) as an indigestible marker from the first day of the experiment. Feed analyzes were performed for dry matter (AOAC 920.39), ash (AOAC 942.05), acid ash (Silva and Queiroz, 2002), crude protein (AOAC 2001.11), and amino acid analysis by HPLC.

At 28 days of age, five birds per pen were randomly selected, euthanized, and immediately performed an abdominal incision exposing the ileum (from Meckel's diverticulum to the ileocecal junction). To collect ileal content, a plastic syringe (30 ml) was attached to the anterior part of the fractionated segment and deionized water was applied until the entire content was poured into a previously identified plastic pot. The collected samples were homogenized, stored in a freezer at -20°C, and lyophilized for 72 hours at -80°C and 10 ATM (Edwards SuperModulo, Thermo Fisher Scientific, Waltham, MA). Then, each sample was ground in a micro mill and subjected to the same analysis as the diets.

To determine the apparent digestibility coefficients (CDIa) the formula was used (Ravindran et al., 2014):

$$AID(\%) = \left(\frac{\frac{AA_{diet}(\%)}{CIA_{diet}(\%)} - \frac{AA_{digest}(\%)}{CIA_{digest}(\%)}}{\frac{AA_{diet}(\%)}{CIA_{diet}(\%)}} \right) \times 100$$

Where AID is the ileal apparent digestibility coefficient, AAdiet is the percentage of amino acid in the diet, AAdigest is the percentage of amino acid in the digest, CIAdiet is the percentage of acid ash in the diet, CIAdigest is the percentage of acid ash in the digest, all the values based on dry matter and to determine the digestibility of CP, the values of AA were replaced by the values of CP analyzed.

Footpad dermatitis and litter quality

Three birds, whose weight represented the average BW per pen, were selected and their feet were analyzed by visual assessment for the presence and degree of a foot injury, following the suggested methodology by Welfare Quality® (2009) at 28 days of age. Thus, scores from 0 (absence) to 4 (severe injuries to the paws and fingers) were considered to qualify the injuries.

At 42 days, litter samples were collected from different places in each pen, avoiding collection at points such as under-feeders and drinkers. Samples were previously weighed (±300g), processed, and analyzed for dry matter (AOAC 920.39), and CP content (AOAC 2001.11).

Statistical analysis

All data were checked for normality and homoscedasticity of errors by the Cramer-Von Mises and Brown and Forsythe tests at 5%, respectively. Data did not show normality in the errors it was log-linearized. A two-way ANOVA was performed using the GLM procedure of SAS (2004), considering the effect of BP level, challenge, and interactions. Tukey's test was used to compare the means considering a significant effect at 5%, and a tendency at 10% of probability.

Scores and oocyst count were evaluated by a one-way nonparametric analysis. For calculating the difference between treatments, the Kruskall-Wallis ranked test for unpaired observations was used, and for post-hoc calculation of pairwise difference between two different levels the Bonferroni two-sample test was used.

Results

Oocyst count and lesions score of Eimeria maxima

The NCH birds did not present oocysts in their excreta (data not shown), thus, only the effect of dietary BP levels within the challenged group was compared (Figure 1). Birds fed with the lowest BP level (6.66% of BP) tended to release a small (P = 0.083) amount of oocyst compared to the highest BP level group (33.30% of BP).

For the intestine health status score (Figure 2), a difference (P<0.05) was observed between the groups CH and NCH, with a high score's incidence in the CH group. For the BP levels a difference was observed between the levels 6.66 and 13.32% from 26.64% and 33.30%, with the high incidence of scores at the highest levels.

Histology and morphometric analysis of the ileum

An interaction was observed for crypt depth and V:C at 20 days of age (six DPI), and for villus length and V:C at 27 days of age (13 DPI) (Table 2). Evaluating the crypt depth at six DPI, lower values were observed in NCH group in broilers fed with 6.66%, 13.32%, and 19.98% of BP dietary levels, and increasing BP levels to 26.64% and 33.3% resulted in a higher crypt depth. CH group had the higher values of crypt depth, except in the first BP level, which differs from others in the same group. For V:C at 20 days of age, increasing dietary BP results in a lower V:C in CH group, being the lower relation at 33.3% of BP, differing (P<0.05) from 6.66% and 13.32% of dietary BP. The NCH group had higher V:C compared to CH, and increasing dietary BP at 26.64% and 33.3% promote a reduction in the V:C (P<0.05) compared to the other levels in the same group.

Increasing dietary BP levels results in a lower V:C relation at 27 days for both groups, and CH reduces the relation for most levels (P<0.05) (except for 19.98 and 33.30%). The villus length at 27 days of age was reduced only in the higher two levels of BP in the CH group and at 33.30% level of BP in the NCH group. When comparing the groups in each level, only at the level of 26.64% of dietary BP the CH reduce the villus length. The number of GC at 20 days of age had a difference only by the main effect of CH, reducing the number (P<0.05) when birds were challenged by *Eimeria maxima*. However, at 27 days of age, no difference was observed for GC. For villus height at 20 days of age, the dietary levels of 13.32%, and 19.98% of BP had the highest height followed by the 6.66% and 26.64% of BP and the lower value was in the higher level. The CH was responsible to reduce (P<0.05) villus height of the birds at 20 days of age.

For crypt depth at 27 days of age with the increase of dietary BP levels a higher depth was observed, showing the lowest value at 6.66% of BP and the higher at 33.3% of BP, and in general, the CH increase the depth of crypt.

Immunity parameters and FITC-dextran

The blood lymphocyte cell count (CD4+, CD8+, and CD4+:CD8+ relation) at 20 days of age (6 DPI) didn't change between BP levels and challenge (Table 3). No interaction (P>0.05) was observed for FITC-dextran analysis, although, a significant (P<0.05) effect was observed for challenge and BP levels factors (Table 3). In general, CH was responsible to increase the amount of FITC-dextran in serum, and the dietary BP levels of 26.64% and 33.3% resulted in a reduced value of FITC-dextran compared to the other levels (P<0.05).

Apparent ileal digestibility (AID) of AA and crude protein

There was an interaction (P<0.05) for BP levels and challenge for AID of the essential AA threonine (**Thr**), arginine (**Arg**), isoleucine (**Ile**), valine (**Val**), phenylalanine (**Phe**) (Table 4) and to the non-essential AA serine (**Ser**), proline (**Pro**), and alanine (**Ala**) (Table 5). Interaction tendency (P<0.10) was observed in lysine (**Lys**), and glutamate (**Glu**). For all these AA, the interaction has resulted from a reduction in AID of the NCH group receiving 26.64% of dietary BP followed by an increase in the last level, being a change in the AID behavior. In general, the lowest BP level (6.66% of BP) results in AA AID reduction, and for Arg, Ile and Glu, the *Eimeria maxima* challenge reduces the AID for birds in this level compared to the NCH group. The other levels did not differ (P>0.05) for AID.

The main effect of BP level was significantly for methionine (**Met**), cysteine (**Cys**), methionine + cysteine (**Met + Cys**), leucine (**Leu**), histidine (**His**), glycine (**Gly**), aspartate (**Asp**), and CP. The difference (P<0.05) for Met, Cys, and Gly was only between the first BP level to the others. For CP the AID of 13.32% of BP was lower than 19.98% and 33.3% of dietary BP. Challenge main effect was significatively only for Met with a reduction in CH group.

Footpad dermatitis and litter quality

A difference (P<0.05) between the dietary BP levels of 6.66% from the others was observed in footpad dermatitis scores (Figure 3), and it was possible to observe high incidence of greater lesions as the BP levels increase (scores between 3 and 4). No significant differences were observed between the challenge factor (data not show).

There was no significant interaction of factors for any of the characteristics evaluated for litter quality (Table 6). For the litter DM the main effects of dietary BP levels and *Eimeria maxima* challenged were significant. The increment of BP levels resulted in a reduction in DM, which means a higher humidity on the litter, being the higher DM at 6.66% and the lowest at 33.30%. The CH was responsible to reduce the DM on litter. For litter CP, only the dietary BP levels were significant, showing a similar CP content in the three first levels (6.66%, 13.32, and 19.98%), and an increase in the levels 26.64% and 33.30%, where the highest level results in a higher litter CP.

Discussion

The challenge model applied was effective, as we expected, being a moderate challenge (dosage of $7x10^3$ oocyst/ml) with an average performance reduction of 11.4% in body weight, 10.1% in feed intake, and an increase of 11.2% in feed conversion rate in challenged broilers (de Freitas et al., *in process*) and not different in mortality (data not show). Analysis of shedding of *Eimeria maxima* oocysts at 20 days of age proves again the challenge efficacy, resulting in oocysts release in all BP levels only in the CH group. In addition, a tendency effect (P = 0.08) was observed for dietary

BP levels resulting in a greater number of oocysts per gram in the highest level (33.30% of BP) compared to the first level (6.66% of BP), indicating an aggravation as dietary BP increases. Corroborating with our findings, a paper published in ’70s by Sharma et al. (1973) testing the effect of different levels of dietary CP on the impact of coccidiosis (promoted by *Eimeria acervulina*), observed a greater oocyst release in the highest level of CP (24% of CP) compared to 20% and 16% of CP. A possible explanation for that is because the greater amount of dietary CP results in higher production of trypsin by the host and it will act directly on the excitation of the oocysts, having greater degradation of the oocyst wall and greater release (Britton et al., 1964). Nonetheless, this response was not always observed, where in a recent study, Taylor et al. (2022) found a greater oocyst release of *Eimeria maxima* in diets with low BP diets, showing an increase of seven times in oocyst shedding in broilers fed with a diet of 12% dietary BP compared to 20% of BP. The authors report that this is possibly due to a lower immune system response, which should be limited due to the smaller amount of nutrients consumed by the challenged birds, reducing their defense capacity. In this way, the controversial results obtained in the literature have to be more investigated to get more precise responses about the dietary protein impact on the oocysts release in broilers.

The presence of inflammation and lesions on the upper intestine observed visually at necropsy could be used as a good inference to disorders in gut health and could be combined with the results of microscopic analysis, such as morphometric and morphologic analysis, to give a gut health status. It was remarkably a high incidence of abnormal characteristics (score 1 and 2) in the CH group compared to NCH, probably due to the *Eimeria maxima* challenge which can promote cell disruption and lesions. The high values of dietary BP levels were also responsible to produce these undesirable characteristics, an example is that high content of BP in the diet is a cofactor to promote dysbiosis because a high amount of CP increases the rate of protein degradation, and the production of substrates such as amines and ammonia, increasing the pH of the gut, resulting in a favorable condition for the propagation of *Clostridium perfringens* and other pathogenic bacterias, generating dysbiosis (Hilliar et al., 2020). At six DPI, an inference could be maybe with the frequency of score 1 and 2 (Figure 2) and the V:C on the same day in the CH group (Table 2). It was observed, that as the dietary BP levels increased a high frequency of score 1 and 2 were evidenced, and in the same way, a reduction in the V:C, which could be related to poor quality of gut health. For the NCH group, a few incidence of score 1 was observed, and these scores were observed at the last two dietary BP levels (26.64% and 33.30%) (data not show), and likewise a lower V:C at 20d compared to the other levels (6.66%, 13.32%, and 19.98%) showing a negative impact of high values of dietary BP in the intestine health status score.

The challenge by *Eimeria maxima* in general was responsible for worsening the gut health characteristics at six and 13 DPI. For both periods evaluated, there was a reduction in villus length and V:C which is related to reduced absorptive capacity and enzymatic actions (Amerah and Ravindran, 2015, Yu et al., 2021), also the CH provide an increase in crypt depth, a process caused by the increase in epithelial turnover, where the organism itself performs it to renew epithelial cells, providing greater expenditure of nutrients for the renewal of the epithelium, related to the increase in the requirement of maintenance (Van Nevel et al., 2005). In the critical period of infection (six DPI), the number of globular cells was reduced, cells that are responsible for the secretion of mucin 2, which is the largest component of mucus, which is responsible for creating the epithelial barrier protecting against pathogens and is important for bird gut health (Reynolds et al., 2020). In such a way, the challenge reduced the intestinal health of the birds, as inflammatory lesions reduce the number of globular cells, producing the mucus production layer, which can increase the chances of other infections, bacterial translocation, and inflammation in the intestine (Gharib-Naseri et al., 2020).

The impact of balanced protein levels on ileum morphology and morphometrics with a huge range as the present study was never investigated. Some authors (Ding et al., 2016 and Ndazigaruye et al., 2019) studied the impact of the reduction of BP in the ileum morphometrics and observed different results, not observing a difference in the variables when reduced only 2% of BP (Ndazigaruye et al., 2019) and found difference only for villus height decreasing with the reduction of dietary BP in 2% (Ding et al., 2016). Different levels of dietary BP are related to different amounts of protein substrates in the intestine, and as commented above, high values of crude protein could result in high values of undigested protein and unabsorbed endogenous protein that will be probably fermented by the microbiota in the intestinal site producing ammonia, amines, indoles and phenols, gases (methane, hydrogen and carbon dioxide) in excess resulting in worse gut health, low performance and high mortality (Qaisrani et al., 2015). Besides, the high production and metabolism of nitrogenous waste products (such as uric acids and ammonia) cause additional energy losses in the body (Qaisrani et al., 2015). In another way, according to Laudadio et al. (2012), low CP levels compared to a standard protein level diet promote good development of intestinal villi, to increase the efficiency of digestion and absorption, which explain the great values for villi height at dietary BP level of 13.32% and 19.98% in both ages evaluated. However, the use of 6.66% of BP level reduces villi height probably due to a lack of nutrients compromising ileum development.

T lymphocytes (CD4+, CD8+) are cells of cellular immunity responsible for establishing a response to *Eimeria* spp. challenge and rapidly enter the intestine after an infection (Rothwell et al., 1995). The CD4+ (T helper) induces an immune response (infection) while CD8+ (cytotoxic T) acts directly on infected cells as an effector system, easy to be observed and quantified in the lamina propria (Mtshali et al., 2020). A variation in the number of some subtype lymphocytes in the gut occurs simultaneously with an opposed variation in the blood easily detected by flow cytometry (Bessay et al., 1996), which was expected to be evaluated in the present trial, however, no differences were observed for all treatments.

When evaluating the FITc-dextran parameter, which was used as an indicator for the assessment of intestinal paracellular permeability, a significant effect of dietary BP levels and the challenge was observed. The action of Eimeria spp. rapidly impacting intestinal permeability is already known, and its damage to the intestinal epithelium begins at three DPI, but with more aggravating lesions on six (for low dosages) DPI (Teng et al., 2021). Thus, cell disruption allows greater intestinal permeability, which was observed in the present study, corroborating with Teng et al., (2021) who showed the greater passage of FITC-dextran in the blood of birds challenged by *Eimeria maxima*. When evaluating the effect of dietary BP levels on FITC-dextran flow, it was observed that lower levels of BP resulted in greater flow of the marker in the serum (6.66%, 13.32, and 19.98%), and in the two highest dietary BP levels (26.64% and 33.30%) a low quantity of the marker in the serum was analyzed. Some studies already verify the effect of different dietary crude protein levels in permeability tests for broilers (Chen et al., 2016 (dual-sugar permeability) and Barekatain et al., 2019 (FITC-d)). In both, low levels of protein result in a high concentration of the indicator in blood compared to high levels as we observed in the present study. According to Barekatain et al. (2019), a possible explanation for that is probably due to an upregulation of the SGLT1 gene at lower levels of protein, which causes it to open tight junctions for the transfer of small molecules and peptides (Arrieta et al., 2006), however, they could not prove that upregulation could account for the passage of molecular size as big as 4,000 MW in the case of FITC-d, need further studies to prove.

A consequence of poor intestinal quality is the reduction of nutrient digestibility, such as amino acids. In a meta-analysis carried out by Kim et al. (2022), it was proved a negative effect of coccidiosis (*Eimeria* spp. mix) in all amino acids AID (except tryptophan, which was not evaluated), reducing the digestibility. In the present study, we did not find similar data at 14 DPI (28 days of age), where only the essential amino acids Met; Met + Cys; Arg, and the non-essential amino acids Ser, and Gly were reduced due to the challenge. A curious fact to note is that the amino acids that we found a difference in digestibility (Met, Met + Cys, and Arg), are the first three most impacted in challenges by coccidiosis according to the authors (Kim et al., 2022). These amino acids are related to the body's defense processes, such as endogenous antioxidants (sulfurized AA) fighting cellular oxidation and the production of nitric oxide (Arg). Similar results have already been found in other studies, such as those by Adedokun et al., (2016) and Rochel et al., (2016) where the authors found a reduction in the ileal digestibility of Met and Met + Cys when broilers were challenged by coccidiosis. According to Bortoluzzi et al. (2020), the amino acids Ser, Thr, and Pro are constituents of mucin, which is produced in greater amounts when birds are challenged by coccidiosis by globular cells, thus inferring a greater endogenous loss of these amino acids used for their synthesis, which leads us to a smaller AID (in the case of Ser). The effect of BP level was mainly caused at 6.66% of BP level, as could be related to poor development of the gut, already commented above, resulting in low AID for amino acids.

For litter quality evaluated at 42 days of age, a worsened response was evaluated as the dietary BP levels were increased, since the CP content in the litter material was higher in the 26.64% and 33.30 % of dietary BP levels, and did not show a difference between groups (CH and NCH). Nagaraj et al., (2007), evaluating different levels of CP in the diet, found a high level of protein in litter material with a higher content of N excreted when increased the dietary CP, corroborating the present study. When evaluating the DM, we obtained a difference for the challenge that resulted in a lower amount of DM (higher moisture in the litter), probably due to the reduction in the use of nutrients and cellular damage, characteristic of the challenge by *Eimeria* spp. (Kipper et al., 2013). The increase in BP levels promotes a reduction of litter DM, which can be explained by the high inclusion of ingredients with high CP composition in the diet, principally soybean meal. This type of ingredient had high values of potassium and oligosaccharides that promote a high intake of water and a high excretion of water by the birds on the litter reducing the DM of the litter material (Youssef et al., 2011). The evaluation of footpad dermatitis lesions at 28 days of age indicates the welfare quality of birds and no effect of challenge was observed (data not show). The frequency of scores with serious lesions (3 and 4) was increased with the increase of BP levels, conditions promoted by poor litter quality, but a significatively difference was observed only for the 6.66% of BP compared to the others, due to a better litter condition and birds thinner compared to the other treatments (Freitas et al., *in progress*). The incidence of footpad dermatitis is related to the moisture content of litter, considered critical above 35% humidity (El-Wahab et al., 2013), and when the ambient temperature is above 20ºC (Wang et al., 1998), in such a way, were the conditions evidenced in the present experiment that results in lesions in all treatments.

Conclusion

The challenge by *Eimeria maxima*, in general, was responsible to impair gut health and reduce AID for Met, Met+Cys, Arg, Ser, and Pro. The association of *Eimeria maxima* challenged and the increase of dietary BP results in an aggravation of the challenge and worsening of the litter quality. High amounts of BP levels were responsible to impair gut health, and for worsening litter quality and footpad dermatitis. The extreme reduction of BP level (6.66%) causes a reduction in morphometrics and worsened AID.

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Table 1. Ingredients (%) and nutrients composition in the low and high balanced protein feeds

Ingredients	Low balanced protein diet	High balanced protein diet
Corn, (7.9% CP)	5.78	28.9
Soybean meal, (45% CP)	8.77	43.8
Corn gluten (60% CP)	3.30	16.5
Soybean oil	6.35	5.56
Dicalcium phosphate	2.16	1.63
Limestone	0.786	0.883
Salt	0.495	0.357
Sodium bicarbonate	0.039	0.193
BioLys® (54.6%)	0.117	0.585
DL – Methionine (99%)	0.055	0.276
Premix Vitamin[1]	0.100	0.100
Premix Mineral[2]	0.100	0.100
Choline Chloride (60%)	0.070	0.070
Potassium Carbonate	1.37	0.000
Corn Starch	44.7	0.000
Inert[3]	4.00	0.000
Celite®	1.00	1.00
Rice Husk	8.84	0.000
Sugar	12.0	0.000
Nutritional composition		
Metabolizable Energy, (Kcal/kg)	3,120	3,120
Crude Protein, (%)	6.66 (7.26)	33.29 (34.80)
Digestible Methionine, (%)	0.16 (0.15)	0.81 (0.80)
Digestible Met + Cys, (%)	0.27 (0.27)	1.33 (1.34)
Digestible Lysine, (%)	0.36 (0.35)	1.80 (1.80)
Digestible Threonine, (%)	0.24 (0.25)	1.20 (1.19)
Digestible Arginine, (%)	0.39 (0.37)	1.93 (1.95)
Calcium, (%)	0.85	0.85
Available phosphorus, (%)	0.43	0.43

Values analyzed on parentheses, total amino acids was analyzed by HPLC and multiplied by digestibility factor proved by AMINOdat5.0®, the CP was evaluated by the AOAC method (2001.11);[1-] Content/kg of premix: Vitamin A =11,000,000 IU; Vitamin D=4,000,000 IU; Vitamin C= 55,000 IU; Vitamin K3=3,000 mg; Vitamin B1= 2,300mg; Vitamin B2=700 mg; Pantothenic acid=12g; Vitamin B6=4,000 mg; Vitamin B12= 25,000 µg; Nicotinic acid= 60g; Folic acid=2,000 mg; Biotin=250 mg and Selenium=300 mg; [2-] Content/kg of premix: Iron=100g; Cupper=20g; Manganese= 130g; Zinc= 130g and Iodine=2,000mg; [3] Inert material was sand.

Table 2. Ileal morphology and morphometric of broilers unchallenged and challenged by *Eimeria maxima* receiving different levels of balanced protein in reference to the lysine digestible at 20 and 27 days of age

Factors: BP levels	Factors: Challenge	GC 20d (n° cells/mm^2)	VH 20d (µm)	CD 20d (µm)	V:C 20d	GC 27d (n° cells/mm^2)	VH 27d (µm)	CD 27d (µm)	V:C 27d
6.66%	Yes	159	1863	483 ^{b}	3.97 ^{bc}	170	2448 ^{bc}	468	4.72 ^{cd}
	No	212	1993	359 ^{a}	5.83 ^{a}	157	2640 ^{ab}	344	7.76 ^{a}
13.32%	Yes	149	2136	637 ^{c}	3.50 ^{bcd}	167	2877 ^{ab}	504	5.12 ^{c}
	No	202	2196	379 ^{a}	6.02 ^{a}	170	2780 ^{ab}	431	6.81 ^{ab}
19.98%	Yes	145	1983	686 ^{c}	3.01 ^{cde}	146	3084 ^{a}	587	5.53 ^{bc}
	No	193	2454	409 ^{ab}	6.17 ^{a}	153	2952 ^{a}	479	6.34 ^{abc}
26.64%	Yes	145	1714	630 ^{c}	2.44 ^{de}	162	2113 ^{c}	630	3.23 ^{d}
	No	202	1997	475 ^{b}	4.28 ^{b}	168	2635 ^{ab}	501	5.72 ^{bc}
33.30%	Yes	142	1534	742 ^{c}	2.28 ^{e}	157	2122 ^{c}	697	3.20 ^{d}
	No	186	1816	475 ^{b}	4.08 ^{bc}	166	2400 ^{bc}	526	4.72 ^{cd}
SEM		8.51	80.3	25.1	0.242	0.041	107	26.3	0.361
Main Factors									
BP levels	6.66%	185	1928 ^{b}	421	4.90	163	2544	406 ^{a}	6.24
	13.32%	176	2166 ^{a}	508	4.76	169	2828	467 ^{ab}	5.96
	19.98%	169	2218 ^{a}	547	4.59	149	3018	533 ^{bc}	5.93
	26.64%	173	1855 ^{bc}	553	3.36	165	2374	566 ^{cd}	4.48
	33.30%	164	1675 ^{c}	608	3.18	161	2261	611 ^{d}	3.96
	SEM	6.01	58.4	17.50	0.176	6.08	75.3	19.5	0.266
Challenge	Yes	148 ^{b}	1846 ^{b}	635	3.04	161	2529	578 ^{a}	4.36
	No	199 ^{a}	2091 ^{a}	419	5.28	160	2681	456 ^{b}	6.27
	SEM	3.80	36.5	11.0	0.111	3.85	47.9	12.7	0.164
P value									
BP levels		0.131	<0.001	<0.001	<0.001	0.217	<0.001	<0.001	<0.001
Challenge		<0.001	<0.001	<0.001	<0.001	0.660	0.027	<0.001	<0.001
BP levels x Challenge		0.936	0.125	0.035	0.024	0.703	0.018	0.484	0.033

Abbreviations: BP, balanced protein; GC, globet cells; VH, villus height; CD, crypt depth; SEM, standard error of mean.
$^{a-b}$ Values within a row with different superscripts differ significantly at $P < 0.05$ by Tukey test.

Table 3. Immunity parameters of broilers at 21 days of age fed with different levels of balanced protein (BP) and challenged or not by *Eimeria maxima*

Factors		CD4+ (nº/mm^3)	CD8+ (nº/mm^3)	Relation CD4+:CD8+	FITc -dextran (µg/ml)
BP levels	6.66%	240.1	1273	0.268	0.573 ^{a}
	13.32%	274.9	1278	0.216	0.559 ^{a}
	19.98%	203.6	1270	0.262	0.607 ^{a}
	26.64%	352.8	1373	0.231	0.377 ^{b}
	33.30%	364.9	1433	0.312	0.323 ^{b}
	SEM	69.16	240.8	0.140	0.024
Challenge	Yes	282.7	1293	0.251	0.530 ^{a}
	No	291.7	1358	0.264	0.446 ^{b}
	SEM	36.42	152.3	0.081	0.016
P value					
BP levels		0.330	0.984	0.980	0.001
Challenge		0.874	0.773	0.870	0.008
BP Levels x Challenge		0.145	0.885	0.811	0.241

Abbreviations: BP, balanced protein.
$^{a-b}$ Values within a row with different superscripts differ significantly at $P < 0.05$ by Tukey test.

Table 4. Apparent ileal digestibility (AID) coefficient (%) of crude protein (CP) and essential amino acids of broilers at d 28 of age receiving different levels of balanced protein and challenge by *Eimeira maxima*

Factors		CP	Met	Cys	Met + Cys	Lys	Thr	Arg	Ile	Leu	Val	His	Phe
BP levels	Challenge												
6.66%	Yes	64.8	83.8	46.4	68.2	77.1 ^{d}	57.3 ^{c}	79.2 ^{c}	56.8 ^{c}	76.8	65.2 ^{c}	75.4	76.6 ^{c}
	No	67.4	85.9	53.6	72.5	80.3 ^{cd}	60.6 ^{c}	84.3 ^{b}	73.1 ^{b}	79.4	70.7 ^{bc}	77.1	81.3 ^{abc}
13.32%	Yes	74.6	89.2	64.4	79.2	84.3 ^{ab}	68.2 ^{ab}	87.2 ^{ab}	79.8 ^{ab}	84.2	77.1 ^{a}	81.5	84.8 ^{ab}
	No	76.4	90.6	66.7	81.0	85.8 ^{a}	70.9 ^{ab}	88.3 ^{a}	78.3 ^{ab}	86.0	78.2 ^{a}	82.0	84.8 ^{ab}
19.98%	Yes	76.6	91.3	61.8	78.1	84.1 ^{abc}	69.9 ^{ab}	90.7 ^{a}	76.5 ^{ab}	83.3	77.1 ^{ab}	83.1	82.8 ^{ab}
	No	79.4	89.5	66.2	81.1	85.4 ^{a}	73.8^{ab}	88.6 ^{a}	77.9 ^{ab}	86.0	80.1 ^{a}	83.6	85.5 ^{a}
26.64%	Yes	78.0	89.7	66.1	80.3	83.2 ^{abc}	72.4 ^{ab}	85.7 ^{ab}	78.8 ^{ab}	82.9	78.2 ^{a}	82.8	82.7 ^{ab}
	No	75.7	89.2	61.5	77.9	81.6 ^{bc}	67.7 ^{b}	85.7 ^{ab}	73.2 ^{b}	81.1	75.4 ^{ab}	81.5	80.7 ^{bc}
33.30%	Yes	79.9	91.2	67.5	81.6	85.0 ^{a}	74.9 ^{a}	86.1 ^{ab}	80.9 ^{a}	84.7	80.1 ^{a}	84.2	84.6 ^{ab}
	No	80.0	90.9	69.4	84.0	85.5 ^{a}	74.4 ^{ab}	87.7 ^{a}	77.9 ^{ab}	84.4	79.9 ^{a}	84.9	84.2 ^{ab}
SEM[1]		1.13	0.66	2.11	1.24	0.788	1.39	0.763	1.30	0.915	1.22	0.851	0.946
Main Factors													
BP levels	6.66%	66.1 ^{c}	84.9 ^{b}	50.0 ^{b}	70.3 ^{c}	78.7	58.9	81.8	64.9	78.1 ^{c}	68.0	76.2 ^{c}	79.0
	13.32%	75.5 ^{b}	89.9 ^{a}	65.5 ^{a}	80.1 ^{ab}	85.1	69.6	87.8	79.0	85.1 ^{a}	77.6	81.8 ^{b}	84.8
	19.98%	78.0 ^{a}	90.4 ^{a}	64.0 ^{a}	79.6 ^{ab}	84.8	71.9	89.6	77.7	84.6 ^{ab}	78.6	83.3 ^{ab}	84.2
	26.64%	76.8 ^{ab}	89.5 ^{a}	63.8 ^{a}	79.1 ^{b}	82.4	70.1	85.7	76.0	82.0 ^{b}	76.8	82.1 ^{b}	81.7
	33.30%	80.0 ^{a}	91.0 ^{a}	68.5 ^{a}	82.8 ^{a}	85.3	74.7	86.9	79.4	84.6 ^{ab}	80.0	84.6 ^{a}	84.4
	SEM	0.80	0.476	1.57	0.916	0.557	1.07	0.502	0.917	0.647	0.865	0.601	0.669
Challenge	Yes	75.8	88.7 ^{b}	61.2	77.5 ^{b}	82.8	68.5	85.9	74.6	82.4	75.5	81.4	82.3
	No	74.8	89.6 ^{a}	63.5	79.3 ^{a}	83.7	69.5	86.9	76.1	83.4	76.8	81.8	83.3
	SEM	0.54	0.356	1.00	0.584	0.366	0.718	0.321	0.603	0.425	0.568	0.395	0.440
P value													
BP levels		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Challenge		0.170	0.032	0.122	0.040	0.080	0.329	0.017	0.083	0.128	0.121	0.474	0.127
Levels x Challenge		0.164	0.134	0.125	0.132	0.074	0.040	<0.001	<0.001	0.103	0.029	0.578	0.014

Abbreviations: BP, balanced protein, CP, crude protein; Met, Methionine; Cys, Cysteine; Lys, Lysine; Thr, Threonine; Arg, Arginine; Ile, Isoleucine; Leu, Leucine; Val, Valine; Hist, Histidine; Phen, Phenylalanine; SEM, Standard error of mean.

$^{a-b}$ Values within a row with different superscripts differ significantly at $P < 0.05$ by Tukey test.

Table 5. Apparent ileal digestibility (AID) coefficient (%) of non-essential amino acids of broilers at 28 days of age receiving different levels of balanced protein and challenge by *Eimeira maxima*

Factors		Gly	Ser	Pro	Ala	Asp	Glu
BP levels	Challenge						
6.66%	Yes	58.90	66.0 [b]	69.5 [c]	69.8 [c]	70.5	80.0 [c]
	No	53.11	67.3 [b]	74.4 [bc]	75.1 [bc]	74.3	83.8 [b]
13.32%	Yes	67.87	77.3 [a]	80.9 [a]	82.0 [a]	77.8	86.8 [ab]
	No	69.00	79.9 [a]	82.9 [a]	82.5 [a]	78.7	87.5 [a]
19.98%	Yes	68.90	75.6 [a]	80.3 [ab]	83.9 [a]	77.7	86.8 [ab]
	No	72.59	81.4 [a]	83.8 [a]	84.7 [a]	79.7	87.7 [a]
26.64%	Yes	70.69	78.0 [a]	81.2 [a]	81.9 [a]	75.4	84.7 [ab]
	No	67.38	76.4 [a]	78.7 [ab]	79.7 [ab]	73.5	83.7 [b]
33.30%	Yes	72.74	80.8 [a]	81.1 [a]	84.0 [a]	77.1	85.8 [ab]
	No	72.93	80.8 [a]	81.4 [a]	83.5 [a]	77.5	86.2 [ab]
SEM		1.65	1.09	1.17	1.13	1.13	0.746
Main Factors							
BP levels	6.66%	56.01 [b]	66.6	72.0	72.4	72.4 [c]	81.9
	13.32%	68.43 [a]	78.6	81.9	82.2	78.3 [a]	87.1
	19.98%	70.74 [a]	78.5	82.0	84.3	78.7 [a]	87.2
	26.64%	69.04 [a]	77.2	80.0	80.8	74.4 [bc]	84.2
	33.30%	72.84 [a]	80.8	81.2	83.7	77.3 [ab]	86.0
	SEM	1.163	0.774	0.826	0.800	0.865	0.528
Challenge	Yes	66.66	75.5	78.6	80.3	75.7	84.8
	No	68.16	77.1	80.2	81.1	76.7	85.8
	SEM	0.765	0.545	0.543	0.527	0.519	0.347
P value							
BP levels		0.001	0.001	0.001	0.001	0.001	0.001
Challenge		0.183	0.044	0.041	0.324	0.184	0.066
BP levels x Challenge		0.108	0.049	0.032	0.034	0.185	0.054

Abbreviations: BP, balanced protein; Gly, Glycine; Ser, Serine; Prol, Proline; Ala, Alanine; Asp, Aspartate; Glu, Glutamate; SEM, Standard error of mean.
[a-b] Values within a row with different superscripts differ significantly at $P < 0.05$ by Tukey test.

Table 6. Effect of increasing levels of balanced protein and challenge by *Eimeria maxima* in the dry matter and crude protein of wood shavings litter

Factors		Litter DM (%)	Litter CP (%)
BP levels	6.66%	68.53 [a]	13.01 [c]
	13.32%	61.54 [b]	13.97 [c]
	19.98%	57.75 [bc]	14.50 [c]
	26.64%	59.45 [bc]	17.54 [b]
	33.30%	54.25 [c]	20.38 [a]
	SEM	1.666	0.575
Challenge	Yes	58.76 [b]	15.96
	No	61.85 [a]	15.80
	SEM	1.054	0.364
BP levels		<0.001	<0.001
Challenge		0.046	0.761
BP levels x Challenge		0.273	0.777

Abbreviations: BP, balanced protein; CP, crude protein; DM, dry matter; SEM, Standard error of mean

[a-b] Values within a row with different superscripts differ significantly at $P < 0.05$ by Tukey test.

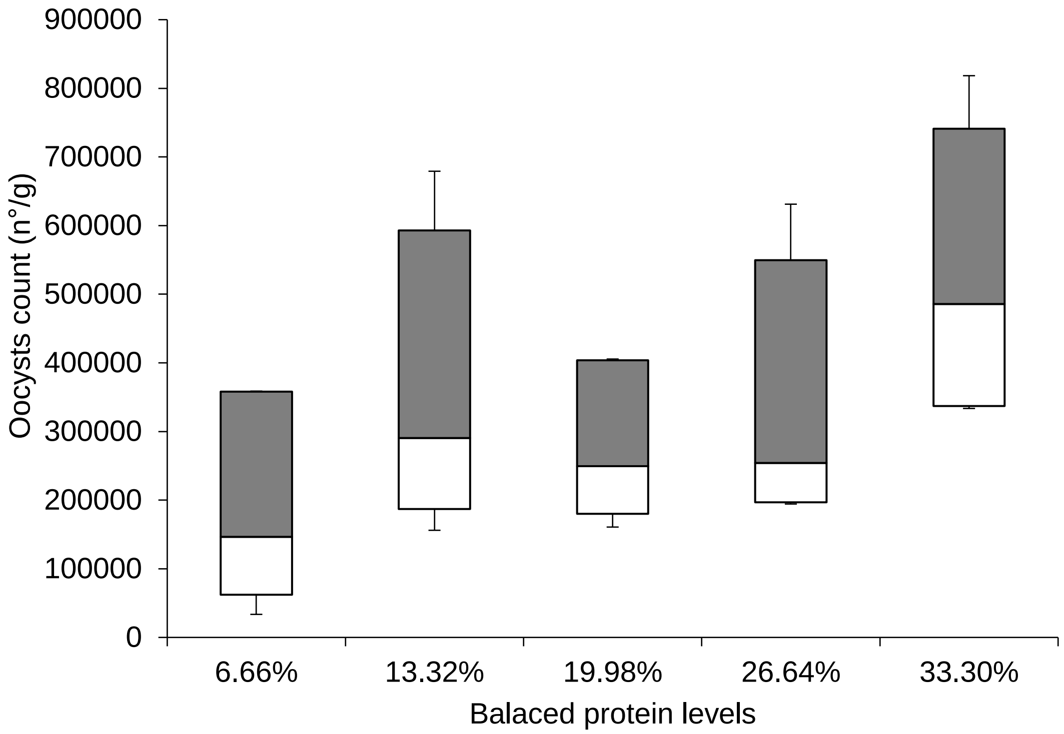

Figure 1. Boxplot of shedding of *Eimeria maxima* oocysts of broilers challenged receiving different dietary levels of balanced protein at 20 days of age. Boxplots with different letters were compared by a non-parametric Kruskal-Wallis test followed by a post hoc Bonferroni test at 5% of significance.

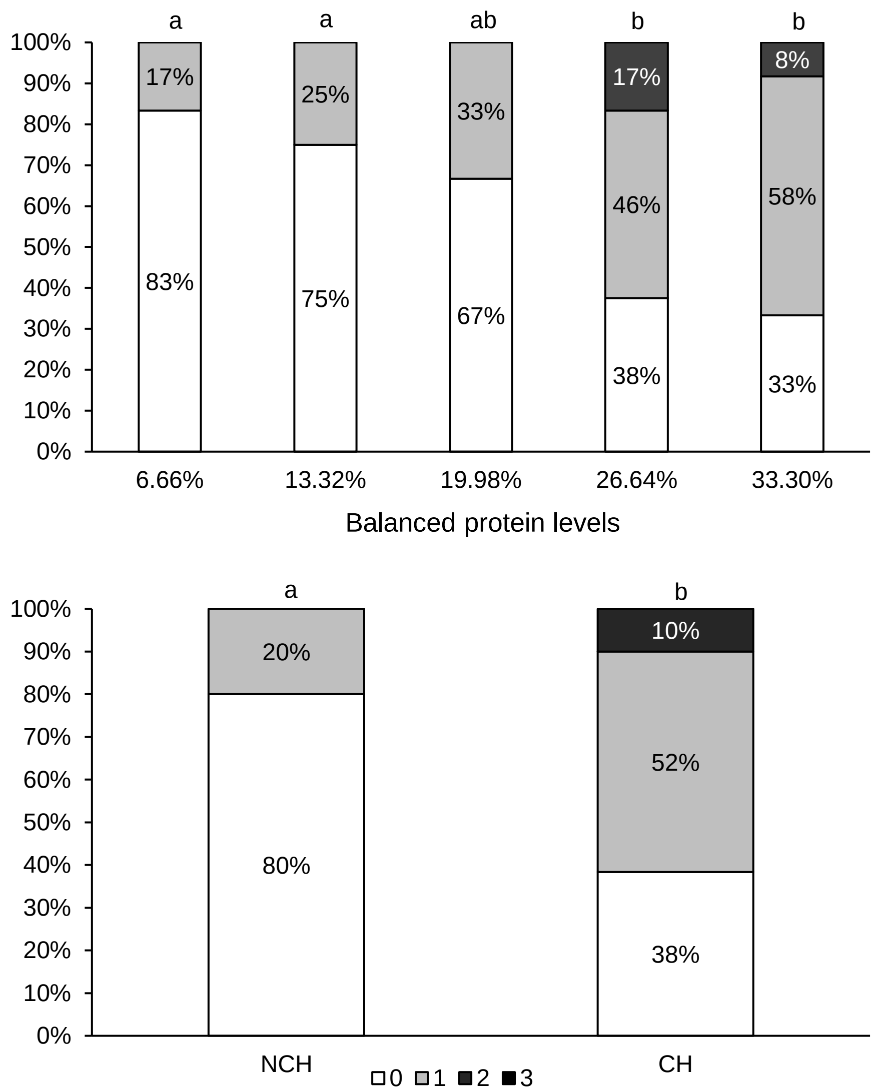

Figure 2. Frequency of visual intestinal health status scores of broilers at 20 days of age fed with different dietary balanced protein levels and challenged (CH) or not (NCH) by *Eimeria maxima*. Columns with different letters differ by non-parametric Kruskal-Wallis Tied Ranks test compared by posthoc Dunn's test at 5%.

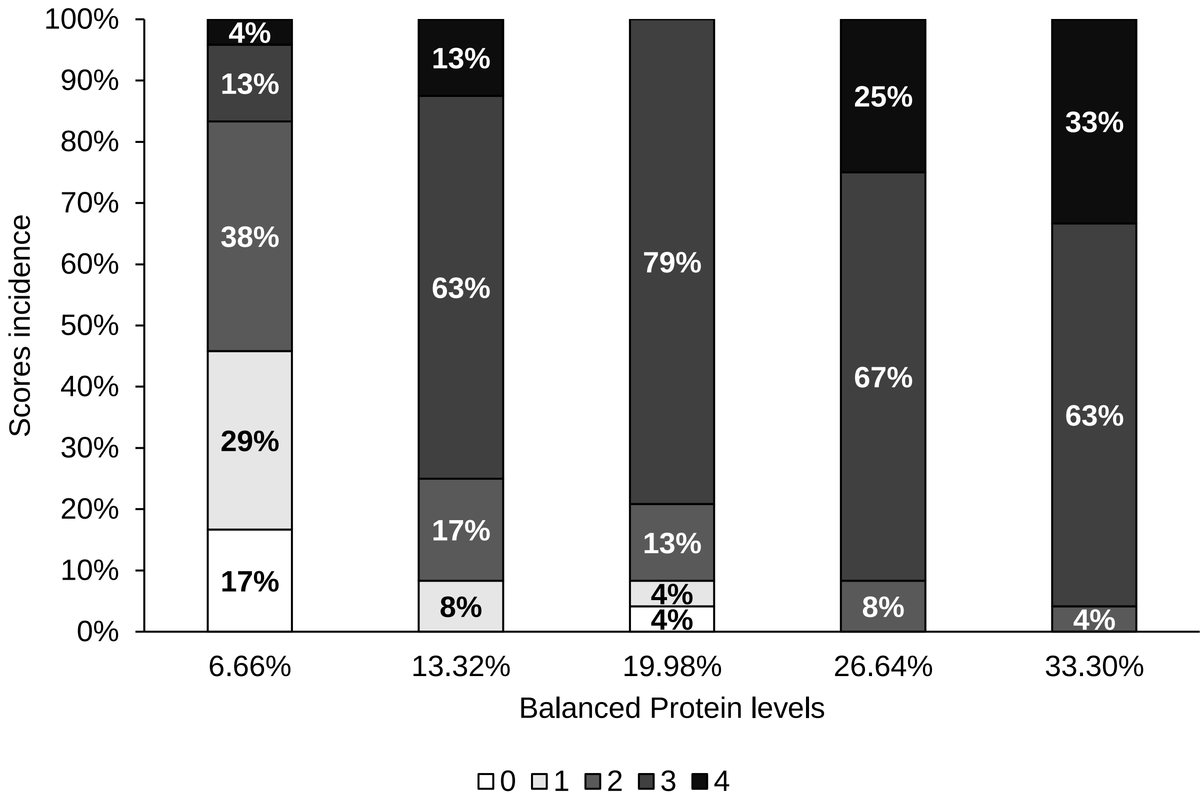

Figure 3. Frequency of footpad dermatitis score according to Welfare® quality scores (2009) of broilers fed with increased balanced protein levels. Columns with different letters differ by non-parametric Kruskal-Wallis Tied Ranks test compared by posthoc Dunn's test at 5%.

CAPÍTULO 5 – IMPLICAÇÕES

A produção de frangos de corte é acometida recorrentemente por desafios sanitários responsáveis por reduzir o desempenho das aves e promover prejuízos econômicos. Desafios entéricos como a coccidiose, causa alteração na resposta metabolica e fisiologica das aves, por promover destruição do tecido epitelial e ativação do sistema imune. Para tentar entender de que forma as aves respondem ao desafio, e quais são os custos que as doenças promovem no crescimento das aves modelos de desafios sanitários estão sendo utilizados e padronizados, como o desafio por inoculação do patógeno.

Várias implicações práticas podem ser obtidas na presente tese, mas como principais temos as modificações nos valores de eficiência de utilização de proteína e na digestibilidade dos aminoacidos que demonstra a alteração na utilização da proteína e aminoácidos quando a ave está em desafio.

Esses valores podem ser aplicáveis em modelos de crescimento de frangos, como o broiler growth model (BGM), que tem como função simular o crescimento de aves em diferentes condições, considerando os principais mecanismos fisiológicos, de forma estocástica e dinâmica. Contudo, para uma visão ampla das alterações metabólicas e fisiológicas, precisa-se determinar também as modificações na utilização de energia, para ser possível simular de forma acurada as modificações no crescimento das aves. Assim, a presente tese tem como sua implicação também, ser um plano piloto para futuros estudos que tem com o objetivo determinar tais modificões nutricionais e interações ambiente x hospedeiro x patógeno, como relatado no capítulo 2.