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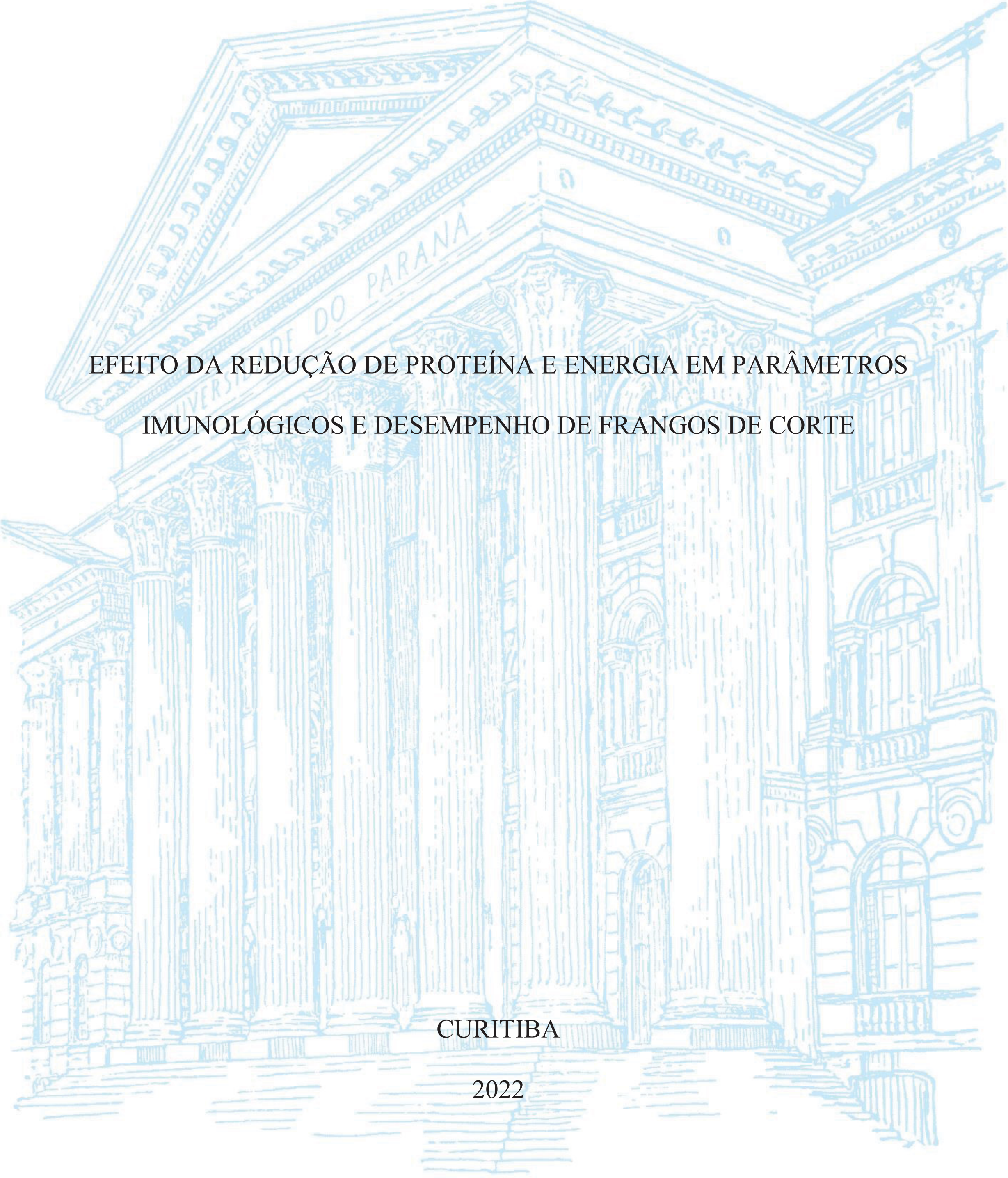

EFEITO DA REDUÇÃO DE PROTEÍNA E ENERGIA EM PARÂMETROS IMUNOLÓGICOS E DESEMPENHO DE FRANGOS DE CORTE

CURITIBA

2022

LEANDRO NAGAE KURITZA

EFEITO DA REDUÇÃO DE PROTEÍNA E ENERGIA EM PARÂMETROS IMUNOLÓGICOS E DESEMPENHO DE FRANGOS DE CORTE

Tese apresentada ao Programa de Pós-graduação em Ciências Veterinárias, Área de Concentração em Biologia integrada, Setor de Ciências Agrárias, Universidade Federal do Paraná, como parte das exigências para obtenção do título de Doutor em Ciências Veterinárias.

Orientadora: Prof. Dr. Simone Gisele de Oliveira
Coorientador: Prof. Dr. Alex Maiorka

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação CIÊNCIAS VETERINÁRIAS da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **LEANDRO NAGAE KURITZA** intitulada: **Efeito da redução de proteína e energia em parâmetros imunológicos e desempenho de frangos de corte**, sob orientação da Profa. Dra. SIMONE GISELE DE OLIVEIRA, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

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Assinatura Eletrônica
09/04/2022 18:27:21.0
SIMONE GISELE DE OLIVEIRA
Presidente da Banca Examinadora

Assinatura Eletrônica
20/04/2022 16:53:12.0
ANTONIO JOÃO SCANDOLERA
Avaliador Externo (UNIVERSIDADE FEDERAL DO PARANÁ)

Assinatura Eletrônica
08/04/2022 16:19:42.0
EVERTON LUIS KRABBE
Avaliador Externo (EMPRESA BRASILEIRA DE PESQUISA AGROPECUÁRIA)

Assinatura Eletrônica
12/04/2022 08:14:56.0
ANA VITORIA FISCHER DA SILVA
Avaliador Externo (UNIVERSIDADE FEDERAL DO PARANÁ)

Assinatura Eletrônica
09/04/2022 20:44:19.0
CHAYANE DA ROCHA
Avaliador Externo (UNIVERSIDADE FEDERAL DO PARANÁ)

Dedico:

À minha querida esposa Ananda Portella Félix, que foi o meu porto seguro, não apenas no doutorado, mas na vida. Também dedico aos meus pais, que me deram oportunidade de poder ter concluído minha graduação e mestrado e ter chegado ao doutorado. E aos meus orientadores, prof. Simone Gisele de Oliveira e prof. Alex Maiorka, pela oportunidade e por todo o aprendizado proporcionado.

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RESUMO

Frangos de corte apresentam grande importância no agronegócio. Por conta disso, diversos experimentos são realizados para definir as exigências nutricionais destes animais. Dentre os principais nutrientes estudados estão os aminoácidos. Além disso, a energia também acaba sendo bastante estudada, uma vez que é de extrema importância para a manutenção do metabolismo dos animais. No entanto, embora sejam bastante estudados, o foco principal do estudo é o desempenho zootécnico, havendo poucas informações sobre os efeitos destes na resposta imunológica das aves. Estudos demonstram que a deficiência de proteína na dieta pode causar alterações na resposta imune, como redução da resposta humoral e celular e que excesso de energia nas dietas pode exacerbar uma resposta imune. Os aminoácidos têm papel fundamental na síntese de várias proteínas específicas, como as citocinas e anticorpos, e regulam rotas metabólicas essenciais para a resposta imune contra patógenos, enquanto a energia é importante para manter o sistema imunológico ativo frente a desafios. Para tentar elucidar melhor o impacto da variação dos níveis de proteína e energia na dieta de frangos de corte foram delineados dois experimentos, nos quais foram trabalhados de forma independente reduções de níveis de proteína e níveis de energia na dieta. Os resultados obtidos demonstraram que a redução da proteína da dieta pode afetar a resposta imune de forma negativa, enquanto a redução da energia pode apresentar efeito benéfico sobre a resposta imune de frangos de corte. No entanto, ainda são necessários mais estudos para melhor estabelecimento das necessidades proteína e energia em frangos de corte frente aos desafios imunológicos do campo.

Palavras-chave: Aminoácidos, imunidade, frangos de corte, resposta imune, níveis de energia

ABSTRACT

Broiler chickens are of great importance in agribusiness. Due to it, several experiments are carried out to define the nutritional requirements of these animals. Among the nutrients studied, one of the most importance are the amino acids. In addition, energy also ends up being extensively studied, since it is extremely important for the maintenance of animal metabolism. However, although they have been extensively studied, the main focus of the studies is related to growth performance, with little information on their effects on the immune response. Studies demonstrate that dietary protein deficiency can cause changes in the immune response, such as reduced humoral and cellular responses, and that excess energy in diets can exacerbate an immune response. Amino acids play a fundamental role in the synthesis of several specific proteins, such as cytokines and antibodies, and regulate metabolic pathways essential for the immune response against pathogens, while energy is important to keep the immune system active in the face of challenges. To try to better elucidate the impact of varying levels of protein and energy in the diet of broilers, two experiments were designed, in which reductions in protein levels and energy levels in the diet of broilers were worked independently. The results obtained showed that the reduction of dietary protein can affect the immune response in a negative way, while the reduction of energy can have a beneficial effect on the immune response of broilers. However, further studies are still needed to better establish the protein and energy requirements of broilers in the face of immunological challenges in the field. Thus, further studies are still needed to better establish the protein and energy requirements in broilers in the face of immunological challenges in the field.

Keywords: Amino acids, immunity, broiler chickens, immune response, energy levels

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LISTA DE ABREVIATURAS

AAE – aminoácidos essenciais

AB – alcian blue

BSA – bovine serum albumin

CP – crude protein

EAA – essential amino acids

FCR – feed conversion ratio

FI – feed intake

GIT – gastrointestinal tract

ME – metabolizable energy

PAS – periodic acid-Schiff

PB – proteína bruta

PBS – phosphate-buffered saline

WG – weight gain

SUMÁRIO

CAPÍTULO I – CONSIDERAÇÕES GERAIS

1. INTRODUÇÃO

A elevada eficiência de produtividade obtida na avicultura de corte deve-se principalmente a quatro principais pilares: nutrição, manejo, ambiência e genética. A seleção genética teve como objetivos selecionar animais que apresentassem melhor desempenho e maior deposição de cortes nobres. No entanto, esta seleção acabou impactando na resposta imune adaptativa dos animais (Cheema et al., 2003). Esta resposta apresenta papel fundamental na remoção total dos patógenos do organismo (Wigley, 2013). Além disso, a melhoria da produção pode ter alterado a forma de atuação do sistema imune, especialmente pelo aumento do número de vacinações, biosseguridade, confinamento, altas densidades de alojamento e uso de antibióticos promotores de crescimento (Krover, 2012).

A nutrição, que representa cerca de 70% dos custos de produção, também apresenta papel importante no desempenho dos animais. As rações para frangos de corte, em sua grande maioria, são formuladas tendo como base as Tabelas brasileiras para aves e suínos (Rostagno et al., 2017), o NRC (1994) e manuais específicos das linhagens. Nessas tabelas e manuais são encontradas as necessidades nutricionais das aves, de acordo com as diferentes fases de produção. No entanto, por se tratar da maior parte dos custos produtivos, as empresas avícolas sempre buscam meios de reduzir custos. A redução dos custos por meio da alteração no valor nutricional das dietas já é uma preocupação desde a década de 80 (Jackson et al., 1982).

As principais alterações nutricionais geralmente se referem a redução dos níveis de proteína bruta (PB) das dietas, associados a inclusão de aminoácidos essenciais (AAE), assim como ajustes nos níveis de energia das dietas para atender as necessidades nutricionais de forma mais acurada. Esta alteração apresenta benefícios do ponto de vista econômico, mas também pode auxiliar na redução da poluição ambiental, por meio da redução da excreção de amônia e nitrogênio (Hilliar et al., 2020; Chrystal et al., 2020). A regulação da excreção de nitrogênio pelo ajuste dos níveis de PB das dietas para frangos foi recentemente revisada (Liu et al., 2021).

Sobre a redução da energia na dieta, diversos autores comprovaram que a redução dos níveis de energia reduz o desempenho dos animais (Cherry et al., 1978; Niu et al., 2009; Golian et al., 2010; Harrington et al., 2015; Govil et al., 2017; Zhao et al., 2017; Hu et al., 2021), mas determinar os níveis ideais é um desafio, uma vez que vários fatores podem influenciar nesta resposta (Lopez e Leeson, 2008).

Além dos pontos descritos acima, é importante ressaltar que as exigências nutricionais descritas em manuais de referência foram elaboradas para atender as necessidades de desempenho produtivo destes animais, sendo que pouco se sabe sobre as exigências nutricionais para ótima resposta do sistema imunológico das aves (Krover, 2012; Kogut, 2017), especialmente no que diz respeito às necessidades de aminoácidos (Wu et al., 2012; Abbasi et al., 2014). Desse modo, a presente revisão objetiva apresentar informações referentes ao papel da proteína e da energia sobre o sistema imunológico das aves.

2. REVISÃO BIBLIOGRÁFICA

2.1 Sistema imunológico das aves

O sistema imunológico das aves pode ser dividido em primário e secundário. O sistema imunológico primário é composto pelo timo e pela bolsa cloacal, antigamente conhecida como Bursa de *Fabricius*; enquanto o sistema imune secundário é composto pelo baço e pelos tecidos linfoides associados a mucosa. Este pode ser dividido em tecido linfoide associado ao olho, composto pela glândula de *Harder* e tecido linfoide associado à conjuntiva, tecido linfoide associado aos brônquios, tecido linfoide genital, tecido linfoide associado a pele e tecido linfoide associado ao trato gastrointestinal, composto pelas placas de *Peyer*, tonsilas esofágicas, pilóricas e cecais e divertículo da gema, antigamente chamado de divertículo de *Meckel* (Nagy et al., 2022). O sistema imunológico primário é responsável pela produção dos linfócitos B, através da bolsa cloacal, e dos linfócitos T, através do timo (Copper et al., 1966), enquanto o sistema imunológico secundário é responsável por organizar a resposta imune, direcionando a resposta das células efetoras (Boehm et al., 2012).

Além da divisão entre órgãos primários e secundários, há outra divisão importante quando falamos de sistema imunológico, que é a imunidade inata e adaptativa. Ambas são extremamente importantes para proteção do organismo, mas cada uma apresenta mecanismos de funcionamento diferentes. A imunidade inata geralmente é considerada como menos específica, mas apresenta ação mais rápida que a imunidade adaptativa, enquanto a imunidade adaptativa apresenta resposta específica contra um antígeno, mas demanda mais tempo para ser executada (Alberts et al., 2002).

A imunidade inata é composta por uma porção hematopoiética e uma porção não-hematopoiética. A porção hematopoiética corresponde às células produzidas quando há resposta inflamatória, enquanto a porção não-hematopoiética corresponde às barreiras do organismo, como a pele, células epiteliais dos tratos gastrointestinal, genitourinário e respiratório. Além disso, a imunidade inata apresenta componentes humorais, compostos pelas proteínas do sistema complemento, proteína C reativa, proteínas de ligação aos lipopolissacarídeos, peptidios antimicrobianos, entre outros (Kaur & Secord, 2019).

Mesmo sendo considerada como menos específica, a imunidade inata apresenta os receptores de reconhecimento de padrões, células efetoras como neutrófilos/heterofilos, células Natural Killer e células dendríticas, assim como a produção de citocinas e quimocinas para regular a resposta inflamatória e induzir a resposta adaptativa (Kaiser, 2010).

Já a imunidade adaptativa apresenta pode ser dividida em celular, através dos linfócitos T, considerada como efetora, e humoral, através dos linfócitos B, relacionada a memória (Bonilla & Ottegen, 2010). Nas aves, a produção dos linfócitos T está associada ao timo (Smith & Göbel, 2022), enquanto a produção dos linfócitos B está associada a bolsa cloacal (Ratcliffe & Härtle, 2022). Dentro da linhagem T, podemos subdividir os linfócitos em T CD4, chamados auxiliares, e T CD8, chamados citotóxicos. Os linfócitos T CD4 são essenciais na regulação da resposta imune das aves (Arstila et al., 1994), atuando como sinalizadores e apresentadores de antígeno para células CD8 e B, enquanto os linfócitos CD8 fazem a destruição das células alvo (Abbas et al., 2000).

2.2 Nutrição e sistema imunológico das aves

O sistema imunológico é composto por órgãos linfoides, células, imunoglobulinas e citocinas que atuam em conjunto formando uma rede de proteção (Parkin e Cohen, 2001). Essa é a principal ferramenta para evitar a entrada de patógenos no organismo de animais e seres humanos. No entanto, para manter o funcionamento normal desse sistema, há a necessidade de dispender nutrientes, muitas vezes destinados à manutenção, crescimento e reprodução (Lochmiller e Deerenberg, 2000). Esses nutrientes são fundamentais para garantir a expansão clonal de linfócitos frente a um desafio, recrutamento de heterofilos e linfócitos, assim como produção de imunoglobulinas e sinalizadores celulares (Adedokun & Olojede, 2019). Quantidades acima ou abaixo das recomendadas de nutrientes podem prejudicar este sistema (Kogut & Klasaing, 2009). Além disso, o acesso precoce ao alimento auxilia na aceleração do desenvolvimento do sistema imunológico dos animais (Taha-Abdelaziz et al., 2018). Desse modo, o entendimento do efeito da nutrição sobre o sistema imunológico pode permitir a manipulação da resposta imune para melhorar as defesas do organismo contra patógenos (Krover, 2012).

O estudo dos nutrientes sobre a resposta imune deve ser cuidadoso, pois a interação destes terá efeito sobre o sistema imune e não apenas um nutriente isolado (Kogut & Klasing, 2009). A interação de diferentes nutrientes capazes de estimular o sistema imunológico pode apresentar papel importante na redução da susceptibilidade a doenças nas aves (Kogut, 2009).

2.2.1 Proteína e o sistema imunológico

As necessidades de PB e aminoácidos para aves de produção já foram amplamente estudadas para otimizar o desempenho produtivo desses animais. No entanto, essas necessidades são determinadas com base em animais sadios (Leeson, 2008) e levando em consideração apenas os aspectos de desempenho zootécnico dos animais (Kidd, 2004). Somando-se a isso, há a crescente tendência de se trabalhar com o conceito de proteína ideal, visando principalmente a redução de custos de produção. Este conceito foi desenvolvido durante as décadas de 50 e 60 na Universidade de Illinois (Emmert e Baker, 1997) e até hoje segue sendo aplicado.

No entanto, este conceito foi desenvolvido visando apenas os aspectos produtivos dos animais, sem levar em conta outros sistemas fisiológicos. Quando há redução dos níveis de proteína da dieta, sem suplementação de aminoácidos, há perda de desempenho por parte dos animais, no entanto também há a redução no desenvolvimento dos órgãos imunes. A perda proporcional de massa dos órgãos imunes acaba sendo maior quando comparados a massa corpórea total perdida, e isso acaba refletindo em menor produção de células imunes (Bell et al., 1976). Esse efeito também pode ser observado em aves alimentadas com restrição proteica, com a redução do tamanho da bolsa cloacal (Glick et al., 1981; Jahanian, 2009), a qual é um dos principais órgãos imunes destes animais (Davison et al., 2008). Como consequência, pode ser observada redução na produção de imunoglobulinas pelos animais (Glick et al., 1981; Jahanian, 2009).

Na busca de evitar problemas no desenvolvimento dos animais, quando é realizada a redução dos níveis de PB da dieta, são feitas suplementações dos AAE de forma individual. Isso garante o fornecimento de níveis adequados dos aminoácidos necessários para a deposição muscular, reduzindo os custos com a dieta e reduzindo o potencial de excreção de nitrogênio, que pode ter um efeito tóxico no ambiente (Han e Lee, 2000; Greenhalgh et al., 2020).

É importante ressaltar que os aminoácidos têm papel fundamental na síntese de várias proteínas específicas, como as citocinas e anticorpos, e regulam rotas metabólicas essenciais para a resposta imune contra patógenos (Li et al., 2007). Isso ganha maior importância quando ocorre um desafio, uma vez que o organismo desvia aminoácidos destinados ao crescimento e produção para os órgãos imunes para responder a uma infecção (Le Floc`h et al., 2004), facilitando a entrada de patógenos no organismo, que poderão prejudicar ainda mais o desempenho destes animais.

No entanto, a redução dos níveis de PB com a suplementação de AAE pode acarretar desequilíbrio nos níveis dos aminoácidos não essenciais, podendo afetar o desempenho dos animais (Awad et al., 2014). Por mais que os aminoácidos não essenciais possam ser sintetizados, se não houver substrato suficiente, estes terão a sua síntese reduzida, podendo tornar-se essenciais, ou condicionalmente essenciais, consequentemente afetando de forma negativa o desempenho produtivo dos animais (Wu et al., 2013; Maia et al., 2021).

Sabe-se também que os aminoácidos não essenciais podem apresentar outras funções no metabolismo dos animais, inclusive sobre o sistema imunológico (Hou et al., 2015; Hope e Salmond, 2021). Como principal exemplo tem-se a glutamina, que é responsável por fornecer energia para as células de defesa do sistema imune (Newsholme et al., 1999). Isso ganha ainda mais importância quando há um desafio, aumentando a inflamação no organismo. Nestes casos há aumento da demanda de glutamina, o que leva a um desbalanço nos níveis deste aminoácido, piorando ainda mais a resposta imune (de Oliveira et al., 2016). Em conjunto com a glutamina, a serina também apresenta efeito modulador sobre a resposta inflamatória dos animais, alterando a expressão de mediadores inflamatórios (Takahashi et al., 2008). Estes mediadores inflamatórios atuam como sinalizadores da resposta imune, estimulando a multiplicação celular (Mizel, 1989; Klasing, 1991). Na Tabela 1 são apresentados os principais efeitos de alguns aminoácidos sobre a resposta imune direta ou indireta de aves.

Tabela 1. Principais efeitos de aminoácidos sobre a resposta imune de aves.

Aminoácido	**Efeito**	**Referência**
Arginina	• Multiplicação dos linfócitos T; • Atividade citotóxica de macrófagos	Le'Floch et al. (2004)
Glutamina	• Fonte de energia para macrófagos, linfócitos, neutrófilos e enterócitos • Aumento da concentração de IgA	Souba (1991) Le'Floch et al. (2004) Li et al. (2007) Bartell & Batal (2007)
Lisina	• Desenvolvimento da bolsa cloacal	Humphrey et al. (2006)
Metionina	• Desenvolvimento dos órgãos imunes	Bang-yuan et al. (2012) Al-Mayah (2006)
Treonina	• Síntese da mucina; • Componente das imunoglobulinas	Slack et al. (2012)

2.2.2 Energia e o sistema imunológico

Assim como a proteína, a energia também apresenta papel importante na imunidade. Embora seja de conhecimento geral que a energia afeta a resposta imune dos animais, o mecanismo por trás desta alteração é pouco entendido. Sabe-se que quando os animais enfrentam um desafio por patógenos, ocorre um aumento nas demandas de energia para estabelecer uma resposta imune eficaz contra estes patógenos (Lochmiller & Deerenberg, 2000). Isso pode estar associado a menor disponibilidade de energia para crescimento. Em um estudo realizado por Martin II et al. (2003), foi observado aumento na taxa metabólica de repouso de animais desafiados com fitohemaglutinina, indicando aumento no consumo de energia desses animais. De acordo com esses autores, a energia despendida com a imunidade foi equivalente a quantidade de energia para a produção de meio ovo por dia. Esse e outros gastos foram compilados por Muehlenbein et al. (2010), demonstrando que o sistema imune apresenta alta demanda energética. Com esse aumento da demanda, há um direcionamento da energia destinada a manutenção, desenvolvimento e produtividade para a resposta imune. Isso ocorre por meio de alterações no metabolismo, priorizando as defesas do organismo (Ganeshan & Chawla, 2014). Curiosamente, quando há outro fator estressor, como a baixa disponibilidade energética, ou mesmo um estresse por frio, os animais podem entrar em um estado de economia de energia, passando a tolerar o patógeno, garantindo maior taxa de sobrevivência dos animais

(Ganeshan et al., 2019). Esse fator foi extremamente importante na evolução das espécies, uma vez que os animais submetidos a outros fatores estressantes durante uma infecção conseguiram se adaptar melhor, apresentando maior taxa de sobrevivência (Ganeshan et al., 2019).

Outro ponto importante é que a alteração nos níveis de energia consumida causa alteração do tipo de resposta imune. Quando há níveis adequados de energia, há melhor resposta contra patógenos intracelulares, e em níveis mais baixos de energia há melhor resposta contra infecções não invasivas (Long & Nanthakumar, 2004). Em passeriformes que realizam processo de migração, foi observada redução na contagem de linfócitos e leucócitos nos animais com menores reservas energéticas ao chegarem ao destino. No entanto, não foi observada diferença nas contagens de imunoglobulinas e na taxa de heterofilos/linfócitos (Owen & Moore, 2008).

Sabe-se que o consumo excessivo de energia, aliado ao baixo gasto energético, está diretamente relacionado ao aumento da obesidade em seres humanos (Stubs & Lee, 2004), e isso acaba impactando de forma negativa no sistema imune (Martí et al., 2001; Matarese e La Cava, 2005; Milner & Beck, 2012). O acúmulo excessivo de gordura corporal está associado à mobilização de citocinas pró-inflamatórias, que são responsáveis pelo aumento do número de macrófagos, tendo relação com a inflamação nestes indivíduos (Weisberg et al., 2003; Lumeng et al., 2007). Em patos, resposta similar também foi observada. O fornecimento de dieta contendo altos níveis de energia foi associado ao aumento nos níveis de mediadores inflamatórios circulantes (Bai et al., 2019).

A redução da energia por tempo prolongado também causa alteração na resposta imune, resultando em redução desta resposta (Lochmiller & Deerenberg, 2000). Isso acaba sendo bastante visível em animais que passam por períodos de restrição alimentar voluntária, como é o caso de vacas pré-parto. Nestes animais há a redução de consumo de alimentos, e isso normalmente está associado a limitação da resposta imune por estes animais (Goff, 2006).

Em frangos, o uso da energia é muito mais restrito, visando melhor desempenho e produtividade, reduzindo ao máximo os custos de produção (Musigwa et al., 2021). Esse equilíbrio as vezes pode representar pequena perda no desempenho dos animais, no entanto, a redução dos custos da dieta acaba compensando esta perda, permitindo a maximização dos ganhos (Aftab, 2019). Embora possam ocorrer diferenças no desempenho destes animais, a resposta imune nem sempre é afetada. Conforme observado por Fanooci & Torki (2010), mesmo com a restrição energética nas dietas dos animais, não houve impacto na resposta de anticorpos contra a doença de Newcastle, assim como não houve alteração no desempenho.

Haunshi et al. (2012) também não encontraram efeito sobre os anticorpos contra a doença de Newcastle, no entanto foram observados efeitos negativos sobre o desempenho dos animais alimentados com restrição energética. Resultados similares foram encontrados por Bhanja & Mandal (2007), não observando diferenças para o peso de órgãos imunes, resposta imune celular e humoral, também com piora no ganho de peso e piora na conversão alimentar das aves alimentadas com restrição energética. Nestes três estudos é importante ressaltar que os níveis de energia foram alterados, mas também houve alteração nos níveis de proteína. Possivelmente, por haver a manutenção da relação entre proteína e energia, não houve prejuízo à resposta imune. Isso é comum quando são trabalhados níveis de proteína e é avaliado o desempenho dos animais (Gous et al., 2018). Praharaj et al. (1997) e Golian et al. (2010) encontraram melhora da resposta imune em frangos de corte quando houve a redução da energia da dieta. Como as condições estavam abaixo do ideal para o melhor desenvolvimento dos animais, possivelmente havia um excesso de nutrientes não utilizados por parte dos animais, e estes nutrientes foram destinados a resposta imune.

2.3 Nutrição e morfometria intestinal das aves

Sabe-se que o intestino delgado é o principal responsável pela digestão e absorção de nutrientes pelas aves (Yamauchi, 2007). Além disso, tem como funções servir como barreira contra patógenos, secretar enzimas e hormônios, abrigar a microbiota e eliminar substâncias não utilizadas pelo organismo (Yegani & Krover, 2008). Para manter todas essas funções, é fundamental que o intestino possua um bom desenvolvimento. Isso é obtido a partir do fornecimento de nutrientes de forma adequada (Abbasi et al., 2014) e no tempo correto, uma vez que o crescimento do intestino é proporcionalmente maior que o crescimento corporal nos primeiros dez dias de vida de frangos (Sklan, 2001). Durante este período, até os 14 dias de vida, é quando o intestino apresenta o maior peso relativo, tendo importante redução após esse período, mas ainda assim mantendo uma taxa mínima de crescimento (Ravindran et al., 2006). Isso demonstra que esse período é o mais crítico para garantir o melhor desempenho dos animais. Restrições de nutrientes neste estágio de vida das aves podem acabar comprometendo o desenvolvimento intestinal destas (Geyra et al., 2001).

Dentre os principais nutrientes necessários para o desenvolvimento intestinal estão os aminoácidos. A restrição de proteína bruta na dieta de frangos de corte foi associada a redução da altura de vilosidade, sendo correlacionado a redução do desempenho destes animais (Laudadio et al., 2012). A redução da proteína também afeta a relação altura de vilo/profundidade de cripta. Valores menores desta relação estão associados a um *turnover* acelerado, indicando que o organismo está tentando compensar essa atrofia acelerando o desenvolvimento (Laudadio et al., 2012), enquanto valores maiores desta relação estão associados ao aumento da digestão e absorção de nutrientes (Khosravinia et al. 2015).

Com relação aos aminoácidos, seu papel fica claro quando é feita redução da proteína bruta da dieta com suplementação de aminoácidos. Em experimento realizado por Teng et al. (2021), a suplementação de treonina, arginina e glicina tiveram efeito positivo sobre altura de vilo e permeabilidade intestinal em frangos de corte recebendo dietas com restrição de proteína bruta e desafiados com *Eimeria spp.*. Os aminoácidos são importantes para a produção das poliaminas, como a espermidina, a espermina e a putrescina, que estão diretamente associadas ao crescimento e diferenciação celular. A baixa disponibilidade destas poliaminas inibe a proliferação migração e apoptose das células intestinais (Fernandes & Murakami, 2010). Isso acaba representando menor altura de vilosidade e menor crescimento intestinal.

Além da proteína e dos aminoácidos, a energia possui papel importante no desenvolvimento intestinal. Miao et al. (2017) encontraram menor altura de vilo aos 21 dias em frangos alimentados com dietas com redução na energia. Embora essa redução tenha ocorrido aos 21 dias, aos 42 dias não foi observada diferença na morfometria intestinal das aves. Isso indica que os animais podem apresentar adaptação aos baixos níveis de energia da dieta. Essa adaptação pode ser sinalizada por meio da redução na deposição do tecido adiposo branco quando há déficit energético no organismo, o qual libera sinalizadores que estimulam o crescimento do intestino e o consumo de alimentos (Nilaweera & Speakman, 2018).

3. CONSIDERAÇÕES FINAIS

Níveis dietéticos de proteína e energia adequados são essenciais para garantir o desenvolvimento das aves, no entanto a resposta imune nem sempre vai ser a ideal quando estes níveis são utilizados. Embora os níveis de proteína e energia já sejam conhecidos para atingir o melhor crescimento dos animais, ainda há pouca informação sobre a sua importância no sistema imune das aves. Alguns efeitos são conhecidos, mas os mecanismos que ativam estes efeitos ainda precisam ser melhor estudados. O desafio atualmente é estabelecer níveis de proteína e energia que otimizem o desempenho produtivo e a resposta imune das aves.

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Capítulo II - EFFECT OF CRUDE PROTEIN REDUCTION IN BLOOD, PERFORMANCE, IMMUNOLOGICAL, AND INTESTINAL HISTOLOGICAL PARAMETERS OF BROILER CHICKENS*

* Artigo aceito para publicação na Animal Science Journal

Research article

Leandro Nagae Kuritza[1]; Leopoldo Malcorra de Almeida[1], Marley Conceição dos Santos[2], Lucas Schmidt Bassi[2], Katiucia Cristine Sonálio[1], Alex Maiorka[1], Simone Gisele de Oliveira[1]

[1]Department of Veterinary Science, Universidade Federal do Paraná, Curitiba, Brazil.

[2]Department of Animal Science, Universidade Federal do Paraná, Curitiba, Brazil.

Correspondence: Leandro Nagae Kuritza, Department of Veterinary Science, Universidade Federal do Paraná, Rua dos funcionários, 1540, 80035-050, Curitiba, Brazil

Email: leandronkmv@gmail.com

Abstract

We aimed to evaluate the effects of the reduction in dietary crude protein (CP) on blood urea, uric acid, performance, immunity, and intestinal histology of broilers. Four diets were formulated with 22.50%, 21.50%, 20.50%, and 19.50% of CP (1 to 21d) and 19.20%, 18.20%, 17.20%, and 16.20% of CP (22 to 42d), meeting the requirements of essential amino acids in all diets. A total of 800 male Ross chicks were randomly allocated to 32 pens, with 25 birds each (n=8). Blood and intestines had been collected for analysis. Uric acid decreased and urea increased with the reduction of CP ($P<0.05$). Reduction in performance and intestinal parameters (villus, crypt, and goblet cells) were observed with the reduction of CP ($P<0.05$). Lower levels of CP resulted in alteration ($P<0.05$) in CD4 and CD8 lineages (21 and 42d). Broken-line models estimated ($P<0.05$) the CP requirement for growth between 21% to 21.3% (1 to 21d) and between 17.2% to 17.4% (22 to 42d) and CP requirements between 17.2% and 18.2% for maximum response of immune cells (42d). Reduction in dietary CP has a negative impact on performance, immune response, and intestinal histology of broilers, even with adequate levels of essential amino acids.

KEYWORDS: Amino acid, Bursa of Fabricius, Immunity, T lymphocyte, Villus

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1 INTRODUCTION

Poultry companies are always looking for ways to reduce production costs, especially related to nutrition. Reducing production costs altering dietary nutrients is a concern since the ’80s (Jackson et al., 1982). To reach these results, nutritionists often reduce dietary crude protein (CP) levels with the inclusion of essential amino acids (EAA) to meet the nutritional requirements of animals. This can also attenuate environmental pollution, with reduction of ammonia and nitrogen excretion (Hilliar et al., 2020; Chrystal et al., 2020). This regulation of nitrogen with reduction in dietary CP on poultry nutrition was widely reviewed recently (Liu et al., 2021). However, although these diets met all the EAA requirements for growth, they may not be sufficient for an optimum immune response (Wu et al., 2012; Abbasi et al., 2014).

It is known that amino acids have a fundamental role in the synthesis of several specific proteins, such as cytokines and antibodies, and regulate metabolic routes essential for the immune response against pathogens (Li et al., 2007). When animals face a challenge, the organism diverts amino acids for growth and production to immune organs to respond to infection (Le Floc'h et al., 2004). This might reduce performance and productivity of birds. Also, if the amounts of amino acids are below recommendation, the immune system may have a reduction in functioning, reducing humoral (Glick et al., 1981), and cell-mediated immunity (Glick et al., 1983). This reduction has been confirmed by some researchers (Tsiagbe et al., 1987; Chen et al., 2003; Rama Rao et al., 2003; Wu et al., 2012), demonstrating that reduction of dietary amino acids can alter immune organs and reduce the immune response. Although some studies have evaluated the effects of low dietary CP on cell-mediated immune response in broilers (Glick et al., 1983; Munir et al., 2009), they did not analyze the adaptive immune response related to T lymphocytes. This response is essential to eliminate intracellular pathogens and regulate humoral immune response through cell signaling (Erf, 2004).

Besides the direct interference on immune cells and organs, reduced dietary levels of amino acids may impair mucus production in the gastrointestinal tract (GIT) (Abbasi et al., 2014), which is the primary immunological barrier, facilitating the exposure of intestinal mucosa to pathogens and damaging the intestinal villi. This damage, associated with the fact that the GIT tissues have high protein requirements to keep the adequate high turnover rate of the intestinal mucosa and its functionality (Abbasi et al., 2014), may aggravate amino acids deficiency and impair broiler performance.

Considering that poultry has a precise nutrition, it is recommended to define the correct balance of protein, and more precisely, amino acids to attend performance needs and also immune response and GIT development and functionality. Thus, the objective of this study was to determine the effects of the reduction of dietary CP levels on blood, performance, immunological, and intestinal histological parameters in broilers.

2 MATERIALS AND METHODS

2.1 Animals

The experiment was approved by the Ethics Committee on Animal Use of Agrarian Sciences Sector of Federal University of Parana, Brazil, under protocol number 088/2018.

A total of 800 male Ross chicks were randomly allocated to 32 pens, with 25 birds each. The chicks were housed in floor pens (1.65 × 1.25 m) containing pine shavings throughout the trial. All pens were equipped with one tubular feeder and nipple drinkers. Before assignment, 80 animals were randomly selected and weighted to determine initial body weight, allowing a homogeneous distribution in weight among treatments. The chicks were maintained at temperatures and lightning according to recommendations from Ross Manual to each age.

The animals were fed experimental starter diets from 1 to 21 d of age and experimental grower diets from 22 to 42 d of age. Experimental diets were formulated to meet the following levels of CP 22.50% starter and 19.20% grower, with reduction of one percentual point for the following treatments (21.50%, 20.50%, and 19.50% from 1 to 21 days and 18.20%, 17.20% and 16.20% from 22 to 42 days). Analyzed values are described in Table 1. Diets were supplemented with the limiting amino acids and were formulated to meet all the nutritional requirements of broilers, except for CP. The CP was estimated using Dumas method, and amino acids analysis was done by standard procedures (AOAC, 1994) using an amino acid analyzer (Biochrom 301, Cambridge, UK). Diets offered and leftovers were weighted weekly to evaluate feed intake (FI) and at 21 and 42 days all birds were weighted to evaluate weight gain (WG) and feed conversion ratio (FCR).

Animals had free access to feed and water during all the experiment, except by the two hours fasting period prior blood collection at 42 days for urea and uric acid analysis. This allows a better response from serum uric acid after amino acid utilization (Powell et al., 2009).

TABLE 1 - Ingredients and analyzed composition of starter and grower diets.

	Starter				Grower			
Item	22.5%	21.5%	20.5%	19.5%	19.2%	18.2%	17.2%	16.2%
Ingredients (kg)								
Corn	520.34	559.44	600.49	639.43	606.60	645.63	685.68	725.64
Soybean meal 46%	403.00	366.00	328.00	291.00	323.00	286.00	248.00	211.00
Soybean oil	43.00	36.00	28.00	21.00	44.00	37.00	30.00	22.00
Limestone 38%	10.30	10.40	10.50	10.70	8.20	8.30	8.40	8.60
Dicalcium phosphate	10.30	10.60	10.90	11.20	7.20	7.50	7.80	8.10
Sodium chloride	5.00	5.00	5.00	5.00	4.50	4.50	4.50	4.50
L-Lysine 99%	1.79	2.95	4.11	5.28	1.33	2.50	3.66	4.82
L-Threonine 98.5%	0.52	1.01	1.50	1.99	0.32	0.81	1.30	1.79
DL-Methionine	3.40	3.69	4.03	4.37	2.50	2.85	3.18	3.52
L-Valine 96.5%	0.00	0.62	1.25	1.87	0.00	0.62	1.25	1.87
L-Tryptophan 98%	0.00	0.19	0.37	0.56	0.00	0.19	0.38	0.56
L-Arginine 96%	0.00	1.12	2.24	3.36	0.00	1.12	2.24	3.36
L-Isoleucine	0.00	0.63	1.26	1.89	0.00	0.63	1.26	1.89
Choline chloride	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Phytase Hiphos 20000GT	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Vitamin premix[a]	1.30	1.30	1.30	1.30	1.30	1.30	1.30	1.30
Mineral premix[b]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Analysed chemical composition								
Crude protein (%)	23.10	22.20	21.49	20.38	19.18	18.52	17.60	16.40
Crude fat (%)	6.78	6.17	5.46	4.84	7.06	6.45	5.84	5.50
Metabolizable energy (Kcal/kg)[c]	3075	3077	3074	3075	3174	3176	3178	3174
Digestible lys (%)	1.41	1.40	1.43	1.41	1.15	1.18	1.14	1.10
Digestible met+cys (%)	0.95	0.97	0.98	0.95	0.82	0.81	0.80	0.81
Digestible thr (%)	0.90	0.88	0.87	0.86	0.75	0.75	0.72	0.71
Digestible trp (%)	0.25	0.25	0.25	0.25	0.21	0.21	0.21	0.21
Digestible arg (%)	1.51	1.51	1.52	1.47	1.25	1.27	1.26	1.22
Digestible gly (%)	0.96	0.90	0.84	0.78	0.81	0.76	0.70	0.63
Calcium (%)	0.98	0.98	0.98	0.98	0.96	0.96	0.96	0.96
Phosphorus (%)	0.56	0.57	0.54	0.49	0.50	0.47	0.45	0.40

[a] Provided per kilogram of diet: vitamin A, 9,000 IU; vitamin D3, 3,000 IU; vitamin E, 30 IU; vitamin K, 1.5 IU; riboflavin, 12 mg; pantothenic acid, 15 mg; niacin, 50 mg; vitamin B12, 0.02 µg; biotin, 0.1 µg; folic acid, 1.5 mg; pyridoxine, 5 mg; and thiamin, 3 mg.

[b] Provided per kilogram of diet: Fe, 55 mg; Mn, 100 mg; Cu, 8 mg; Se, 0.15 mg; Zn, 80 mg; I, 1 mg, as ferrous sulfate monohydrate, manganese sulfate, copper sulfate, sodium selenite, zinc sulfate, calcium iodate, respectively.

[c] Calculated according to the equations for metabolizable energy described in Brazilian Tables for Poultry and Swine (Rostagno, 2017)

2.2 Blood analysis

At 21- and 42-days, one bird per replicate was selected for blood collection. These collections were performed by trained people, through direct puncture of the brachial vein with heparinized syringes to collection of 4 ml of blood from each bird. For flow cytometry analysis, blood was stored in heparinized tubes and transported to the laboratory to be processed, as described by Beirão et al. (2012). Briefly, single staining was performed using the antibodies with a 1:10 dilution of the primary antibody (0.5 mg/mL) in PBS (pH 7.4). Twenty microliters of this dilution were then mixed with 10^6 mononuclear cells and kept at room temperature in the dark for 20 min. After this incubation period, cells were washed with 2 mL of PBS and centrifuged at 400×g for 7 min. Cells received secondary antibody (5 µL of 1:200 dilution) or streptavidin FITC (diluted 1:10 in PBS) according to the protocol. Cells were kept for 20 min at room temperature in the dark and then washed with 2 mL PBS and centrifuged. The final pellet was resuspended in 250 µL of PBS with 1% BSA. All samples went through cytometry within 2 h of staining. Double staining was performed for the assessment of T cell subsets of animals. Flow cytometry was performed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Green fluorescence (from FITC) was detected on the FL1 channel (530/30 nm), and orange fluorescence was detected on the FL2 channel (585/42 nm). Cells were analyzed at up to 10,000 events in the lymphocyte gate based on forward and side scatter, including contaminating thrombocytes (Bohls et al., 2006). Data were analyzed with FlowJo software (TreeStar, Inc, Ashland, OR, USA).

For uric acid and urea analysis, at 42-days, another bird from each replicate was selected, and blood was collected, following the same procedure described before and sent to a hematological laboratory for analysis. Uric acid and urea were determined in individual samples in an automatic analyzer (Mindray®, BS-200, São Paulo-SP, Brazil), and using commercial kits (Quibasa – Quimica Básica LTDA, Belo Horizonte, Brazil).

2.3 Histology

Also, at 21 and 42-days, another bird per replicate was euthanized by cervical dislocation for bursa of Fabricius collection and weighting and for intestinal collection and analysis. Samples of duodenum, jejunum and ileum, 2 cm each, were collected and stored in 10% buffered formaldehyde solution and sent to the laboratory for preparation. Segments were then dehydrated in a graded alcohol series (70%–100%), cleared in xylol and embedded in paraffin. Transverse and longitudinal 5-µm thick sections were prepared using microtome, deparaffinized with xylene, rehydrated, and stained with hematoxylin-eosin.

For goblet cells counting, slides were stained for neutral and acid mucins using Alcian blue (AB) and periodic acid-Schiff (PAS). Slices were deparaffinized in xylene, dehydrated through a graded series of ethanol, and then treated with AB G8X for 5 min. After washing in running tap water, the slices were washed in distilled water and treated with 1% periodic acid for 5 min and kept in dark condition. Then, the slices were washed again in running tap water, treated with Schiff's reagent for 30 min and kept in dark condition. Slices were lightly counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene, and mounted.

Images were obtained with a computer-supported imaging system connected to a light microscope (Leica DM1000, Leica Microsystem, Cambridge, United Kingdom). These measures were made using ToupView 3.7 (ToupTek Photonics, Zhejiang, China) image analysis software.

A total of 10 villi were examined from different sections in each animal. The parameters evaluated were villus height, measured from the tip of the villus to the valley between individual villus, excluding crypt; villus width, measured at one-third and two-thirds of the length of the villus; crypt depth, measured from the valley between individual villus to the basolateral membrane; villus height to crypt depth ratio; villus area, calculated according to Nain et al. (2012), and number of goblet cells per villus. For each slide, five villi were considered. The average of these values per replication was used for statistical analysis.

2.4 Statistical analysis

Data obtained were tested for normality (Shapiro-Wilk) and homogeneity of variance (Bartlett) and when these assumptions were accepted, data were analyzed as a completely randomized design, with eight replicates per treatment. Data were submitted to regression analysis (linear and quadratic) at 5% probability level using the PROC REG of SAS 9.3 (SAS Inst. Inc., Cary, NC). Based on these results, straight broken-line models were estimated for the variables considered potential candidates using the PROC NLIN of SAS. The iterative procedure makes repeated estimates for coefficients and minimizes residual error until the best-fit lines are achieved. The following equation was used:

$Y = L + U \times (R-x)$

Where x = independent variable (dietary CP concentrations), R = CP requirement, y = dependent variable, L = asymptote, and U = slope. R-x is defined as zero when $x > R$. The coefficient of determination of the model (R^2 value) was determined as follows: $R^2 = 1 -$ (residual sum of squares/corrected total sum of squares).

3. RESULTS

Lower levels of dietary CP resulted in a linear increase ($P>0.05$) in the concentration of urea and a linear decrease ($P>0.05$) in the concentration of uric acid at 42 days (Table 2).

TABLE 2 – Urea and uric acid concentrations (mg/dl) in blood of chickens at 42 days.

Item	% Crude protein				SEM^a	P*	
	19	18	17	16		L**	Q***
Urea	1.64	1.71	1.87	2.06	0.066	0.015	0.614
Uric acid	1.43	1.37	1.38	0.97	0.051	0.002	0.060

* P value, ** Linear effect, *** Quadratic effect, ^{a}Standard error of the mean

For performance, reduction in dietary CP levels resulted in a linear decrease on WG and FCR ($P<0.05$) from 1 to 21 days, from 22 to 42 days and during the whole period of the experiment (from 1 to 42 days, Table 3). Despite these linear reductions on performance, the broken-line model found a breakpoint for CP requirement ($P<0.05$) for BWG and FCR at 21 days, estimated to be 21.0058±0.477% and 21.3570±0.434%, respectively (Table 8). In a similar way, at 42 days, the CP requirement for BWG, FCR, and uric acid was estimated to be 17.225±0.3861%, 17.4083±0.414%, and 17.2317±0.252%, respectively ($P<0.05$, Table 8).

TABLE 3 – Performance of broilers from 1 to 21 days, 22 to 42 days, and 1 to 42 days.

Item[a]	WG[c] 1-21	FI[d] 1-21	FCR[e] 1-21	WG[c] 22-42	FI[d] 22-42	FCR[e] 22-42	WG[c] 1-42	FI[d] 1-42	FCR[e] 1-42
19/22.5	969.87	1237.9	1.277	2211.7	3818.9	1.730	3136.0	5005.1	1.598
18/21.5	954.82	1236.6	1.296	2007.6	3691.8	1.849	2962.4	4928.3	1.666
17/20.5	941.98	1239.1	1.315	2103.1	3769.7	1.794	3045.0	5008.8	1.646
16/19.5	901.98	1219.1	1.352	1977.8	3733.6	1.890	2879.5	4952.6	1.721
SEM[b]	10.851	12.169	0.031	41.109	36.311	0.031	45.985	41.729	0.019
P-L*	<0.001	0.340	<0.001	0.005	0.330	0.011	0.002	0.705	<0.001

[a]Dietary crude protein levels for 1-21 days/22-42 days, [b]Standard error of the mean, [c]Weight gain, [d]Feed intake, [e]Feed conversion ratio, *P value for linear effect, P values for quadratic and cubic effects were not significant ($P>0.05$).

In Tables 4 and 5 are presented the results related to immunity at 21 and 42 days, respectively. Reduction in dietary CP resulted in a linear decrease in size of bursa of Fabricius at 42 days ($P<0.05$), but not at 21 days ($P>0.05$). At 21 days, it was observed a linear reduction in CD4-CD8+, CD8+TCRVb1-, CD8+TCRVb1+, and CD8-TCRVb1+ T cells ($P<0.05$). At 42 days, CD4+CD8-, CD8+TCRVb1-, CD8-TCRVb1+, CD8+CD28- T cells presented a quadratic behavior ($P<0.05$), and CD4+CD8+ and CD8+CD28+ T cells presented a linear reduction ($P<0.05$). Other cells did not differ at 21 or 42 days ($P>0.05$). At 42 days, the dietary CP requirement for maximum CD8+TCRVb1-, CD8+CD28-, and CD8+CD28+ T cells response was estimated to be 17.7657±0.886%, 17.2561±0.308%, and 18.2000±1.085%, respectively, by the broken-line model ($P<0.05$, Table 8).

TABLE 4 - Proportion of immune cells in blood and weight of bursa of Fabricius of broilers at 21 days.

Item	% Crude protein				SEM^a	P*	
	22.5	21.5	20.5	19.5		L**	Q***
Cells (%)							
CD4+CD8-	19.07	20.28	19.42	17.55	0.657	0.369	0.258
CD4+CD8+	8.66	8.37	8.86	8.25	0.578	0.890	0.895
CD4-CD8+	6.88	4.71	6.00	3.73	0.426	0.025	0.951
CD8+TCRVb1-	3.19	2.48	1.91	1.41	0.273	0.016	0.837
CD8+TCRVb1+	5.40	4.37	3.76	2.91	0.316	0.004	0.880
CD8-TCRVb1+	21.91	21.04	18.94	16.43	0.693	0.002	0.507
CD8+CD28-	2.62	2.22	3.21	2.08	0.225	0.748	0.415
CD8+CD28+	15.84	17.37	18.55	15.62	0.947	0.950	0.261
CD8-CD28+	6.18	5.99	6.01	4.96	0.456	0.392	0.648
CD4:CD8 ratio	3.55	4.93	3.66	4.96	0.304	0.183	0.815
Leucocytes (µl)	8062	6568	5301	6340	932.9	0.077	0.069
Bursa of Fabricius (g)	2.54	2.39	2.50	2.36	0.097	0.653	0.976

*P value, **Linear effect, ***Quadratic effect, ^{a}Standard error of the mean

TABLE 5 - Proportion of immune cells in blood and weight of bursa of Fabricius of broilers at 42 days.

Item	% Crude protein				SEM^a	P*	
	19	18	17	16		L**	Q***
Cells (%)							
CD4+CD8-	10.93	13.17	11.36	9.49	0.510	0.162	0.040
CD4+CD8+	2.56	2.39	2.13	1.44	0.200	0.047	0.513
CD4-CD8+	5.39	5.31	5.34	4.54	0.427	0.533	0.686
CD8+TCRVb1-	6.19	13.39	8.08	5.05	0.797	0.104	0.001
CD8+TCRVb1+	5.37	5.80	4.65	3.45	0.414	0.065	0.320
CD8-TCRVb1+	8.65	9.53	9.40	6.76	0.430	0.117	0.037
CD8+CD28-	2.44	3.42	2.83	1.00	0.260	0.017	0.003
CD8+CD28+	4.19	5.30	2.83	1.44	0.423	0.002	0.087
CD8-CD28+	12.65	17.27	14.19	12.61	0.811	0.648	0.055
CD4:CD8 ratio	2.37	2.94	2.68	2.14	0.203	0.611	0.189
Leucocytes (µl)	18245	15131	15380	14106	2184.7	0.277	0.231
Bursa of Fabricius (g)	6.39	5.81	5.36	5.06	0.216	0.024	0.743

*P value, **Linear effect, ***Quadratic effect, ^{a}Standard error of the mean

At 21 days there was a linear reduction on villus height and area, crypt depth, villus/crypt ratio, and on number of goblets cells with the reduction of dietary CP levels ($P<0.05$) in duodenum. The same changes were observed in the other intestinal segments, with exception of villus/crypt in jejunum ($P=0.317$) and number of goblet cells ($P=0.647$) in ileum (Table 6). The CP requirement for villus height (duodenum) was estimated to be

20.7386±0.3667% ($P<0.05$, Table 8). However, for villus area, the estimated CP requirement was higher than the levels tested in this study, making impossible to determine the breakpoint.

TABLE 6 - Means of intestinal mucosal morphometry and goblet cells count of broilers at 21 days.

Item	% Crude protein				SEM[a]	P-L*
	22.5	21.5	20.5	19.5		
Duodenum						
Villus height (µm)	1350.2	1236.2	1240.6	1016.1	24.12	<0.001
Villus area (mm^2)	1.48	1.15	0.99	0.82	0.081	0.004
Crypt depth (µm)	179.6	161.0	150.1	148.5	6.34	0.042
Villus/Crypt	9.07	8.20	8.67	6.89	0.38	0.017
Goblet cells[b]	130	110	108	77	5.8	<0.001
Jejunum						
Villus height (µm)	888.0	892.1	859.2	837.4	21.88	0.042
Villus area (mm^2)	0.81	0.72	0.66	0.64	0.076	0.013
Crypt depth (µm)	140.2	125.1	121.4	117.5	7.42	0.028
Villus/Crypt	7.00	7.40	7.40	7.30	0.24	0.317
Goblet cells[b]	100	103	86	89	4.8	0.027
Ileum						
Villus height (µm)	567.5	579.2	559.1	521.6	12.42	0.013
Villus area (mm^2)	0.47	0.46	0.45	0.40	0.044	0.042
Crypt depth (µm)	97.4	118.6	124.1	145.2	3.84	<0.001
Villus/Crypt	6.10	5.10	4.70	3.80	0.188	<0.001
Goblet cells[b]	74	74	81	74	5.1	0.647

[*] P value for linear effect, P values for quadratic and cubic effects were not significant ($P>0.05$), [a]SEM: standard error of mean, [b]Number of cells per villus.

Similar to what happened at 21 days, at 42 days there was a reduction on villus height and area, crypt depth, and on number of goblets cells with the reduction of dietary CP levels ($P<0.05$) in duodenum and jejunum. The same changes were observed in ileum, with exception of the crypt depth, which presented a linear increase ($P=0.015$) and villus/crypt ratio, which presented a quadratic trend ($P=0.043$) with the reduction of dietary CP levels (Table 7). For villus height and area, and goblet cells counts, the breakpoint was not reached in duodenum, jejunum, and ileum, demonstrating that the CP requirements for the maximum response of these variables are greater than the dietary CP levels evaluated.

TABLE 7 - Means of intestinal mucosal morphometry and goblet cells count of broilers at 42 days.

Item	% Crude protein				SEM[a]	P*	
	19	18	17	16		L**	Q***
Duodenum							
Villus height (µm)	1710.6	1533.2	1429.7	1472.9	34.49	<0.001	0.002
Villus area (mm^2)	3.53	1.88	1.58	1.76	0.129	<0.001	<0.001
Crypt depth (µm)	188.6	190.6	173.5	165.7	7.50	<0.001	0.355
Villus/Crypt	9.52	8.38	8.77	8.91	0.525	0.393	0.084
Goblet cells[b]	180	167	139	142	9.9	<0.001	0.326
Jejunum							
Villus height (µm)	1064.7	974.4	939.6	903.7	14.8	<0.001	0.064
Villus area (mm^2)	1.15	0.89	0.75	0.72	0.042	<0.001	0.004
Crypt depth (µm)	146.6	125.4	128.3	106.3	3.46	<0.001	0.815
Villus/Crypt	7.43	7.84	7.61	8.73	0.292	<0.001	0.406
Goblet cells[b]	162	135	116	88	3.6	<0.001	0.879
Ileum							
Villus height (µm)	637.4	591.0	529.4	509.3	12.03	<0.001	0.059
Villus area (mm^2)	0.55	0.45	0.40	0.40	0.024	<0.001	0.197
Crypt depth (µm)	127.6	138.8	143.9	141.2	4.19	0.015	0.098
Villus/Crypt	5.09	4.38	3.72	3.84	0.146	<0.001	0.043
Goblet cells[b]	134	93	86	81	3.8	<0.001	<0.001

[*]P value, [**]Linear effect, [***]Quadratic effect, [a]Standard error of the mean, [b]Number of cells per villus.

A summary of the estimated requirements for CP and the regression equations for the one-slope broken-line models are presented in Table 8 and in supplementary figures 1-9. Results that did not show a breakpoint or presented a low correlation were not presented.

TABLE 8 - Estimated dietary crude protein requirement based on linear broken line model analyses.

Item	Intercept (L)	Slope (U)	Breakpoint (R)	P-value	R^2
21 days					
Weight gain (g)	962.30±7.81	-40.26±15.61	21.0058±0.477	<0.001	0.983
Feed conversion (g)	1.286±0.01	0.035±0.01	21.3570±0.434	<0.001	0.997
Villus height (µm)	1293.60±40.11	-223.70±80.23	20.7386±0.3667	0.002	0.899
42 days					
Weight gain (g)	3049.20±36.29	-165.60±72.59	17.225±0.3861	<0.001	0.650
Feed conversion (g/g)	1.632±0.01	0.075±0.03	17.4083±0.414	0.007	0.788
CD8+TCRVb1- (%)	9.77±0.74	-3.03±0.32	17.7657±0.886	0.047	0.733
CD8+CD28- (%)	2.93±0.31	-1.83±0.63	17.2561±0.308	0.003	0.998
CD8+CD28+ (%)	4.60±0.50	-1.50±1.00	18.2000±1.085	0.002	0.871
Uric acid (mg/dl)	1.40±0.06	-0.41±0.12	17.2317±0.252	<0.001	0.906

4 DISCUSSION

In the present study, it was observed that lower levels of dietary CP resulted in a linear increase in blood urea and a linear decrease in uric acid concentrations. The reduction in blood uric acid concentration was previously observed in other studies, when broilers were fed reduced amounts of dietary CP (Corzo et al., 2005; Namroud et al., 2008; Awad et al., 2015). These results indicate an alteration in protein catabolism when broilers were fed diets with low CP levels. Ammonia is one of the products obtained from amino acid degradation in the liver, and due to its toxicity, it must be converted to less toxic compounds, as uric acid in birds (Campbell, 1991). Lower dietary CP may reduce uric acid concentration in the blood due to a reduction in amino acid degradation in the liver, prioritizing their usage to protein synthesis (Corzo et al., 2005). Besides, it is possible that the reduction in dietary CP resulted in an insufficient amount of glycine to formation of uric acid (Powell et al., 2009).

The linear increase in blood urea concentration may had occurred due to the supplementation of tryptophan in the experimental diets, causing an imbalance between this amino acid and glycine. Similar results were observed by Yuan et al. (2012), studying variation of glycine levels in diets with reduced CP to broilers.

In this study, based on the estimated breakpoints of dietary CP for growth response (1-21 and 22-42 days), we can infer that slightly lower CP levels than the recommendations still can result in the same performance. Until certain reduction in dietary CP, animals can reduce the nitrogen excretion, increasing the available nitrogen for amino acids synthesis, maintaining the same performance (Deschepper & deGroote, 1995). However, with an excessive reduction in dietary CP, even with the supplementation of EAA, animals can present a reduction in performance, as observed in this and other studies (Corzo et al., 2005; Jiang et al., 2005; Awad et al., 2015; Maia et al., 2021). This may indicate that non-essential amino acids (NEAA) have an essential role in muscle deposition, especially glycine (Jiang et al., 2005; Incharoen et al., 2010; Siegert & Rodehutscord, 2019), when dietary nitrogen is limited. EAA requirements were established considering a minimum dietary CP. When the dietary CP is not sufficient to provide nitrogen to NEAA synthesis, it may impair performance and physiological responses (Liu et al., 2021). NEAA can be synthesized from EAA and from the nitrogen pool, however, defining the optimal EAA to NEAA ratio is challenging, varying according to different physiological demands (Herger, 2003; Hou et al., 2015). If the proportion of EAA to NEAA is higher, as expected with the supplementation of EAA in a low CP diet, this may impair performance, probably due to the lack of nonspecific nitrogen needed to synthesize NEAA, increasing EAA utilization for this synthesis (Maia et al., 2021).

The lack of effects of dietary CP restriction on FI demonstrates that broilers could not compensate this deficit increasing consumption, probably due to physical limitations of GIT. Similar results were described by Incharoen et al. (2010) evaluating low-CP diets fed to broiler chickens.

Dietary CP reduction negatively impacted not only performance, but also immune organs weight, as the bursa of Fabricius (Glick et al., 1981; Jahanian, 2009). Related to this reduction, Jahanian (2009) also observed a significant decrease in lymphocytes in peripheral blood, and lower antibodies levels against Newcastle Disease in chickens receiving a reduced level of dietary CP. In our study, although no difference in bursa of Fabriciuis weight had been observed at 21 days, at 42 days, a linear reduction was observed in this organ, in response to decreasing levels of dietary CP. This is one of the main immune organs of chickens and is responsible for antibodies response (Davison et al., 2008).

Even without a significant difference at 21 days in bursa of Fabricius and total leucocytes, a linear reduction in CD4-CD8+, CD8+TCRVb1-, CD8+TCRVb1+, and CD8-TCRVb1+ T cells in blood was observed, following the reduction in dietary CP levels. These cells are cytotoxic T lymphocytes and are associated to pathogens cell death (Zhang & Bevan, 2011).

At 42 days, CD4+CD8-, CD8+TCRVβ1-, CD8-TCRVβ1+, CD8+CD28- T cells presented a quadratic behavior, and CD4+CD8+ and CD8+CD28+ T cells presented a linear reduction with the reduction on dietary CP levels. CD8+TCRVβ1- cells are peripheral cytotoxic cells and are associated with direct attack against infected cells (Davison et al., 2008), and CD8-TCRVβ1+ are considered mucosal helper, inducing IgA production (Cihak et al., 1991). Double positive (CD4+CD8+) cells are immature cells when in thymocytes, but in peripheral blood, little is known about their role (Overgaard et al., 2015). We also found different estimate levels for CP requirements for maximum response of CD8+TCRVβ1-, which represents peripheral cytotoxic cells, indicating differences in peripheral and mucosal immunity in healthy animals (as CD8+TCRVβ1+ presented a trend to an increased requirement). This was observed in quails, which no effect on the amount of intestinal intraepithelial lymphocytes could be observed, probably reflecting the fact that mucosal immunity responds to stimuli quite differently from the peripheral immune system (Zardo et al., 2015). Also, CD8+CD28+ presented the highest requirements. This is the group of naïve CD8 T cells that requires activation to allow the expansion of memory CD8+ T cells, generation of maximal secondary response, and effective clearing of virus. A failure on the activation of these cells could affect secondary responses generated from memory CD8+ T cells induced by infection but also from vaccines and tumors (Borowski et al., 2007).

In rats, when reduced levels of CP are given, it is possible to observe reduction in the ability of replication of CD8 cells, reducing the immune response (Iyer et al., 2012). In mice, immune depletion due to low protein inclusion in the diet implies in severe damage to thymic function, and lymphocytes that are derived therefrom. In this species, the thymus presents an imbalance between the amount of CD4 and CD8 lymphocytes. However, this is not reflected in the same way in the peripheral blood (Lee & Woodward, 1996). Likewise, in the present study the ratio of these lymphocyte types in the blood did not reflect the change in protein in the diet, although it is possible that the thymus underwent major modifications. We did not find other studies that evaluated the effects of reduced dietary CP levels on T lymphocytes on broilers, but these results may indicate a lower ability of these animals to respond to an infection challenge when fed with lower dietary CP levels. With the supplementation of EAA, is expected a reduction in NEAA when CP is reduced. Lower levels of NEAA may impair immune response, once they are involved in the regulation of this response (Hou et al., 2015)

Another fact that may have had a greater impact of dietary CP limitation on animals was the reduction in villus height and area, limiting the intestinal absorptive surface area and nutrient absorption. In this study, we did not find a breakpoint for villus height and area at 21 (with exception of duodenum) and 42 days, making impossible to estimate the CP requirements for maximum response of these variables. Thus, these requirements are probably above the dietary CP levels evaluated. Other studies evaluating the impact of dietary CP restriction in broilers also found a reduction in villus height, with reduced growth response, represented by a lower body weight at slaughter age (Laudadio et al., 2012). However, results about crypt depth and villus to crypt depth ratio are controversial among studies (Sterling et al., 2005; Laudadio et al., 2012; Abbasi et al., 2014) . Deeper crypt and a lower villus to crypt ratio may indicate faster tissue turnover to permit renewal of the villus, which suggests that the host's intestinal response mechanism is trying to compensate for normal sloughing or atrophy of villi due to the limited amino acids content from diet (Laudadio et al., 2012). However, these were observed only in the ileum (mainly at 42 days of age), while a lower crypt dept was found in duodenum and jejunum of broilers fed lower CP diets both at 21 and 42 days in the present study.

Gastrointestinal tissues have high protein requirements to keep the adequate high turnover rate of intestinal epithelium and its functionality (Abbasi et al., 2014). Amino acids play an important role on villus integrity and function mainly due to the production of polyamines, as spermidine, spermine, and putrescine. These polyamines are directly associated with growth and cellular differentiation of the intestine and are responsive to dietary protein levels. Low production of these polyamines inhibits proliferation, migration, and apoptosis of intestinal cells (Fernandes & Murakami, 2010). So, we can assume that the reduced CP diets evaluated were not sufficient to provide optimum villus growth, reflecting on performance, as discussed previously in this study.

The reduction in goblet cells number only in duodenum and jejunum, but not in ileum at 21 days, of broilers fed the lower CP diets are consistent with the other histological findings, being explained due to dietary amino acids restriction. Goblet cells are mucin-producing, which are glycosylated proteins secreted along the intestinal epithelium and are involved in the diffusion and absorption of nutrients and the protection of intestinal mucosa against pathogens and toxins (Abbasi et al., 2014). Considering that the ileum is the small intestinal segment with the greatest immunological challenge from bacteria and endotoxins, due to its proximity of the large intestine, it is possible that it keeps the same amount of goblet cells independently of the dietary CP level (among the CP levels evaluated in this study) to mucosa protection. However, this fact was not observed at 42 days, demonstrating that the compensatory mechanisms may have reached their limit to try to compensate the dietary CP restriction. In this light, ileum from broilers fed the lower CP diets probably presented deeper crypt also to try to compensate goblet cells production.

In this study we observed that estimated CP requirements are lower than that usually recommended by the literature, considering growth response. We found an estimated requirement of 21% for BWG and FCR from 1 to 21 days, while NRC recommendations are 23% (National Research Council [NRC], 1994) and Brazilian Tables for Poultry and Swine are around 23 to 24% (Rostagno et al., 2017) in this initial phase. From 22 to 42 days, we observed the same effect, with an estimated recommendation of 17.2 and 17.4% for BWG and FCR, respectively, while NRC presented a recommendation of 20% (NRC, 1994) and Brazilian Tables for Poultry and Swine are around between 18.5 and 20% (Rostagno et al., 2017). These levels are also above recommendations for some immune response, as observed in this study, even for CD8+CD28+, that had an estimated requirement of 18.2% of CP at 42 days. However, recommended levels by the two major genetic companies in the world are closer to the levels found in this study (17 to 18% CP for Cobb and 18.3% CP for Ross).

In animals in a homeostasis situation, the slight dietary CP restriction with the supplementation of EAA may not represent a big challenge for the immune system, but this may change when animals face a pathogen. This alteration in amino acids and protein needs was reviewed by Le Floc'h et al. (2004) and indicates that body can redistribute amino acids to away from protein production (growth, lactation, etc.) towards tissues involved in inflammation and immune response to synthesize inflammatory and immune proteins, to support immune cell proliferation, and to synthesize other compounds important for body defense functions. This may result in an impaired growth response, or inefficient immune response against pathogens. It is understandable that higher levels of CP may be associated to an increase in nitrogen excretion, with a pollutant effect in the environment (Liu et al., 2021), but an excessive reduction in CP levels may cause other issues that may reduce growth and immune response.

Under the conditions in which this study was conducted, the CP requirements could be slightly reduced, as for WG, FCR, and some immune responses. However, other variables, as villus height and area demonstrated that the CP levels evaluated were not sufficient to present a maximum response. Further studies with higher dietary CP levels are needed to better adjust the requirements for these variables.

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CONFLICT OF INTEREST

Authors declare that there is no Conflict of Interests that could be perceived as prejudicing the impartiality of the research reported.

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CAPÍTULO III - EFFECT OF DIFFERENT DIETARY ENERGY DENSITIES ON BLOOD UREA AND URIC ACID, PERFORMANCE, CARCASS YIELD, IMMUNITY, AND INTESTINAL MORPHOLOGY OF BROILERS*

*Artigo nas normas do Animal Science Journal

Abstract: The present study aimed to investigate the effects of reduction of dietary metabolizable energy (ME) levels on blood urea and uric acid, performance, carcass yield, immune response, and intestinal morphology in broilers. Three different diets were formulated, with normal (3050, 3100, and 3200 kcal/kg), medium (2975, 3000, and 3075 kcal/kg), and low (2900, 2900, and 2950 kcal/kg) ME levels to starter (1-14 days), grower (15-28 days), and finisher (29-42 days) phases, respectively. A total of 750 male Ross chicks were randomly allocated to 30 pens, with 25 birds each, totaling 8 replicates per treatment. Performance was evaluated at 21 and 42 days. Blood and intestine (duodenum, jejunum, ileum) were collected for histology at 21 and 42 days. Carcass yield was evaluated at 42 days. Broilers fed the diets with lower ME levels presented higher concentrations of uric acid in the blood, lower weight gain, higher feed conversion ratio, and lower abdominal fat deposition ($P<0.05$). They also presented higher total leucocytes, CD4+CD8+, CD4-CD8+, CD8+CD28-, CD8+CD28+, CD8-CD28+, CD8+TCRVb1, CD8+TCRVb1+, and CD8-TCRVb1+ at 42 days ($P<0.05$). At 21 days there was a reduction in the villus height and area and crypt depth in the duodenum and jejunum in broilers fed the diets containing the medium and low ME level ($P<0.05$). In conclusion, the reduction in the dietary ME content has a negative impact on broiler performance and on the development of the intestine at the initial growth phase. However, it seems to increase the cellular immune response at 42 days.

Keywords: leucocytes; metabolizable energy; poultry nutrition.

INTRODUCTION

The energetic demands of poultry have been largely studied, due to its importance on performance (Leeson et al., 1996; Rosa et al., 2007). Dietary energy derived from carbohydrates, lipids, and proteins is responsible for about 70% of the production costs. Thus, the variation on dietary energy density becomes even more important when nutritionists need to adjust the formulation to reduce costs. This has been a practice since the 80`s (Jackson et al., 1982), however, besides reducing dietary costs it also may affect the growth response of animals.

It is known that relatively slightly lower levels of dietary energy below the recommendation may impair the performance of broilers, reducing the growth rate (Leeson et al., 1996; Rosa et al., 2007), while higher levels of dietary energy may increase the growth rate (Waldroup, 1981). Besides, the dietary energy restriction may also impact the development of the intestinal mucosa, considering the high energetic demand of the gut (Miao et al., 2017). This demonstrates that maintaining the correct levels of dietary energy is not a simple task, and small changes can impact the dietary cost and broiler performance. Besides, varying levels of dietary energy are related to different deposition rates of crude protein and fat in the carcass (Rosa et al., 2007). A higher level of fat in the carcass is a non-desirable characteristic for the consumers, and this may reduce the carcass value (Tůmová e Teimouri, 2010).

Besides affecting performance, intestinal development, and carcass composition, the energy density of the diet may also influence the immune response. Mounting an immune response when a challenge is present has some energetic costs, and the expenditure of this energy may affect other metabolic processes (Demas et al., 1997; Amat et al., 2006). When an immune challenge occurs, the energetic expenditure is increased (Martin II et al., 2003), allowing an adequate response. However, this may impair the growth of animals (Benson et al., 1993; Lochmiller e Deerenberg, 2000). Although some studies had evaluated the impact of diets with different energy densities on broiler performance and immunity, most of the studies evaluated multiple factors besides energy, such as different protein or amino acids to energy ratios (Golian et al., 2010; Mirzaaghatabar et al., 2011; Fosoul et al., 2016; Perween et al., 2016) and different energy sources (van Heugten et al., 1996; Reuter et al., 2008; Taleb et al., 2017; Omidi et al., 2020). Thus, it is important to isolate the effects of diets with different energy densities alone to better understand its impact on the immune response of broilers. Based on this, the present study was designed to address the effects of reduction of dietary energy levels on blood urea and uric acid, performance, carcass yield, immune response, and intestinal morphology in broilers.

MATERIALS AND METHODS

The experiment was approved by the Ethics Committee on Animal Use of Agrarian Sciences Sector of Federal University of Parana, Brazil.

A total of 750 male Ross chicks were randomly allocated to 30 pens, with 25 birds each. The chicks were housed in floor pens (1.65 × 1.25 m) containing pine shavings throughout the trial. All pens were equipped with one tubular feeder and nipple drinkers. Before assignment, 80 animals were randomly selected and weighted to determine initial body weight, allowing a homogeneous distribution in weight among treatments. The chicks were maintained at temperatures and lightning according to recommendations from Ross Manual to each age.

The animals were fed the experimental diets divided into three phases: starter (1-14 days), grower (15-28 days), and finisher (29-42 days). Experimental diets were formulated with three different metabolizable energy (ME) levels according to the recommendations of Rostagno et al. (2017), followed by reductions of 75 and 150 kcal/kg in starter diets, 100 and 200 kcal on grower diets, and 125 and 250 kcal/kg on finisher diets. Energetic levels are: for the diets with normal levels of ME (3050, 3100, and 3200 kcal/kg to starter, grower, and finisher diets, respectively), for the first reduction (2975, 3000, and 3075 kcal/kg to starter, grower, and finisher diets, respectively), and for the higher reduction (2900, 2900, and 2950 kcal/kg to starter, grower, and finisher diets, respectively). Diets were formulated to meet all the nutritional requirements of broilers, as recommended by Rostagno et al. (2017), except for ME in the reduced ME diets (Table 1). Diets offered and leftovers were weighted weekly to evaluate feed intake (FI) and at 21 and 42 days all birds were weighted to evaluate weight gain (WG) and feed conversion ratio (FCR).

Table 1 - Ingredients and calculated chemical composition of starter, grower, and finisher diets containing different metabolizable energy (ME) levels.

	Starter (1-14 days)			Grower (15-28 days)			Finisher (29-42 days)		
Item	Normal	Medium	Low	Normal	Medium	Low	Normal	Medium	Low
Ingredients (kg)									
Corn	591.16	608.44	600.46	618.75	639.85	652.35	669.15	698.15	708.9
Soybean meal	356.00	353.00	354.00	323.50	321.50	317.00	272.50	267.50	262.7
Soybean oil	20.00	5.7	0.00	27.00	8.00	0.00	34.00	10.00	4.00
Limestone	9.50	9.50	9.50	10.90	10.90	10.90	9.80	9.80	9.80
Dicalcium phosphate	8.40	8.40	8.40	6.40	6.40	6.40	4.20	4.20	4.20
Sodium chloride	4.70	4.70	4.70	4.50	4.50	4.50	4.20	4.20	4.20
L-Lysin	2.74	2.79	2.76	2.25	2.25	2.25	1.40	1.45	1.45
L-Threonine	0.90	0.89	0.89	1.10	1.05	1.05	0.55	0.55	0.55
DL-Met	3.45	3.43	3.44	3.25	3.20	3.20	2.25	2.20	2.20
Choline chloride	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Phytase	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Caulim	0	0	12.70	0	0	0	0	0	0
Salinomycin	0.50	0.50	0.50	0	0	0	0	0	0
Vitamin premix[†]	1.50	1.50	1.50	1.20	1.20	1.20	0.80	0.80	0.80
Mineral premix[‡]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Calculated chemical composition (%)									
Crude protein	22.01	22.00	21.98	20.72	20.78	20.79	18.50	18.50	18.49
Fat	5.00	3.65	3.05	5.43	3.65	3.04	6.22	3.96	3.20
ME (Kcal/kg)[§]	3050	2975	2900	3100	3000	2900	3200	3075	2950
Lysine	1.28	1.28	1.28	1.15	1.15	1.15	0.96	0.96	0.96
Met+cys	0.95	0.95	0.95	0.87	0.87	0.87	0.73	0.73	0.73
Threonine	0.84	0.84	0.84	0.76	0.76	0.76	0.64	0.64	0.64
Tryptofan	0.23	0.23	0.23	0.22	0.22	0.22	0.19	0.19	0.19
Arginine	1.38	1.38	1.38	1.24	1.24	1.24	1.09	1.09	1.09
Calcium	0.84	0.84	0.84	0.80	0.80	0.80	0.70	0.70	0.70
Phosphorus	0.45	0.45	0.45	0.40	0.40	0.40	0.35	0.35	0.35

† Provided per kilogram of diet: vitamin A, 9,000 IU; vitamin D3, 3,000 IU; vitamin E, 30 IU; vitamin K, 1.5 IU; riboflavin, 12 mg; pantothenic acid, 15 mg; niacin, 50 mg; vitamin B12, 0.02 µg; biotin, 0.1 µg; folic acid, 1.5 mg; pyridoxine, 5 mg; and thiamin, 3 mg. ‡Provided per kilogram of diet: Fe, 55 mg; Mn, 100 mg; Cu, 8 mg; Se, 0.15 mg; Zn, 80 mg; I, 1 mg, as ferrous sulfate monohydrate, manganese sulfate, copper sulfate, sodium selenite, zinc sulfate, calcium iodate, respectively. § Calculated according to the equations for metabolizable energy described by Rostagno (2017). Amino acids as digestible content.

Animals had free access to feed and water during the experiment, except by the two hours fasting period prior to blood collection at 42 days for urea and uric acid analysis. This allows a better response from serum uric acid after amino acid utilization (Powell et al., 2009). At 21- and 42-days, one bird per replicate was selected for blood collection. These collections were performed by trained people, through direct puncture of the brachial vein with heparinized syringes to collection of 4 ml of blood from each bird. For flow cytometry analysis, blood was stored in heparinized tubes and transported to the laboratory to be processed, as described by Beirão et al. (2012). Briefly, single staining was performed using the antibodies with a 1:10 dilution of the primary antibody (0.5 mg/mL) in PBS (pH 7.4). Twenty microliters of this dilution were then mixed with 10^6 mononuclear cells and kept at room temperature in the dark for 20 min. After this incubation period, cells were washed with 2 mL of PBS and centrifuged at 400×g for 7 min. Cells received secondary antibody (5 µL of 1:200 dilution) or streptavidin FITC (diluted 1:10 in PBS) according to the protocol. Cells were kept for 20 min at room temperature in the dark and then washed with 2 mL PBS and centrifuged. The final pellet was resuspended in 250 µL of PBS with 1% BSA. All samples went through cytometry within 2 h of staining. Double staining was performed for the assessment of T cell subsets of animals. Flow cytometry was performed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Green fluorescence (from FITC) was detected on the FL1 channel (530/30 nm), and orange fluorescence was detected on the FL2 channel (585/42 nm). Cells were analyzed at up to 10,000 events in the lymphocyte gate based on forward and side scatter, including contaminating thrombocytes (Bohls et al., 2006). Data were analyzed with FlowJo software (TreeStar, Inc, Ashland, OR, USA).

For uric acid and urea analysis, at 42-days, another bird from each replicate was selected, and blood was collected, following the same procedure described before and sent to a hematological laboratory for analysis. Uric acid and urea were determined in individual samples in an automatic analyzer (Mindray®, BS-200, São Paulo-SP, Brazil), and using commercial kits (Quibasa – Quimica Básica LTDA, Belo Horizonte, Brazil).

Also, at 21 and 42-days, another bird per replicate was euthanized by cervical dislocation for intestinal collection and analysis. Samples of duodenum, jejunum, and ileum, 2 cm each, were collected and stored in 10% buffered formaldehyde solution and sent to the laboratory for preparation. Segments were then dehydrated in a graded alcohol series (70%–100%), cleared in xylol and embedded in paraffin. Transverse and longitudinal 5-µm thick sections were prepared using microtome, deparaffinized with xylene, rehydrated, and stained with hematoxylin-eosin.

For goblet cells counting, slides were stained for neutral and acid mucins using Alcian blue (AB) and periodic acid-Schiff (PAS). Slices were deparaffinized in xylene, dehydrated through a graded series of ethanol, and then treated with AB G8X for 5 min. After washing in running tap water, the slices were washed in distilled water and treated with 1% periodic acid for 5 min and kept in dark condition. Then, the slices were washed again in running tap water, treated with Schiff's reagent for 30 min and kept in dark condition. Slices were lightly counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene, and mounted.

Images were obtained with a computer-supported imaging system connected to a light microscope (Leica DM1000, Leica Microsystem, Cambridge, United Kingdom). These measures were made using ToupView 3.7 (ToupTek Photonics, Zhejiang, China) image analysis software.

A total of five villi were examined from different sections in each animal. The parameters evaluated were villus height, measured from the tip of the villus to the valley between individual villus, excluding crypt; villus width, measured at one-third and two-thirds of the length of the villus; crypt depth, measured from the valley between individual villus to the basolateral membrane; villus height to crypt depth ratio; villus area, calculated according to Nain et al. (2012); and number of goblet cells per villus. For each slide, five villi were considered. The average of these values per replication was used for statistical analysis.

At 42 days carcass traits were also evaluated in the euthanized birds. The eviscerated carcass weight was calculated by removing the feathers, blood, head, feet, and organs. The breast and leg muscle were removed from the carcass, trimmed of adipose tissue, and weighed. The abdominal fat was also removed and weighted. The breast, leg muscle, and abdominal fat were expressed as a percentage of the eviscerated carcass weight.

Data obtained were tested for normality (Shapiro-Wilk) and homogeneity of variance (Bartlett) and when these assumptions were accepted, data were analyzed as a completely randomized design, with eight replicates per treatment using the PROC GLM of SAS 9.4 (SAS Inst. Inc., Cary, NC). Means were compared by Tukey`s test.

RESULTS

The lowest level of dietary ME resulted in an increase in serum uric acid ($P<0.05$) when compared to the other treatments. However, no difference ($P>0.05$) was observed for serum urea (Table 2).

Table 2. Urea and uric acid concentrations (mg/dl) in the blood of chickens at 42 days fed with diets containing different levels of metabolizable energy (ME).

Item	Dietary ME (kcal/kg)*			SEM**	P***
	Normal	Medium	Low		
Urea	1.4	1.1	1.2	0.15	0.645
Uric acid	2.6^{b}	2.6^{b}	3.2^{a}	0.44	<0.001

^{a,b} Means in the same row with different superscript letters differ by Tukey`s test (P<0.05).

*Dietary ME level: normal (3050, 3100, and 3200 kcal/kg to starter, grower, and finisher diets, respectively), medium (2975, 3000, and 3075 kcal/kg to starter, grower, and finisher diets, respectively), and low (2900, 2900, and 2950 kcal/kg to starter, grower, and finisher diets, respectively).

**Standard error of the mean, ***P-value.

For performance, reduction in dietary ME levels resulted in a decrease in the WG and an increase in the FCR from 1 to 21 days (P<0.05) and from 22 to 42 days (P<0.05). No difference for FI was observed during the entire experimental period (Table 3). At the 42 days, the broilers fed diets with lower ME presented lower abdominal fat deposition (P<0.05, Table 3).

Table 3. Performance from 1 to 21 and from 22 to 42 days and carcass yield at 42 days (%) of broilers fed with diets containing different levels of metabolizable energy (ME).

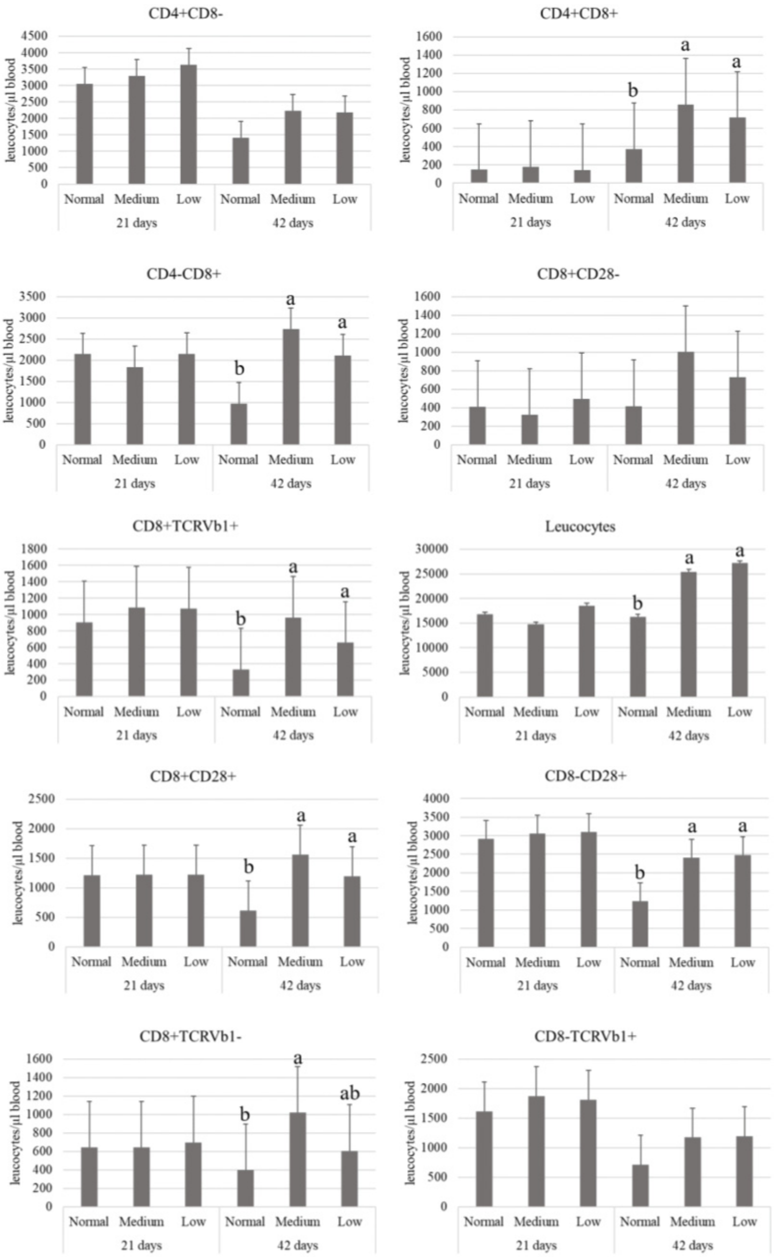
Item	Dietary ME (kcal/kg)*			SEM**	P***
	Normal	Medium	Low		
0-21 days					
Weight gain	789.33^{a}	740.07^{b}	722.17^{b}	12.345	0.003
Feed intake	1076.11	1079.37	1083.87	17.080	0.949
Feed conversion ratio	1.39^{b}	1.44^{ab}	1.50^{a}	0.018	<0.001
22-42 days					
Weight gain	3012.48^{a}	2858.69^{b}	2730.36^{c}	31.668	<0.001
Feed intake	4498.97	4484.26	4448.42	63.148	0.845
Feed conversion ratio	1.52^{b}	1.55^{b}	1.63^{a}	0.011	<0.001
Carcass yield at 42 days					
Breast	38.17	39.48	38.77	1.079	0.494
Leg	26.28	26.44	25.21	0.583	0.280
Abdominal fat	1.64^{a}	1.27^{ab}	1.09^{b}	0.163	0.037

^{a,b} Means in the same row with different superscript letters differ by Tukey`s test ($P<0.05$).

*Dietary ME level: normal (3050, 3100, and 3200 kcal/kg to starter, grower, and finisher diets, respectively), medium (2975, 3000, and 3075 kcal/kg to starter, grower, and finisher diets, respectively), and low (2900, 2900, and 2950 kcal/kg to starter, grower, and finisher diets, respectively).

**Standard error of the mean, ***P-value

The results obtained for immunity are presented in Figure 1. The main effects of energetic restriction on immunity were observed at 42 days. It was observed an increase ($P<0.05$) in total leucocytes, CD4+CD8+, CD4-CD8+, CD8+CD28-, CD8+CD28+, CD8-CD28+, CD8+TCRVb1, CD8+TCRVb1+, and CD8-TCRVb1+ in broilers receiving diets with lower ME levels.



^{a,b} Means with different superscript letters at the same age differ by Tukey`s test ($P<0.05$).

Figure 1. Total leucocytes, CD4, CD8, CD28, and TCRVb1 concentrations in the blood of broiler chickens at 21 and 42 days fed with diets containing different levels of metabolizable energy (ME).

At 21 days there was a reduction in the villus height and area and crypt depth ($P<0.05$) in the duodenum and jejunum in broilers fed the diets containing the medium and low ME level

(Table 4). No differences were observed in the ileum ($P>0.05$). At 42 days, no difference was observed among treatments for any intestinal segment ($P<0.05$, Table 5).

Table 4. Means of intestinal mucosal morphometry and goblet cells count of broilers at 21 days fed with diets containing different levels of metabolizable energy (ME).

Item	ME (kcal/kg)*			SEM**	P***
	Normal	Medium	Low		
Duodenum					
Villus height (μm)	2109^{a}	1836^{b}	1865^{b}	73.9	0.031
Villus area (mm^2)	1.30^{a}	1.04^{b}	1.07^{b}	0.114	0.022
Crypt depth (μm)	295.5^{a}	223.9^{b}	233.2^{b}	15.06	0.006
Villus/Crypt	7.3	8.4	8.1	0.48	0.256
Goblet cells****	138	141	136	9.4	0.921
Jejunum					
Villus height (μm)	1078^{a}	792^{b}	785^{b}	55.9	0.016
Villus area (mm^2)	0.55^{a}	0.30^{b}	0.29^{b}	0.095	<0.001
Crypt depth (μm)	169.8^{a}	112.1^{b}	120.6^{b}	10.16	0.001
Villus/Crypt	6.6	7.1	6.7	0.45	0.678
Goblet cells****	89	91	90	7.9	0.986
Ileum					
Villus height (μm)	543	518	518	27.5	0.753
Villus area (mm^2)	0.21	0.19	0.20	0.014	0.569
Crypt depth (μm)	110.9	108.8	112.7	8.00	0.941
Villus/Crypt	5.0	4.8	4.6	0.24	0.628
Goblet cells****	68	62	71	3.7	0.246

[a,b] Means in the same row with different superscript letters differ by Tukey`s test ($P<0.05$).

*Dietary ME level: normal (3050, 3100, and 3200 kcal/kg to starter, grower, and finisher diets, respectively), medium (2975, 3000, and 3075 kcal/kg to starter, grower, and finisher diets, respectively), and low (2900, 2900, and 2950 kcal/kg to starter, grower, and finisher diets, respectively).

**Standard error of the mean, ***P-value, ****Number of cells per villus.

Table 5. Means of intestinal mucosal morphometry and goblet cells count of broilers at 42 days fed with diets containing different levels of metabolizable energy (ME).

Item	ME (kcal/kg)*			SEM**	P***
	3200	3075	2950		
Duodenum					
Villus height (μm)	1716	1765	1646	58.1	0.363
Villus area (mm^2)	0.83	0.82	0.77	0.05	0.616
Crypt depth (μm)	191.7	169.9	165.0	9.64	0.139
Villus/Crypt	9.1	10.7	10.2	0.69	0.260
Goblet cells****	127	140	141	8.4	0.453
Jejunum					
Villus height (μm)	1175	1091	1133	49.9	0.505
Villus area (mm^2)	0.53	0.43	0.45	0.04	0.192
Crypt depth (μm)	143.3	140.9	141.0	7.10	0.966
Villus/Crypt	8.4	7.8	8.1	0.44	0.665
Goblet cells****	115	98	113	7.3	0.253
Ileum					
Villus height (μm)	603	606	607	34.9	0.996
Villus area (mm^2)	0.27	0.25	0.26	0.020	0.840
Crypt depth (μm)	116.0	119.3	110.1	8.36	0.738
Villus/Crypt	5.3	5.1	5.7	0.29	0.411
Goblet cells****	83	82	82	7.0	0.997

^{a,b} Means in the same row with different superscript letters differ by Tukey`s test ($P<0.05$).

*Dietary ME level: normal (3050, 3100, and 3200 kcal/kg to starter, grower, and finisher diets, respectively), medium (2975, 3000, and 3075 kcal/kg to starter, grower, and finisher diets, respectively), and low (2900, 2900, and 2950 kcal/kg to starter, grower, and finisher diets, respectively).

**Standard error of the mean, ***P-value, ****Number of cells per villus.

DISCUSSION

Our study demonstrated that dietary energy restriction affects the performance and some physiological and morphological responses of broiler chickens. For example, an increase in blood uric acid was observed with the reduction of dietary energy. Similar results were observed by Hu et al. (2021) evaluating diets with different ME densities for broiler chickens. Uric acid is the major avian nitrogenous waste product and an important antioxidative agent (Dawson et al., 1991; Harr, 2002; Juráni et al., 2004). It is known that feed restriction may result in an increase of serum uric acid, indicating that amino acids are being mobilized to compensate for the energy deficit (Pastro and Hunt, 1971; Mbugua et al., 1985). The increase in blood uric acid may be related to an imbalance between energy and protein in the diet. This imbalance usually alters performance and muscle deposition (Zaman et al., 2008), being the increase in the serum uric acid a possible indicator of it. In fact, a reduction in the WG and the highest FCR were observed in broilers fed with diets containing lower ME level. Similar results were observed by other authors (Cherry et al., 1978; Niu et al., 2009; Golian et al., 2010; Harrington et al., 2015; Govil et al., 2017; Zhao & Kim, 2017; Hu et al., 2021), indicating that energy is crucial to ensure the expected growth of chickens. Besides, in the present study, the animals were not able to increase the FI to compensate for the dietary ME restriction. Usually, higher levels of dietary ME stimulate higher consumption (Golian et al., 2010), but lower levels do not affect FI (Govil et al., 2015). This may be related to their genetic selection for high FI, are not able to regulate the ingestion according to the dietary energy density (Massuquetto et al., 2020).

Regarding the immune response, the results bring some interesting data. In general, higher levels of dietary energy resulted in lower counts of immune cells, especially those from CD8 lineage. This may indicate that excessive energy may impair immune response, reducing the capacity of animals to defend against pathogens. Only a few works correlate energy levels and immune response in chickens (Fanooci & Torki, 2010; Yang et al., 2015; Taleb et al., 2017). In humans, as higher levels of energy consumption are associated to obesity (Ledikwe et al., 2006), and it has a direct effect on immunity (de Heredia et al., 2012). This is an important issue and usually is correlated to several diseases in humans and companion animals. In this case, higher levels of dietary energy induce an inflammatory status, that increases proinflammatory cytokines, associated with higher circulating immune cells (Karalis et al., 2009). This was also observed in ducks, with an increase in proinflammatory cytokines in animals fed high-energy diets, when challenged with lipopolysaccharides (Bai et al., 2019). In farm animals, usually, the energy density of the diet is controlled to provide enough energy for maintenance, growth, and/or production with the best possible cost. Sometimes this energy may be reduced to control excessive weight, to compensate for the low availability of raw materials, or even to reduce production costs. In chickens, the reduction of dietary energy may not affect (Fanoooci e Torki, 2010; Haunshi et al., 2012), or even reduce immune response (Hangalapura et al., 2005). Taleb et al. (2017) also found similar results, with no effect on lymphocytes and reduction on other immune cells in broilers fed low-energy diets. The main effect observed by these authors was the reduction in antibodies against Newcastle Disease and Infectious Bursal Disease with the lowest level of energy. All these results differ from the result obtained in the present study. Only two other studies found reduction in immunity related to lower levels of energy (Praharaj et al., 1997; Golian et al., 2010), but in this case, protein levels are also changing, and this may also impair immune response. In the case of Praharaj et al. (1997), there was also observed a higher mortality rate in animals receiving high energy and protein in diets, and this may be related to the higher growth, or to a better capacity of adaptation of animals receiving lower dietary nutrients (Ganeshan et al., 2019)

As described before, we observed an important reduction in cell counts when higher levels of dietary energy were fed, while would be expected an increase in immune response due to inflammatory status caused by the adipocytes, as observed in humans (Karalis et al., 2009; de Heredia et al., 2012), or even no difference among the levels of dietary energy (Fanoooci e Torki, 2010). The reduction mainly affects the CD8 T cell lineage (CD4+CD8+, CD4-CD8+, CD8+TCRVb1+, CD8+CD28+, and CD8+TCRVb1-), that are cytotoxic T lymphocytes and are associated to pathogens cell death (Zhang & Bevan, 2011). The most interesting result found is the increase in CD8+TCRVβ1- T cells, that are peripheral cytotoxic cells and are associated with direct attack against infected cells (Davison et al., 2008). These cells, in conjunction with the CD4-CD8+ and CD8+CD28+ may indicate that these animals are facing a challenge, and due to it, present higher levels of CD8 subset.

The energetic costs of immune response in birds may vary between 5 and 15% of the maintenance energy needs (Hasselquist e Nilsson, 2012), and a prolonged period with reduced dietary energy may result in a readaptation of the immune system to increase cell production. One possibility for this change may be related to the selection of lineages of broiler chickens for growth performance. When a new lineage is compared to an old one, the relative weight of immune organs of the old lineage is higher (Cheema et al., 2003). This suggests that actual lineages had been selected for a higher growth performance, and with the adequate nutrition, animals will preferably direct nutrients for muscle deposition. However, with an imbalance between protein and energy, that in this experiment is represented by the increase in uric acid, animals may utilize the excessive protein to produce energy and create a more consistent immune response, as observed in this study. Thus, is important that further studies better evaluate the effects of dietary energy density in the immune response of broilers.

The different dietary energy densities also affected the intestinal morphology of broilers at 21 days, with a significative reduction in villus height and crypt depth. Miao et al. (2017) also found lower villus height in broilers receiving lower dietary energy at same age. Zou et al. (2013) found lower crypt depth and higher villus/crypt ratio in the duodenum and higher villus height and villus/crypt ratio in the ileum of broilers, differing from our results. However, these authors utilized lower dietary energy levels and utilized some feed enzymes as part of the study. Alteration in villus height and crypt depth of broilers receiving lower levels of dietary energy were observed by other authors at 39 (Ivanovich et al., 2017) and 42 days (Ceylan et al., 2021), differing from our results. However, in the study of Ceylan et al. (2021), they have utilized guanadinoacetic acid, that have a beneficial effect on intestinal growth, and this may have improved the intestinal health of animals receiving lower levels of dietary energy. In the case of Ivanovich et al. (2017), the dietary energy was reduced until 10 days of age, indicating that the animals can present a compensatory growth of intestines when normal levels of energy are reestablished. Yang et al. (2009) found lower intestinal weight at 14 days, but no difference was observed at 42 days. Also, Palo et al. (1995) found lower jejunal protein, RNA and DNA content in jejunum of chickens with feed restriction from 7 to 14 days, but no difference was observed at 41 days. These results are similar to those found in the present study, indicating that animals can compensate the lower intestinal development that initially occurred due to the lower levels of dietary energy. When lower dietary energy is available, the white adipose tissue is mobilized, and this is associate with the release of signalizing molecules that will stimulate feed intake and intestinal growth (Nilaweera & Speakman, 2018).

It is important to point out that the growth of the intestines is higher when compared to the whole body at the first 10 days of life of chickens (Sklan, 2001). Also, the relative weights of intestinal segments are maximum during the first two weeks of the life of broilers, having a rapid decline after. But even with this decline, the intestinal mass maintains a slower growth rate (Ravindran et al., 2006). Any nutritional restrictions in this period may impair intestinal development (Geyra et al., 2001), causing a reduction in performance.

CONCLUSION

Lower dietary energy than the recommendations negatively affect the performance in the overall period and the intestinal development at 21 days of the broilers. However, the immune response seems to be higher in broilers fed diets with lower ME content.

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