

RAFAEL DE SOUSA FERREIRA

**PROTEIN LEVELS AND ESSENTIAL-TO-TOTAL NITROGEN RATIO IN DIETS
FOR BROILER CHICKENS**

Thesis submitted to the Animal Science Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Arele Arlindo Calderano

Co-advisers: Luiz Fernando Teixeira Albino
(*in memoriam*)

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Arele Arlindo Calderano
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To God, my family, and Luiz Albino.

BIOGRAPHY

Rafael de Sousa Ferreira, son of Maria de Fátima Domingos de Sousa and José Ribamar Alves Ferreira, was born in Sobral, Ceará state, Brazil, on February 25th 1994.

He started studying for an undergraduate degree in Animal Science at Universidade Estadual Vale do Acaraú (Sobral, Ceará state, Brazil) in July 2012, obtained a Bachelor of Science in Animal Science in July 2017.

In August 2017, started the Master's degree with a major in Animal Production in Animal Science at Universidade Federal do Ceará (Fortaleza, Ceará state, Brazil) and defended his dissertation in September 2019.

In August 2020, he started his PhD program in Animal Science with a major in Poultry and Swine nutrition and production at UFV. On March 22nd 2024, he submitted his defense to the thesis committee to obtain the Doctor of Philosophy degree in Animal Science.

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Seus pensamentos são como cavalos alados,
aprenda a dominá-los senão tomarão rumos
indefinidos

Autor desconhecido

ABSTRACT

Ferreira, Rafael de Sousa, D.Sc., Universidade Federal de Viçosa, march, 2024.
Protein levels and essential-to-total nitrogen ratio in diets for broiler chickens.
Adviser: Arele Arlindo Calderano. Co-advisers: Luiz Fernando Teixeira Albino

Two experiments were carried out to evaluate the effects of essential: total nitrogen ratios (eN-to-tN) in reduced crude protein (CP) diets supplemented with non-essential amino acid (NEAA) (experiment I), and NEAA mixture and mixture and non-protein nitrogen (experiment II) on performance, nitrogen retention, metabolizable energy values, and amino acid digestibility from broiler chickens from 12 to 23 days of age. In the first experiment, 576 broilers were used and allocated to eight treatments: T1 (positive control) - 220 g/kg CP and 0.50 eN-to-tN ratio; T2 - 200 g/kg CP (0.54 eN-to-tN); T3- 200 g/kg CP + 9.84 g/kg NEAA mixture (0.52 eN-to-tN); T4 - 200 g/kg CP + 18.51 g/kg NEAA mixture (0.50 eN-to-tN); T5 - 190 g/kg CP (0.50 eN-to-tN); T6 - 190 g/kg CP + 14.92 g/kg NEAA mixture (0.54 eN-to-tN); T7 - 190 g/kg CP + 22.93 g/kg NEAA mixture (0.52 eN-to-tN); T8 - 190 g/kg CP + 31.58 g/kg NEAA mixture (0.50 eN-to-tN). Decreasing eN-to-tN ratio from 0.58 to 0.50 improves the final body weight, body weight gain, and feed conversion ratio of broilers from 12 to 23 d post-hatching. A high retained nitrogen was observed on broilers fed diet with eN-to-tN ratio of 0.58 or 0.54 when compared to broilers fed eN-to-tN ratio of 0.50. Reducing CP from 220 to 200 or 190 g/kg with supplementation of NEAA, reaching eN-to-tN ratio of 0.58 to 0.50 resulted in higher metabolizable energy utilization and improved amino acid digestibility. In the second experiment, 648 broilers were used and allocated to eight treatments. The first treatment was a positive control with 220 g/kg CP and eN-to-tN ratio of 0.50. The second treatment was a negative control with 190 g/kg CP and an eN-to-tN ratio of 0.58. From negative control, other seven dietary treatments were formulated, supplementing the diets with 190 g/kg CP with different levels of NEAA mixture (60% Ala, 20% Gly, 20% Glu) and non-protein nitrogen (urea, ammonium acetate, monoammonium phosphate). The results show that reducing CP from 220 to 190 g/kg without supplementation of NEAA mixture or non-protein nitrogen compromised ($P < 0.05$) the growth performance of broilers. However, a decrease in nitrogen intake and nitrogen excretion was observed in the negative control treatment. Metabolizable energy values decreased when negative control treatment was supplemented with NEAA mixture combined with ammonium acetate or

monoammonium phosphate. Apparent and standardized ileal digestibility of some amino acids increased when there was a reduction of CP when compared to those fed with positive control treatment. The supplementation of NEAA and/or non-protein nitrogen seems to be efficient in a diet with reduced CP, allowing a reduction from 220 to 190 g/kg of CP and achieve an eN-to-tN of 0.50.

Keywords: amino acids; broilers; ideal protein; nitrogen

RESUMO

Ferreira, Rafael de Sousa Ferreira, D.Sc., Universidade Federal de Viçosa, março de 2024. **Níveis de proteína e relação nitrogênio essencial: nitrogênio total em dietas para frangos de corte.** Orientador: Arele Arlindo Calderano. Coorientadores: Luiz Fernando Teixeira Albino

Dois experimentos foram realizados para avaliar os efeitos das relações de nitrogênio essencial: total (Ne:Nt) em dietas com redução de proteína bruta (PB) suplementadas com aminoácidos não essenciais (AANE) (experimento I) e mistura de AANE e nitrogênio não proteico (experimento II) sobre desempenho, utilização de nitrogênio, valores de energia metabolizável e digestibilidade de aminoácidos em frangos de corte de 12 a 23 dias de idade. No primeiro experimento foram utilizados 576 frangos de corte distribuídos em oito tratamentos: T1 (controle positivo) - 220 g/kg de PB e relação 0,50 Ne:Nt; T2 - 200 g/kg de PB (0,54 Ne:Nt); T3 - 200 g/kg de CP + 9,84 g/kg de mistura de AANE (0,52 Ne:Nt); T4 - 200 g/kg de CP + 18,51 g/kg de mistura de AANE (0,50 Ne:Nt); T5 - 190 g/kg de PB (0,50 Ne:Nt); T6 - 190 g/kg de CP + 14,92 g/kg de mistura de AANE (0,54 Ne:Nt); T7 - 190 g/kg de CP + 22,93 g/kg de mistura de AANE (0,52 Ne:Nt); e T8 - 190 g/kg de CP + 31,58 g/kg de mistura de AANE (0,50 Ne:Nt). A redução da relação Ne:Nt de 0,58 para 0,50 melhorou o peso corporal final, o ganho de peso corporal e a conversão alimentar de frangos de corte de 12 a 23 dias de idade. Observou-se maior retenção de nitrogênio em frangos alimentados com dieta com proporção Ne:Nt de 0,58 ou 0,54 quando comparados a frangos alimentados com proporção Ne:Nt de 0,50. A redução da PB de 220 para 200 ou 190 g/kg com suplementação de AANE, atingindo a relação Ne:Nt de 0,58 a 0,50 resultou em maior utilização de energia metabolizável e melhor digestibilidade de aminoácidos. No segundo experimento foram utilizados 648 frangos de corte distribuídos em oito tratamentos: T1 (controle positivo) - 220 g/kg de PB e relação Ne:Nt de 0,50; T2 – (controle negativo) - 190 g/kg de PB e uma relação Ne:Nt de 0,58. A partir do controle negativo, foram formulados outros sete tratamentos dietéticos, complementando as dietas com 190 g/kg de PB com diferentes níveis de mistura de AANE (60% alanina, 20% glicina, 20% glutamato) e nitrogênio não proteico (uréia, acetato de amônio, fosfato monoamônico). A redução da proteína bruta de 220 para 190 g/kg sem suplementação com mistura AANE ou nitrogênio não proteico comprometeu ($P < 0,05$) o desempenho de crescimento dos frangos de corte. No entanto, foi observada uma

diminuição na ingestão e excreção de nitrogênio no tratamento controle negativo. Os valores de energia metabolizável diminuíram quando o tratamento controle negativo foi suplementado com mistura de AANE combinada com acetato de amônio ou fosfato monoamônico. A digestibilidade ileal aparente e padronizada de alguns aminoácidos aumentou quando houve redução da proteína bruta quando comparados aos alimentados com tratamento controle positivo. A suplementação de AANE e/ou nitrogênio não proteico parece ser eficiente em uma dieta com PB reduzida, permitindo redução de 220 a 190 g/kg e alcance de Ne:Nt de 0,50.

Palavras-chave: aminoácidos; frango de corte; nitrogênio; proteína ideal

SUMMARY

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INTRODUCTION

The current broiler chicken presents faster growth and an expressive weight gain and this all happened to a better knowledge of the productive processes and nutrient metabolism, such as amino acids (Applegate and Angel, 2014; Bailey 2020). Amino acids can be classified as essential (EAA) and non-essential amino acids (NEAA). EAA (lysine, methionine, threonine, valine, etc) are described as amino acids, that animals cannot produce, or they cannot synthesize at their maximum performance. Therefore, these amino acids should be added to the diet, while NEAA (glycine, glutamine, alanine, glutamate) can be produced by animals and do not need to be added to the diet (Lopez, 2022; Hou and Wu, 2017).

Aiming to reduce feed costs, related to less environmental impact and positive productive effect in the production of broilers, “the ideal protein concept” has been used, where diets with dietary reduction of crude protein can present an ideal balance of EAA for birds, through the dietary addition of crystalline amino acids (Wu et al., 2013). The use of the ideal protein concept was initially proposed by Wa et al. (1951) aiming at ideal proportions and amounts of EAA in the diet of chickens. Apart from this concept, there has been a great deal of interest in increasing the inclusion rates of crystalline amino acids such as lysine and methionine in low protein diets. (Liu and Selle, 2017). However, some studies have shown that birds fed diets with reduced protein and the addition of crystalline EAA did not show the same results as those fed a high-protein diet (Deschepper and Groote, 2007; Van Harn et al., 2019; Chrystal et al., 2020).

According to Aftab et al. (2006), the performance of birds can suffer damage with the reduction of crude protein due to factors such as: changes in the electrolyte balance of the diet due to the reduction in potassium concentrations caused by the reduction in soybean meal; antagonism caused by competition for the site of intestinal absorption; deficiency of EAA; low non-specific nitrogen supply for the synthesis of NEAA; reduction of voluntary consumption; low supply of NEAA such as glycine.

Therefore, the ideal protein concept has been changed. It is necessary to supplement diets with reduced dietary protein levels with EAA and also including NEAA. Stranks et al. (1998) redefined this conception as being a “correct balance between the EAA and NEAA.” The supply of NEAA in these diets is important because the non-specific nitrogen is reduced when the level of protein it is reduced, where non-

specific nitrogen is used for the synthesis of NEAA (Heger, 2003). EAA can be diverted from protein synthesis to catabolism playing a role as a nitrogen source (Wu et al., 2013). However, Wu and Li (2022) mention that EAA is not an efficient source of non-specific N for the synthesis of NEAA, and when EAA is used for it, it can result in a negative effect on the growth performance of broilers.

Some studies show the effect of supplying reduced crude protein diet with NEAA. Supplementing low-crude protein diet with a mixture of NEAA (glycine, glutamate, proline, alanine, and aspartate), Awad et al. (2015) reported that it can improve broilers' performance when compared to those fed low crude protein diet without this supplementation. To Dean et al. (2006) the supplementation individually of glutamate, proline, alanine, and aspartate in low crude protein diet (160 g/kg) improved the feed conversion ratio of broilers from 1 to 18 d post hatch when comparable to birds receiving a higher-CP diet (220 g/kg CP). According to He et al. (2021), a mixture of NEAA seems to be a more efficient source of non-specific nitrogen in a diet with reduced dietary crude protein.

Another source of non-specific nitrogen that can be used in low crude protein diet is called "non-protein nitrogen", as urea. Using non-protein nitrogen in non-ruminant nutrition has been reported by some authors, but there are disagreements about its use. Some authors related that using urea at higher levels presents a toxic effect on the growth performance of broilers (Shahzad et al., 2012), and others mentioned that using urea at low levels can have a positive effect on the growth performance of chicks (Chowdhury et al. 1996). Given this information, these works aim to evaluate the effects of supplementing diets with reduced crude protein with a mixture of amino acids (glycine, glutamate, and alanine) and non-protein nitrogen (urea, ammonium acetate, monoammonium phosphate) on productive parameters, as well as on nitrogen utilization, metabolizable energy, and amino acid digestibility in broiler chickens aged 12 to 23 days.

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Manuscript 1

Response of broiler chickens to essential-to-total nitrogen ratio in reduced crude protein diet supplemented with non-essential amino acids

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Simples Summary

Reducing dietary protein in broiler feed and adding essential amino acids is an established strategy to reduce costs and impact on the environment. However, some studies have shown that the addition of non-essential amino acids is needed to prevent a decrease in the growth performance of the broilers. Thus, the aim of this study was to evaluate the effect of essential-to-total nitrogen ratios in reduced protein diets supplemented with non-essential amino acids on the performance and nitrogen retention of broilers, as well as, metabolizable energy values and amino acids digestibility of the diets. Broilers fed essential-to-total nitrogen (eN:tN) ratio of 0.58, 0.54, and 0.52 show a lower final body weight, body weight gain, and a worse feed conversion ratio when compared to broilers fed diet with 0.50 eN:tN ratio. The nitrogen retention was higher on diet with 0.58 or 0.54 eN:tN ratio. Metabolizable energy and amino acids digestibility were higher on diets with a reduction of crude protein and supplementation with a mixture of glutamine, glycine, and alanine, reaching different eN:tN ratio. Based on the results, it is possible to reduce dietary crude protein from 220 to 190 g/kg. However, these diets need to be supplemented with non-essential amino acids to reach an optimum eN:tN ratio of 0.50 without deleterious consequences on live performance of chicks.

Abstract

This study aimed to evaluate the effects of essential-to-total nitrogen ratios in low-protein diets supplemented with non-essential amino acids on the performance, nitrogen retention, metabolizable energy values, and amino acid digestibility of broiler chickens from 12 to 23 d post hatch. 576 male broilers were fed eight dietary treatments with different essential-to-total nitrogen (eN:tN) ratio (0.58, 0.54, 0.52, and 0.50). Decreasing eN:tN ratio from 0.58 to 0.50 improved the final body weight, body weight gain, and feed conversion ratio of broilers from 12 to 23 d post hatching. A high retained nitrogen was observed on broilers fed diet with eN:tN ratio of 0.58 or 0.54 when compared to broilers fed eN:tN ratio of 0.50. Reducing crude protein from 220 to 200 or 190 g/kg with supplementation of non-essential amino acids, reaching eN:tN ratio of 0.58 to 0.50 resulted in higher metabolizable energy utilization and improved amino acids digestibility. We can reduce the crude protein in up to 30 g/kg of the diet of broilers without compromising their productive efficiency, but a non-specific source

of nitrogen must be supplemented and low crude protein diet must have an eN:tN ratio of 0.52 or 0.50.

Keywords: amino acids; low protein diet; ideal protein; nitrogen efficiency

1. Introduction

The ideal protein concept used in the nutrition of non-ruminants helped to reduce feed costs and decrease nitrogen excretion, by providing the animal a diet with lower crude protein (CP) content and ideal amounts of amino acids [1]. However, when dietary CP is reduced and just essential amino acids (EAA) are added, non-essential amino acids (NEAA) can become limiting. Although cells can synthesize NEAA and their supplementation is considered dispensable [2,3], the nitrogen from the EAA for this synthesis can be not sufficient to meet the ideal metabolic and functional needs under normal or stress conditions [4]. Then, the deficiency of some NEAA, as glutamate and glycine, may result in lower animal productive efficiency [2,3,4,5,7].

According to some studies, the supplementation of NEAA in low CP diets is necessary to obtain good performance parameters (weight gain and feed conversion ratio) [4,8] in addition to contributing to animal health and welfare [9,10]. Dean et al. [11] report that the supplementation of Gly, Glu and Ala in diets with a CP reduction of 160 g/kg formulated based on corn and soybean meal, can improve the growth performance of broilers, when compared with diets with high protein content (220 g/kg CP). Thus, low-CP diets must have a correct ratio of EAA and NEAA [12,13,14,15], defined by an optimum essential-to-total nitrogen (eN:tN) ratio.

Defining eN:tN ratio it is important to indicate a sufficient amount of EAA and NEAA in low protein diets aimed at obtaining maximum growth related to better protein utilization [13]. To attain an optimum eN:tN ratio, the knowledge of dietary N concentration is needed where high and low eN:tN ratios have an adverse effect on performance [13]. There are few studies aimed at estimating an optimum eN:tN ratio in chicks. Stucki and Harper [15] using proline, glycine, and glutamic acid concluded the optimum eN:tN ratio to broilers was 0.57. Heger [15] attributes better weight gain and greater nitrogen retention in broilers fed diets containing an optimum eN:tN ratio from 0.50 to 0.60. In a recent study with broiler chickens aged from 8 to 21 days, fed diets supplemented with a mixture of glycine (gly), glutamate (glu), and alanine (ala),

Maia et al. [16] reported the low CP diets should have an optimum eN:tN ratio of 0.50 to maintain the growth performance and nitrogen utilization of broilers.

Although there are studies addressing the eN:tN ratio on productive and nitrogen efficiency in broiler chickens, the effects of the eN:tN ratio on metabolizable energy and amino acid digestibility have not yet been studied. This study hypothesizes that an optimal eN:tN ratio to improve performance, nitrogen retention, metabolizable energy, and amino acids digestibility can be reached by reducing dietary CP and supplementing this diet with a mixture of NEAAs. Thus, an experiment was carried out to evaluate the effects of eN:tN ratios in reduced CP diets supplemented with NEAAs on performance, nitrogen retention, metabolizable energy values, and amino acid digestibility from broiler chickens from 12 to 23 days of age.

2. Materials and Methods

The Ethics Committee in the Use of Farming Animal at the Federal University of Viçosa (CEUA – UFV) approved all animal handling procedures (protocol number 22/12.05.2020), in compliance with the ethical principles of animal experimentation established by the Conselho Nacional de Controle de Experimentação Animal (CONCEA).

Birds, Diets and Experimental Design

One-day-old male broilers Cobb 500 were obtained from a commercial hatchery where all chicks were vaccinated against Marek's Disease (day 18 via in ovo administration) and Newcastle Disease and Infectious bronchitis (via coarse pray at hatch). They were reared on floor pens covered by litter until the 11th day and fed with a standard starter diet (3,065 kcal/kg, 220 g/kg CP) based on corn-soybean meal as mash, formulated following the recommendations of Rostagno et al. [17]. From d12 to d23, 576 birds were distributed, according to their body weight ($0,378 \pm 39$ g), in a completely randomized design with eight treatments, twelve replications per treatment, and six birds per experimental unit. They were housed in wire floor cages (500 cm²/bird) in a four-level battery equipped with a trough feeder and nipple drinker. The temperature was maintained thermostatically at $32 \text{ }^{\circ}\text{C} \pm 1 \text{ }^{\circ}\text{C}$ for the first week; thereafter, the temperature was routinely lowered according to the Cobb® guideline recommendation.

The experimental diets were based on corn, soybean meal, and crystalline amino acids to reach the recommendation, except for CP [17] (Table 1). These diets contained three different levels of CP (220, 200, and 190 g/kg) and three different eN:tN ratios varying from 0.50 to 0.58. The first treatment was the positive control with 220 g/kg CP and 0.50 eN:tN ratio. The second, 200 g/kg CP (0.54 eN:tN); Third, 200 g/kg CP + 9.84 g/kg NEAA mixture (0.52 eN:tN); Fourth, 200 g/kg CP + 18.51 g/kg NEAA mixture (0.50 eN:tN); Fifth, 190 g/kg CP (0.58 eN:tN); Sixth, 190 g/kg CP + 14.92 g/kg NEAA mixture (0.54 eN:tN); Seventh, 190 g/kg CP + 22.93 g/kg NEAA mixture (0.52 eN:tN); Eighth, 190 g/kg CP + 31.58 g/kg NEAA mixture (0.50 eN:tN). A mixture of NEAA was prepared by mixing 60% Ala, 20% Gly, and 20% Glu. The levels of NEAA mixture and NPN were used to simulate an increase in dietary CP from 190 to 220 g/kg and an eN-to-tN of 50%.

The experimental diets were formulated by the addition in the basal feed an appropriate amount of the crystalline amino acids (L-Lysine Sulphate, 546 g/kg; DL-Methionine, 990 g/kg; L-Threonine, 980 g/kg; L-Valine, 965 g/kg; Glycine, 990 g/kg; L-Arginine, 985 g/kg; L-Isoleucine, 990 g/kg; L-Tryptophan, 990 g/kg) provided by Evonik Ltd., São Paulo, Brazil, CJ Bio Ltd., São Paulo, Brazil, and Ajinomoto Ltd., São Paulo, Brazil. Treatments were formulated by supplementing the basal diet with NEAA (L-Gly, L-Ala, and L-Glu) provided by Ajinomoto Ltd., São Paulo, Brazil, as a replacement for corn starch.

Dietary essential nitrogen content was obtained by multiplying broilers requirement for EAA by their respective N content, and total nitrogen was calculated by multiplying the N content in the dietary components by its respective inclusion in the diet. Digestible lysine was fixed at 11.7 g/kg in the starter phase (12 to 23 d) to avoid excess N from EAA, and the ideal protein concept was used for the remaining EAA. To maintain the same dietary electrolyte balance (DEB, approximately 200 mEq/kg) and pH in the diets, sodium bicarbonate was used. The protein-free diet (PFD) (Table 2) was formulated to determine endogenous amino acid losses in the ileum of birds. In all experimental diets, 10 g/kg of Celite TM (acid-insoluble ash – AIA) was added.

Growth performance, metabolizable energy, and amino acid digestibility

Growth performance was evaluated during the experimental period (from 12 to 22 day post-hatch). At 22 day, broilers and feed in each experimental unit were weighed to determine the final body weight (FBW), body weight gain (BWG), feed

intake (FI), and feed conversion ratio (FCR). The body weights of any dead birds were recorded daily to correct feed intakes on a cage basis and adjust FCR calculations.

To determine apparent metabolizable energy (AME), nitrogen-corrected apparent metabolizable energy (AMEn), and nitrogen parameters, total excreta was collected twice a day from 18 to 22 d. The collected excrements from each cage and day were pooled and homogenized at the end of the experimental period and 200 g samples were pre-dried at 55 °C for 72 h and ground in a ball mill (Tecnal Equipamentos para Laboratório, TE-350, São Paulo, Brazil) for 5 min, until a fine mixture is obtained [18]. To determine apparent and standardized ileal amino acids digestibility, at 23 days of age, all birds used in the study were slaughtered by cervical dislocation their abdominal cavity was opened and all intestinal contents were found 40 cm away from the terminal ileum, anterior to the ileocecal junction, were collected [19]. Ileal digesta from the animals used in each replication were pooled to form a composite sample for each treatment.

Chemical Analysis and calculations

To obtain the values of dry matter (DM) and CP, feed and excreta were analyzed by AOAC's methods [20]. The Kjeldahl method was used to determine nitrogen levels, both in diets and excreta, based on official analysis methods [20]. Calculating excreted nitrogen (EN) included multiplying the total amount of excretion (measured in DM) by the nitrogen rate discovered in the excretion (also measured in DM). N in the diet was multiplied by the amount of DM consumed to determine nitrogen intake (NI). Based on the amount of nitrogen that was consumed minus the amount that was excreted, nitrogen balance (NB) was determined. By subtracting the amount of nitrogen that was consumed from the nitrogen balance, the retained nitrogen rate (%NR) was computed [18].

Feed and excreta of each experimental group had their gross energy (GE) analyzed by using a C5001 adiabatic calorimetric pump (IKA-Werke GmbH & Co. KG, Stauffen, Germany). AME and AMEn values were calculated using the equations provided by Sakomura and Rostagno [18] based on GE values recorded for feed and excreta:

$$\text{AME} = (\text{GE}_{\text{ing}} - \text{GE}_{\text{exc}}) / \text{DM}_{\text{ing}} \text{ and}$$

$$\text{AMEn} = (\text{GE}_{\text{ing}} - \text{GE}_{\text{exc}} - (8.22 * \text{NB})) / \text{DM}_{\text{ing}}$$

in which GE_{ing} = gross energy ingested, GE_{exc} = gross energy excreted, NB = nitrogen balance, and DM_{ing} = dry matter ingested.

According to the AOAC's recommended procedure [20], ileal digesta samples were lyophilized under vacuum, at -40 °C for 72 h, and laboratory analyses were performed to determine the amino acid content using HPLC (high-performance liquid chromatography). The digesta and diet samples were analyzed for DM and ash [20]. Based on Joslyn [21], insoluble acid ash and indigestibility ratio were calculated. The AA and CP content of the diets and digesta were analyzed by AMINOLab (Evonik, Germany). The following equations were used to calculate the apparent and standardized ileal amino acid digestibility using the methods suggested by Sakomura and Rostagno [18]:

$$\text{CDAAapa} = ((\text{AAing} - (\text{AAdig} * \text{IF1}))/\text{AAing}) * 100$$

$$\text{CDAAsta} = (\text{AAing} - ((\text{AAdig} * \text{IF1}) - (\text{AAend} * \text{IF2}))/\text{AAing}) * 100$$

in which CDAA_{apa} = apparent amino acid digestibility coefficient; AA_{ing} = ingested amino acid; AA_{dig} = digesta amino acid; IF₁ = indigestibility factor 1, IF₁ = acid-insoluble ash (AIA) diet/AIA digesta; IF₂ = indigestibility factor 2, IF₂ = AIA protein-free diet/AIA digesta; CDAA_{sta} = standardized amino acid digestibility coefficient; and AA_{end} = endogenous amino acid.

Statistical Analysis

Data were initially submitted to the Kolmogorov-Smirnov normality test to confirm normal distribution of residuals. When data were not normally distributed, they were transformed into Log 10. Then the data were analyzed via one-way ANOVA, using ExpDes.pt from the R statistical package (version 4.0.4). To compare each treatment to the control treatment (treatment 1) Dunnett's multiple comparisons were used. The level of significance was set at P < 0.05 for all the tests.

Results

The results from the experimental diets analysis are presented in Table 3. These results were in close agreement with calculated levels.

Growth Performance

The different ratios of eN:tN in broiler's diet had an effect on FBW, BWG, and FCR parameters when compared to the control treatment ($p < 0.05$; Table 4). There was a decrease in FBW and BWG when broilers were fed with 200 g/kg CP (eN:tN 0.54), 190 g/kg CP (eN:tN 0.58), and 190 g/kg CP (eN:tN 0.54) when compared to a group fed with 220 g/kg CP (eN:tN 0.50). Broilers receiving a diet with 200 g/kg CP (eN:tN 0.54 and 0.52) and 190 g/kg CP (eN:tN ratio of 0.58 and 0.54) had a worse FCR ($p < 0.05$) when compared to animals receiving a diet with 220 g/kg CP and eN:tN ratio of 0.50. There was no significant effect ($p > 0.05$) on FI when broilers were fed diets with 200 and 190 g/kg CP and eN:tN ratio of 0.58, 0.54, and 0.52 when compared with the PC treatment.

Nitrogen Utilization

Nitrogen utilization parameters (NI, NE, NB, and %RN) results are present in Table 5. Based on Dunnett's test, birds submitted to treatments with 200 g/kg CP and eN:tN ratios of 0.58, and 190 g/kg CP (eN:tN of 0.54 and 0.52) had lower ($p < 0.05$) NI and NE (g/bird/day) than those fed with 220 g/kg CP and eN:tN of 0.50. There was an increase ($p < 0.05$) of 12% in the NB in the treatment with 190 g/kg CP and eN:tN of 0.54 when comparing the result obtained in the positive control treatment. %RN increased 14% and 10% when a group received a diet with 190 g/kg CP and eN:tN ratio of 0.58 and 0.54, respectively, when compared to the birds fed diet with 220 g/kg CP and eN:tN of 0.50.

Metabolizable Energy

Supplementation with 18.51 g/kg NEAA mixture in a diet with 200 g/kg CP and eN:tN ratio of 0.50 increased ($p < 0.05$) dry matter intake (Table 6) than those of the PC treatment. The AME, AMEn and AMEnNM values showed higher values ($p < 0.05$) in the treatments with 200 g/kg CP and eN:tN ratio of 0.50, and in the treatments with 190 g/kg CP (0.58, 0.54, 0.52, and 0.050 eN:tN ratio) when compared to the treatment with 220 g/kg CP and 0.50 eN:tN ratio.

Apparent and Standardized Ileal Amino Acid Digestibility

There was an increase ($p < 0.05$) in the apparent ileal digestibility (AID) values of the amino acids: methionine + cysteine, valine, glycine, and alanine in the treatments with 200 and 190 g/kg CP and supplementation with NEAA mixture, resulting in eN:tN

ratios of 0.58, 0.54, and 0.52, when compared to the treatment where there was no dietary CP of 220 g/kg and an eN:tN ratio of 0.50 (Table 7). On the other hand, the AID values of the Arg and Pro decreased in groups fed with 200 and 190 g/kg CP and eN:tN ratio of 0.58 and 0.50, even when supplemented with NEAA mixture, when compared to PC treatment. Comparing the AID value of Gly between birds fed diet with 220 g/kg CP (eN:tN 0.50) to a group fed diet with 190 g/kg CP (eN:tN 0.58), the birds fed diet with 190 g/kg CP and 0.58 eN:tN ratio had higher ($p < 0.05$) AID value of Gly than those group fed with PC treatment. Broilers fed PC diet had a lower ($p < 0.05$) AID value in Ala than those fed diet with 200 and 190 g/kg CP and NEAA mixture supplementation, resulting in a variation of eN:tN ratio from 0.50 to 0.54.

Methionine + cystine, valine, glycine, and alanine showed an increase ($p < 0.05$) in standardized ileal digestibility (SID) in treatments with 200 and 190 g/kg CP and eN:tN varying from 0.50 to 0.58, comparing to PC treatment (Table 8). Broilers fed diet with 190 g/kg CP associated with an addition of 22.93 g/kg NEAA mixture and eN:tN ratio of 0.52 increased ($p < 0.05$) the SID value for Met + Cys and Val when comparing to the results obtained in the treatment with eN:tN of 0.50 and 220 g/kg CP. Varying the eN:tN ratio from 0.58 to 0.50 associated with dietary reduction of CP (200 and 190 g/kg CP) and supplementation of NEAA mixture increased the SID value of Gly and Ala when compared to PC treatment.

Discussion

Dietary reduction in CP is a common strategy aiming to reduce soybean meal inclusion through the addition of crystalline EAA [22]. However, diets containing only EAA can result in different ratios of eN:tN due to an incorrect balance between EAA and NEAA. High or low eN:tN ratio is observed in diet with excess or lack of EAA [13]. Broilers fed with high or low eN:tN ratio have a negative effect on growth performance due to several factors, such as: the ineffective synthesis of some NEAA, such as glutamine and glycine, due to a non-specific nitrogen deficiency for the production of NEAA [14]. Berres et al. [23] suggested that it is vital to provide non-essential nitrogen to birds that are fed low-CP diet, particularly in the early growth stages, as NEAA can become limiting below a certain level of dietary protein. Thus, Rose et al. [24] and Greenstein et al. [25] reported the inclusion of a NEAA mixture is necessary to obtain maximum growth performance.

Different proportions of NEAA mixture were added to the diets to obtain different eN:tN ratios. We observed broilers fed a 0.54 and 0.58 eN-to-tN ratio had a significant effect on broilers' performance, decreasing the FBW and BWG and worsening FCR, when compared with diets with a 0.50 eN:tN ratio. The growth performance of broilers was maintained in dietary treatment with eN:tN ratio of 0.52. Authors such as Stucki and Happer, [14] and Sugahara and Ariyoshi, [26] reported broilers' performance was maximized when the eN:tN ratio was about 0.66 and 0.57, respectively. However, from the results of this study, we can verify feeding broilers with 190 g/kg CP resulted in eN:tN ratio of 0.58 and it was not enough to improve the growth of broilers. The diet with reduced CP and EAA supplementation resulted in a higher eN:tN ratio due to the decrease in CP content and the high concentration of EAA, which induces a low dietary concentration of N [13]. The negative effect of high eN:tN ratio on growth rate can be explained because the amount of non-essential N is limited and some EAA is not efficient in converting into NEAA, such as lysine and arginine [13,27,].

The supplementation of NEAA, such as glutamate, glycine, and alanine, in a reduced CP diet, seems to be necessary to obtain a maximum growth performance of broilers, reaching an optimum eN:tN ratio of 0.50. Nomroud et al. [28] observed that NEAA (Glu + Gly) mixture supplementation in low-CP diets resulted in animal performance improvement when compared to those birds that did not receive this supplementation. Award et al. [29] mention that addition of NEAA mixture (Gly, Glu, Pro, Ala, and Asp) in diets with reducing protein can improve the poor productive efficiency of broilers submitted to low-CP diets without NEAA addition.

In addition to the NEAA mixture, individual NEAA supplementation can also improve the performance of birds on a low-CP diet. Gly, for example, is a limiting NEAA, especially in the initial phase of broilers necessary for the excretion of ammonia into uric acid [7]. Glycine supplementation in reduced protein diets can improve the productive efficiency of broilers [30], obtaining a better FCR [31,32] and greater BWG [7]. Nonetheless, according to Hofmann et al. [33] the individual effect of NEAA on body weight gain will depend on the concentration of CP in the diet, whereas in diets with 160 g/kg CP individual NEAA supplementation does not seem to affect weight gain in broiler chickens.

Dietary changes in the eN:tN ratio in the diet of broilers, in addition to having an impact on their performance, can also affect the efficiency of nitrogen in poultry nutrition. Low-CP diets typically reduce the excretion of N into the environment due to

N being a limited factor in low-CP diet, resulting in better nitrogen efficiency [34,35]. In this study, there is an increase in the eN:tN ratio as there is a dietary reduction in dietary CP, where diets with 190 and 200 g/kg CP presented respectively 0.58 and 0.54 eN:tN ratio. Animals submitted to a diet with an eN:tN ratio of 0.58 and 0.54 had lower NI and NE when compared to a diet with a 0.50 eN:tN ratio. An increase in %RN was observed in animals subjected to treatments 0.58 and 0.54 eN:tN ratio than those subjected to treatment with 0.50 eN:tN ratio. Heger et al. [13] observed that there was greater nitrogen retention in pigs when these animals were fed a diet containing eN:tN ratio of 0.48. This same author attributes better nitrogen retention in diets with an eN:tN ratio of 0.48 due to the EAA-sparing effect, where the nitrogen of these amino acids is not used for the production of NEAA, mentioning that nitrogen is better retained in diets with proportions of 0.48 eN:tN ratio [13].

As mentioned, the excess nitrogen will be excreted as uric acid. This excretion generates an energy expenditure of 3.75 ATPs/mol of N, which is greater than the excretion of N in the form of urea (2.0 ATPs) in mammals [36]. It occurs mainly in high-CP diet, due to the excess of AA present in this diet, resulting in less energy availability and lower productive efficiency of broilers. In this study, we also evaluated the effect of reducing CP on AME and AMEn utilization in broiler chickens. A reduction of 20 g/kg CP and supplementation of 1.851% NEAA mixture increased the AME, AMEn, and AMEnMN values available for the birds, even when there was a reduction of 30 g/kg CP with or without NEAA addition compared to treatment with 220 g/kg CP. According to Liu et al. [37] and Chrystal et al. [38] these ME values may be linked to the increase in the corn starch content of the diet and the decrease in the lipid content generated from the reduction in the protein level. This can be observed in this study, where there was an increase in the addition of corn and a reduction in the inclusion of soybean oil when dietary CP was reduced. Kamran et al. [39] reported birds fed low-CP diets used carbohydrates as an energy source rather than free fatty acids resulting in higher plasma triglyceride levels. According to the research carried out for the production of this work, there are no studies that identify the effect of protein reduction on metabolizable energy utilization for broilers. In some cases, some studies seek to identify the effects of a diet with reduced CP and different energy levels. In these studies, it is possible to observe that the reduction of CP combined with the reduction of energy levels in the diet have a negative effect on growth performance [40,41,42].

The protein utilization efficiency of diets for broilers can be influenced according to the energy content of the diets. Gous et al. [41] reported the efficiency of using dietary crystalline amino acids is linked to AME values. According to Kyriazakis and Emmas [43], the efficiency of protein utilization is dependent on the metabolizable energy: dietary CP (AMEn:DCP) ratio of the food. Thus, the animals must have access to the required amount of energy and protein for their growth, without excess or lack of nutrients. In this study, we evaluated the effect of CP reduction on apparent and standardized ileal digestibility of essential and non-essential amino; we can notice that the reduction of CP affects the digestibility of some amino acids, essential or non-essential.

Amino acids such as Met+Cys, Val, Gly, and Ala show an increase in the AID and SID values when there was a dietary reduction of CP and addition of NEAA mixture, compared to the PC treatment. Alanine was on the amino acids that showed the highest AID and SID when dietary protein was reduced. On the other hand, Liu et al. [37] report the reduction of CP in broilers diets did not affect the AID of alanine. These authors reported protein reductions can interfere positively or negatively with the amino acid digestibility coefficient. Lemme et al. [44] mention an increase in the amino acid digestibility coefficient can be explained by the addition of crystalline amino acids; that are more rapidly absorbed than protein-bound [54]. Ravindran et al. [19] report reductions in dietary CP can attenuate endogenous amino acid fluxes, which increase their AID coefficients. Hossain et al. [46] suggest that factors like diet composition, fiber levels, antinutritional factors, ingredient diversity, protein quality, and physicochemical characteristics of the protein can affect the ileal amino acid digestibility in birds. According to Liu et al. [37], few studies have explored the impact of protein reduction on amino acid digestibility.

Conclusions

Reduction of 20 to 30 g/kg of the dietary CP from a diet with 220 g/kg CP and supplementation of NEAA mixture (Glu, Gly, and Ala) seems do not impair the performance of broiler chickens from 12 to 23 d post hatching. However, it is important that the eN:tN ratio be 0.52 or less to obtain these results.

Author Contributions

Conceptualization, H.S.R., and L.F.T.A.; formal analysis, R.d.S.F.; funding acquisition, H.S.R, and L.F.T.A.; investigation, R.d.S.F., R.A.R.d.L., R.N.A., H.R.S.; methodology, H.S.R., L.F.T.A., R.d.S.F., and V.D.N.; project administration, R.d.S.F., and L.F.T.A.; supervision, L.F.T.A., and A.A.C.; writing—original draft, R.d.S.F.; writing—review and editing, R.d.S.F., R.N.A., H.R.S., A.A.C., M.I.H., and V.D.N

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Informed Consent Statement

Not applicable.

Data Availability Statement

Not applicable

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Conflicts of Interest

The authors declare no conflict of interest. The co-author is an employee of Evonik Ltd., the supplier of some amino acids, which does not mean competing interests.

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Table 1. Ingredient and nutrient composition of dietary treatments fed to broilers male from 12 to 23 d of age (g/kg).

Treatments	1	2	3	4	5	6	7	8
CP (g/kg)	220	200	200+Mix	200+Mix	190	190+Mix	190+Mix	190+Mix
eN:tN ratio	0.50	0.54	0.52	0.50	0.58	0.54	0.52	0.50
Ingredients								
Corn	491.37	568.23	568.23	568.23	621.99	621.99	621.99	621.99
Soybean meal, (450 g/kg CP)	392.10	320.00	320.00	320.00	268.90	268.90	268.90	268.90
Soybean oil	44.60	30.00	30.00	30.00	18.80	18.80	18.80	18.80
Dicalcium phosphate	17.00	17.50	17.50	17.50	17.80	17.80	17.80	17.80
Limestone	8.40	8.80	8.80	8.80	8.90	8.90	8.90	8.90
Salt	4.10	3.10	3.10	3.10	2.80	2.80	2.80	2.80
Sodium bicarbonate	1.50	3.00	3.00	3.00	3.50	3.50	3.50	3.50
L- Lysine BioLys (546 g/kg)	1.39	4.34	4.34	4.34	6.43	6.43	6.43	6.43
DL-Methionine (990 g/kg)	2.81	3.41	3.41	3.41	3.82	3.82	3.82	3.82
L-Threonine (980 g/kg)	0.28	1.20	1.20	1.20	1.84	1.84	1.84	1.84
L-Valine (965 g/kg)	-	1.02	1.02	1.02	1.87	1.87	1.87	1.87
Glycine (990 g/kg)	-	1.80	1.80	1.80	3.38	3.38	3.38	3.38
L-Arginine (985 g/kg)	-	0.70	0.70	0.70	2.07	2.07	2.07	2.07
L-Isoleucine (990 g/kg)	-	0.45	0.45	0.45	1.25	1.25	1.25	1.25
L-Tryptophan (990 g/kg)	-	-	-	-	0.20	0.20	0.20	0.20
Premix ¹	4.45	4.45	4.45	4.45	4.45	4.45	4.45	4.45
Corn Starch	32.00	32.00	22.16	13.49	32.00	17.08	9.07	0.42
NEAA mixture (154.5 g/kg N) ²	-	-	9.84	18.51	-	14.92	22.93	31.58
AIA ³	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Calculated composition								
Crude protein, g/kg	220.0	200.0	209.5	217.9	190.0	204.4	212.1	220.5
Metabolizable energy, kcal/kg	3.065	3.065	3.065	3.065	3.065	3.065	3.065	3.065
eN:tN ratio	0.497	0.545	0.520	0.500	0.582	0.540	0.520	0.500
Calcium, g/kg	8.78	8.78	8.78	8.78	8.78	8.78	8.78	8.78
Available phosphorus, g/kg	4.19	4.19	4.19	4.19	4.19	4.19	4.19	4.19
Sodium, g/kg	2.18	2.18	2.18	2.18	2.18	2.18	2.18	2.18
SID Lysine, g/kg	11.70	11.70	11.70	11.70	11.70	11.70	11.70	11.70
SID Methionine, g/kg	5.73	6.04	6.04	6.04	6.44	6.44	6.44	6.44
SIDMet + Cys, g/kg	8.66	8.66	8.66	8.66	8.66	8.66	8.66	8.66
SID Threonine, g/kg	7.72	7.72	7.72	7.72	7.72	7.72	7.72	7.72
SID Valine, g/kg	9.19	9.01	9.01	9.01	9.01	9.01	9.01	9.01
SID Isoleucine, g/kg	8.61	7.84	7.84	7.84	7.84	7.84	7.84	7.84
SID Tryptophan, g/kg	2.53	2.17	2.17	2.17	2.11	2.11	2.11	2.11
SID Arginine, g/kg	13.86	12,52	12,52	12,52	12,52	12,52	12,52	12,52
SID Gly+Ser, g/kg	17.79	17.20	17.20	17.20	17.20	17.20	17.20	17.20
DEB (mEq/kg) ⁴	231	203.88	203.88	203.88	206	206	206	206

pH	5.92	6.06	6.04	6.01	5.97	6.02	6.05	6.04
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¹Mineral Premix (amount per kg diet): manganese – 77.0 mg, iron – 55.0 mg, zinc – 71.5 mg, copper – 11.0 mg, iodine – 1.10 mg, selenium – 0.33 mg . Vitamin Premix (amount per kg diet): vitamin A - 8250 IU, vitamin D3 - 2090 IU, vitamin E - 31.0 IU, vitamin B1 - 2.20 mg, vitamin B6 - 3.08 mg, pantothenic acid - 11.0 mg, biotin - 0.077 mg, vitamin K3 - 1.65 mg, folic acid - 0.77 mg, nicotinic acid - 33.0 mg, vitamin B12 - 0.013 mg, choline chloride (60%)- 1 g; butylated hydroxytoluene - 0.1 g; salinomycin sodium (12%) -0.55 g.

²Non-essential amino acids mixture (60% Alanine, 20% Glycine, 20% Glutamic acid = 154.5 g/kg N).

³Acid-insoluble ash (AIA). Cellite source.

⁴DEB mEq/kg calculated as $10,000 \times (\text{Na}^+ + \text{K}^+ - \text{Cl}^-)$.

Table 2. Composition of protein-free diet (PFD).

Ingredient	PFD (g/kg)
Corn starch	827.50
Sugar	50.00
Soybean oil	50.00
Dicalcium phosphate	16.20
Limestone	8.00
Salt	4.50
Corn cob	30.0
Mineral supplement ¹	0.50
Vitamin supplement ²	1.30
Choline chloride (60%)	2.00
Inerte Indigestible	10.0
Total	100.000

¹Mineral supplement - guaranteed levels per kg of feed: selenium, 0.375 mg; manganese, 88 mg; iron, 62.5 mg; zinc, 81.3 mg; copper, 12.5 mg; iodine, 1.25 mg.

²Vitamin supplement - guaranteed levels per kg of feed: vitamin A, 9,375 IU; vitamin D3, 2,375 IU; vitamin E, 35 IU; vitamin B1, 2.50 mg; vitamin B2, 6.25 mg; vitamin B6, 3.5 mg; vitamin B12, 0.015 mg; nicotinic acid, 37.5 mg; B.C. pantothenic acid, 12.5 mg; vitamin K3, 1.88 mg; B.C. folic acid, 0.875 mg; biotin, 0.088 mg.

Table 3. Analyzed crude protein (CP), dry matter (DM), and total amino acid level (g/kg) of experimental diets fed to broilers from 12 to 23 days of age¹.

Treatments	1	2	3	4	5	6	7	8
CP (g/kg)	220	200	200+Mix	200+Mix	190	190+Mix	190+Mix	190+Mix
eN:tN ratio	0.50	0.54	0.52	0.50	0.58	0.54	0.52	0.50
DM	877.7	876.6	874.7	880.4	873.3	870.3	874.5	874.0
CP	233.2	205.8	215.9	225.2	198.50	209.3	214.9	223.5
Lys	13.54	12.12	13.15	12.44	13.21	12.17	13.00	13.17
Met	5.66	5.58	6.17	6.02	5.85	6.19	6.51	6.08
Cys	3.42	3.03	3.12	2.98	2.88	3.01	2.99	2.98
Met+Cys	9.08	8.61	9.29	9.00	8.73	9.20	9.50	9.06
Thr	8.63	8.47	8.36	8.19	7.83	9.35	7.70	8.24
Ile	9.99	8.81	9.12	8.68	8.76	8.63	8.69	8.51
Val	10.70	10.08	10.40	9.02	10.83	10.10	10.23	10.07
Arg	15.30	13.31	13.74	13.13	13.26	13.25	13.40	13.80
Leu	18.62	16.46	16.85	15.91	15.48	15.51	15.27	15.20
His	5.88	5.11	5.25	4.99	4.75	4.75	4.73	4.65
Gly	9.33	9.57	11.91	13.10	10.81	13.37	14.98	16.57
Ala	10.82	9.65	13.96	21.71	9.35	17.19	21.56	26.04
Glu	39.64	33.97	36.57	36.41	31.40	34.45	35.85	36.53
Pro	12.55	11.18	11.47	10.91	10.62	9.67	9.40	9.25
Asp	23.44	19.45	19.97	18.88	17.58	17.98	17.92	17.53
Phe	11.32	9.61	9.89	9.31	8.85	9.05	8.98	8.94
Ser	10.90	9.37	9.52	9.07	8.58	8.85	8.74	8.59

¹Samples were analyzed in duplicate by Evonik Operations GmbH, Baden-Württemberg Germany.

Table 4. Final body weight (FBW), Body weight gain (BWG), Feed intake (FI), and feed conversion ratio (FCR) of broiler male chicks from 12-23 days of age submitted to different levels of essential to non-essential amino acid ratio.

Treatments	eN:tN ratio	FBW (kg)	BWG (kg)	FI (kg)	FCR
220 g/kg CP (Control)	0.50	1.060	0.683	0.849	1.243
200 g/kg CP	0.54	1.012*	0.635*	0.856	1.350*
200 g/kg CP + 0.984% Mix	0.52	1.023	0.645*	0.852	1.322*
200 g/kg CP + 1.851% Mix	0.50	1.035	0.657	0.861	1.310
190 g/kg CP	0.58	1.002*	0.623*	0.866	1.390*
190 g/kg CP + 1.492% Mix	0.54	1.010*	0.632*	0.847	1.344*
190 g/kg CP + 2.293% Mix	0.52	1.036	0.658	0.850	1.298
190 g/kg CP + 3.158% Mix	0.50	1.047	0.669	0.836	1.250
<i>P</i> -value		0.004	0.001	0.667	<0.001
SEM		0.012	0.011	0.011	0.025

CP: crude protein; Essential nitrogen:total nitrogen (eN:tN).

MIX: non-essential amino acids mixture (60% alanine, 20% glycine, and 20% glutamic acid).

SEM: standard error mean.

Means followed by * in the same column differ from T1 based on Dunnett's test at 5% significance level.

Table 5. Effect of crude protein (CP) reduction and addition of non-essential amino acid mixture (Mix) on the nitrogen content of excreta (NE), nitrogen intake (NI), nitrogen balance (NB) and percentage of retained nitrogen (%RN) in broiler male chicks 12- 23 days of age.

Treatments	eN:tN ratio	NI (g/bird/d)	NE (g/bird/d)	NB (g/bird/d)	%RN
220 g/kg CP (Control)	0.50	3.14	1.19	1.95	61.60
200 g/kg CP	0.54	2.83*	0.97*	1.86	65.21
200 g/kg CP + 0.984% Mix	0.52	2.86*	1.11	1.75	61.01
200 g/kg CP + 1.851% Mix	0.50	3.15	1.08	2.07	65.70
190 g/kg CP	0.58	2.88*	0.81*	2.07	71.74*
190 g/kg CP + 1.492% Mix	0.54	3.19	0.99*	2.20*	68.99*
190 g/kg CP + 2.293% Mix	0.52	3.23	1.15	2.08	63.98
190 g/kg CP + 3.158% Mix	0.50	3.25	1.14	2.11	64.97
<i>P</i> -value		<0.001	<0.001	<0.001	<0.001
SEM		0.069	0.050	0.069	1.579

Essential nitrogen:total nitrogen (eN:tN).

SEM: standard error mean.

Means followed by * in the same column differ from T1 based on Dunnett's test at 5% significance level.

Table 6. Dry matter intake (DMI), apparent metabolizable energy (AME) and corrected by nitrogen balance (AMEn) in dry and natural matter (AMEnNM) of excreta of broilers male chicks from 12-23 days of age.

Treatments	eN:tN ratio	DMI (kg)	AME (kcal/kg)	AMEn (kcal/kg)	AMEnNM (kcal/kg)
220 g/kg CP (Control)	0.50	2.74	3292	3097	2718
200 g/kg CP	0.54	2.84	3357	3196	2804
200 g/kg CP + 0.984% Mix	0.52	2.69	3322	3162	2774
200 g/kg CP + 1.851% Mix	0.50	2.94*	3470*	3297*	2892*
190 g/kg CP	0.58	2.88	3491*	3314*	2894*
190 g/kg CP + 1.492% Mix	0.54	2.86	3456*	3266*	2852*
190 g/kg CP + 2.293% Mix	0.52	2.77	3416*	3233*	2823*
190 g/kg CP + 3.158% Mix	0.50	2.76	3443*	3254*	2842*
<i>P</i> -value		0.004	<0.001	<0.001	<0.001
SEM		0.051	34.960	33.920	29.089

CP: crude protein; Essential nitrogen:total nitrogen (eN:tN).

MIX: non-essential amino acids mixture (60% alanine, 20% glycine and 20% glutamic acid).

SEM: standard error mean.

Means followed by * in the same column differ from T1 based on Dunnett's test at 5% significance level.

Table 7. Effect of crude protein (CP) reduction and addition of non-essential amino acid mixture (Mix) on apparent ileal amino acid digestibility (%) of essential and non-essential amino acids of broilers male chicks 12-23 days of age.

Treatments	1	2	3	4	5	6	7	8	P-Value	SEM
CP (g/kg)	220	200	200+Mix	200+Mix	200+Mix	200+Mix	200+Mix	200+Mix		
eN:tN ratio	0.50	0.54	0.52	0.50	0.58	0.54	0.52	0.50		
Essential Amino Acids										
Lysine	90.36%	89.67%	88.34%	88.28%	90.22%	90.93%	92.37%	89.91%	0.068	0.007
Methionine	93.88%	94.36%	93.36%	93.65%	94.11%	95.54%	90.53%	92.85%	0.389	0.014
Methionine + Cistine	88.51%	89.19%	88.18%	88.22%	89.11%	90.22%	91.06%*	89.32%	0.026	0.006
Threonine	80.52%	82.28%	78.92%	79.00%	80.29%	84.32%	81.76%	81.21%	0.247	0.011
Arginine	93.11%	92.31%	91.67%	91.07%*	92.57%*	92.67%	92.82%	91.27%	0.013	0.005
Valine	82.50%	84.59%	82.40%	80.59%	85.30%	85.96%*	87.16%*	85.36%	<0.001	0.011
Leucine	85.46%	85.11%	82.88%	82.58%	83.85%	85.64%	86.34%	84.58%	0.258	0.009
Isoleucine	83.95%	83.58%	81.43%	81.17%	83.34%	85.01%	86.29%	83.80%	0.146	0.019
Histidine	88.78%	89.03%	87.60%	87.38%	88.11%	88.84%	89.53%	88.19%	0.362	0.007
Phenylalanine	87.24%	86.24%	84.60%	84.53%	85.23%	87.06%	87.91%	86.80%	0.078	0.008
Non-essential Amino Acids										
Aspartic Acid	85.92%	85.49%	83.88%	83.16%	84.16%	84.71%	85.54%	83.36%	0.254	0.009
Glutamic Acid	90.03%	89.23%	88.64%	90.78%	88.58%	90.75%	91.65%	90.22%	0.056	0.007
Serine	85.44%	84.75%	82.65%	82.24%	83.90%	84.09%	84.45%	82.54%	0.218	0.009
Glycine	81.11%	83.36%	84.59%	86.34%*	84.85%*	88.65%*	90.53%*	90.53%*	<0.001	0.011
Cistine	79.40%	81.92%	79.86%	79.99%	81.40%	81.75%	82.64%	81.09%	0.336	0.010
Alanine	84.60%	83.88%	87.30%*	92.18%*	83.36%	92.19%*	94.11%*	94.36%*	<0.001	0.014
Proline	85.80%	85.22%	83.64%	83.17%	83.37%	83.49%	83.40%	81.39%*	0.030	0.009

SEM: standard error mean.

Means followed by * in the same column differ from T1 based on Dunnett's test at 5% significance level.

Table 8. Effect of crude protein (CP) reduction and addition of non-essential amino acid mixture (Mix) on standardized ileal amino acid digestibility of essential and non-essential amino acids.

Treatments	1	2	3	4	5	6	7	8	<i>P</i> -Value	SEM
CP (g/kg)	220	200	200+Mix	200+Mix	200+Mix	200+Mix	200+Mix	200+Mix		
eN:tN ratio	0.50	0.54	0.52	0.50	0.58	0.54	0.52	0.50		
Essential Amino Acids										
Lysine	94.56%	94.36%	92.65%	92.87%	94.61%	95.56%	96.73%	94.73%	0.177	0.007
Methionine	101.78%	102.25%	100.47%	101.05%	101.75%	102.61%	97.31%	100.19%	0.197	0.014
Methionine + Cistine	94.78%	95.80%	94.31%	94.54%	95.62%	96.40%	97.04%*	95.60%	0.047	0.006
Threonine	87.11%	88.98%	85.70%	85.97%	87.69%	90.35%	89.12%	88.08%	0.159	0.011
Arginine	96.83%	96.58%	95.79%	95.41%	96.94%	96.93%	97.05%	95.60%	0.064	0.005
Valine	87.82%	90.23%	87.85%	86.92%	90.65%	91.54%*	92.70%*	90.98%	0.003	0.019
Leucine	88.52%	88.56%	86.24%	86.17%	87.60%	89.28%	90.05%	88.30%	0.333	0.009
Isoleucine	89.64%	90.03%	87.64%	87.74%	89.96%	91.55%	92.81%	90.45%	0.214	0.010
Histidine	98.97%	100.14%	98.40%	98.81%	100.31%	100.71%	101.51%	100.36%	0.218	0.007
Phenylalanine	92.26%	92.15%	90.33%	90.66%	91.78%	93.29%	94.22%	93.14%	0.087	0.008
Non-essential Amino Acids										
Aspartic Acid	88.35%	88.41%	86.72%	86.18%	87.46%	87.84%	88.70%	86.59%	0.391	0.009
Glutamic Acid	91.47%	90.91%	90.19%	92.35%	90.43%	92.39%	93.23%	91.77%	0.079	0.007
Serine	90.66%	90.81%	88.60%	88.53%	90.65%	90.46%	90.94%	89.13%	0.368	0.009
Glycine	87.21%	89.30%	89.34%*	90.70%*	90.22%*	92.86%*	94.32%*	93.94%*	<0.001	0.010
Cistine	96.13%	98.65%	96.59%	96.72%	98.12%	98.48%	99.37%	97.82%	0.336	0.010
Alanine	89.85%	89.77%	91.36%	94.80%*	89.56%	95.47%*	96.74%*	96.54%*	<0.001	0.010
Proline	90.34%	90.30%	88.58%	88.40%	88.83%	89.32%	89.43%	87.52%	0.326	0.009

SEM: standard error mean.

Means followed by * in the same column differ from T1 based on Dunnett's test at 5% significance level.

Manuscript 2

Effect of supplementation of non-essential amino acids and non-protein nitrogen on growth rate, nitrogen parameters, metabolizable energy, and amino acid digestibility of broiler chickens fed reduced protein diet

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Simples Summary

When reducing dietary crude protein and adding essential amino acids, some studies suggest that animal performance will not be as efficient as imagined when using the concept of ideal protein in poultry nutrition. It occurs because the nitrogen of essential amino acid will not be sufficient for the synthesis of so-called non-essential amino acids, probably because other non-essential amino acids are growth-limiting. Thus, this study aimed to evaluate the effect of reduced crude protein diet supplemented with a mixture of non-essential amino acids and a non-protein nitrogen source on animal performance, nitrogen utilization, metabolizable energy, and amino acids digestibility of broiler from 12 to 23 days post hatch. Reducing crude protein to 190 g/kg without supplementation of mixture of non-essential amino acids and a source of non-protein nitrogen resulted in decrease ($P>0.05$) of final body weight, body weight gain, and feed intake when compared to group fed diet with 220 g/kg CP. The reduction of dietary CP from 220 to 190 g/kg allows better nitrogen utilization, decreasing nitrogen intake and excretion. These results found in this study allow reducing dietary crude protein in poultry nutrition, without compromising broiler performance and reducing the impact of nitrogen excretion on the environment.

Abstract

This study shows the effect of supplementing reduced crude protein diet with non-protein nitrogen (NPN) source and non-essential amino acids (NEAA) mixture in order to provide the best productive performance, in addition to evaluating N utilization, metabolizable energy, and amino acids digestibility of broiler chickens from 12 to 23 d of age. 648 Cobb 500 male broilers were fed nine dietary treatments. A positive control (PC) and negative control (NC) diet were formulated with 220 and 190 g/kg CP, respectively. From negative control, other seven dietary treatments were formulated, supplementing with different levels of NEAA mixture (60% Ala, 20% Gly, 20% Glu) and NPN (urea, ammonium acetate, monoammonium phosphate). To compare each treatment to the positive control (treatment 1) Dunnett's multiple comparisons was used. The results show that reducing crude protein from 220 to 190 g/kg without supplementation of NEAA mixture or NPN compromised ($P<0.05$) the growth performance of broilers. However, a decrease in nitrogen intake and nitrogen excretion was observed in NC treatment. Metabolizable energy values decreased when NC treatment was supplemented with NEAA mixture combined with ammonium acetate or

monoammonium phosphate. Apparent and standardized ileal digestibility of some amino acids increased when there was a reduction of CP when compared to those fed with PC treatment. The supplementation of NEAA or NPN seems to be efficient in diet with reduced CP, allowing a reduction from 220 to 190 g/kg CP and achieving an eN:tN of 0.50.

Keywords: amino acids; ideal protein; nitrogen utilization; urea

1. Introduction

Supplementation of broilers' diet with essential amino acids (EAA) allows a decrease in dietary crude protein (CP) and has been related to less environmental impact and increase protein utilization in broiler nutrition [1,2]. Reducing dietary CP has a positive effect on nitrogen (N) utilization, reducing the N excretion and ammonia emission from broiler houses [3]. However, the use of exclusively EAA in reduced-CP diet is not enough to achieve broilers' maximum growth [4] because EAA is not an efficient source of non-specific N used for the synthesis of non-essential amino acids (NEAA) [5], resulting in an imbalance of EAA and NEAA [2].

Therefore, to obtain a maximum growth rate is necessary to supplement reduced CP diet with additional N by adding sources of non-specific N as glutamate, glycine, alanine, or a non-protein nitrogen (NPN) source [6,2]. Reduced CP diet supplemented with EAA can result in a variation in the essential-to-total nitrogen (eN:tN) ratio [2]. Supplementing this diet with a source of non-specific nitrogen is important to achieve a correct balance between EAA and NEAA and an optimum eN:tN ratio. An eN:tN ratio of about 0.60 is associated with a poor growth performance [2], however, the addition of a mixture of NEAA (Glu, Gly, and Ala) in low-CP diet resulted in improved growth rate when the eN:tN ratio was 0.50 [7]. Glutamate and glycine are an excellent source of non-specific nitrogen and carbon for the synthesis of amino acids in chicks via amination and amidation [8,2]. According to D'Mello [6] and He et al. [8] supplementation of low-CP diet with a mixture of NEAA seems to be a more effective source of non-specific nitrogen, where the dietary supply of glutamate and glycine is essential for the growth performance of broilers.

The addition of NPN, as urea, in reduced CP diet can be an efficient source of non-specific nitrogen and provide an optimum eN:tN ratio to broilers. In ruminant nutrition, the use of NPN is already well described in the literature [9], However, in

poultry nutrition, there is a lack of information about the possibility of using NPN as a partial substitute of protein [10]. Shanzad et al. [11] report urea at higher levels present a toxic effect, decreasing the growth rate of broilers. However, Chowdhury et al. [12] using urea at low levels reported a positive effect on the efficiency productive chicks.

Thus, we hypothesized that reducing dietary CP would result in impairments in broiler chickens' performance and worsened N utilization, metabolizable energy, and amino acid digestibility. However, by supplementing reduced CP diets with NEAA mixture and NPN source, these negative effects could be mitigated. The objective of this study, therefore, was to evaluate the effect of reduced protein diets for broiler chickens from 12 to 23 d of age, supplemented with NPN source and NEAA mixture, to provide the best productive performance, in addition to evaluating N utilization, metabolizable energy, and amino acids digestibility.

2. Material and methods

The Ethics Committee in the Use of Farming Animal at the Federal University of Viçosa (CEUA – UFV) approved all animal handling procedures (protocol number 22/12.05.2020), in compliance with the ethical principles of animal experimentation established by the Conselho Nacional de Controle de Experimentação Animal (CONCEA).

Bird Husbandry, Experimental design, and Diets

One-day-old male chicks (Cobb 500) were obtained from a commercial hatchery where all chicks were vaccinated against Marek's Disease (day 18 via in ovo administration) and Newcastle Disease and Infectious bronchitis (via coarse pray at hatch). Until 11 d of age, they were reared on floor pens covered by litter and fed with a standard starter diet (3065 kcal/kg, 220 g/kg CP) based on corn-soybean meal in a mash, to meet or exceed the requirements of broiler chicks [13]. At day 12, 648 broilers were distributed to 108 equal cages (equipped with a trough feeder and nipple drinker) (six birds/0.05 m²/bird) across 9 treatments and 12 replications, according to their body weight (0,244 ± 29 g), in a completely randomized design. The temperature was maintained thermostatically at 32°C and gradually decreased to 25 °C at 23 d of age. It was provide a photoperiod at 24L for the first week post-hatching and 20L:4D was maintained from 8 to 23 d of age. Florescent lamps were used to provide artificial light

at an intensity of 10-lux. Feed and water were provided ad libitum throughout the experimental period.

A positive control (PC) (220 g/kg CP) diet was formulated based on corn, soybean meal, and crystalline amino acids in order to reach the recommendation [20] (Table 1), and assumed to contain a dietary eN:tN ratio of 0.50. A negative control (NC) was formulated reducing the dietary CP (190 g/kg) and an eN-to-tN ratio of 0.58. From NC, other seven dietary treatments were formulated, supplementing the diets with 190 g/kg CP with different levels of NEAA mixture (60% Ala, 20% Gly, 20% Glu) and NPN (urea, ammonium acetate, monoammonium phosphate). The levels of NEAA mixture and NPN were used to simulate an increase in dietary CP from 190 to 220 g/kg CP and an eN-to-tN of 50%. Whole soybean was added in the fourth (1kg) and fifth (0.5 kg) on top. The experimental diets were formulated by the addition in the basal feed (PC and NC experiment diet) an appropriate amount of the crystalline amino acids (L-Lysine Sulphate, 546 g/kg; DL-Methionine, 990 g/kg; L-Threonine, 980 g/kg; L-Valine, 965 g/kg; Glycine, 990 g/kg; L-Arginine, 985 g/kg; L-Isoleucine, 990 g/kg; L-Tryptophan, 990 g/kg) provided by Evonik Ltd., São Paulo, Brazil, CJ Bio Ltd., São Paulo, Brazil, and Ajinomoto Ltd., São Paulo, Brazil. Treatments were formulated by supplementing the basal diet with NEAA (L-Glycine, L- Alanine, and L-Glutamate) provided by Ajinomoto Ltd., São Paulo, Brazil, as a replacement for corn starch.

The eN was obtained by multiplying broilers requirement for EAA by their respective N content, and tN was calculated by multiplying the N content in the dietary components by its respective inclusion in diet. The ideal protein concept was used for the remaining EAA and to avoid a possible excess of N from EAA, digestible lysine was fixed at 11.7 g/kg in the starter phase (12 to 23 d). Sodium bicarbonate was used to maintain the same dietary electrolyte balance (DEB, approximately 200 mEq/kg) and pH in the diets. The protein-free diet (PFD) (Table 2) was formulated to determine endogenous amino acid losses in the ileum of birds. In all experimental diets, 10 g/kg of Celite TM (acid-insoluble ash – AIA) was added.

Live Bird Performance

During the experimental period (from 12 to 23 day post-hatch) growth rate was determinate. Mortalities were recorded and the feed conversion ratio (FCR) was calculated with correction for mortalities by adding the weight of dead birds to live birds for all experimental period. At 22 day, broilers and feed in each experimental unit were

weighed to determine the final body weight (FBW), body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR).

Nitrogen Utilization and Metabolizable Energy Analysis

N utilization, apparent metabolizable energy (AME), and N-corrected apparent metabolizable energy (AMEn) analysis were conducted from 18 to the 22 d posthatch, collecting total excreta twice a day during this period. The excrement collected in each cage was homogenized at the end of the experimental period and 200 g samples were pre-dried at 55 °C for 72 h [14], and ground in a ball mill (Tecnal Equipamentos para Laboratório, TE-350, São Paulo, Brazil) for 5 min, until a fine mixture is obtained.

Dry matter (DM) of the diets and excreta were determined in duplicate on a 2-g sample by drying in a forced-air oven at 103°C for 16 h. The N content of diets and excreta were determined in duplicate on a 0.2-g sample by the Kjeldahl method according to the [15]. The N and DM content of the diets and excreta were used to determinate N utilization parameters and metabolizable energy.

N utilization was determinate nitrogen intake (NI), nitrogen excreted (NE), nitrogen balance (NB), and retained nitrogen rate (%RN). NI was calculated by multiplying the N present in diet by the dry matter intake. NE was calculated by multiplying the total excretion amount (in DM) by the %RN found in the excretion (also in DM). Nitrogen balance (NB) was obtained based on the amount of NI minus the NE. The Retained nitrogen rate (%RN) was calculated by divided the NB from the amount of N that was consumed [16].

Gross energy (GE) values contents of feed and excreta were determined in duplicate on 1-g samples using a C5001 adiabatic calorimetric pump (IKA-Werke GmbH & Co. KG, Staufen, Germany). Feed consumption and excreta weights during the collection period were used to calculate energy, NI, and NE. Values of AME and AMEn were calculated based on GE values recorded for food and excreta by using the equations described by Sakomura and Rostagno [16]:

$$\text{AME} = (\text{GEing} - \text{GEexc})/\text{DMing} \text{ and}$$

$$\text{AMEn} = (\text{GEing} - \text{GEexc} - (8.22 * \text{NB}))/\text{DMing}$$

in which GEing = gross energy ingested, GEexc = gross energy excreted, NB = nitrogen balance, and DMing = dry matter ingested.

Amino acid apparent and standardized ileal digestibility analysis

From 21 to 23 days of experiment was determined the apparent (AIAAD) and standardized (SIAAD) ileal amino acid digestibility. At 23-days age, 648 birds were slaughtered by cervical dislocation and their abdominal cavity was opened and all intestinal contents found 40 cm away from the terminal ileum, anterior to the ileocecal junction, were removed [17]. Ileal digesta from the animals used in each replication were combined to form a composite sample for each treatment.

Ileal digesta samples were lyophilized under vacuum, at -40°C for 72 hours. Samples of digesta and diets were analyzed for DM and ash [15]. Acid insoluble ash (AIA) and indigestibility factor were determined according to Joslyn [18]. The AA and CP content of the diets and digesta were analyzed by AMINOLab (Evonik, Germany). Calculations of apparent and standardized ileal digestibility of amino acids were performed using the methodology proposed by Sakomura and Rostagno [16], according to the equations below:

$$CDAAapa = ((AAing - (AADig * IF1))/AAing) * 100$$

$$CDAAsta = (AAing - ((AADig * IF1) - (AAend * IF2))/AAing) * 100$$

in which CDAAapa = apparent amino acid digestibility coefficient; AAing = ingested amino acid; AAdig = digesta amino acid; IF1 = indigestibility factor 1, IF1 = acid-insoluble ash (AIA) diet/AIAdigesta; IF2 = indigestibility factor 2, IF2 = AIA protein-free diet/AIAdigesta; CDAAsta = standardized amino acid digestibility coefficient; and AAend = endogenous amino acid.

Statistical Analysis

Data were initially submitted to the Kolmogorov-Smirnov normality test to confirm normal distribution. When data were not normally distributed, they were transformed into Log 10. Then the data were analyzed via one-way ANOVA, using ExpDes.pt from the R statistical package (version 4.0.4). To compare each treatment to positive control (treatment 1) Dunnett's multiple comparisons was used. The level of significance was set at $P < 0.05$ for all the tests.

3. Results

Analyzed CP, DM, and amino acid content in the experimental diets are present in Table 3. The experimental dietary composition results showed that almost all the analyzed values of the AA were comparable with calculated values.

Live Bird Performance

Performance measurements obtained in the trial are shown in Table 4. Significant difference was observed in FBW, BWG, and FI between NC and PC treatment. There was no significant difference ($p > 0.05$) on FCR when comparing PC treatment to other treatments in this trial. Broilers fed with 190 g/kg CP without the supplementation of NEAA mixture and NPN sources show a lower ($p < 0.05$) FBW than broilers fed with 220 g/kg CP. Reducing dietary CP from 220 to 190 g/kg without the addition of non-specific N source resulted in a decrease ($p < 0.05$) on the BWG of broilers when compared with dietary treatment with 220 g/kg CP. FI from 12 to 22 d was higher ($p < 0.05$) for chickens fed with PC treatment than those fed with NC treatment. In this trial, the optimum eN-to-tN ratio for maximum growth performance was obtained with 0.50, when compared to treatment with eN-to-tN ratio of 0.58.

Nitrogen Utilization

NI per g/bird/d decreased ($p < 0.05$) when was reduced the dietary CP from 220 to 190 g/kg, even with the supplementation of EAA in the NC treatment (Table 5). Lower ($p < 0.05$) NE was observed in the broilers fed with NC with or without the supplementation of urea when compared to PC treatment. Reducing 30 g/kg CP and supplementation of NEAA mixture and monoammonium phosphate increase ($p < 0.05$) NE, when compared to broilers fed 220 g/kg. Broilers fed with NC diet show a lower ($p < 0.05$) NB than those fed with PC treatment. %RN from 12 to 22 d was lower when broilers were fed with 190 g/kg CP and supplemented with 15.63 g/kg of ammonium acetate and 16.18 g/kg NEAA mixture when compared to PC treatment.

Metabolizable energy

The treatments with reduction of 30 g/kg CP with addition of MP + NEAA mixture show a higher ($p < 0.05$) DMI than the result observed in PC treatment (Table 6). Low-CP diet (190 g/kg) with the addition of AANH₃ or MP + NEAA mixture decreased ($p < 0.05$) AME content when compared to PC diet (220 g/kg). Fed broilers with 190 g/kg CP and supplementation of 16.18 g/kg ammonium acetate + 15.63 g/kg NEAA mixture

had a significant effect ($p < 0.05$) on AMEn value. In this group, was observed a lower AMEn value when compared to the group fed with 220 g/kg CP. The addition of urea in a diet with 190 g/kg CP increased the AMEnMN value when compared to PC treatment. However, broilers fed with 190 g/kg CP and supplemented with ammonium acetate + NEAA mixture show a lower ($p < 0.05$) AMEnNM value than broilers fed with 220 g/kg CP.

Amino acid digestibility

The AIAAD of EAA and NEAA are reported in Table 7. Significant interaction between PC and NC with or without supplementation of NEAA mixture and NPN sources was observed in this trial for some amino acids as: lysine, methionine + cystine, threonine, valine, leucine, isoleucine, glutamic acid, glycine, cystine, and alanine ($p < 0.05$). PC diet decreased ($p < 0.05$) AIAAD value of lysine, methionine, methionine + cystine, valine, and glycine when compared to the result obtained with dietary reduction of CP from 220 to 190 g/kg, even when NEAA mixture and NPN sources were added. AIAAD values of the threonine, isoleucine, and cystine increase ($p < 0.05$) in groups fed with 190 g/kg CP, without supplementation with NEAA mixture and NPN, when compared to PC treatment. Glutamic acid and Alanine show a higher AIAAD value in the treatments with 190 g/kg CP and addition of NEAA mixture associated or not with NPN than those obtained in PC treatment.

All EAA, except arginine, show a significant effect ($p < 0.05$) on SIADD value when comparing the NC treatments with PC treatment (Table 8). Broilers receiving a diet with 190 g/kg CP and supplementation or not with NEAA mixture and NPN sources show an increase on SIADD value of lysine, methionine, methionine + cystine, threonine, valine, leucine, isoleucine, histidine, and phenylalanine. Reducing dietary protein to 190 g/kg resulted in higher ($p < 0.05$) SIAAD value of glycine and cystine, than those results obtained in PC treatment, even when supplemented reduced CP diet with non-specific nitrogen sources. Alanine shows an increase in SIAAD value only in the treatments with reduction of CP and supplementation of NEAA mixture with or not NPN source addition when compared to PC treatment.

4. Discussion

Reduced-CP diets supplemented only with EAA (Met, Lys, and Thr) may have a direct influence on performance parameters of broilers, resulting in lower BWG and

higher FRC [19,20]. It occurs especially when the birds are in the grower phase and when this dietary reduction is less than 190 g/kg CP, as the protein in this phase play a fundamental role in the development of the gastrointestinal tract (GIT) of the birds [21,22]. The GIT, if well developed, contributes positively to the productive efficiency of the birds, though better digestion and absorption of nutrients, resulting in greater bioavailability of nutrients for growth of broilers [23].

In this study, fed broilers with 190 g/kg CP and eN:tN ratio of 0.58, even with EAA supplementation, resulted in lower FBW, BWG, and FI when compared to the group fed PC dietary treatment. However, broilers fed 190 g/kg CP and eN:tN ratio of 0.50, with NEAA or NPN supplementation, maintained the FBW, BWG, and FI. Yuan et al. [24] and Ospina-Rojas et al. [19] attribute this negative effect of reduced-CP diet on growth performance to the insufficient amount of non-specific nitrogen present in these diets, nitrogen necessary for the synthesis of NEAA. According to Dean et al. [25], when broilers are fed reduced CP diet with only EAA supplementation the nitrogen from the EAA can be used for the synthesis of NEAA, which is not enough N to carry out this synthesis, also resulting in less availability of EAA for animal growth. These authors mention this is more evident in the dietary reduction of CP above 30 g/kg. To Aftab et al. [26] another effect of reducing CP level is related to the FI of the broilers. These authors mention that the FI can be reduced when the broilers are fed with reduced-CP diet. In this study, when the group was fed 190 g/kg without supplementation of none non-specific N source, the FI was significantly decreased from those who were fed diet with 220 g/kg or 190 g/kg supplemented with NEAA or NPN. Aftab et al. [26] explain that this ben occurs for some reasons as: reduced levels of NEAA and an unbalanced plasma amino acid profile.

To avoid these negative effects, an additional source of non-specific N is needed for the NEAA synthesis. NEAA supplementation, such as glutamine, glycine, and alanine, or a source of NPN seems to be an adequate strategy in reduced CP diets, as these NEAA or NPN will provide the non-specific N necessary for the synthesis of other NEAA [27], which leads to improved animal performance [6]. Glutamine is an important NEAA capable of converting into other NEAA [2], such as glutamine, arginine, and proline [28]. It is transported from the lumen to the enterocyte where it undergoes transamination, producing alpha-ketoglutarate where it acts directly in the citric acid cycle providing energy for cell turnover intestinal [29]. According to Porto et al. [28], glutamine supplementation promotes protein synthesis,

in addition to reducing the negative effects of heat stress. Thus, glutamine supplementation is essential for the maximum growth performance of broilers, improving growth and feed efficiency, even broilers under stress conditions [28,30,31]. Corzo et al. [32] supplemented 0.6% of glutamine to a diet with 18% CP for broilers from 6-21 days of age and observed enhanced BWG and FCR when compared to the group fed 180 g/kg CP without supplementation of glutamine.

Glycine is another source of non-specific N and plays multiple metabolic functions in the organism, such as protein synthesis and it is an integral part of creatine, uric acid, DNA, and RNA [24]. It is considered a NEAA for some authors [8,32,33] but, for young chicks, it is considered a semi-EAA, because it has a substantial effect on poultry nutrition, mainly in the initial phase of birds, due to its play on uric acid metabolism [34,19]. Supplementation of Gly in low-CP diets can improve BWG and FCR mainly in the early phases of growth [35]. Ospina-Roja et al. [19] and Siegert and Rodehutschord [33] mention that reducing dietary CP without decreasing BGW is limited due to insufficient glycine concentration in the diet, even with EAA supplementation.

Therefore, NEAA supplementation in diets with reduced protein combined with supplementation with EAA brings benefits when compared to diets with reduced protein and with supplementation only with EAA, resulting in an improvement in the productive parameters of chickens [1,4,19,20]. On the other hand, the combination of some NEAA can also act to improve animal performance, as we can see in this work, where each amino acid will have different concentrations of N to enhance the supply of N necessary for the synthesis of other amino acids by half of the available N [6,25].

Other sources of non-specific nitrogen used in this study were urea, ammonium acetate, and monoammonium phosphate. The addition of one of these NPN in the diet with 190 g/kg CP was not significantly different on FBW, BWG, and FI when compared to those fed with 220 g/kg CP without supplementation of any source of non-specific nitrogen. Therefore, it is possible to reduce the protein level in the diet of broiler chickens without negative effects on animal performance, when combined with the addition of a NPN source. Mansilla et al. [36] reported that ammonia provided by NPN can be used for NEAA synthesis, particularly when NEAA are limiting. Koyabayashi et al. [37] mention that in non-ruminant nutrition, the utilization of urea has no nutritional value for poultry and it is unable its utilization. On the other hand, Suncio et al. [38] assign that the supplementation of urea may contribute to replacing some NEAA in the diet of non-ruminants. For the utilization of any source of NPN the gut microorganism

plays an important role in the utilization of N from these sources, being responsible for the growth-promoting effect of urea in chicks [39]. Okomura et al. [40] studying the supplementation of urea in germ-free chicks did not observe a benefit effect in these animals. However, when these authors supplemented urea in conventional birds, FCR was significantly better.

When we studied the effect of NPN on nitrogen utilization, we observed that broilers fed 190 g/kg CP and supplementation of mixture of NEAA and a monoammonium phosphate increased the NE and the use of ammonium acetate showed a lower %RN when compared to broilers fed with PC diet. Karasawa et al. [41] related that it occurs may high activity of cecal ammonia production from a source of NPN, as monoammonium phosphate, causing higher ammonia contents in excreta than birds fed without a NPN source.

Diets formulated according to the ideal protein concept can improve animal performance, by increasing protein retention, and contributing to reducing N excretion [42,43,44]. High-CP diets can result in lower efficiency nitrogen due to high concentration of AA. The excess of AA will be utilized for deamination, which results in increased excretion of N as uric acid in the bird's excreta [45], resulting in a negative impact on the environment due to increased excretion of N. Thus, a lower excretion of nitrogen is required to reduce the impacts of this compound on the environment [3,46]. In this study, reducing the dietary CP from 220 to 190 g/kg resulted in lower NI and NE. However, a lower NB was observed when a group was fed 190 g/kg CP without any source of N, when compared to those fed PC diet. Some studies corroborate with the results found in this study, such as those found by Macelline et al. [47] who observed a reduction of 45% on NE when there was a reduction from 205 to 170 g/kg CP and supplementation with EAA in broiler chickens in a period of 15 to 35 d of age. Kriseldi et al. [48] found a reduction of 14.1% in N excretion when the birds were fed 24% CP diet. In this experiment, the group fed with low-CP diet supplement or not with NEAA mixture resulted in greater nitrogen retention when compared to the result obtained in PC treatment. For Belloir et al. [49] and Hilliar et al. [1] reducing CP tends to increase nitrogen efficiency in broilers, such that birds fed a diet <160 g/kg CP had nitrogen retention greater than 70%.

A better nitrogen efficiency can provide better welfare to the birds, due to a lower emission of ammonia [50], since diets with a high protein induce the bird to consume more water, which leads to an increase in the moisture of the litter [51]. This higher

moisture can result in footpad dermatitis and breast blisters, in addition to increased risks of contamination by pathogens such as coccidiosis [52]. Thus, reduced CP diet can help lower N excretion into the environment, as well as provide better welfare to the birds. However, further studies are needed to evaluate the efficiency of nitrogen in low-CP diet supplemented with EAA and NEAA, as the broiler's performance results obtained in this work demonstrate that the reduction of CP without the addition of NEAA resulted in lower growth performance. Thus, to better use of low-CP diet on poultry nutrition, studies are needed to improve the efficiency of nitrogen and do not decrease the growth performance of broilers.

5. Conclusions

The supplementation of NEAA or NPN seems to be efficient in diet with reduced CP, allowing a reduction from 220 to 190 g/kg CP and achieving an eN:tN of 0.50 having a beneficial effect on live performance, less environmental impact, and better values of AME and digestibility of amino acids.

Author Contributions

Conceptualization, H.S.R., and L.F.T.A.; formal analysis, R.d.S.F., and T.F.D.; funding acquisition, H.S.R., and L.F.T.A.; investigation, R.d.S.F., R.A.R.d.L., L.F.T.A.; methodology, H.S.R., L.F.T.A., R.d.S.F., and V.D.N.; project administration, R.d.S.F., and L.F.T.A.; supervision, L.F.T.A., and A.A.C.; writing—original draft, R.d.S.F.; writing—review and editing, R.d.S.F., J.K.V., A.A.C., M.I.H., and V.D.N

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Informed Consent Statement

Not applicable.

Data Availability Statement

Not applicable

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Conflicts of Interest

The authors declare no conflict of interest. The co-author is an employee of Evonik Ltd., the supplier of some amino acids, which does not mean competing interests.

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Crude protein, g/kg	220	190	220.5	220.5	220.5	220.5	220.5	220.5	220.5
Metabolizable energy, kcal/kg	3.065	3.065	3.065	3.065	3.065	3.065	3.065	3.065	3.065
eN:tN ratio	0.497	0.582	0.500	0.500	0.500	0.500	0.500	0.500	0.500
Calcium, g/kg	8.78	8.78	8.78	8.78	8.78	8.78	8.78	8.78	8.78
Available phosphorus, g/kg	4.19	4.19	4.19	4.19	4.19	4.19	4.19	4.19	4.19
Sodium, g/kg	2.18	2.18	2.18	2.18	2.18	2.18	2.18	2.18	2.18
SID Lysine, g/kg	11.70	11.70	11.70	11.70	11.70	11.70	11.70	11.70	11.70
SID Met + Cys, g/kg	8.66	8.66	8.66	8.66	8.66	8.66	8.66	8.66	8.66
SID Threonine, g/kg	7.72	7.72	7.72	7.72	7.72	7.72	7.72	7.72	7.72
SID Valine, g/kg	9.19	9.01	9.01	9.01	9.01	9.01	9.01	9.01	9.01
SID Isoleucine, g/kg	8.61	7.84	7.84	7.84	7.84	7.84	7.84	7.84	7.84
SID Tryptophan, g/kg	2.53	2.11	2.11	2.11	2.11	2.11	2.11	2.11	2.11
SID Arginine, g/kg	13.86	12.52	12.52	12.52	12.52	12.52	12.52	12.52	12.52
SID Gly+Ser, g/kg	17.79	17.20	17.20	17.20	17.20	17.20	17.20	17.20	17.20
DEB (mEq/kg)	231	206	206	206	206	206	206	206	206

¹Mineral Premix (amount per kg diet): manganese – 77.0 mg, iron – 55.0 mg, zinc – 71.5 mg, copper – 11.0 mg, iodine – 1.10 mg, selenium – 0.33 mg . Vitamin Premix (amount per kg diet): vitamin A - 8250 IU, vitamin D3 - 2090 IU, vitamin E - 31.0 IU, vitamin B1 - 2.20 mg, vitamin B6 - 3.08 mg, pantothenic acid - 11.0 mg, biotin - 0.077 mg, vitamin K3 - 1.65 mg, folic acid - 0.77 mg, nicotinic acid - 33.0 mg, vitamin B12 - 0.013 mg, choline chloride (60%)- 1 g; butylated hydroxytoluene - 0.1 g; salinomycin sodium (12%) -0.55 g.

²Acid-insoluble ash (AIA). Cellite source.

³Non-essential amino acids mixture (60% Alanine, 20% Glycine, 20% Glutamic acid = 15.45% N).

eN-to-tN: essential to total nitrogen; mix: non-essential amino acids mixture; AANH³: ammonium citrate; MP: monoammonium phosphate.

Table 2. Composition of protein-free diet (PFD)

Ingredient	PFD (g/kg)
Corn starch	827.50
Sugar	50.00
Soybean oil	50.00
Dicalcium phosphate	16.20
Limestone	8.00
Salt	4.50
Corn cob	30.0
Mineral supplement. ¹	0.50
Vitamin supplement. ²	1.30
Choline chloride (60%)	2.00
Bookmark (Celite)	10.00
Total	1000

¹Mineral supplement - guaranteed levels per kg of feed: selenium, 0.375 mg; manganese, 88 mg; iron, 62.5 mg; zinc, 81.3 mg; copper, 12.5 mg; iodine, 1.25 mg.

²Vitamin supplement - guaranteed levels per kg of feed: vitamin A, 9,375 IU; vitamin D3, 2,375 IU; vitamin E, 35 IU; vitamin B1, 2.50 mg; vitamin B2, 6.25 mg; vitamin B6, 3.5 mg; vitamin B12, 0.015 mg; nicotinic acid, 37.5 mg; B.C. pantothenic acid, 12.5 mg; vitamin K3, 1.88 mg; B.C. folic acid, 0.875 mg; biotin, 0.088 mg

Table 3. Analyzed crude protein (CP), dry matter (DM), and total amino acid level (g/kg) of experimental diets fed to broilers from 12 to 23 days of age.

Treatments	1	2	3	4	5	6	7	8	9
CP (g/kg)	220	190	190+Mix	190+Urea	190+Mix +Urea	190+ AANH ³	190+Mix +AANH ³	190+Mix +2%MP	190+Mix +1%MP
eN:tN ratio	0.50	0.58	0.50	0.50	0.50	0.50	0.50	0.50	0.50
DM	900.7	886.6	903.2	900.6	902.3	877.7	887.4	897.6	896.0
CP	240	198.8	227.3	221.8	231.1	227.8	222.4	231.3	236.3
Lys	12.97	13.38	13.23	14.04	12.68	13.26	12.9	14.02	14.39
Met	5.87	5.67	6.19	6.17	6.54	5.68	6.20	5.97	6.28
Cys	3.41	2.89	2.85	2.93	2.83	2.97	2.90	2.98	3.02
Met+Cys	9.28	8.56	9.04	9.10	9.37	8.65	9.10	8.95	9.30
Thr	8.95	8.04	8.04	8.56	8.04	8.20	8.45	8.56	9.07
Ile	9.95	8.79	8.48	8.88	8.45	8.59	8.50	7.92	8.05
Val	10.73	10.49	10.47	10.75	10.69	10.99	10.46	11.45	12.13
Arg	15.57	13.55	13.48	14.07	13.56	13.52	13.29	13.72	14.05
Leu	18.59	16.42	15.85	16.28	15.58	16.20	15.96	16.28	16.43
His	5.86	4.91	4.78	4.94	4.71	4.86	4.79	4.94	5.02
Gly	9.25	10.39	16.46	10.98	14.12	10.43	14.12	14.03	15.54
Ala	10.98	9.54	25.07	9.68	17.85	9.55	17.41	18.28	24.38
Glu	40.05	33.31	37.49	33.32	34.72	33.20	35.50	36.81	38.63
Pro	12.62	11.05	10.63	11.07	10.48	10.27	10.20	10.49	10.44
Asp	23.54	18.55	17.79	18.56	17.60	18.27	17.78	18.62	19.16
Phe	11.26	9.28	8.95	9.20	8.83	9.37	9.13	9.61	9.70
Ser	10.87	8.95	8.49	8.96	8.55	8.88	8.71	9.04	9.28

eN-to-tN: essential to total nitrogen; mix: non-essential amino acids mixture; AANH³: ammonium citrate; MP: monoammonium phosphate.

Table 4. Final body weight (FBW), Body weight gain (BWG), Feed intake (FI) and feed conversion ratio (FCR) of broiler male chicks from 12-23 days of age submitted to crude protein (CP) reduction and addition of non-essential amino acid mixture (Mix) and non-protein sources of nitrogen.

Treatments	eN:tN ratio	FBW (kg)	BWG (kg)	FI (kg)	FCR
220 g/kg CP (PC)	0.50	1.065	0.820	1.012	1.234
190 g/kg CP (NC)	0.58	1.012*	0.767*	0.952*	1.241
190 g/kg CP + 3.158% Mix	0.50	1.029	0.800	0.968	1.216
190 g/kg CP + 1.111% Urea	0.50	1.050	0.80	0.992	1.231
190 g/kg CP + 1.618% Mix+ 0.556% Urea	0.50	1.054	0.809	1.021	1.262
190 g/kg CP + 3.125% AANH ³	0.50	1.056	0.809	1.024	1.266
190 g/kg CP + 1.618% Mix + 1.563% AANH ³	0.50	1.056	0.814	1.025	1.260
190 g/kg CP + 1.812% Mix + 2% MP	0.50	1.059	0.832	1.031	1.240
190 g/kg CP + 2.524% Mix + 1% MP	0.50	1.081	0.823	1.059	1.286
<i>P</i> -value		0.006	0.050	0.001	0.182
SEM		0.012	0.013	0.019	0.028

Positive control (PC); Negative control (NC); Essential nitrogen:total nitrogen (eN:tN); Ammonium acetate 16% nitrogen (AA); Monoammonium phosphate 11% nitrogen. 21% phosphor (MP).

MIX: non-essential amino acids mixture (60% alanine. 20% glycine and 20% glutamic acid).

SEM: standard error mean.

Means followed by * in the same column differ from T1 based on Dunnett's test at 5% significance level.

Table 5. Effect of crude protein (CP) reduction, addition of mixed non-essential amino acids (Mix) and non-protein N sources on the nitrogen intake (NI), nitrogen excreted (NE), nitrogen balance (NB) and percentage of retained nitrogen (RN) in broiler male chicks 12- 23 days of age.

Treatments	eN:tN ratio	NI (g/bird/d)	NE (g/bird/d)	NB (g/bird/d)	%RN
220 g/kg CP (PC)	0.50	3.36	1.08	2.28	67.89
190 g/kg CP (NC)	0.58	2.62*	0.83*	1.79*	67.74
190 g/kg CP + 3.158% Mix	0.50	3.16	1.01	2.15	67.26
190 g/kg CP + 1.111% Urea	0.50	3.07	0.91*	2.16	70.09
190 g/kg CP + 1.618% Mix+ 0.556% Urea	0.50	3.32	0.99	2.33	70.03
190 g/kg CP + 3.125% AANH ₃	0.50	3.23	1.13	2.10	64.95
190 g/kg CP + 1.618% Mix + 1.563% AANH ₃	0.50	3.18	1.21	1.97	61.93*
190 g/kg CP+ 1.812% Mix + 2% MP	0.50	3.47	1.24*	2.23	64.06
190 g/kg CP + 2.524% Mix + 1% MP	0.50	3.50	1.27*	2.23	63.46
<i>P</i> -value.		<0.001	0.001	<0.001	<0.001
SEM		0.108	0.057	0.097	1.629

Positive control (PC); Negative control (NC); Essential nitrogen:total nitrogen (eN:tN); Ammonium acetate 16% nitrogen (AA); Monoammonium phosphate 11% nitrogen. 21% phosphor (MP).

SEM: standard error mean.

Means followed by * in the same column differ from T1 based on Dunnett's test at 5% significance level.

Table 6. Effect of crude protein (CP) reduction, addition of mixed non-essential amino acids (Mix) and non-protein N sources on dry matter intake (DMI), apparent metabolizable energy (AME) and corrected by nitrogen balance (AMEn) on the dry and natural (AMEnNM) matter of the excreta of broilers male chicks 12-23 days of age.

Treatments	eN:tN ratio	DMI (kg)	AME (kcal/kg)	AMEn (kcal/kg)	AMEnNM (kcal/kg)
220 g/kg CP (PC)	0.50	2.863	3424	3326	2956
190 g/kg CP (NC)	0.58	2.834	3427	3350	2990
190 g/kg CP + 3.158% Mix	0.50	2.727	3427	3331	2975
190 g/kg CP + 1.111% Urea	0.50	2.870	3502	3409	3042*
190 g/kg CP + 1.618% Mix+ 0.556% Urea	0.50	3.064	3484	3390	3022
190 g/kg CP + 3.125% AANH ³	0.50	2.964	3415	3328	2953
190 g/kg CP + 1.618% Mix + 1.563% AANH ³	0.50	2.920	3301*	3218*	2855*
190 g/kg CP + 1.812% Mix + 2% MP	0.50	3.100*	3326*	3237	2912
190 g/kg CP + 2.524% Mix + 1% MP	0.50	3.180*	3408	3280	2940
<i>P</i> -value		<0.001	<0.001	<0.001	0.001
SEM		0.083	38.194	36.610	32.545

Positive control (PC); Negative control (NC); Essential nitrogen:total nitrogen (eN:tN); Ammonium acetate 16% nitrogen (AA); Monoammonium phosphate 11% nitrogen. 21% phosphor (MP).

SEM: standard error mean.

Means followed by * in the same column differ from T1 based on Dunnett's test at 5% significance level.

Table 7. Effect of crude protein (CP) reduction and addition of non-essential amino acid mixture (Mix) and non-protein N on apparent ileal amino acid digestibility of essential and non-essential amino acids of broilers male chicks 12-23 days of age (%).

Treatments	1	2	3	4	5	6	7	8	9	P-Value	SEM
CP (g/kg)	220	190	190+Mix	190+Urea	190+Mix+Urea	190+AANH ³	190+Mix+AANH ³	190+Mix+2%MP	190+Mix+1%MP		
eN:tN ratio	0.50	0.58	0.50	0.50	0.50	0.50	0.50	0.50	0.50		
Essential Amino Acids											
Lysine	87.83%	91.84%*	91.01%*	88.74%	90.80%*	90.96%*	90.43%*	90.60%*	90.64%*	<0.001	0.006
Methionine	92.89%	95.06%*	95.02%*	92.53%	94.91%*	93.85%	94.79%*	94.08%	94.14%	<0.001	0.004
Methionine + Cistine	88.30%	92.25%*	91.71%*	81.18%*	91.40%*	90.34%	91.26%*	90.59%*	90.81%*	<0.001	0.011
Threonine	81.20%	85.54%*	84.01%	79.94%	83.64%	83.07%	84.16%	83.81%	83.75%	0.003	0.010
Arginine	93.12%	93.18%	92.58%	92.82%	92.83%	92.94%	92.32%	91.78%	91.73%	0.103	0.004
Valine	84.60%	89.05%*	87.99%*	83.36%	87.72%*	87.90%*	87.48%	87.64%*	88.18%*	<0.001	0.009
Leucine	87.78%	90.10%	88.69%	85.21%	78.58%*	88.54%	88.67%	87.88%	87.66%	<0.001	0.012
Isoleucine	86.61%	89.55%*	88.09%	84.38%	87.83%	87.77%	88.00%	86.05%	85.82%	0.002	0.008
Histidine	90.24%	92.13%	91.32%	89.01%	91.33%	91.46%	91.08%	90.76%	90.99%	0.187	0.006
Phenylalanine	89.57%	91.14%	89.99%	87.28%	89.82%	90.28%	89.73%	89.24%	89.46%	0.293	0.007
Non-essential Amino Acids											
Aspartic Acid	88.36%	88.98%	87.51%	86.17%	87.32%	87.77%	86.99%	86.72%	87.01%	0.158	0.007
Glutamic Acid	92.82%	93.44%	93.73%	91.81%	93.30%	92.94%	93.12%	92.72%	93.11%*	0.052	0.004
Serine	88.13%	88.68%	86.92%	85.86%	87.34%	88.02%	87.18%	86.77%	86.58%	0.118	0.007
Glycine	83.59%	89.25%*	92.47%*	85.30%	91.26%*	87.44%*	91.06%*	87.29%*	91.07%*	<0.001	0.010
Cistine	80.39%	86.73%*	84.21%	77.15%	83.30%	83.63%	83.70%	83.61%	83.90%	<0.001	0.012
Alanine	86.49%	87.10%	94.63%*	85.50%	92.82%*	86.57%	92.24%*	91.95%*	93.80%*	<0.001	0.011
Proline	90.20%	91.32%	90.08%	89.26%	90.44%	90.16%	89.68%	89.10%	88.95%	0.088	0.006

SEM: standard error mean.

Means followed by * in the same column differ from T1 based on Dunnett's test at 5% significance level.

eN-to-tN: essential to total nitrogen; mix: non-essential amino acids mixture; AANH₃: ammonium citrate; MP: monoammonium phosphate.

Table 8. Effect of crude protein (CP) reduction and addition of non-essential amino acid mixture (Mix) and non-protein N sources on standardized ileal amino acid digestibility of essential and non-essential amino acids (%).

Treatments	T1	T2	T3	T4	T5	T6	T7	T8	T9	P-Value	SEM
CP (g/kg)	220	190	190+Mix	190+Urea	190+Mix+Urea	190+AANH ³	190+Mix+AANH ³	190+Mix+2%MP	190+Mix+1%MP		
eN:tN ratio	0.50	0.58	0.50	0.50	0.50	0.50	0.50	0.50	0.50		
Essential Amino Acids											
Lysine	91.16%	95.08% *	94.29% *	91.83%	94.20% *	94.18% *	93.77%*	93.72%*	93.66%*	<0.001	0.006
Methionine	100.25%	102.71%*	102.03%*	99.56%	101.50%	101.37%	101.75*	101.39%	101.08%	<0.001	0.005
Methionine + Cistine	92.95%	97.31%*	96.51%*	88.21%*	96.01% *	95.28%*	96.00%*	95.47%*	95.50%*	<0.001	0.009
Threonine	86.02%	90.93%*	89.41%	85.00%	89.01%	88.27%	89.27%	88.92%	88.56%	0.009	0.010
Arginine	95.95%	96.38%	95.80%	95.90%	96.02%	96.10%	95.57%	94.96%	94.83%	0.139	0.004
Valine	88.62%	93.18%*	92.13%*	87.39%	91.76%*	91.78%*	91.60%	91.47%	91.77%*	<0.001	0.009
Leucine	90.10%	92.74%	91.43%	87.87%	83.68%*	91.18%	91.37%	90.56%	90.31%	<0.001	0.010
Isoleucine	90.95%	94.49%*	93.21%	89.26%	92.94%	92.74%	93.08%	91.56%	91.24%	0.002	0.008
Histidine	97.61%	100.97%*	100.40%*	97.79%	100.50%*	100.25%*	100.08%*	99.60%*	99.67%*	<0.001	0.009
Phenylalanine	93.40%	95.81%*	94.84%	91.99%	94.71%	94.84%	94.45%	93.78%	93.96%	0.005	0.007
Non-essential Amino Acids											
Aspartic Acid	90.26%	91.40%	90.04%	88.59%	89.86%	90.20%	89.51%	89.15%	89.32%	0.225	0.007
Glutamic Acid	93.90%	94.74%	94.89%	93.11%	94.54%	94.23%	94.34%	93.91%	94.24%	0.064	0.004
Serine	92.10%	93.53%	92.04%	90.70%	92.39%	92.82%	92.13%	91.60%	91.28%	0.168	0.007
Glycine	88.26%	93.42%*	95.11%*	89.25%	94.32%*	91.53%*	94.12%*	91.45%*	93.87%*	<0.001	0.006
Cistine	93.05%	101.74%*	99.44% *	91.95%	98.55%*	98.01%*	98.57%*	98.26%*	98.32%*	<0.001	0.013
Alanine	90.42%	91.65%	96.36%*	89.98%	95.24%*	91.04%	94.72%*	94.34%*	95.59%*	0.039	0.008
Proline	93.62%	95.25%	94.16%	93.17%	94.55%	94.32%	93.91%	93.26%	93.12%	0.127	0.006

SEM: standard error mean.

Means followed by * in the same column differ from T1 based on Dunnett's test at 5% significance level.

eN-to-tN: essential to total nitrogen; mix: non-essential amino acids mixture; AANH₃: ammonium citrate; MP: monoammonium phosphate.

GENERAL CONCLUSIONS

1. Reduction of 20 to 30 g/kg of the dietary CP from a diet with 220 g/kg CP and supplementation of non-essential amino acids mixture (Glu, Gly, and Ala) seems do not impair the performance of broiler chickens from 12 to 23 d post hatching, when compared with dietary protein reductions without adding a certain amount of non-essential amino acids. However, it is important that the essential nitrogen-to-total nitrogen ratio be 0.52 or less to obtain these results. Low-CP diets with the addition of non-essential amino acids and eN:tN ratio from 0.58 to 0.50 can improve nitrogen retention, AME, AMEn values, and amino acid digestibility.
2. Reducing dietary CP reduces final body weight, body weight gain, and feed intake of broilers from 12 to 23 d of age when this diet was not supplemented with a source of non-specific nitrogen, as NEAA or NPN and eN:tN ratio was 0.58. The supplementation of NEAA or NPN seems to be efficient in diet with reduced CP, allowing a reduction from 220 to 190 g/kg CP and achieving an eN:tN of 0.50. Reduced-CP diet seems to have a beneficial effect on environmental impact being decreased NI and NE even when were added NEAA mixture and urea. Furthermore, AME and AMEn values seem not to be compromised with the reduction of CP, with or not supplementation of NEAA mixture of urea. Apparent and standardized ileal digestibility of some amino acids as: methionine, lysine, threonine, and glycine increase when there is a reduction of CP, even when was not supplemented with NEAA mixture and NPN.



UNIVERSIDADE FEDERAL DE VIÇOSA
 COMISSÃO DE ÉTICA NO USO DE ANIMAIS DE PRODUÇÃO
 CEUAP/UFV

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Viçosa, 12 de agosto de 2020

CERTIFICADO

Certificamos que o projeto intitulado "Níveis de proteína e relação nitrogênio essencial: nitrogênio não essencial em frangos de corte", protocolo n° 022/2020, sob a responsabilidade de Luiz Fernando Teixeira Albino - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo chordata, subfilo vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei n° 11.794, de 8 de outubro de 2008, do decreto n° 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi apreciado pela Comissão de Ética no Uso de Animais de Produção da Universidade Federal de Viçosa (CEUAP-UFV) em reunião de 23 de Junho de 2020.

Finalidade: (x) Pesquisa () Ensino

Vigência do Projeto: de 14 de agosto de 2020 a 20 de agosto de 2021

Espécie/linhagem: Frango de corte (*Gallus domesticus*) Nº de animais: 3528

Peso: 0,035Kg Idade: 01 dia Sexo: Macho Origem: Incubatorio Rivelli CNPJ/CPF: 478.715.616-49 /

Endereço: Rua Leão José, 257 Mateus Leme, MG Responsável : Maria Cecilia CRMV: 10595

CERTIFICATE

We certify that the project entitled "Protein level and essential to non-essential nitrogen ratio in broiler diets", protocol n° 022/2020, under the responsibility of Luiz Fernando Teixeira Albino - which involves the production, maintenance and/or use of animals belonging to the phylum chordata, subphylum vertebrata (except man), for scientific research purposes (or education) - is in accordance with the law n°. 11.794, of October 8, 2008, Decree n°. 6899 of July 15, 2009, and the rules issued by the Brazilian National Council for Animal Experimentation Control (CONCEA), and was approved by the Ethics Commission on the use of farm animals of Universidade Federal de Viçosa (CEUAP-UFV) in its meeting on June, 23 of 2020.

Finality: (x) Research () Education

Duration of the Project: from Aug. 14th, of 2020 to Aug. 20th, 2021.

Species / strain: Broiler (*Gallus domesticus*) Nº of animals: 3528

Weight: 0,035Kg Age: 01 day Sex: Male Source: Incubatorio Rivelli CNPJ/CPF: 478.715.616-49 /

Endereço: Rua Leão José, 257 Mateus Leme, MG Responsável : Maria Cecilia CRMV: 10595

Luciana Navajas Rennó

Coordenadora da CEUAP/UFV