ANA CLARA BAIÃO MENEZES

USE OF ^{15}N TO ESTIMATE MICROBIAL CONTAMINATION AND PROTEIN DEGRADATION OF CONCENTRATE FEEDS AND THE EFFECT OF DECREASING DIETARY CRUDE PROTEIN ON METHANE EMISSION AND NITROGEN LOSSES IN NELLORE BULLS

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

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Approved: February, 16, 2016.

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“You can judge a man's true character by the way he treats his fellow animals.”
Paul McCartney

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BIOGRAPHY

Ana Clara Baião Menezes, daughter of José Menezes and Terezinha Baião Menezes, was born in Ubá, Minas Gerais – Brazil on June 2, 1991.

She started bachelor´s degree in Animal Science at the Universidade Federal de Viçosa in 2009 and obtained Bachelor of Science in Animal Science in March of 2014.

In March of 2014, she started her Master degree with a major in ruminant nutrition and beef cattle production at the Universidade Federal de Vicosa.

SUMMARY

ABSTRACT

MENEZES, Ana Clara Baião, M.Sc., Universidade Federal de Viçosa, February of 2016. **Use of ^{15}N to estimate microbial contamination and protein degradation of concentrate feeds and the effect of decreasing dietary crude protein on methane emission and nitrogen losses in Nellore bulls.** Adviser: Sebastião de Campos Valadares Filho. Co-advisers: Edenio Detmann and Mario Luiz Chizzotti.

This study was developed based on two experiments conducted on the feedlot and Animal Laboratory of Animal Science Department of Universidade Federal de Viçosa, and the results are shown in two chapters. The aim of the first study was to use ^{15}N to estimate the microbial contamination (**MC**) of crude protein (**CP**) fractions that were soluble (**a**) as well as insoluble but potentially degradable (**b**), and the digestion rate of fraction b (**kd**), as well as to determine the incubation time necessary to estimate the rumen degradable protein (**RDP**) of energy and protein feeds when considering two outflow rates (0.05 h^{-1} and 0.08 h^{-1}). Twelve types of feeds were evaluated, with six energy concentrates: wheat bran, rice meal, ground corn, ground sorghum, ground corn cob, and soybean hulls; and six protein concentrates: cottonseed meal 38% CP, soybean meal, ground bean, peanut meal, sunflower meal, and corn gluten meal. The feeds were divided into 4 groups and were incubated in the rumens of 4 crossbred bulls. The feed samples were incubated for 0, 2, 4, 8, 16, 24, 48, and 72 h. To determine the MC of the incubated residues, ruminal bacteria were labeled with ^{15}N via continuous intraruminal infusion of $^{15}(NH_4)_2SO_4$. Ruminal digesta were collected for the isolation of bacteria before the first infusion of ^{15}N during the acclimation period, and after the infusion of ^{15}N during the collection period. There was no difference ($P > 0.05$) in the parameters a, b, and kd, corrected and uncorrected, for all feeds that were evaluated. All of the feed tests followed an exponential model of degradation and the model fitted well to the data, except for corn gluten meal, probably because the maximum incubation time that was used (72 h) was not long enough to allow for an

accurate estimation of the degradation profile. The cluster analysis (R^2 = 0.944) allowed for the grouping of feeds into three different groups according to the necessary incubation time to estimate RDP. The first was formed by the high-starch energy concentrates (15.4 ± 0.46 h), the second by the low-starch energy concentrates (6.8 ± 0.60 h), and the third by the protein concentrates (9.9 ± 0.41) while considering a kp of 0.05h^{-1}. In conclusion, the microbial contamination was low and non-significant; therefore, correction of ruminal protein degradation is irrelevant with regards to the concentrate that was studied. However, the chemical composition of this feeds resulted in different incubation times to estimate RDP content, and this has to be taken into account in the techniques that are used to determine CP digestibility in the rumen and intestines. The aim of the second experiment was to evaluate whether a reduction in dietary crude protein (CP) content affects animal performance, energy and protein requirements, N losses, and enteric methane emission in finishing Nellore bulls. Twenty-six animals, with an average age of 20 ± 1.0 months and initial body weight (BW) of 296 ± 8.1 kg were used in this experiment. Four animals were used as baseline reference animals and were slaughtered at the beginning of the experiment. Four animals were fed at maintenance level (MAIN), whereas 18 bulls were divided into 3 groups (*n* = 6 in each group) and were randomly assigned to the treatments consisting of three levels of CP in the diets: 10, 12, and 14% of CP. At the end of the experiment, all animals were slaughtered to evaluate their chemical body composition, energy and protein requirements, and carcass characteristics. A linear effect was observed for dietary CP level on CP intake and digestibility, while greater values were obtained for animals that were fed 14% CP. Nitrogen metabolism was affected by CP levels, where animals that were fed 12 and 14% CP had greater urinary N losses than those that were fed 10% CP. There was no effect of CP level on retained N, animal performance, and carcass characteristics among diets, and there was no effect of CP level on microbial efficiency and CH_4 emissions. Thus, this study showed that for finishing bulls, the level of dietary CP did not interfere with muscle deposition and greenhouse gas emissions. The reduction of CP content in diets does not affect DM intake, animal performance, and carcass characteristics, thereby suggesting that the use of 10% of CP in diets for finishing bulls reduces their environmental impact due to a lower urinary N excretion than 12 and 14% CP-based diets. Animals that were fed 10, 12, and 14% CP diets had emissions equivalent to 3,893; 3,755; and 4,255 g d^{-1} of CO_2, respectively, and no difference was observed among diets. Furthermore, methane emission is not affected by CP levels ranging between 10 to 14% which, on average, is 16.3 g kg^{-1} of DM intake. Our study found that a decreased CP level did not influence animal performance, but it did decrease N losses in manure without affecting methane emissions. However, it is important to highlight that more studies are necessary to confirm these results.

RESUMO

MENEZES, Ana Clara Baião, M.S., Universidade Federal de Viçosa, Fevereiro de 2016. **Uso do ^{15}N para estimar a contaminação microbiana e a degradação proteica de alimentos concentrados e o efeito da redução dos níveis de proteína bruta na dieta de machos Nelore não castrados sobre a emissão de metano e excreção de nitrogênio.** Orientador: Sebastião de Campos Valadares Filho. Coorientadores: Edenio Detmann e Mário Luiz Chizzotti.

Este trabalho foi desenvolvido baseado em 2 experimentos conduzidos no confinamento experimental do laboratório animal do Departamento de Zootecnia da Universidade Federal de Viçosa, e os resultados são mostrados em dois capítulos. O objetivo do primeiro experimento foi utilizar o ^{15}N para estimar a contaminação microbiana (CM) das seguintes frações da proteína bruta (PB): fração solúvel (a), insolúvel potencialmente degradável (b) e a taxa de digestão da fração b (kd). Assim como determinar os tempos de incubação necessários para estimar a proteína degradável no rúmen (PDR) de concentrados energéticos e proteicos, considerando duas taxas de passagem (0,05 h^{-1} e 0,08 h^{-1}). Doze alimentos foram avaliados, sendo seis concentrados energéticos (farelo de trigo, farelo de arroz, fubá de milho, sorgo moído, milho desintegrado com palha e sabugo e casca de soja) e seis concentrados proteicos (farelo de algodão 38% PB, farelo de soja, feijão moído, farelo de amendoim, farelo de girassol e farelo de glúten de milho). Os alimentos foram divididos e quatro grupos e incubados no rúmen de quatro touros fistulados. Os alimentos foram incubados por 0, 2, 4, 8, 16, 24, 48 e 72 horas. Para determinar a CM dos resíduos incubados, as bactérias ruminais foram marcadas com ^{15}N através de infusões ruminais contínuas de $^{15}(NH_4)_2SO_4$. Digesta ruminal foi coletada para isolamento de bactéria antes da primeira infusão de ^{15}N, durante o período de adaptação, e após a infusão de ^{15}N nos períodos de coleta. Não foi observada diferença (P > 0,05) nos parâmetros a, b e kd corrigidos e sem correção para todos os alimentos avaliados. Todos os alimentos seguiram um modelo exponencial de degradação, exceto o glúten de milho.

Provavelmente porque o tempo máximo de incubação utilizado (72 horas) não foi longo o suficiente para permitir uma estimativa acurada do perfil de degradação. A análise cluster (R^2 = 0.944) permitiu agrupar os alimentos em três diferentes grupos de acordo com o tempo necessário parra estimar a PDR. O primeiro grupo foi formado por concentrados energéticos com alto teor de amido (15.4 ± 0.46 h), o segundo por concentrados energéticos com baixo teor de amido (6.8 ± 0.60 h) e o terceiro por concentrados proteicos (9.9 ± 0.41), considerando taxa de passagem de 0,05 h^{-1}. Como conclusão, a contaminação microbiana foi baixa e não significativa, assim a correção da degradação proteica torna-se irrelevante para os concentrados utilizados neste estudo. No entanto a composição química dos alimentos resultou em diferentes tempos de incubação para estimar o conteúdo de PDR, e isto precisa ser levado em consideração nas técnicas utilizadas para estimar a digestibilidade ruminal e intestinal da PB. O objetivo do segundo experimento foi avaliar se a redução nos níveis de proteína bruta (PB) da dieta afeta o desempenho animal, exigências de energia e proteína, perdas nitrogenadas e emissão de metano entérico em machos Nelore não castrados na fase de terminação. Vinte e seis animais com idade média de 20 ± 1 mês e peso corporal inicial de 296 ± 8,1 kg foram utilizados neste experimento. Quatro animais foram designados ao grupo referência, sendo abatidos ao início do experimento. Quatro animais foram alimentados a nível de mantença, enquanto dezoito animais foram divididos em três grupos (n = 6 em cada grupo), e foram aleatoriamente designados a tratamentos consistindo de três níveis de PB na dieta: 10, 12 e 14% de PB. Ao final do experimento todos os animais foram abatidos para determinação de sua composição química corporal, exigências de energia e proteína e características de carcaça. Foi observado efeito linear do nível de PB da dieta sobre o consumo e digestibilidade da PB, onde os maiores valores foram obtidos para animais alimentados com 14%PB. O metabolismo do nitrogênio foi afetado pelos níveis de PB, onde animais alimentados com 12 e 14 % PB tiveram maior excreção urinaria de N que aqueles alimentados com 10% PB. Não houve efeito do nível de PB dietético sobre retenção de N, desempenho animal e características de carcaça, e também não foi observado efeito do nível de PB da dieta sobre a eficiência microbiana e emissão de CH_4. Este estudo mostrou que para bovinos em terminação o nível de PB da dieta não influencia a deposição muscular e a emissão de gases do efeito estufa. A redução do nível de PB das dietas não afetou o consumo de MS, desempenho e características de carcaça, sugerindo que o uso de 10% PB em dietas de terminação reduz o impacto ambiental devido a menor excreção urinária de N que dietas de 12 e 14% PB. Animais alimentados com 10, 12 e 14% de PB nas dietas tiveram emissões de 3.893, 3.755 e 4.255 g/d de equivalente CO_2 respectivamente e não foram observadas diferenças entre as dietas. Além disso a emissão de metano não foi afetada por níveis de PB da dieta variando entre 10 e 14%, sendo em média igual a 16,3 g/kg do consumo de MS. Este estudo mostrou que a redução do nível de PB da dieta não influenciou o desempenho animal e reduziu as perdas nitrogenadas nas fezes e urina sem afetar a emissão de metano. Porém é importante destacar que mais estudos são necessários para confirmar esses resultados.

INTRODUCTION

In situ methods are widely used to estimate the ruminal degradability of crude protein (CP), but an important source of errors in this method is the microbial contamination (MC) of the residual particles of incubated feeds, resulting in underestimation of CP degradability (Wulf and Südekum, 2005), as well as overestimation of the ruminal undegradable protein (RUP) content.

As ^{15}N is not naturally found in protein of feeds, it is widely used as a microbial marker. The ^{15}N can be incorporated by fertilization of cultivated feeds with ammonium sulfate enriched with ^{15}N (Wanderley et al., 1993; Kamoun et al., 2007) or by ruminal infusion (Dixon and Chanchai., 1999; Machado et al., 2013; Rotta et al., 2014).

There are few data in the literature regarding the effect of MC in the *in situ* evaluation of concentrate feeds, and they are controversial. Most authors evaluated one (González et al., 2006; Stefánski et al., 2013) or few feeds (Rodríguez et al., 2008; Arroyo and González, 2011), and most of them did not evaluate only concentrate. Researches developed on Brazil by Machado et al (2013) showed that MC is an important source of errors for tropical forages; these authors also concluded that protein and fiber level influence the dimension of contamination. González et al. (1998) and Wanderley et al. (1993) also showed that the chemical composition of feeds could influence the dimension of MC.

In Brazil, most of feedlots use 79% of concentrate in finishing diets (Oliveira and Millen., 2014). Corn, sorghum, soybean hulls, soybean meal, sunflower meal and cottonseed meal are the main ingredients used to formulate diets, so it is important to know the availability of rumen degradable protein (RDP) of these feeds. Inadequate supply of RDP relative to fermentable carbohydrate supply results in negative associative effects on fiber digestion that may result in “energy spilling” (Van Kessel and Russell, 1996; Klevesahl et al., 2003) and decreased microbial efficiency (Hoover and Stokes, 1991). So, the knowledge about RDP content and its availability on ruminal environment is important to attempt maximize the synthesis of microbial protein.

The mobile nylon bag technique consists in ruminal incubation of 16 h to access RDP content and consequently RUP content, to simulate intestinal digestibility (Casamiglia and Stern., 1995; Paz et al., 2014). But the use of a same incubation time for different feedstuffs can overestimate or underestimate the intestinal digestibility.

Accurate prediction of N supply is important to reach the requirements of rumen microflora and minimize feed costs and N waste (Brooks et al., 2012). The environmental impact of feeding animals in feedlots is a growing concern (Cole et al., 2006; Patra and Lalhriatpuii., 2016). Most of nutrients that are absorbed from feeds to feedlots are excreted as feces and urine, and cattle commonly retain only 10 to 20% of their nutrient intake (McBride et al., 2003). In addition, between thirty to fifty percent of N from feedstuffs may be lost via volatilization, mainly in the form of ammonia (Bierman et al., 1999; Todd et al., 2005), and this amount of ammonia can be affected by dietary crude protein (CP) level (Burgos et al., 2010). Furthermore, protein is considered to be the most expensive nutrient in a ruminant diets (Russell et al., 1992). Thus, unbalanced diets contribute to negative environmental impacts and represent significant economic losses.

According to the NRC (1996), CP requirements decrease during the finishing phase, however according to Oliveira and Millen (2014), Brazilian feedlots adopt up to 16.6% CP in their diets for finishing cattle to stimulate DM intake and to slaughter younger animals.

In addition, The Food and Agriculture Organization of the United Nations (FAO) reported that greenhouse gas emissions from livestock sector represent 14.5% of global human-induced greenhouse gas emissions and that the emissions from beef production represent 41% of sector's (Gerber et al., 2013).

Methane emissions (CH_4) by livestock may be affected by diet, genetic, individual differences among animals, which corresponds to 15 to 20% of human activities (Martin et al., 2008). Recent studies have demonstrated higher dietary protein contents are related to improved dry matter (DM) intake (Berends et al., 2014) and increased feed intake causes an increase in CH_4 production (Shibata and Terada., 2010; Chaokaur et al., 2015).

Methane production was positively related to diet digestibility and negatively related to dietary fat concentration, whereas dietary carbohydrate composition had only minor effects (Chianese et al., 2009). Production of CH_4 has a negative impact on animal productivity, resulting in energy losses ranging from 2% to 12% of the animal's gross energy intake (Ramin and Huhtanen., 2013; Haarlem et al., 2008). So, reduce CH4 emissions is an important way to decrease environmental pollution and minimize energy losses.

Thus, on the first experiment we hypothesized that MC is not relevant in concentrate feeds and it may not affect the estimated CP degradability; also, the incubation time necessary to estimate RDP may vary between feeds. In this way, our objectives were to use ^{15}N to estimate the MC of crude protein fractions that are soluble (a) and insoluble but potentially degradable (b) and the rate of digestion of the fraction b (kd), as well as to quantify the necessary incubation time to estimate RDP of energy and protein concentrates. On the second experiment we hypothesized that reducing CP levels in diets will reduce N losses and CH_4 emissions without affecting animal performance, and the objectives were to evaluate how the reduction in dietary CP contents affects animal performance, nutrient requirements, nitrogen losses, and methane emissions in finishing Nellore bulls.

Chapters 1 and 2 were writing according to the guidelines of Animal Journal and Journal of Agriculture, Ecosystems and Environmental, respectively.

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CHAPTER 1

Does microbial nitrogen contamination affect the estimation of crude protein degradability of concentrate feeds?

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Short Tittle: Protein degradation in feeds for cattle

Abstract

The effects of microbial contamination (**MC**) on crude protein (**CP**) degradability of concentrate feeds are still controversial. Moreover, the incubation time can influence the estimation of rumen degradable protein (**RDP**), a relevant parameter on beef cattle nutrition. The aim of this study was to use ^{15}N to estimate the MC of CP fractions that were soluble (**a**), insoluble but potentially degradable (**b**), and the digestion rate of fraction b (**kd**), as well as to determine the incubation time necessary to estimate the RDP of energy and protein feeds when considering two outflow rates (0.05 h^{-1} and 0.08 h^{-1}).Twelve types of feeds were evaluated, with six energy concentrates: wheat bran, rice meal, ground corn, ground sorghum, ground corn cob, and soybean hulls; and six protein concentrates: cottonseed meal 38% CP, soybean meal, ground bean, peanut meal, sunflower meal, and corn gluten meal. The feeds were divided into 4 groups and were incubated in the rumens of 4 crossbred bulls. The feed samples were incubated for 0, 2, 4, 8, 16, 24, 48, and 72 h. To determine the MC of the incubated residues, ruminal bacteria were labeled with ^{15}N via continuous intraruminal infusion of $^{15}(NH_4)_2SO_4$. Ruminal digesta were collected for the isolation of bacteria before the first infusion of ^{15}N during the acclimation period, and after the infusion of ^{15}N during the collection period. There was no difference ($P > 0.05$) in the parameters a, b, and kd, corrected and uncorrected, for all feeds that were evaluated. All of the feed tests followed an exponential model of degradation and the model fitted well to the data, except for corn gluten meal, probably because the maximum incubation time that was used (72 h) was not long enough to allow for an accurate estimation of the degradation profile. The cluster analysis (R^2 = 0.944) allowed for the grouping of feeds into three different groups according to the necessary incubation time to estimate RDP. The first was formed by the high-starch energy concentrates (15.4 ± 0.46 h), the second by the low-starch energy concentrates (6.8 ± 0.60 h), and the third by the protein concentrates (9.9 ± 0.41) while considering a kp of $0.05h^{-1}$. In conclusion, the microbial contamination was low and non-significant; therefore, correction of ruminal protein degradation is irrelevant with regards to the concentrate that was studied. However, the chemical composition of this feeds resulted in different incubation times to estimate RDP content, and this has to be taken into account in the techniques that are used to determine CP digestibility in the rumen and intestines.

Keywords: energy, incubation time, microbial contamination, protein, rumen degradable protein

Implications

The knowledge about the amount of rumen degradable protein of concentrate feeds is important to formulate precise diets for ruminants. Considering this, the correction for microbial contamination for concentrate feeds is not recommended because there is a small, but non-significant contribution of microbial contamination on crude protein fractions in the feeds mostly used in tropical conditions and it does not influence the crude protein degradability and rumen undegradable protein values. The recommendation is to incubate protein feeds for 9.9 hours and the energy feeds for 15.4 and 6.8 hours depending on the starch content.

Introduction

In situ methods are widely used to estimate the ruminal degradability of CP, but an important source of errors in this method is the microbial contamination of residual particles of incubated feeds, thereby resulting in the underestimation of CP degradability (Wulf and Südekum, 2005; Westreicher-Kristen *et al*., 2013) as well as the overestimation of rumen undegradable protein (**RUP**) content. Machado *et al*. (2013) showed that microbial contamination (**MC**) is an important source of errors for tropical forages, and concluded that dietary protein and fiber content influence the degree of contamination. However, concentrate incubation residues are less likely to be contaminated when compared to forage residues due to the lower microbial adhesion associated with a lower fiber content, and the microbial protein tends to be diluted in feeds with higher levels of CP (Rodriguéz and González, 2006).

The accurate prediction of N supply and the requirements of rumen microflora are important in order to minimize feed costs and N waste (Brooks *et al.,* 2012). The mobile bag technique (De Boer *et al.,* 1987) is used to assess rumen degradable protein (**RDP**) and RUP content, and to simulate intestinal digestibility. This technique considers a ruminal incubation time of 16 h (Paz *et al.,* 2014), which is a necessary step to estimate the intestinal digestibility of RUP, and lower incubation times are associated with higher values of intestinal digestibility of feeds (Beckers *et al.,* 1996).

However, a great diversity of concentrate feeds, differing by their fiber and N contents, may be incorporated into ruminant diets, and it can affect the dimension of MC and the incubation time that is necessary to provide an accurate estimate of RDP. For this reason, we hypothesized that MC is not relevant in concentrate feeds and it may not affect the estimated CP degradability; also, the incubation time necessary to estimate RDP may vary between feeds. Therefore, our objectives were to use ^{15}N to estimate the MC of crude protein fractions that are soluble (**a**) and insoluble but potentially degradable (**b**) and the rate of digestion of the fraction b (**kd**), as well as to quantify the necessary incubation time to estimate RDP of energy and protein concentrates.

Materials and methods

Characterization of concentrate samples

The experiment was carried out at the Animal Science Department of the Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil. The procedures for the human care and handling of the animals are in agreement with the Ethic Commission in Use of Production Animals of the Universidade Federal de Viçosa and with actual Brazilian legislation (protocol number 96/2014). Twelve types of concentrates were evaluated with six energy concentrates: wheat bran (*Triticum aestivum*), rice meal (*Oryza sativa*), ground corn (*Zea mays L.*), ground sorghum (*Sorghum vulgare*), ground corn cob (*Zea mays L.*), and soybean hulls (*Glycine max (L.) Merr*); and six protein concentrates: cottonseed meal 38% CP (*Gossypium hirsutum*), soybean meal (*Glycine max (L.) Merr*), ground bean (*Phaseolus vulgaris L.*), peanut meal (*Arachis* *hypogaea L*.), sunflower meal (*Helianthus annuss*), and corn gluten meal (*Zea mays L.*).

All samples were ground in a Wiley mill (TECNAL, Piracicaba, São Paulo, Brazil) with a 1-mm sieve for chemical analyses and a 2-mm sieve for *in situ* ruminal incubation. These samples were analyzed for DM, organic matter, N, and ether extract according to the AOAC (2012; method number 934.01, 930.05, 981.10, and AOAC, 2006; method number 945.16, respectively). The neutral detergent fiber and neutral detergent fiber corrected for ash and protein analyses were performed according to the technique described by Mertens *et al.* (2002) without the addition of sodium sulfite, but with the addition of thermostable alpha-amylase to the detergent. The determination of neutral detergent insoluble nitrogen followed the technique described by Licitra *et al.* (1996). The lignin was extracted with 72% sulfuric acid while following the recommendations of Van Soest and Wine (1967). The non-fibrous carbohydrates were calculated according to Detmann and Valadares Filho (2010). The chemical composition of feeds can be found in Table 1.

Table 1

Incubation and sampling procedures

The twelve feeds were divided into four groups, with three different types of concentrate in each one, and these feeds were ruminally incubated in four crossbred bulls while following a Latin square design (4 × 4). Within each period, each concentrate group was incubated in the rumen of a different bull

(Figure 1). As reported by Machado *et al.* (2013), the objective of the Latin square was to assist and organize the information that is collected in the field, while allowing for measurements of degradation of different feeds without confounding the effect of the animal, as well as controlling sources of variation and avoiding bias without estimating the variability.

The bulls were fed *ad libitum* with a diet based on a 50:50 (DM basis) mixture of corn silage and concentrate. The diet presented with 120 g CP/kg of DM. The concentrate was composed of 790 g/kg ground corn, 86 g/kg soybean meal, 60 g/kg wheat bran, 14 g/kg urea, 1.5 g/kg ammonium sulfate, 9.0 g/kg salt, 9.0 g/kg mineral mix, and 15 g/kg sodium bicarbonate. The animals were adapted to the experimental diet for 21 days prior to the incubations.

Individually identified nylon bags (Sefar Nitex; Sefar, Thal, Switzerland; porosity of 50 μm and 8 × 15 cm) were used, to which 6.0 g of each feed, previously ground at 2 mm, were added. The incubation times were 0, 2, 4, 8, 16, 24, 48, and 72 h. The number of bags varied as a function of the time of incubation in order to obtain enough residue for laboratory analyses: one bag for 0 and 2 h, two bags for 4 and 8 h, three bags for 16 h, four bags for 24 h and five bags for 48 and 72 h, with a total of twenty-two bags per feed and sixty-six incubated bags per animal (excluding time 0).

The samples were ruminally incubated and attached to a steel chain with a weight at the end, thus allowing for immersion within the ruminal contents. Bags were placed in the rumen in the reverse order, so all bags were removed at the same time and then washed in running water (Machado *et al.,* 2013). Bags for time 0 were not incubated in the rumen, but they were rinsed in running water, together with the incubated bags. Bags were oven-dried at 55℃ for 72 h, after which they were placed in an oven at 105 ℃ for 2 hours, placed in a desiccator, and then weighed.

The ^{15}N infusion was performed according to Rotta *et al*. (2014), which is briefly described here: the bulls received a solution containing 7.03 g of ammonium sulfate enriched with 10% of ^{15}N atoms [$(^{15}NH_4)_2SO_4$; Sigma Aldrich (Isotec), Miamisburg, OH], while providing 200 mg of daily ^{15}N to each animal. The amount of NH_4^+ infused into the salt was 1.99 g. Considering a rumen volume of 80 L and a N-ammonia pool of 5 mmol/L, there would be a total N-ammonia pool of approximately 400 mmol/d (Machado *et al.,* 2013). When considering 14 mg/mmol of N-NH3, there was a potential daily pool of 5.6 g N-NH3. Therefore, it should be noted that 1.99 g of NH_4^+ infused per day is not a negligible value when compared to the 5.6 g of N-NH3, the potential daily pool of ruminal N-ammonia (Machado *et al.,* 2013). This solution was continuously infused via a peristaltic pump (model 646; Milan, Scientific Equipments, Colombo, Paraná, Brazil) and adapted hoses connected to a ruminal fistula, which was surgically placed as described by Dougherty (1981). The pump was constantly regulated so that the volume of the solution was infused over a period of 24 h. After this time, the containers holding the enriched ^{15}N solution were replaced with containers holding a new solution, always at the same concentration. The infusion of ammonium sulfate was initiated 60 h before the first incubation day and continued until the last day of incubation to ensure a uniform distribution of ^{15}N and incorporation of $^{15}NH_3$ into the ruminal microbial pool (Broderick and Merchen, 1992).

Ruminal digesta were collected for the isolation of bacteria before the first infusion of ^{15}N to quantify the ^{15}N background, and ruminal digesta also were obtained for the isolation of bacteria after the infusion of ^{15}N on the second day of each collection period. Three collections were performed each day, with the first collection occurring before feeding and the other collections performed at 2 and 4 h after feeding. The procedure that was used for the isolation of bacteria was developed by Reynal *et al*. (2005) and adapted by Krizsan *et al.* (2010) as follows: 1 L of the ruminal digesta sample was filtered through a 100-μm nylon filter with a 44% pore surface area (Sefar Nitex 100/44; Sefar, Thal, Switzerland), and the material that remained in the filter was washed with 800 mL of 0.90% (wt/vol) saline solution (NaCl). The remaining phase on the filter was saved for isolation of the bacteria associated with the particles (**BAP**). The filtered material was processed for the isolation of bacteria that were associated with the liquid phase (**BAL**). After centrifugation (1,000 × g for 10 min, 5 °C), the pellet was saved for BAP isolation, and the supernatant was centrifuged at 11,250 × g for 30 min at 5 °C. After that, 200 mL of McDougall's buffer (McDougall, 1948) was added to this centrifugation pellet, and this material was again centrifuged at 16,500 × g for 20 min at 5 °C. The resulting pellet from this centrifugation consisted of the BAL and was stored in aluminum trays for subsequent lyophilization. To isolate the BAP, 700 mL of 0.90% (wt/vol) saline solution with 0.1% Tween-80 (vol/vol) was added to plastic containers with the above mentioned samples, which were then homogenized with a glass rod for 30 s and stored in the refrigerator at 4 °C for 12 h for subsequent centrifugation. After 12 h, the samples were filtered by using a 100-μm nylon filter with a 44% pore surface area (Sefar Nitex 100/44; Sefar, Thal, Switzerland). The filtrate was centrifuged at 1,000 × g for 10 min at 5 ℃, and the supernatant was then centrifuged at 11,250 × g for 30 min at 5 ℃. The pellet resulting from this centrifugation was combined with 200 mL of McDougall buffer and was centrifuged at 16,250 × g for 20 min at 5 ℃. The BAP of this last centrifugation was stored in aluminum trays for subsequent lyophilization. Thirty-two samples were obtained after lyophilization for ^{15}N analyses with 4 periods and 4 bulls for BAP and BAL (4 × 4 × 2).

Chemical analyses

The incubation residues were analyzed with regard to crude protein content, and BAL and BAP samples with regard to OM and CP content according to previously described methods.The enrichment with ^{15}N atoms was measured by using an isotope ratio mass spectrometer (Delta S; Finnigan MAT, Bremen, Germany). Samples were prepared for each incubation time, consequently resulting in samples of 1 g each, and were placed in 5 by 8 mm capsules for future readings. The ratios of stable isotopes of the same chemical element (^{15}N:^{14}N) were evaluated in terms of Δ per thousand, according to international standards, and were converted to percentages of atoms in excess.

Statistical Analyses

The chemical composition, ^{15}N enrichment, and its background in BAL and BAP were compared by using an analysis of variance according to the following model:

$$Y_{ijk} = \mu + A_i + P_j + AP_{ij} + B_k + \varepsilon_{ijk}$$

where μ is the general constant, A_i is the effect of animal I (random), P_j is the effect of experimental period j (random), AP_{ij} is the interaction between animal I and period j (random), B_k is the effect of the bacteria sampled from the different phases of digesta k (fixed), and ε_{ijk} is the random error. The different phases of digesta were considered as repeated measures. The data was analyzed by using the MIXED procedure of SAS 9.4.

The degradation profiles of CP were interpreted by using the asymptotic model of Ørskov and McDonald (1979), which was adapted to compare the corrected and uncorrected parameters for MC. The following model was used to estimate the parameters of CP degradability:

$$CPd_t = D1 \times \left(a1 + b1 \times \left(1 - e^{(-kd1 \times t)}\right)\right) + D2 \left(a2 + b2 \times \left(1 - e^{(-kd2 \times t)}\right)\right) + \varepsilon$$

Where CPd_t = the percentage of CP degraded at time t; D1 and D2 are dummy variables corresponding to the procedure that was used when the protein degradation was corrected or not: D1 = 0 and D2 = 1 correspond to degradation without correction for the MC, and D1 = 1 and D2 = 0 correspond to degradation corrected for the MC; t = the effect of time on the variables (h); a = the soluble fraction of the CP (%); b = the insoluble fraction that is potentially degradable (%); and kd = the degradation rate of "b" (h^{-1}); and ε = random error assumed to yield an asymptotic normal distribution.

The NLIN procedure of SAS 9.4 was used. Restricted and full models were compared by a likelihood ratio test (Rao, 1973). In this case, two different adjustments were performed for each feed. In the first adjustment, it was assumed that the dimensions of the soluble, insoluble but potentially degradable fractions, and the fractional degradation rate were similar in the corrected and non-corrected profiles. The model that arose from this adjustment was called the restricted model. In the second adjustment, those parameters were supposed to be different for the corrected and non-corrected profiles, and the model was called the complete model. From this information, the statistical comparison was performed by using the χ^2 distribution as follows:

$$\chi^2_{calc.} = -n \times \ln(\frac{RSSc}{RSSr})$$

$$\text{d.f.} = p(c) - p(r)$$

where $\chi^2_{calc.}$ is the calculated value of χ^2 statistics, n is the number of observations that were used for adjusting the degradation profiles, RSS*c* is the residual sum of squares of the complete model, RSS*r* is the residual sum of squares of the restricted model, d.f. is the number of degrees of freedom that were used to perform the test, p(c) is the number of parameters that were considered in the adjustment of the complete model, and p(r) is the number of parameters that were considered in the adjustment of the restricted model. For all feeds, p(c) = 6, p(r) = 3, and d.f. = 3.

Following the model adjustment, the RDP was calculated as follows:

$$RDP = a + b \times \frac{kd}{kd + kp}$$

where kp is the ruminal outflow rate (h^{-1}). The other terms were previously defined.

Two outflow rates of 0.05 h^{-1} and 0.08 h^{-1} were used to estimate RDP values and to estimate the necessary incubation time to estimate feeds' RDP

(Steingass *et al.,* 2013). A ruminal outflow rate of 0.05 h^{-1} was used to simulate the outflow rate for calves, low-yielding dairy cows, and beef cattle (medium rate), while 0.08 h^{-1} was used to simulate the outflow rate for high yielding dairy cows (high rate) according to the AFRC (1993).

The necessary incubation time (h) to estimate the RDP of each feed by using a single point incubation was quantified as the incubation time when the degraded fraction of CP becomes equal to the RDP estimate. The following equation was used:

$$t = -\ln\left[\frac{1-\left(\frac{RDP-a}{b}\right)}{kd}\right]$$

Additionally, in order to identify concentrate subgroups with similar incubation times to estimate the RDP, the necessary incubation times that were obtained by using both outflow rates were submitted to a multivariate non-hierarchical clustering procedure (Katthree and Naik, 2000) by using the FASTCLUS procedure of SAS 9.4. All statistical procedures were conducted by using 0.05 as the critical level for the probability of a type I error.

Results

Bacteria associated with the liquid phase and bacteria associated with the particles ^{15}N content

There was no difference ($P > 0.05$) between BAL and BAP ^{15}N content; thus, an average content was used to estimate the MC in incubation residues (Table 2).

Table 2

Effect of microbial contamination on crude protein degradation

Crude protein degradation parameters from energy and protein concentrates, corrected and non-corrected for MC, are presented in Tables 3 and 4, respectively. All of the feeds followed an exponential model of degradation and the model fitted well to the data, except for corn gluten meal. The degradation pattern of this feed was not regular and the asymptote estimation was not possible because the model did not converge. Probably, the maximum incubation time that was used here (72 h) was not long enough to allow for an accurate estimation of the degradation profile. For this reason, corn gluten meal was no longer considered in any part of the discussion. There was no difference ($P > 0.05$) between the fractions a, b, and kd corrected and not corrected for MC. Rumen degradable protein values were obtained by considering outflow rates of 0.05 h^{-1} and 0.08 h^{-1}. There was a small, but non-significant effect of MC on CP degradation (Figures 2 and 3), resulting in no difference ($P > 0.05$) on CP degradation parameters, corrected and not corrected for MC.

Table 3

Table 4

Incubation time to estimate rumen degradable protein content

The cluster analysis allowed for the grouping of feeds into three different groups (feeds with high starch content, low starch content, and protein concentrates) according to the necessary incubation time to estimate RDP. It should be highlighted that overall R^2 of the clustering procedure was high (R^2 = 0.944).

Discussion

Bacteria associated with the liquid phase and bacteria associated with the particles ^{15}N content

According to Olubobokun and Craig (1990) and Beckers *et al.* (1995), the capture of ^{15}N by ruminal microorganisms can be different from BAL and BAP because BAP is more predominant in rumen content than BAL (Legay-Carmier and Bauchart., 1989). Nevertheless, we did not observe differences ($P > 0.05$) between BAL and BAP ^{15}N content. Results in this present study are similar to those obtained by Machado et al. (2013) and Rotta *et al.* (2014), with no difference in ^{15}N:^{14}N ratio of BAP and BAL, but different from Rodríguez *et al.* (2000) who found lower concentrations of ^{15}N in BAP than in BAL.

Effect of microbial contamination on crude protein degradation:

Literature data are variable with respect to the effects of microbial contamination on the degradation of crude protein and its fractions (a, b, kd) for concentrate feed, as well as for the microbial marker that is used. Results that are similar to this study were obtained by Stefánski *et al.* (2013), who did not observe differences in CP degradation for MC contamination when studying canola meal while using a maximum of 10 hours of incubation. However, González *et al.* (2011), when using ^{15}N as a microbial marker, found that MC is an important source of errors in CP degradation of ryegrass and sunflower meal, and Alexandrov (1998) observed a significant effect of MC on CP degradation as a function of incubation time for sunflower meal when using DAPA as a microbial marker.

Rodríguez and González (2006) reported a small but non-significant effect for fraction a when evaluating 14 feeds such as ground corn, sunflower meal, soybean meal, soybean hulls, and wheat bran; however, these small effects resulted in an increase in fraction b values. Beckers *et al.* (1995), when studying meat and bone meal, soybean meal, and wheat bran, found a significant effect of MC on the parameters a, b, and kd of wheat bran. These authors reported that the greatest neutral detergent fiber content of wheat bran leads to a similar pattern as forages (Craig *et al.,* 1987; Olubobokun *et al.,* 1990; Wanderley *at al.,* 1993), where the high bacterial colonization of fibrous feeds led to the greatest underestimation of CP effective degradability when the correction was omitted. Mathers and Aitchison (1981) and Beckers *et al.* (1995) considered that for concentrate feeds that are rich in protein and without fiber, such as meat, bone meal, and fish meal, the MC may be nutritionally unimportant.

Some authors (Valadares Filho *et al.,* 1992; Ould-Bah *et al.,* 1998) suggested that concentrate feeds present with low to moderate values of MC when compared to roughage feeds. According to Krawielitzki *et al.* (2006), the binding and access of microorganisms to feed particles is supported by fibrous substances. Rodríguez and González (2006) relayed that the microbial contamination would be directly related to the cellulose content of the feed. However, according to Wanderley *et al.* (1993), feeds with a low CP content would present with greater MC effects, and the microbial protein tends to be diluted in feeds with higher levels of CP (Rodriguéz and González, 2006). Machado *et al.* (2013) studied tropical forages and observed significant effects of MC, and these authors developed equations while considering the content of fiber and CP in roughages. In this scenario, the source of feed may affect the extension of MC. In summary, the low fiber content of feeds that were evaluated in this study was related to a non-significant contribution in MC.

Incubation time to estimate rumen degradable protein content

Knowledge about the RDP content of feeds is necessary to formulate diets to meet the requirements of beef cattle. Ruminants have particularities with regards to their protein nutrition because most of their amino acids and absorbable proteins (50 to 80%) are from microbial proteins that are synthesized in the rumen (Bach *et al.,* 2005). In this study, we also evaluated the necessary incubation time to estimate RDP of each feed and identified concentrate subgroups with similar incubation times while considering two outflow rates, 0.05 h^{-1} (moderate) and 0.08 h^{-1} (fast), according to the AFRC (1993).

The cluster analysis allowed for the grouping of feeds into three different groups. The first was formed by the high-starch energy concentrates (ground corn, ground sorghum, and ground corn cob), the second by the low-starch energy concentrates (wheat bran, rice meal, and soybean hulls), and the third by the protein concentrates (Table 5). For the high starch energy concentrates, more hours of incubation were needed to estimate the RDP content (15.4 ± 0.46 h, kp = 0.05 h^{-1} or 10.4 ± 0.12 h, kp = 0.08 h^{-1}) than for the other groups. This occurred because of the structural features of starch and interactions with other components like proteins or lipids (Svihus *et al.,* 2005). The presence of a protein matrix around the starch beads hampers the microorganisms' access and the digestible enzymes that are necessary to digest the feed. Moreover, corn and sorghum plants that are commonly found in Brazil have a harder endosperm, which therefore indicates a greater binding between protein and starch (McAllister *et al.,* 1990).

Table 5

In line with this thought, the low starch energy concentrates, composed of feeds with low starch contents, required the lowest incubation hours (6.80 ± 0.60 h, kp = 0.05 h^{-1} or 5.40 ± 0.41 h, kp = 0.08 h^{-1}). The third group, composed of protein concentrates, reached intermediate values of incubation hours that were needed to estimate RDP (9.90 ± 0.41 h, kp = 0.05 h^{-1} or 7.50 ± 0.25 h, kp = 0.08 h^{-1}). Data from the literature (Paz *et al.,* 2014) considered 16 hours of ruminal incubation as a necessary step in the mobile nylon bag technique in order to assess RDP content, and consequently estimate RUP content (de Boer *et al.,* 1987). According to our data, 16 h of ruminal incubation are indicated only for ground corn, ground sorghum, and ground corn cob, while considering an outflow rate of 0.05 h^{-1}. For the other feeds that were evaluated in this study, 16 hours of incubation can overestimate the intestinal digestibility, because theoretically, less RUP arrived in the small intestine. So, the majority of concentrated feeds will not be retained for 16 h in the rumen because of the chemical composition, particle size, and when considering the passage rate, as they rapidly flow to the intestine. Thus, we present the incubation hours in Table 5 as those needed to estimate RDP content from concentrate feeds.

Conclusions

The microbial contamination was low and non-significant, so the correction of ruminal protein degradation is irrelevant for the concentrates that were studied. However, the chemical composition of these feeds resulted in different incubation times to estimate RDP content, and it needs to be considered in techniques that are used to determine CP digestibility in the rumen and intestines.

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Table 1 *Chemical composition of feeds that were used in the experiment*

	Compounds, %DM										
Feed	DM	OM	CP	EE	NDF	NDFap	NDIP	NDIA	iNDF	LIGNIN	NFC
					Energy concentrates						
Wheat bran	86.9	95.1	19.8	3.11	33.8	30.3	3.36	0.07	11.8	4.55	41.9
Rice meal	86.5	90.4	16.4	17.8	21.2	17.9	3.10	0.14	10.9	5.19	38.3
Ground corn	86.5	98.7	9.63	3.28	8.02	5.62	2.36	0.04	2.36	1.40	80.2
Ground	86.3	98.7	10.4	2.12	9.59	6.57	2.88	0.13	1.56	2.09	79.7
Ground corn	87.5	96.7	7.65	4.91	32.4	29.9	2.15	0.36	14.5	3.90	52.5
Soybean hulls	87.5	95.5	14.6	2.01	65.5	58.8	5.73	1.03	3.71	4.16	20.1
					Protein concentrates						
Cottonseed	89.1	93.4	41.3	2.76	33.7	19.2	13.8	0.67	15.3	4.11	30.2
Soybean meal	87.9	93.7	52.5	2.37	20.2	18.6	1.47	0.13	1.99	2.00	20.3
Ground bean	93.5	95.1	26.5	1.63	19.4	15.0	3.78	0.54	1.60	1.30	51.9
Peanut meal	88.2	96.2	52.1	2.17	12.6	10.3	1.64	0.62	10.8	2.52	31.6
Sunflower meal	85.1	93.7	32.2	1.49	47.5	45.1	1.59	0.85	29.6	6.35	14.9
Corn gluten	91.8	95.6	65.6	1.47	26.6	16.5	4.43	0.51	3.68	3.23	47.9

OM = organic matter; EE = ether extract; NDFap = NDF corrected for ash and protein; NDIP = neutral detergent insoluble protein; NDIA = neutral detergent insoluble ash; iNDF = indigestible NDF; NFC = non fibrous carbohydrates.

Table 2 *Descriptive statistics for ^{15}N:^{14}N ratio (Δ per thousand) and bacterial N content (% OM) obtained from bacteria associated with particles and bacteria associated with the liquid phase*

	Bacteria associated with particles		Bacteria associated with liquid phase	
Item	^{15}N:^{14}N[1]	N (% OM basis)	^{15}N:^{14}N[1]	N (% OM basis)
Mean	304	5.89	322	5.46
True mean[2]	295	-	314	-
Minimum	180	5.12	195	5.01
Maximum	467	7.04	507	5.91
Standard deviation	129	-	142	-
SEM	53.8	1.04	56.9	0.96
n	32	32	32	32

OM = organic matter

[1]Means for ^{15}N:^{14}N ratio and bacterial N content did not differ by *F* test (*P* = 0.71). The mean value between BAP and BAL was 313.32 Δ per thousand for ^{15}N:^{14}N which was used to calculate the microbial contamination. Average bacterial N content before infusion: 6.10% (OM basis).

[2]True mean = mean – background ^{15}N:^{14}N (*n* = 32 Δ per thousand).

Table 3 *Fractions a and b of crude protein and degradation rate of fraction b (kd), corrected or not corrected for microbial contamination in energy concentrates*

Feed	Parameters[1]	No correction	Corrected	RDP 5	RDP 8	*P*-value[2]
	a	31.2 ± 2.02	31.1 ± 2.02			
Wheat bran	b	63.7 ± 2.21	63.3 ± 2.21	85.9	81.9	0.993
	kd	0.332 ± 0.0260	0.324 ± 0.0250			
	a	36.9 ± 2.63	36.9 ± 2.63			
Rice meal	b	44.8 ± 2.87	44.0 ± 2.87	75.2	72.5	0.995
	kd	0.336 ± 0.0490	0.335 ± 0.0500			
	a	30.9 ± 1.60	30.3 ± 1.58			
Ground corn	b	68.8 ± 3.39	69.7 ± 5.77	58.4	50.9	0.910
	kd	0.037 ± 0.0050	0.033 ± 0.0030			
	a	35.1 ± 1.48	34.6 ± 1.46			
Ground sorghum	b	64.3 ± 2.29	64.7 ± 2.49	52.8	47.1	0.973
	kd	0.021 ± 0.0020	0.020 ± 0.0020			
	a	24.3 ± 2.51	24.2 ± 2.47			
Ground corn cob	b	70.1 ± 4.41	69.9 ± 4.88	54.8	47.1	0.958
	kd	0.043 ± 0.0070	0.039 ± 0.0070			
	a	17.3 ± 3.87	17.2 ± 3.87			
Soybean hulls	b	67.3 ± 4.27	66.4 ± 4.27	70.0	64.3	0.997
	kd	0.200 ± 0.0300	0.200 ± 0.0310			

a = soluble fraction of CP (%CP); b = insoluble but potentially degradable fraction of CP (%CP); kd = degradation rate of b fraction of CP (%/h); RDP 5 = Ruminal degradable protein considering 5% kp; RDP 8 = Ruminal degradable protein considering 8% kp

[1]Each degradation parameter was obtained by using 8 incubation times repeated in 4 separate periods.

[2]P-value obtained from parameters data, corrected and not corrected for microbial contamination

Table 4 *Fractions a and b of crude protein and degradation rate of fraction b (kd), corrected or not corrected for microbial contamination, in protein concentrates*

Feed	Parameters[1]	No correction	Corrected	RDP 5	RDP 8	*P*-value[2]
	a	27.3 ± 2.07	27.2 ± 2.07			
Cottonseed meal	b	62.2 ± 2.31	61.6 ± 2.31	73.7	67.7	0.997
	kd	0.154 ± 0.0150	0.154 ± 0.0150			
	a	27.0 ± 2.25	27.0 ± 2.25			
Soybean meal	b	70.6 ± 2.51	70.5 ± 2.51	80.1	73.2	0.999
	kd	0.152 ± 0.0140	0.152 ± 0.0140			
	a	23.8 ± 2.82	23.7 ± 2.82			
Ground bean	b	73.4 ± 3.43	73.6 ± 3.43	71.2	62.9	0.999
	kd	0.092 ± 0.0120	0.091 ± 0.0120			
	a	26.8 ± 3.35	26.7 ± 3.34			
Peanut meal	b	65.0 ± 3.79	64.9 ± 3.79	73.9	67.2	0.999
	kd	0.134 ± 0.0210	0.132 ± 0.0200			
	a	18.0 ± 4.61	30.1 ± 3.67			
Sunflower meal	b	63.8 ± 4.95	50.2 ± 4.44	65.7	60.4	0.738
	kd	0.145 ± 0.0280	0.121 ± 0.0290			

a = soluble fraction of CP (%CP); b = insoluble but potentially degradable fraction of CP (%CP); kd = degradation rate of b fraction of CP (%/h); RDP 5 = Ruminal degradable protein considering 5% kp; RDP 8 = Ruminal degradable protein considering 8% kp

[1]Each degradation parameter was obtained by using 8 incubation times repeated in 4 separate periods.

[2]*P*-value obtained from parameters data, corrected and not corrected for microbial contamination

Table 5 *Incubation time necessary to estimate rumen degradable protein of each feed when considering two outflow rates*.

Cluster	Feeds	Rumen outflow rate									
		0.05 h^{-1}					0.08 h^{-1}				
					Confidence interval					Confidence interval	
		IIT	AIT	SEM	Lower	Upper	IIT	AIT	SEM	Lower	Upper
1	Ground corn	15.2	15.4	0.46	13.4	17.4	10.3	10.4	0.12	9.80	10.9
	Ground sorghum	16.3					10.6				
	Ground corn	14.8					10.2				
2	Wheat bran	6.20	6.80	0.60	4.20	9.30	5.00	5.40	0.41	3.60	7.10
	Rice meal	6.10					4.90				
	Soybean hulls	7.90					6.20				
	Cottonseed	9.10					7.00				
3	Soybean meal	9.20	9.90	0.41	8.80	11.1	7.00	7.50	0.25	6.80	8.20
	Ground bean	11.4					8.30				
	Peanut meal	9.80					7.40				
	Sunflower meal	10.2					7.60				

IIT = Individual incubation time; AIT = Average incubation time


Bull # 1
Bull # 2
Bull # 3
Bull # 4
P1
P2
P3
P4
G1
G2
G3
G4
G2
G3
G4
G1
G3
G4
G1
G2
G4
G1
G2
G3

Figure 1

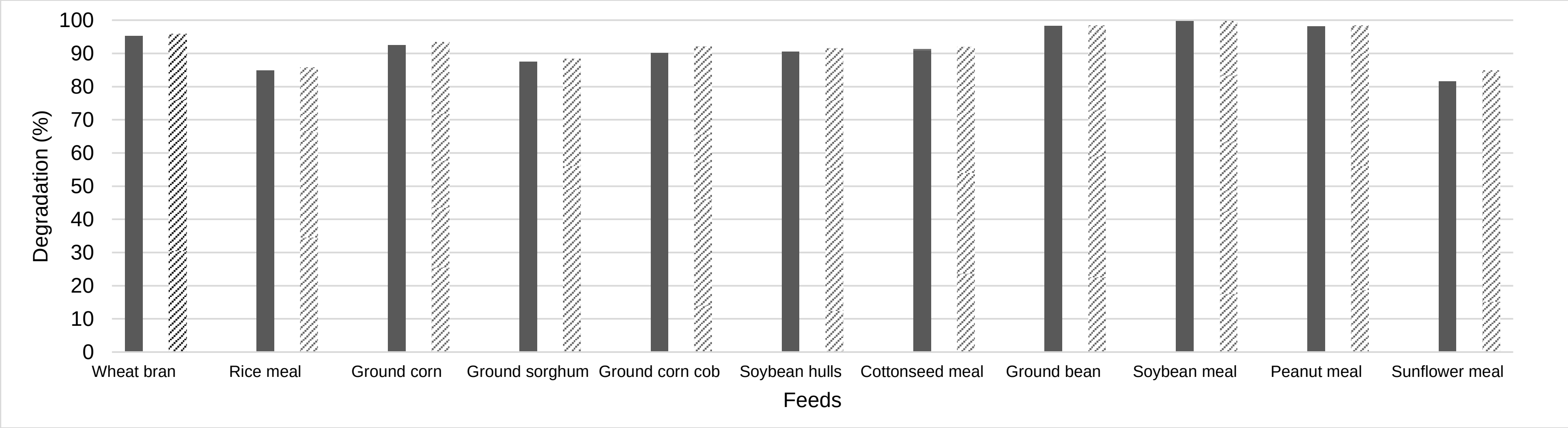
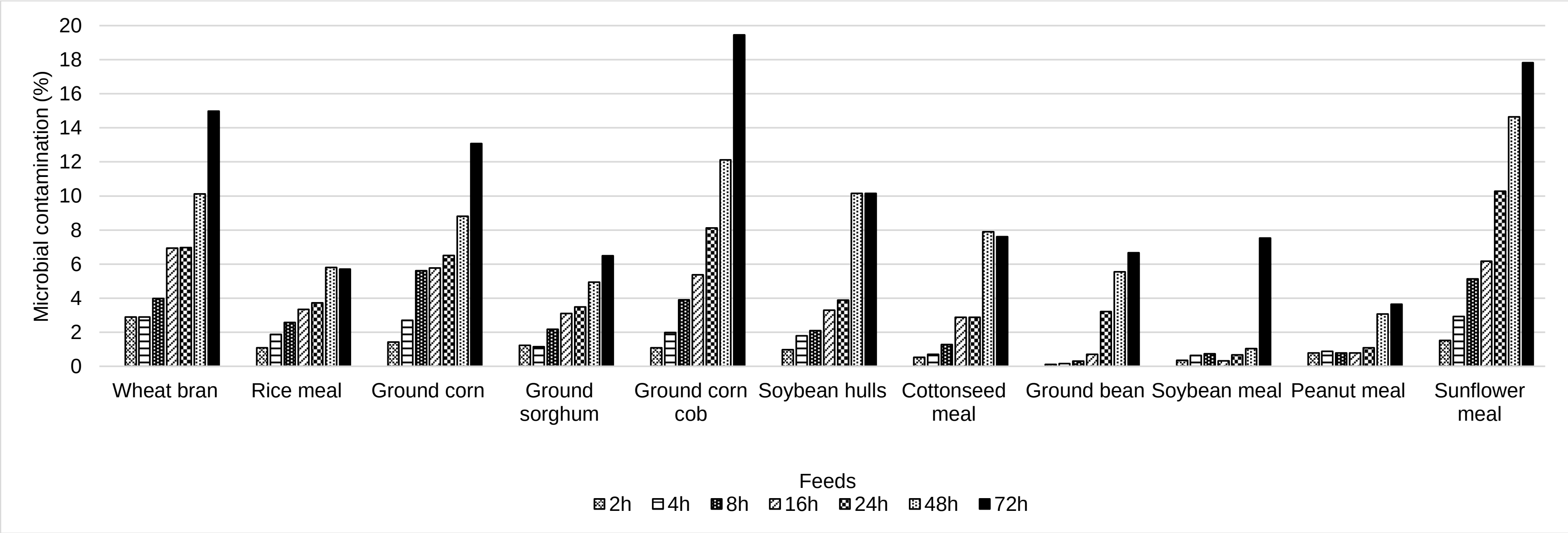

100
90
80
70
60
50
40
30
20
10
0
Degradation (%)
Wheat bran
Rice meal
Ground corn
Ground sorghum
Ground corn cob
Soybean hulls
Cottonseed meal
Ground bean
Soybean meal
Peanut meal
Sunflower meal
Feeds

Figure 2



Microbial contamination (%)
0
2
4
6
8
10
12
14
16
18
20
Wheat bran
Rice meal
Ground corn
Ground sorghum
Ground corn cob
Soybean hulls
Cottonseed meal
Ground bean
Soybean meal
Peanut meal
Sunflower meal
Feeds
2h
4h
8h
16h
24h
48h
72h

Figure 3

List of figure captions

Figure 1 Schematic representation of the bag incubation procedure of 12 concentrates, divided into 4 groups. Each concentrate group was incubated for 4 times in the rumen of a different bull for each period [P1 (Experimental period 1), P2 (Experimental period 2), and P3 (Experimental period 3)]. G1 [(feed group 1: ground sorghum, wheat bran, soybean meal), G2 (feed group 2: sunflower meal, ground corn, ground bean), G3 (feed group 3: rice meal, ground corn cob, peanut meal), G4 (feed group 4: cottonseed meal 38% CP, soybean hulls, corn gluten meal)]. Adapted from Machado et al. (2013).

Figure 2 Crude protein degradation (%) non-corrected and corrected for microbial contamination.

Figure 3 Microbial contamination (%) of each concentrate feed as a function of incubation tim

CHAPTER 2

Does a reduction in dietary crude protein content affect performance, nutrient requirements, nitrogen losses, and methane emissions in finishing Nellore bulls?

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ABSTRACT

An experiment was conducted to evaluate whether a reduction in dietary crude protein (CP) content affects animal performance, energy and protein requirements, N losses, and enteric methane emission in finishing Nellore bulls. Twenty-six animals, with an average age of 20 ± 1.0 months and initial body weight (BW) of 296 ± 8.1 kg were used in this experiment. Four animals were used as baseline reference animals and were slaughtered at the beginning of the experiment. Four animals were fed at maintenance level (MAIN), whereas 18 bulls were divided into 3 groups (n = 6 in each group) and were randomly assigned to the treatments consisting of three levels of CP in the diets: 10, 12, and 14% of CP. At the end of the experiment, all animals were slaughtered to evaluate their chemical body composition, energy and protein requirements, and carcass characteristics. A linear effect was observed for dietary CP level on CP intake and digestibility, while greater values were obtained for animals that were fed 14% CP. Nitrogen metabolism was affected by CP levels, where animals that were fed 12 and 14% CP had greater urinary N losses than those that were fed 10% CP. There was no effect of CP level on retained N, animal performance, and carcass characteristics among diets, and there was no effect of CP level on microbial efficiency and CH_4 emissions. Thus, this study showed that for finishing bulls, the level of dietary CP did not interfere with muscle deposition and greenhouse gas emissions. The reduction of CP content in diets does not affect DM intake, animal performance, and carcass characteristics, thereby suggesting that the use of 10% of CP in diets for finishing bulls reduces their environmental impact due to a lower urinary N excretion than 12 and 14% CP-based diets. Animals that were fed 10, 12, and 14% CP diets had emissions equivalent to 3,893; 3,755; and 4,255 g d^{-1} of CO_2, respectively, and no difference was observed among diets. Furthermore, methane emission is not affected by CP levels ranging between 10 to 14% which, on average, is 16.3 g kg^{-1} of DM intake. Our study found that a decreased CP level did not influence animal performance, but it did decrease N losses in manure without affecting methane emissions. However, it is important to highlight that more studies are necessary to confirm these results.

Keywords: Methane; Nellore; Performance; Protein; Requirements

1. Introduction

The environmental impact of feeding animals in a feedlot is a growing concern (Cole et al., 2006; Staerfl et al., 2012; Patra and Lalhriatpuii, 2016). The majority of nutrients that are absorbed from feedstuffs on feedlots are excreted as feces and urine, and cattle commonly retain only 10 to 20% of their nutrient intake (McBride et al., 2003). In addition, between thirty to fifty percent of N from feedstuffs may be lost via volatilization, mainly in the form of ammonia (Bierman et al., 1999; Todd et al., 2005), and this amount of ammonia can be affected by dietary crude protein (CP) level (Burgos et al., 2010). Furthermore, protein is considered to be the most expensive nutrient in a ruminant´s ration. Thus, unbalanced diets contribute to negative environmental impacts and represent significant economic losses.

According to the NRC (1996), CP requirements decrease during the finishing phase; however, commercial feedlots use high CP levels to encourage greater intake and in order to slaughter animals earlier. In addition, methane (CH_4) emissions by livestock may be affected by diet, genetic, or individual differences among animals in a herd, which corresponds to 15% to 20% of human activities (Martin et al., 2008).

Recent studies have demonstrated greater protein levels are related to increased dry matter (DM) intake (Berends et al., 2014) and increased feed intake leads to an increase in CH_4 production (Shibata and Terada, 2010; Chaokaur et al., 2015), which could also impact the reduction of CH_4 emissions by society and affect market pressure.

Therefore, we hypothesized that reducing CP levels in diets will reduce N losses and CH_4 emissions without affecting animal performance. Thus, an experiment was conducted to evaluate whether the reduction in dietary CP contents affects animal performance, nutrient requirements, N losses, and CH_4 emissions in finishing Nellore bulls.

2. Materials and methods

2.1. Animals, experimental design, and treatments

The experiment was conducted at the Experimental Feedlot of the Animal Science Department at the Universidade Federal de Viçosa (UFV), Viçosa, Minas Gerais, Brazil. Animal care and handling followed guidelines set by the UFV (process 96/2014). Twenty-six Nellore bulls, at 20 ± 1.0 months of age and with an average initial body weight (BW) of 296 ± 8.1 kg, were divided into the following groups: four animals were randomly selected as the baseline group to be slaughtered at the beginning of the experiment in order to evaluate initial body composition, four animals were fed at maintenance level (12 g kg^{-1} BW) while receiving a 12% CP-based diet, and eighteen bulls were fed *ad libitum* and were randomly selected to receive one of three diets with different levels of CP, either 10, 12, or 14% CP (n = 6 in each treatment).

The animals were housed in 30-m^2 individual pens with a concrete floor, which were equipped with feeders and concrete drinkers. The animals were submitted to a 21-d acclimation period to the experimental conditions, at which time animals with endo and ectoparasites were identified and treated. During this period, the animals were acclimated to Greenfeed equipment while aiming to evaluate CH_4 emissions in Nellore bulls. At the end of this period, the animals were weighed after a 16-hour solid fasting period, and the treatments were randomly assigned to the animals. The experiment lasted for 112 days with four 28-d experimental periods. During the 1st period, we conducted CH_4 emission evaluations, during the 2nd period we conducted nutrient digestibility trials, and during the 3rd and 4th periods we monitored animal performance. The bulls were weighed at the beginning and end of the experiment to evaluate their average daily gain (ADG).

Diets were formulated according to the Brazilian Tables of Nutrient Requirements of Zebu beef cattle - BR CORTE system (Valadares Filho et al., 2010) to achieve an ADG of 1.0 kg. These diets consisted of corn silage and a concentrate that was formulated with ground corn, wheat bran, soybean meal, urea, ammonium sulfate, sodium bicarbonate, common salt, and mineral mix (Table 1) while using a roughage:concentrate ratio of 50:50. Fresh feed was weighed daily and provided after removing the orts from the previous day. The feed was offered twice a day for the animals; at 0700 h, the roughage was supplied in full with half of the amount of concentrate, and at 1500 h, the remaining concentrate was provided according to procedures described by Pazdiora et al. (2011).

The feeding was adjusted on a daily basis to maintain orts at approximately 5 to 10% of the total feed and was supplied *ad libitum* to cattle. The difference between the amount of offered feed and the amount of orts was recorded as the amount of daily feed intake. Drinking water was continuously available to the animals. The amount of feedstuffs was recorded daily; additionally, the ingredients in the concentrate were sampled each time the concentrate was manufactured.

Samples of corn silage and orts were collected every day, and then stored in a freezer at - 20°C. Weekly, a composite sample of corn silage and orts from each animal were submitted to be dried in a forced ventilation oven at 55°C and were ground in a Wiley mill using 1-mm mesh sieves. Then, composite samples of corn silage and orts were obtained per experimental period.

2.2. Apparent nutrient digestibility

To evaluate nutrient digestibility, spot samples of feces were obtained from each animal at the following hours: 0600 h, 1200 h, and 1800 h on the 53rd, 54th, and 55th days respectively. Fecal samples were dried in a forced ventilation oven at 55°C and were ground using a knife mill with a 2-mm mesh sieve. Subsequently, a composite sample per animal was performed. Indigestible neutral detergent fiber (iNDF) was used as an internal marker to estimate the total amount of feces produced on a DM basis.

2.3. Methane and urine collections

During the first experimental period, each group of animals (n = 6) was confined for a 7-d period to a pen provided with Greenfeed equipment (C-Lock Inc, South Dakota, USA) that was used to measure enteric CH_4 production in grams per day. Methane production was estimated through continuous analysis point samples of excreted air via respiration and eructation of animals during feedings throughout the day. The Greenfeed equipment contained small feed portions of pelleted concentrate; this was used to attract the animals in order to perform measurements and record CH_4 emissions at regular intervals throughout the day, six times a day every 4 hours, while the equipment provided 5 drops of approximately 35 grams of a commercial pelleted concentrate ration to each animal in intervals of approximately 5 seconds. The chemical composition of this ration was as follows: 89.63% DM, 92.05% organic matter (OM), 19.70% CP, 2.95% ether extract (EE), 33.73% neutral detergent fiber (NDF), and 35.65% non-fiber carbohydrates. A commercial ration was used with the Greenfeed equipment because this equipment only accepts pelleted rations, while the three concentrates that were used in this study were finely ground.

Spot urine samples were collected from each group at the end of each week during the first period at 0600 h. A 5-mL urine sample was diluted in 20-mL of 0.036 N sulfuric acid solution to avoid bacterial destruction of allantoin. A 30-mL urine sample was used to measure urea, uric acid, and creatinine concentrations. These samples were stored at −20°C for further laboratory analyses.

Allantoin, creatinine, and uric acid were analyzed using an HPLC (Agilent 1100 series, Agilent Technologies, Waldbronn, Germany) as described by George et al. (2006). The total excretion of purine derivatives, calculated as the sum of allantoin and uric acid, was expressed in millimoles per day. Absorbed purines (X, in millimoles per day) were calculated based on the excretion of purine derivatives (Y, in millimoles per day) using the following equation: $Y = (X – (0.30 \times BW^{0.75}))/0.80$, where 0.80 was assumed to be the recovery of purines absorbed as purine derivatives, while $0.30 \times BW^{0.75}$ was assumed to be the daily endogenous purine excretion per kilogram of metabolic weight per day (Barbosa et al., 2011). Ruminal synthesis of N compounds

(Y, in grams of N per day) was calculated as a function of absorbed purines (X, in millimoles per day) by utilizing the following equation proposed by Barbosa et al. (2011): $Y = (70 \times X)/(0.93 \times 0.14 \times 1,000)$, where 70 was the N content of purines (in milligrams of N per mole), 0.14 was the presumed purine N/total N ratio of the bacteria, and 0.93 was an estimate of the true digestibility of microbial purines.

Nitrogen balance was calculated according to Cole et al. (2006) and Cole and Todd (2009), where urine N was obtained based on the difference of N intake, fecal N, and retained N.

Nitrous oxide (N_2O g d^{-1}) excreted in feces and urine was calculated according to the guidelines of IPCC (2006), where 2% of the N excreted in livestock manure (feces and urine) was the emission factor that was adopted to find the amount of N_2O emitted.

The carbon dioxide equivalent was calculated by considering that CH_4 and N_2O are greenhouse gases with 25 and 298 times the Global Warming Potential of CO_2, respectively (IPCC, 2006; Forster et al., 2007). The carbon dioxide equivalent per kilogram of product was calculated by considering the amount of CH_4 emitted in grams per day and the amount of muscle on the carcass, multiplied by a factor of 25.

All of the data that was obtained in this study was on a per animal basis and not on a farm level basis. However, to extrapolate data under Brazilian conditions, we choose tier 1 from IPCC (2006) to estimate CH_4 emissions from livestock manure. Tier 1 was chosen because this is a simplified method that only requires livestock population data per animal species/category and climate region or temperature, in combination with IPCC default emission factors to estimate emissions. The following equation was used:

$$CH_{4\,MANURE} = \sum_T \frac{(EF_T \times N_T)}{10^6}$$

Where, $CH_{4Manure}$ = CH_4 emissions from manure management, for a defined population, Gg CH_4 yr^{-1}

EF_T = emission factor for the defined livestock population, kg CH4 $head^{-1}$ yr^{-1}

N_T = the number of head of livestock species/category *T* in the country

T = species per category of livestock

2.4. Slaughter and samplings

Prior to slaughter, all animals were fasted from solids for 16-h to estimate their shrunk body weight. Animals were slaughtered via captive bolt followed by bleeding. After bleeding, the gastrointestinal tract contents (i.e., rumen, reticulum, omasum, abomasum, and small and large intestines) were removed and washed. The weights of the heart, lungs, liver, spleen, kidneys, KPH fat, diaphragm, mesentery, tails, trimmings, and the washed gastrointestinal tract were added to the other parts of the body (i.e., carcasses, head, hide, limbs, and blood) to determine the empty body weight (EBW).

The rumen, reticulum, omasum, abomasum, small and large intestines, KPH fat, mesentery, liver, heart, kidneys, lungs, tongue, spleen, diaphragm, esophagus, trachea, and reproductive tract were ground for 20 min by using an industrial cutter to create a homogeneous sample of organs + viscera. A sample of blood was obtained during the course of bleeding. The head and all of the limbs, after removal of the hide, were ground by using a grinding machine to reduce the size of the bones. The hide was sampled in parts, where 2 parts represented the shoulder, 3 parts represented the dorsal line, 2 parts represented the ventral line, 2 parts represented the rear, 1 part was used to represent each foot, and 1 part was used to represent the head, which overall represented the entire hide.

After slaughter, the carcass of each animal was separated into 2 half-carcasses that were chilled at 4ºC for 18-h. After this period, the half-carcasses were weighed and the section between the 9th and 11th ribs was obtained from the left half-carcass according to procedures described by Hankins and Howe (1946). This section was dissected into muscle, fat, and bone, and each portion was separately weighed. The muscle and fat of each animal were homogenized and ground in order to obtain a composite sample of muscle and fat proportional to the natural weight of each portion. Bones from each animal were sliced to obtain a sample of the bones. All of the samples were lyophilized and then were ground in a knife mill with a 1-mm mesh sieve to evaluate the DM, OM, CP, and EE contents.

2.5. Laboratory analysis

Samples of corn silage, concentrate ingredients, orts, and feces were quantified in terms of DM, OM, N, and EE according to the AOAC (2012; method number 934.01, 930.05, 981.10 and AOAC, 2006; method number 945.16, respectively). Neutral detergent fiber was analyzed according to the technique described by Mertens et al. (2002) without the addition of sodium sulfite, but with the addition of thermostable alpha-amylase to the detergent (Ankom Tech. Corp., Fairport, NY). Indigestible neutral detergent fiber was used as an internal marker to estimate fecal excretion in conjunction with an *in situ* incubation of 288 hours (Casali et al., 2008). Non-fiber carbohydrates were calculated according to Detmann and Valadares Filho

(2010), where NFC (% DM) = 100 – [CP – (CP derived from U + U) + NDF + EE + ash]. The total digestible nutrient (TDN) content of the diets was estimated as the sum of digestible nutrients, where TDN = $CP_{digestible}$ + 2.25 × $EE_{digestible}$ + $NDF_{digestible}$ + $NFC_{digestible}$ (NRC, 2001). Digestible energy (DE) intake was obtained by multiplying digestible nutrients by their respective energy values (NRC, 2001): DE = 5.6 × $CPI_{digestible}$ + 9.4 × $EEI_{digestible}$ + 4.2 × $NDFI_{digestible}$ + 4.2 × $NFCI_{digestible}$. The concentration of metabolizable energy intake (MEI) was estimated as 82% DE (NRC, 1996).

Samples of muscle + fat and bones from the section between the 9th and 11th ribs and the non-carcass were quantified in terms of DM, OM, CP, and EE as previously described.

2.6. Calculations

The empty body chemical composition was estimated by using the equations described by Marcondes et al. (2012) for Nellore bulls, which were validated by Costa e Silva et al. (2013a):

Crude protein (%): CP_{EBW} = 10.78 + 0.47 × % CP_{Cor} - 0.21 × % VF

Ether extract (%): EE_{EBW} = 2.75 + 0.33 × % EE_{Cor} + 1.80 × % VF

Water (%): W_{EBW} = 38.31 + 0.33 × % W_{Cor} - 1.09 × % VF + 0.50 × % OV,

where CP_{Cor} = % CP in the 9th to 11th rib section; EE_{Cor} = % EE in the 9th to 11th rib section; EBW = empty body weight; VF = % visceral fat (renal, pelvic, cardiac, and mesenteric fat) in EBW; W_{Cor} = % water in the 9th to 11th rib section; and OV = % organs and viscera in EBW.

The relationship between the shrunk body weight (SBW) and EBW was calculated for all animals to convert SBW to EBW, while the relationship between the ADG and empty body gain (EBG) was calculated to convert ADG to EBG. Body energy content was obtained from body protein and fat contents and their respective caloric equivalents were determined according to the ARC (1980):

Energy content (MJ) = (5.6405 × body protein (kg) + 9.3929 × body fat (kg)) × 4.184, where, 4.184 is the conversion from Mcal to MJ.

Thus, the heat production was calculated based on the difference between MEI and energy content in the body, by using the equation above. Then, the net energy requirement for maintenance (MJ/EBW$^{0.75}$/d) was estimated to be the intercept (β_0) of the exponential regression between heat production (HP) and MEI. The following model was utilized:

$$HP = \beta_0 \times e^{(\beta 1 \times MEI)},$$

where HP = heat production (MJ/EBW$^{0.75}$/d), MEI = metabolizable energy intake (MJ/EBW$^{0.75}$/d), β_0 and β_1 are regression parameters, and ‘e’ is the Euler number (2.718281). Also, the ME requirement for maintenance (MEm, MJ/EBW$^{0.75}$/d) was estimated by the iterative method, with MEm considered to be the value where MEI equals HP.

The efficiency of ME utilization for maintenance (km) was calculated as the ratio between the net energy and ME for maintenance. The net energy requirement for growth (NEg) was estimated from the regression between NEg, EBG, and metabolic EBW by using the following model:

$$NEg = a \times EBW^{0.75} \times EBG^{b},$$

where NEg = the net energy for growth represented as the energy retained in the body (MJ d^{-1}), $EBW^{0.75}$ = metabolic empty body weight, and EBG = empty body gain (kg d^{-1}).

Metabolizable protein for maintenance was obtained based on the linear regression between metabolizable protein intake and empty body gain (EBG) divided by average $EBW^{0.75}$, while the net protein requirement for growth (NPg) was estimated by a model involving EBG and retained energy in the body:

$$NPg = \beta_1 \times EBG + \beta_2 \times RE,$$

where NPg = retained protein or the net protein requirement for growth (g d^{-1}), EBG = empty body gain (kg d^{-1}), RE = retained energy (MJ d^{-1}), and β_1 and β_2 are regression parameters. The MPg was estimated by dividing the NPg by the efficiency of metabolizable protein utilization for growth (k), according to the equation proposed by Valadares Filho et al. (2010).

2.7. Statistical analyses

Intake and nutrient digestibility, animal performance, microbial protein synthesis, N balance, and CH_4 emissions were evaluated as mixed models through PROC MIXED (SAS Inst. Inc., Cary, NC). The heterogeneous first-order autoregressive matrix (ARH (1)) was used; this is the variance and covariance matrix of random effects for all of the variables that were analyzed. Linear and quadratic effects were evaluated by orthogonal contrasts as a function of the levels of CP in the diet.

Data of REF and MAIN animals were only utilized to estimate energy and protein requirements for finishing Nellore bulls, while data from MANT animals were not used to calculate protein requirements. A linear regression model between metabolizable protein intake and EBG was analyzed in order to estimate the net protein requirements for maintenance by using PROC REG (SAS Inst. Inc., Cary, NC). To estimate the net protein requirements for gain and the net energy requirement for maintenance and gain, data were analyzed by using non-linear models through PROC NLIN (SAS Inst. Inc., Cary, NC) and were adjusted by the Gauss–Newton method. For all comparisons, the level of 0.05 was established as the critical level to test the probability of a type I error.

3. Results and discussion

3.1. Intake and nutrient digestibility

As expected, a linear effect ($p < 0.05$) was observed for the dietary CP level on CP intake (Table 2), where a greater intake was observed in animals that were fed 14% CP (1.22 kg/d), an intermediate intake in animals that were fed 12% CP (1.09 kg/d), and a lower intake in animals that were fed a 10% CP-based diet (0.89 kg/d). However, there was no effect ($p > 0.05$) for the intakes of DM, OM, EE, NDF, and NFC. According to Oliveira and Millen (2014), Brazilian feedlots commonly adopt CP levels between 9.3% to 16.6%, with average values of 13.5% CP. The high-protein levels of finishing diets can increase DM digestion and intake, thereby leading to a greater ADG and improved feed efficiency (Véras et al., 2007). Thus, based on data that was obtained in this study, we suggest that intake was not affected by CP level, so reducing the level of dietary CP during the finishing phase will result in less costs associated with feedstuffs; however, it is important to consider that the present study used 6 animals per treatment and our power to detect differences was limited. The excess CP intake in the 14% CP-based diet in relation to the 10% CP diet was 330 g d^{-1} and is equivalent to 733 g d^{-1} of soybean meal or 131 g d^{-1} of urea that could be saved.

A linear effect ($p < 0.05$) was observed between the dietary CP levels of CP digestibility (Table 2), which was greater for 14% CP (74.3%) and similar for 10% (70.7%) and 12% CP-based diets (70.0%). There was no effect ($p > 0.05$) of the digestibility of DM, OM, EE, NDF, and NFC. Similar results were observed by Obeid et al. (2006) when they evaluated different dietary CP levels (9, 11, 13, and 15%) and observed that intakes of DM, OM, NDF, and NFC were not influenced by CP levels in diets, although they observed a linear increase in the intakes of CP and EE (kg/d) with an increase in dietary CP levels. Digestibility of DM, OM, EE, NDF, and NFC were not influenced by CP levels, but the apparent CP digestibility coefficient increases linearly with the increased CP in diets. Similar results were also observed by Cavalcante et al. (2005) and Silva et al. (2005), where increasing dietary CP levels of finishing Nellore cattle did not influence intake and nutrient digestibility, except for CP, as there was a greater intake and nutrient digestibility for diets with greater CP levels.

3.2. Energy and protein requirements

Energy and protein requirements (Table 6) were estimated according to the BR CORTE system (Valadares Filho et al., 2010). The nutrient balance was calculated based on the difference between nutrient intake and requirements (Table 6), as described by Rotta et al. (2014), and negative values mean that the requirement was overestimated. A negative value was obtained for CP; this result shows that the BR CORTE system (Valadares Filho et al., 2010) overestimates the CP requirements by

45.2, 24.4, and 11.2% for the 10%, 12%, and 14% CP-based diets, respectively. This overestimation occurs because the BR CORTE system did not consider N recycling in the rumen and considered 10% to be the net ruminal losses of N. Similar results were obtained by Prados et al. (2015) when using growing crossbred bulls, as they estimated that values for CP intake (1,020 g/d) were 17.2% greater than the observed values for CP intake (870 g d^{-1}). Amaral et al. (2014) observed, for finishing crossbred bulls, a CP intake (1,580 g d^{-1}) predicted by Valadares Filho et al. (2010) that was 17.0% greater than the value that was observed (1,348 g d^{-1}). Costa e Silva et al. (2013a) found that CP intake was overestimated by 16.8% in relation to the average values that were observed for finishing Nellore bulls.

The relationship between EBW and BW in this study was 0.915, which is similar to those values reported by Costa e Silva et al. (2012; 0.914) and greater than the 0.895 and 0.891 suggested by Valadares Filho et al. (2010) and the NRC (1996), respectively. The NRC (1996) also considers that this ratio can vary from 0.85 to 0.95. The average relationship between EBG and ADG was 0.986, which was greater than the values proposed by Valadares Filho et al. (2010; 0.936) and the NRC (1996; 0.951), but lower than those reported by Costa e Silva et al. (2012) and Marcondes et al. (2013), at 1.013 and 1.014 respectively.

The relationship between HP and MEI was described by the following equation: $HP = 0.3182 \times e^{(0.8533 \times MEI)}$ (Figure 1) where NEm was estimated as 318.2 kJ/kg $EBW^{0.75}$/d for finishing Nellore bulls, which was close to the 320.1, 310.5, and 313.8 kJ/kg $EBW^{0.75}$/d proposed by Chizzotti et al. (2008), Valadares Filho et al. (2010), and Costa e Silva et al. (2012); however, it was greater than the value obtained in a respirometric chamber, which was 288.7 kJ/$EBW^{0.75}$/d (Poczopko, 1971). The metabolizable energy for maintenance was 478.7 kJ/kg $EBW^{0.75}$/d, which was similar to the 476.3 reported by Costa e Silva et al. (2012), while NEg (MJ/EBW$^{0.75}$/d) was estimated by the following equation: RE = 0.9825× EBW$^{0.75}$ × EBG$^{0.8262}$.

The efficiency of the use of ME was divided into maintenance (km) and gain (kg) as recognized by several systems (AFRC, 1993; NRC, 1996; CSIRO, 2007). The value of km (NEm/MEm) was 66.5% and was similar to those proposed by Chizzotti et al. (2008) and Costa e Silva et al. (2012), which was 67% for both. The value of kg was obtained based on the linear regression between RE and MEI, and this value of 34.4% (Figure 2) was similar to the 33.0% obtained by Costa e Silva et al. (2012). Several factors such as age, composition, or condition of feed can affect the efficiency of the use of energy for maintenance and gain (Blaxter et al., 1966; Garrett, 1980; Gionbelli et al., 2012), and kg and EBG were the most important variables that affected km (Marcondes et al., 2013). The majority of the information regarding these variables comes from data involving growing Nellore cattle (Tedeschi et al., 2002; Chizzotti et al., 2007; 2008; Costa e Silva et al., 2015), so it is important to conduct more studies with Nellore bulls during the finishing phase to more accurately predict their requirements.

Metabolizable protein for maintenance was 4.07 g/BW$^{0.75}$, and this value was obtained based on a linear regression between metabolizable protein intake and EBG divided by the average metabolic EBW (Figure 3). Wilkerson et al. (1993) found that MPm was 3.80 g/BW$^{0.75}$, which has been adopted by the NRC (1996), while 4.13 g/BW$^{0.75}$ was the value adopted by Valadares et al. (1997); Valadares Filho et al. (2010) considered 4.00 g/BW$^{0.75}$ similar to the value that was obtained in this study. Net protein for gain (g d^{-1}) was estimated by the following equation: NPg = 188.5 × EBG – 3.33 × RE, while Valadares Filho et al. (2010) suggested the following equation for Nellore bulls: NPg = 238.79 × EBG – 15.68 × NEg. According to Fox and Black

(1984), the net protein requirements for finishing bulls is dependent on the measurement of the body composition of animals; thus, the requirements vary with BW, rate of weight gain, breed, sex, dietary effects, and nutritional management. The net protein requirements for gain are lower in bulls that are late maturing rather than early maturing cattle (Geay, 1984) and this is because bulls deposit more lean tissue than steers (Vanderwert et al., 1985).

3.3. Nitrogen balance, microbial efficiency, and CH_4 emissions

A linear effect ($p < 0.05$) was observed for dietary CP levels on urinary N excretion, where animals that were fed 14% CP had greater ($p < 0.05$) urinary N excretion (130.29 g d^{-1}) than animals that were fed 10% (93.23 g d^{-1}) or 12% CP (122.05 g d^{-1}; Table 3 and Figure 4). There was no effect ($p > 0.05$) of dietary CP levels on retained N (g d^{-1}) among animals, thus showing that N intake does not interfere with muscle deposition in finishing bulls. A beef cattle animal that was fed 12% and 14% CP excreted 28.8 and 37.1 g N/d more, respectively, than animals that were fed 10% CP, which results in greater N excretion of 2.42 and 3.11 kg per animal when fed 12% and 14% CP, respectively, while considering an 84-d feedlot period. Moreover, considering that there were 4,500,000 cattle raised on Brazilian feedlots in 2014 (ANUALPEC, 2015) and the average dietary CP content is 13.5% (Oliveira and Millen, 2014), the amount of excess N excretion can reach 13.9 thousand tons for an 84-d period. The 84-d period is the average feedlot period in Brazil and before the feedlot period, animals are raised on pastures.

Cole et al. (2005) and Todd et al. (2006) observed that urinary N losses were greater for finishing bulls that received 13% CP than those that were fed 11.5% CP (from 24 to 50%). The route of N excretion, such as fecal N and urinary N, was dependent on diet composition and was greater than 75% of N excretion that was found in urine when high protein and high concentrate-based diets were used (Swensson, 2003; Cole et al., 2005; Hristov et al., 2011). Cole et al. (2003) evaluated three CP levels in diets where steers were fed as follows: constant 12% CP, constant 14% CP, and oscillating 10 or 14% CP at 2-d intervals. The authors observed a greater N excretion in animals that were fed constant 14% CP. In this study, the 10% CP diet resulted in 22.1% and 28.4% less urinary N losses than the 12% and 14% CP-based diets, respectively, thereby resulting in a smaller environmental impact.

There was no effect ($p > 0.05$) on microbial efficiency (Table 4), so we considered the average value obtained from the three treatments to be 119.8 g CP/kg TDN. This value was close to the 120 g CP/kg TDN proposed by Valadares Filho et al. (2010), and was lower than the 130 g CP/kg TDN proposed by the NRC (1996). Costa e Silva et al. (2013b), when evaluating Nellore bulls, found 142 g CP/kg TDN, and this value was greater than that found in this study.

According to Moore et al. (1999), the relationship between the TDN:CP ratio of diets is important when it comes to evaluating the balancing of the ration. A TDN:CP ratio lower than 7 means that there is sufficient N in the diet; however, a deficit of energy is associated with a TDN:CP ratio greater than 7, and means that there is a deficit in protein and an excess of energy, therefore making protein supplementation necessary. So, based on the idea proposed by Moore et al. (1999), we can infer that a TDN:CP ratio equal to 7 means that a diet supplies enough energy and protein. In this study, we observed that the TDN:CP ratio varied among dietary CP levels as follows: 7.36, 6.23, and 5.67 for 10, 12, and 14% CP-based diets, respectively. Therefore, we suggest that CP levels between 10 and 12% can be used for finishing Nellore bulls.

There was no effect ($p > 0.05$) of dietary CP level on CH_4 emissions. However, it is worth noting, that a small sample size was a limitation of this study. Methane excreted by cattle comes from ruminal fermentation and is related to intake and digestibility of feedstuffs (Rivera et al., 2010). According to Lassey et al. (1997), around 87% of the variations in CH_4 emissions are related to differences between animals, and only 13% of these variations are related to differences in DM intake. So, individual characteristics are the most important reason for the variations in CH_4 emissions (Zotti and Paulino, 2009). According to Lassey (2002), these variations can occur in *Bos indicus*, *Bos taurus* cattle, and their crossbreeds, and it can be related to characteristics such as rumen volume, ability to select feedstuffs, retained time of feed in the rumen, and factors that affect fiber digestion.

According to Shibata and Terada (2010), CH_4 production (g d^{-1}) tends to decrease as the protein content of feedstuff increases, but in this study, despite the increasing protein levels, there was no effect on CH_4 emission. Data that was obtained in this study showed an average CH_4 production of 158.7 g d^{-1}; this value was intermediate when compared to those obtained for Mercadante et al. (2015), which were 144.0 g d^{-1} for Nellore bulls with low residual feed intake and 163.0 g d^{-1} for Nellore bulls with high residual feed intake. However, Possenti et al. (2008) observed lower CH_4 emissions (147.5 g d^{-1}), and Johnson and Johnson (1995) suggested greater values for beef cattle (from 164.0 to 194.0 g d^{-1}). According to Shibata and Terada (2010), increased feed intake causes an increase in CH_4 production because of a greater supply of substrates for ruminal fermentation and the consequent increase in the supply of substrates for methanogenesis (Hegarty et al., 2007). Enteric CH_4 production in ruminants is a well-understood process that is closely related to the production of volatile fatty acids in the rumen (Hungate, 1982; Johnson and Johnson, 1995). The primary substrate for methanogenesis is H_2, which is generated primarily during the fermentation of plant cell wall carbohydrates to acetate and butyrate (Moss et al., 2000).

In contrast, the fermentation of starch and other nonstructural carbohydrates favors propionate production, which serves as a competitive pathway for H_2 use in the rumen (Benchaar et al., 2001). In this study, DMI did not differ among treatments and the roughage:concentrate ratio was constant throughout the experiment, which can be explained by the lack of effect that was observed for g CH_4/kg DMI, g CH_4/kg TDNI, and g CH_4/kg NDF intake with average values of 16.3 g kg^{-1}, 36.9 g kg^{-1}, and 53.2 g kg^{-1}, respectively. The value of 16.3 g kg^{-1} DMI was lower than those reported by Mercadante et al. (2015) of 24.4 and 25.7 g kg^{-1} DMI for low- and high-residual feed intake Nellore bulls, respectively. Nevertheless, a similar result was obtained by Woodward et al. (2002; 19.5 g kg^{-1} DMI), while Aguerre et al. (2011) observed a greater value (25.9 g kg^{-1} DMI) for dairy cows that were fed a corn silage-based diet. The lack of differences in CH_4 emissions in this study can be explained by the quality of the diets due to their digestibility and the homogeneity of animals that were used (same breed, same sex, and same age).

The CH_4 emissions rate did not exceed the 6.5% default value that is recommended by the IPCC (2006) for cattle in developing countries. However, Chaokaur et al. (2015) reported CH_4 emission rates ranging from 7.3 to 11.5% in mature Brahman cattle with body weights ranging from 300 to 420 kg and fed a tropical forage-based diets. This study also indicated that an increase in CP level in the diet did not change the CH_4 emission rate in beef cattle. However, it is important to point out that the present study had a limited number of animals ($n = 26$) and that we may not be able to identify possible effects on CH_4 emission.

A linear effect ($p < 0.05$) was observed for dietary CP levels on urinary N_2O (Table 3). Variation in dietary N intake will particularly affect the excretion of urinary N, which is much more vulnerable to losses than is fecal N (Dijkstra et al., 2013). Cattle urine has been shown to stimulate N_2O production to a larger extent than dung due to the dual effect of a large pool of readily available N and C and increased soil water content (Allen et al., 1996; van Groenigen et al., 2005; Boon et al., 2014).

Considering that the main factors affecting CH_4 emissions are the amount of manure that is produced and the portion of the manure that decomposes anaerobically, we did an extrapolation, following the IPCC (2006) tier 1 guidelines, to estimate the amount of CH_4 that is produced by manure from feedlots on a national basis. Thus, considering that Brazil is located in Latin America, whose average temperatures range between 20 ºC and 25 ºC (INMET, 2016) and that there were 4,500,000 cattle raised on Brazilian feedlots in 2014 (ANUALPEC, 2015) we determined that an emission factor of 1 (kg CH_4 $head^{-1}$ yr^{-1}) results in an amount of 4.5 G g CH_4 yr^{-1}.

Furthermore, CH_4 and N_2O are important greenhouse gases with 25 and 298 times the Global Warming Potential of CO_2, respectively (Forster et al., 2007). A linear ($p < 0.05$) effect was observed for the dietary CP level on the CO_2 equivalent of urinary N_2O and there was no effect ($p > 0.05$) of dietary CP level on CO_2 equivalent of daily enteric CH_4, fecal N_2O and CO_2 equivalent per kilogram of product (Table 5).

3.4. Animal performance and carcass characteristics

We believe that is important to note that the present study used six animals per treatment. This number was established according to the recommendations of the Ethic Commission in Use of Production Animals of the Universidade Federal de Viçosa, and was in agreement with the Ethical Principles for Animal Research established by the National Council of Animal Experimentation Control (CONCEA) and with actual Brazilian legislation. Following these guidelines, we calculated the number of animals that were necessary to develop the study and to detect differences among treatments, thereby concluding that six animals were sufficient for this experiment.

There was no effect ($p > 0.05$) of CP levels on animal performance and carcass characteristics. In contrast to this study, Cole et al. (2003) observed that animals that were fed 14% CP had a greater ADG and a greater G:F ratio than those that were fed 12% CP or oscillating CP levels of 10 and 14% at a 2-d interval. For growing animals, CP levels influence weight gain (Winchester et al., 1957), although, according to Cavalcante et al. (2005) and Amaral et al. (2014), the same behavior was not observed for finishing animals. This occurs because the dietary protein requirement decreases when considered as the percentage of the diet (NRC, 1996) when cattle reach maturity, as these animals deposit more fat tissue and thereby increase the fat:muscle ratio.

According to Oliveira and Millen (2014), Brazilian feedlots adopt up to 16.6% CP in their diets for finishing cattle to stimulate DM intake, but it can cause an excess of N, thereby resulting in environmental contamination. According to Bouwman et al. (1997), ammonia emissions by ruminants are greater than that of other domestic animals, which in turn contribute to eutrophication and acidification of ecosystems (Cowling and Galloway, 2001). Moreover, as protein is considered to be the most expensive nutrient in diets (Russel et al., 1992), we suggest, based on data from this study, that the CP level can be reduced without affecting animal performance, consequently avoiding damage to the environment and economical losses, but this must be confirmed with productive assays while using a greater number of animals. This concern coincides with the recommendations of Thomson et al. (1995) and Galyean and Gleghorn (2001), who suggested feedlot diets should have a CP level between 12 and 13%.

4. Conclusions

The reduction in the CP content of diets does not affect DM intake, animal performance, and carcass characteristics, thereby suggesting that the use of 10% of CP in diets for finishing bulls reduces the environmental impact due to a decrease in urinary N excretion. This value was 28.8 and 37.1 g N/animal/d lower for the 10% CP-based diet when comparing with the 12% and 14% CP-based diets, respectively. Furthermore, CH_4 emission may not be affected by CP levels which, on average, is 16.3 g/kg of DM intake. However, more studies are necessary to attest these results, because this study had a limited number of animals per treatment.

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Table 1 Proportions of ingredients in each concentrate and composition of each concentrate and corn silage.

Item	Concentrate			Corn silage
	10% CP[a]	12% CP	14% CP	
Proportion of ingredients (% DM)				
Ground corn	79.0	79.0	80.0	-
Soybean meal	3.6	8.6	13.7	-
Wheat meal	12.0	6.0	0.0	-
Urea	0.59	1.4	2.2	-
Ammonium sulfate	0.07	0.16	0.25	-
Common salt	0.90	0.90	0.91	-
Mineral mix[b]	0.90	0.90	0.91	-
Sodium bicarbonate	1.5	1.5	1.5	-
Magnesium oxide	0.50	0.50	0.50	-
Chemical composition (% DM)				
Dry matter (%)	86.4	86.5	87.5	31.1
Organic matter	89.3	89.4	90.4	94.4
Ether extract	2.92	2.86	2.83	2.5
Crude protein	12.7	16.3	20.0	7.3
Neutral detergent fiber	13.9	11.5	9.3	50.2
Indigestible neutral detergent fiber	3.0	2.4	1.9	12.6
Non-fiber carbohydrates	59.8	58.7	58.3	34.4

[a] CP, crude protein.

[b] 266 g kg^{-1} calcium (calcium carbonate source); 147 g kg^{-1} phosphorus (dicalcium phosphate source); 7 g kg^{-1} magnesium; 3 g kg^{-1} potassium; 2 g kg^{-1} sodium (sodium chloride source); 7 g kg^{-1} sulfur (cobalt sulfate and zinc sulfate source); 1,191 mg kg^{-1} copper (copper chelate source); 5,070 mg kg^{-1} iron (iron sulfate source); 1,728 mg kg^{-1} manganese (manganese chelate source); 4,198 mg kg^{-1} zinc (zinc sulfate source); 136 mg kg^{-1} cobalt (cobalt sulfate source); 118 mg kg^{-1} chromium.

Table 2 Dry matter, nutrient intake (kg DM/d), and apparent digestibility (%) for bulls fed with different crude protein levels.

Item	Diets			SEM[b]	Contrasts	
	10%	12%	14%		Linear	Quadrati
Dry matter						
Intake (kg d^{-1})	8.7	9.1	9.0	0.45	0.683	0.5686
Apparent digestibility	73.3	73.4	75.0	0.97	0.230	0.5451
Organic matter						
Intake (kg d^{-1})	8.3	8.7	8.6	0.43	0.625	0.6013
Apparent digestibility	74.7	74.8	76.5	0.92	0.186	0.4484
Crude protein						
Intake (kg d^{-1})	0.89	1.09	1.22	0.05	0.000	0.5714
Apparent digestibility	70.7	70.0	74.3	1.12	0.041	0.0925
Neutral detergent fiber						
Intake (kg d^{-1})	2.8	3.0	2.9	0.14	0.725	0.6434
Apparent digestibility	60.4	63.8	62.9	1.18	0.167	0.1538
Ether extract						
Intake (kg d^{-1})	0.25	0.25	0.24	0.01	0.693	0.7567
Apparent digestibility	83.5	81.4	79.9	1.92	0.200	0.9032
Non fiber						
Intake (kg d^{-1})	4.2	4.4	4.2	0.22	0.890	0.5815
Apparent digestibility	84.2	83.2	86.4	0.85	0.107	0.0636

[a] CP, crude protein.

[b] SEM, standard error of mean.

Table 3 Calculated nitrogen balance of bulls fed with different crude protein levels.

Item	Diets			SEM[b]	Contrasts	
	10%	12%	14%		Linea	Quadrati
N intake (g d^{-1})	167.1	205.4	209.8	8.13	0.003	0.1160
N digested (% intake)	72.8	73.5	75.6	1.70	0.302	0.4741
Fecal N (g d^{-1})	45.5	54.4	51.2	3.08	0.231	0.1355
Urine N (g d^{-1})	93.2	122.1	130.3	8.32	0.009	0.3370
Urine N (% of excreted	67.2	69.2	71.8	2.13	0.226	0.3583
Retained N (g d^{-1})	28.4	28.9	28.3	2.10	0.978	0.8243
Retained N (% of N	17.2	14.2	13.5	1.28	0.072	0.5002
Fecal N_2O (g d^{-1})	0.91	1.09	1.02	0.06	0.181	0.4194
Urinary N_2O (g d^{-1})	1.87	2.44	2.61	0.16	0.009	0.5086

[a] CP, crude protein.

[b] SEM, standard error of mean.

Table 4 Mean values of excretion of purine derivatives, microbial protein production, and microbial efficiency.

Item	Diets			SEM[b]	Contrast	
	10%	12%	14%		Linear	Quadrati
Allantoin (mmol d^{-1})	196.8	161.8	184.0	16.0	0.5998	0.1733
Uric acid (mmol d^{-1})	21.0	18.2	26.1	2.30	0.1522	0.0828
PDE[c] (mmol d^{-1})	217.7	180.0	210.1	17.7	0.7770	0.1466
Microbial nitrogen (g	132.6	106.4	126.7	11.9	0.7417	0.1405
ME[d] (g $_m$CP per kg	127.4	113.0	119.0	9.99	0.5790	0.4273
kg TDNI per kg CPI[e]	7.4	6.2	5.7	0.08	<0.000	0.0141

[a] CP, crude protein.

[b] SEM, standard error of mean.

[c] PDE, purine derivative excretion.

[d] ME, microbial efficiency.

[e] kg TDNI per kg CPI, total digestible nutrients intake per crude protein intake.

Table 5 Methane and CO_2 equivalent emissions and its relation with different parameters.

Item	Diets			SEM[b]	Contrast	
	10%	12%	14%		Linea	Quadrati
CH_4 (g d^{-1})[c]	155.7	150.2	170.2	8.83	0.286	0.2653
CH_4 per kg NDFI[d]	53.2	50.0	56.3	4.10	0.611	0.3702
CH_4 per kg TDNI[e]	37.6	34.6	38.6	2.80	0.805	0.3304
CH_4 per kg DMI[f]	16.1	15.2	17.6	1.02	0.335	0.2151
CH_4 per kg ADG[g]	115.2	104.1	118.0	8.56	0.824	0.2618
CH_4-CO_2 eq per kg	10,075	9,652	11,117	888.3	0.417	0.3936
CH_4-CO_2 eq. (g d^{-1})[i]	3,893	3,755	4,255	220.8	0.336	0.9554
Fecal CO_2 eq. (g d^{-1})[j]	271.2	324.2	305.2	19.29	0.181	0.4194
Urinary CO_2 eq. (g d-	555.5	727.7	776.6	50.16	0.009	0.5086
Total CO_2 eq. (g d^{-1})[l]	4,719	4,807	5,337	234.9	0.104	0.8964
MJ per 100 MJ GEI[m]	5.6	5.1	6.0	0.48	0.570	0.2306

[a] CP, crude protein.

[b] SEM, standard error of mean.

[c] CH_4 (g d^{-1}), daily enteric methane production in grams per day.

[d] CH_4 per kg NDFI, daily enteric methane production per kilogram of consumed neutral fiber detergent.

[e] CH_4 per kg TDNI, daily enteric methane production per kilogram of consumed total digestible nutrients.

[f] CH_4 per kg DMI, daily enteric methane production per kilogram of consumed dry matter.

[g] CH_4 per kg ADG, daily enteric methane production in relation to average daily gain.

[h] CH_4-CO_2 eq per car, daily enteric methane production per kg muscle carcass.

[i] CH_4-CO_2 eq (g d^{-1}), equivalent CO_2 of daily enteric methane production in grams per day.

[j] Fecal CO_2 eq. (g d^{-1}), equivalent CO_2 of daily N_2O excreted on feces in grams per day.

[k] Urinary CO_2 eq. (g d^{-1}), equivalent CO_2 of daily N_2O excreted on urine in grams per day.

[l] Total CO_2 eq. (g d^{-1}), equivalent CO_2 of daily enteric methane production plus daily N_2O excreted on feces and urine in grams per day.

[m] MJ per 100 MJ GEI, methane emission rate.

Table 6 Animal performance, nutrient balance, and carcass characteristics of bulls fed with 3 different crude protein levels.

Item	Diets			SEM[b]	Contrast	
	10% CP[a]	12% CP	14% CP		Linear	Quadratic
Animal Performance						
Initial BW[c] (kg)	324	325	329			
Final BW[c] (kg)	470	479	477	9.13	0.5682	0.6386
ADG[d] (kg)	1.3	1.5	1.5	0.06	0.6386	0.5324
G:F[e]	0.162	0.174	0.172	0.003	0.0544	0.1264
ME[f] intake (MJ d^{-1})	99.3	102.1	103.2	1.26	0.6231	0.8942
Nutrient requirements						
ME (MJ d^{-1})	92.8	97.7	97.2			
CP (kg d^{-1})	1.29	1.36	1.36			
Nutrient balance						
ME (MJ d^{-1})	6.5	4.4	6.0			
CP (kg d^{-1})	-0.40	-0.26	-0.14			
Carcass characteristics						
Cold carcass weight (kg)	278	284	279	6.07	0.8988	0.5250
Hot carcass weight (kg)	286	288	285	6.42	0.8982	0.7226
12th-rib fat (mm)	5.0	5.6	4.4	0.72	0.5852	0.2981
Hot carcass dressing (%)	60.9	60.1	59.6	0.58	0.1439	0.8993
Cold carcass dressing (%)	59.2	59.2	58.5	0.43	0.2410	0.4927

[a] CP, crude protein.

[b] SEM, standard error of mean.

[c] BW, body weight.

[d] ADG, average daily gain.

[e] G:F, gain-to-feed ratio.

[f] ME, metabolizable energy.

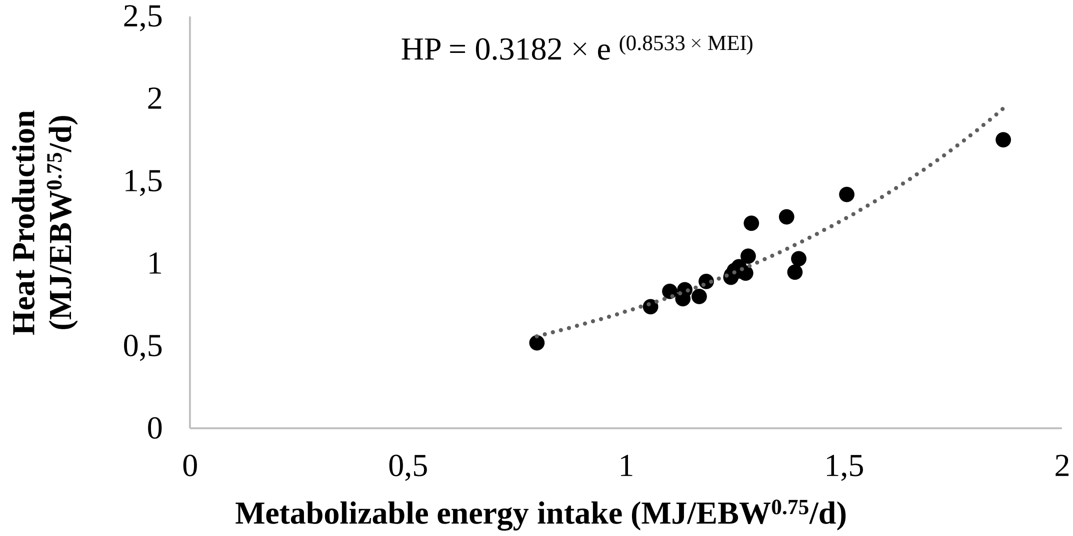

Fig. 1. Relationship between heat production and metabolizable energy intake (MEI)

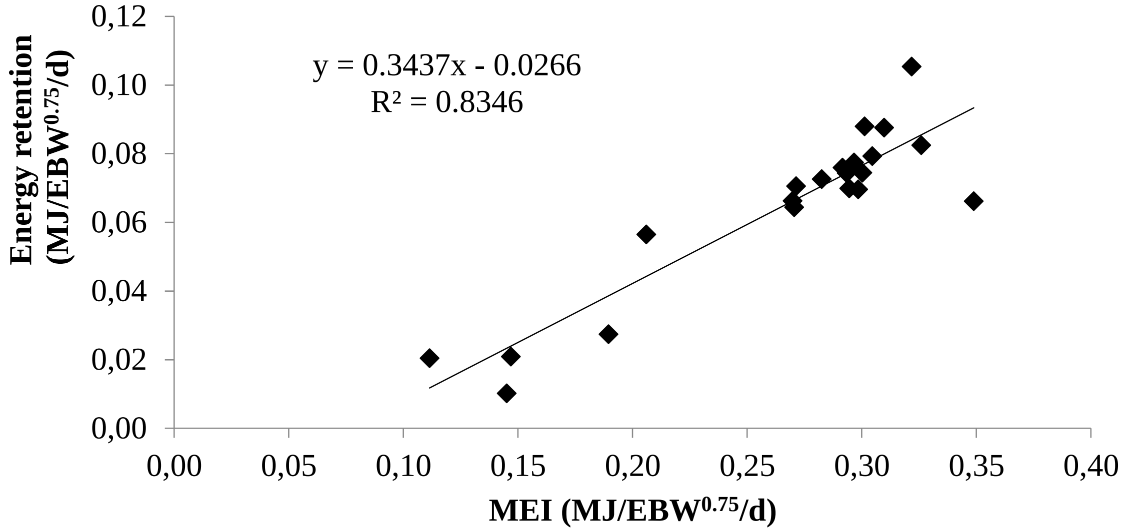

Fig. 2. Relationship between retained energy and metabolizable energy intake (MEI).

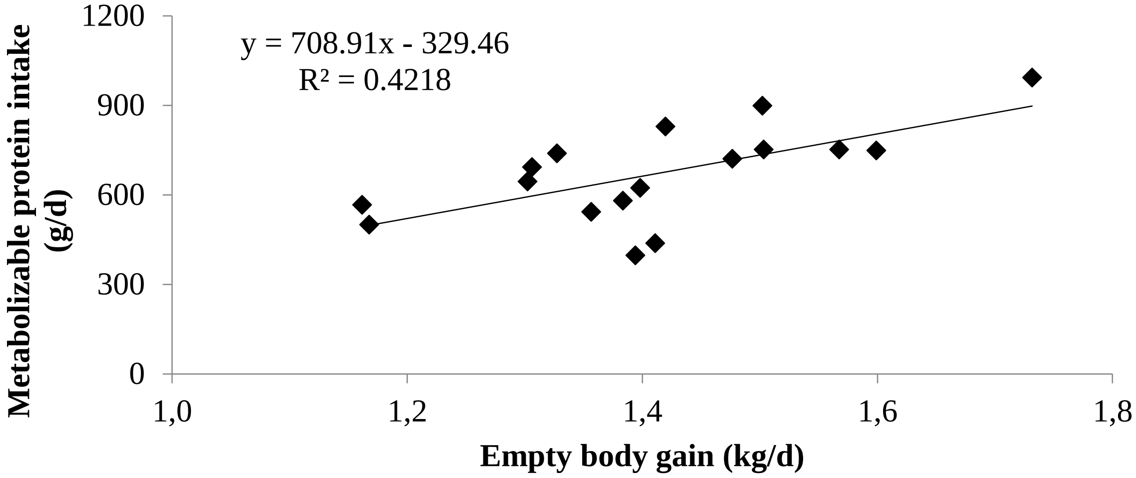

Fig. 3. Relationship between metabolizable protein intake (MPI) and empty body gain.

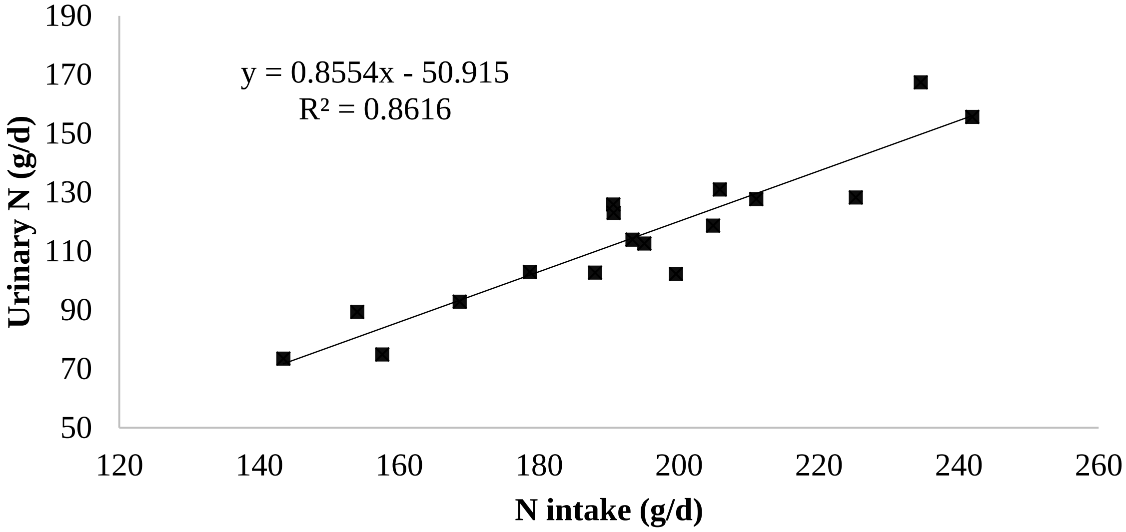

Fig. 4. Relationship between urinary N (g/d) and N intake (g/d).