WILLIAM LIMA SANTIAGO DOS REIS

# INVESTIGATION ON DAILY OR EVERY THREE DAYS SUPPLEMENTATION WITH PROTEIN OR PROTEIN AND STARCH OF CATTLE FED TROPICAL GRASS

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

VIÇOSA
MINAS GERAIS – BRAZIL
2018

**Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa**

T

R375i
2018

Reis, William Lima Santiago dos, 1987-
Investigation on daily or every three days supplementation with protein or protein and starch of cattle fed tropical grass / William Lima Santiago dos Reis. - Viçosa, MG, 2018.
xi, 47f. : il. ; 29 cm.

Inclui apêndices.
Orientador: Edenio Detmann.
Tese (doutorado) - Universidade Federal de Viçosa.
Referências bibliográficas: f.30-36.

1. Bovinos - Alimentação e rações. 2. Suplemento dietético. 3. Proteínas na nutrição animal. 4. Amido. I. Universidade Federal de Viçosa. Departamento de Zootecnia. Programa de Pós-graduação em Zootecnia. II. Título.

CDD 22 ed. 636.20852

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APPROVED: February 5th, 2018

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(Advisor)

# ACKNOWLEDGEMENTS

I would like to thank:

God for the gift of life. For help me, in these last years, to get back home. Things that were somewhere in my heart and soul have been revisited in this journey and I'm enjoying it.

Professor Edenio, for all the teachings given to me, for taking animal science and research seriously, with feverous excitement and for the inspiring classes. As an old hand, I also have to say that you had changed a bit in this journey, you got closer to the students creating the opportunity to take more teachings from you and making work easier and productive. I hope you have enjoyed these changes too.

Friends of the lab. Malber, João Lovatti, Tadeu, Cris, Larissa, Amanda, João Pacheco, Alininha, Naime, Gisele. You were there for all rumen evacuations, for the lab work, for a talk, for a beer. You really did the work easier guys.

Elsa, Déborah, Sophia and the others friends of the rumen microbiology lab for always been there when I needed most.

Janaina, my future wife, for all support and care. You've been listening to my lamentations since the undergraduation, but you are always supportive, always have a kind word, a wisdom advice. From every part of Viçosa, from Diamantina, from Rondônia (you are almost a foreign) and even from Rio, I keep in my memory (believe me, I have it) many special moments we´ve lived together. I love you.

My family; my parents, Otavio and Marluce; my siblings, Otavia, Thaís, Renata, Meredites e Paula. For always support my decisions. Thanks a lot, I love you all. I would like also to thank my nieces, girls you have brought a new flame of happiness to our family. Uncle love you.

My friends from Viçosa and Sericita. Life is not giving me many opportunities to see you, but it's always a pleasure when we met.

The national council of research (CNPq), for the research grant and for the research founding's. I would not be finishing this graduation without it.

The Universidade Federal de Viçosa, for the opportunity of learn lots of things, for give me friends that I will keep with me for the entire life.

Professor Luciana, for the surgeries in the animals, the collaboration in blood analyses and for the overall advices.

The members of the thesis committee, for all the advices and collaboration.

# BIOGRAPHY

William Lima Santiago dos Reis, son of Otavio dos Reis Filho and Marluce Lima Santiago dos Reis, was born in Ponte Nova/MG-Brazil on September 7, 1987. He started an undergraduate degree in Animal Science at the Universidade Federal de Viçosa in 2007 and obtained a Bachelor of Science in Animal Science in 2012. In 2012, he started the M.S. program with a major in ruminant nutrition and production at the Universidade Federal de Vicosa. In February of 2014, he obtained the M.S. degree in Animal Science. Then, in the same year, he started his D.S. program in Animal Science with a major in ruminant nutrition and production at the Universidade Federal de Viçosa. On 5th February 2018, he submitted his dissertation to the thesis committee to obtain the Doctor *Scientiae* degree in Animal Science.

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# ABSTRACT

REIS, William Lima Santiago dos, D.Sc., Universidade Federal de Viçosa, February, 2018. **Investigation on daily or every three days supplementation with protein or protein and starch of cattle fed tropical grass.** Advisor: Edenio Detmann. Co-advisors: Sebastião de Campos Valadares Filho and Cláudia Batista Sampaio.

Effects of daily or every three days supplementation with protein or protein and starch on intake, digestion, rumen dynamics, urinary excretion characteristics, nitrogen (N) balance and liver function of Nellore heifers fed high-quality tropical forage were evaluated. Five Nellore heifers (299 ±7.5 kg of body weight, BW) fitted with ruminal and abomasal cannulas were used in a 5 x 5 Latin square design. Treatments included: control, non-supplemented; 200 g of crude protein (CP) daily; 200 g of CP and 400 g of starch daily; 600 g of CP every three days; 600 g of CP and 1200 g of starch every three days. Therefore, over a three-day supplementation cycle, on average, supplemented animals received 200 of CP, or 200 g of CP plus 400 g of starch. Supplements, on average, enhanced ($P<0.02$) CP intake, digested organic matter (DOM) intake and the ratio of CP intake to DOM intake (CP:DOM ) when comparing to control. Among supplemented animals, every three days (infrequent) supplementation decreased ($P<0.05$) forage intake and therefore the intake of several fractions of the diet. Starch addition to supplements also had a negative effect on forage intake, noted as a decrease($P<0.04$) on NDF intake (g/kg BW). Supplements enhanced ($P<0.01$) total organic Matter (OM) and CP digestibility. Ruminal CP digestibility was also enhanced ($P<0.01$) by supplements, changing from negative in control to positive in supplemented animals. The same pattern was observed for rumen N balance (RNB). In situ degradability of potentially digestible NDF (pdNDF), on average of the three-day supplementation cycle, was not affected ($P\geq0.26$) by any treatment. However, there was ($P<0.02$) an interaction between days of the supplementation cycle and treatments. Control and frequently supplemented animals presented stable in situ rate of fiber digestion throughout the three-day supplementation cycle. Nevertheless, for animals supplemented with protein and starch every three days, there was an impairment to fiber digestion on the first day of the supplementation cycle (day 1), day in which these animals had access to supplements, followed by a recuperation of fiber digestion rate in the following two days (day 2 and day3), when they were not supplemented. Furthermore, for animals supplemented with protein every three days, inversely, in situ fiber digestion rate was greater on day 1 and then gradually dropped in the day 2 and day 3, following rumen ammonia N (RAN) pattern observed in these animals. On average, supplements enhanced ($P<0.01$) RAN from 4,2 to 8,7 mg/dL, respectively, for control and supplemented. Among supplemented animals, infrequent supplementation enhanced ($P<0.04$) RAN level when compared to daily supplementation. An interaction ($P<0.01$) between days of the supplementation cycle and treatments pointed out that RAN levels were constant over days for control and frequently supplemented animals, but for infrequent supplemented animals it was greater on day 1 and then decreased in the two following days reaching, on day 3, values similar those ones observed for control. Supplementation did not ($P\geq0.21$) affected pH or volatile fat acids concentration in rumen fluid when compared to control, but among supplemented animals, infrequent supplementation reduced ($P<0.02$) acetate-to-propionate ratio and starch addition reduced ($P<0.02$) acetic molar proportion. Supplements promoted an 90% increment in N balance. It raised from 8 (control) to 15 g/day (supplemented). Among supplemented animals, there was no ($P\geq0.43$) differences in N balance. Microbial flow and microbial production efficiency were not affected ($P\geq0.18$) by treatments. On average, supplemented animals presented greater ($P<0.01$) Urinary-N excretion (UNE) than control. Supplements, on average, also enhanced ($P<0.01$) serum urea-N (SUN). Among supplemented animals, addition of starch to supplements reduced ($P<0.04$) UNE, but it did not result in greater N retention. There was ($P<0.01$) an interaction between days of the supplementation cycle and treatments for SUN and the urinary excretion characteristics. Non-supplemented and daily supplemented animals presented constant values for these variables among the three-day supplementation cycle. However, in animals supplemented every three days changes in SUN levels resulted in an untypical decoupling between SUN and RAN throughout the supplementation cycle. While RAN was decreasing between day 1 and day 2, SUN peaked on day 2, which seem to be supported by the greater salvage of the urea-N filtered in kidneys, on day 2. In other words, the changes in SUN and urinary excretion characteristics pointed out that the they after supplementation seem to be important for N saving in ruminants infrequently supplemented. The greater N retention of supplemented animals was associated with greater ($P<0.02$) blood concentrations of IGF-1, and Insulin. Liver function, accessed through dousing transaminases activities in blood, was not affected ($P\geq0.07$) by treatments. In summary, Animals supplemented have greater N balance than non-supplemented animals. Starch addition to protein supplements does not enhance N balance. In spite of no changes in N balance between supplemented animals, animals receiving different supplements applied distinct mechanisms, in regard of N transactions along rumen, blood and kidneys.

# RESUMO

REIS, William Lima Santiago dos, D.Sc., Universidade Federal de Viçosa, fevereiro de 2018. **Estudo da suplementação diária ou a cada três dias com proteína ou proteína e amido em bovinos alimentados com gramínea tropical.** Orientador: Edenio Detmann. Coorientadores: Sebastião de Campos Valadares Filho e Cláudia Batista Sampaio.

Foram avaliados os efeitos da suplementação diária ou a cada três dias, com proteína ou proteína e amido, sobre o consumo, a digestão, a dinâmica ruminal, as características de excreção urinaria, o balanço de nitrogênio (N) e a função hepática de novilhas nelore alimentadas com forragem tropical de alta qualidade. Cinco novilhas (299 ±7,5 kg, Peso corporal, PC) fistuladas no rúmen e no abomaso foram utilizadas em um delineamento em quadrado latino 5 x 5. Foram avaliados os seguintes tratamentos: Controle, não suplementado; 200 g de proteína bruta (PB) diariamente; 200 g de PB e 400 g de amido diariamente; 600 g PB a cada três dias; 600 g PB e 1200 g de amido a cada três dias. Desta forma ao longo do ciclo de suplementação de três dias, em média, animais suplementados receberam 200g de PB, ou 200g de PB e 400 g/dia de amido. Em média, a suplementação aumentou ($P<0.02$) o consumo de PB, de matéria orgânica digerida (MOD) e a relação entre o consumo de PB e de MOD (PB:MOD), quando comparado ao controle. Entre animais suplementados, a suplementação a cada três dias (infrequente) reduziu ($P<0.05$) o consumo de forragem, reduzindo então o consumo de diversas frações da dieta. A adição de amido aos suplementos também afetou negativamente o consumo de forragem, como pode-se depreender da redução ($P<0.04$) no consumo de FDN (g/kg PC) ocasionada pela adição do amido. A suplementação aumentou ($P<0.01$) o coeficiente de digestibilidade total da matéria orgânica (MO) e da PB. O coeficiente de digestibilidade ruminal da PB também foi ampliado ($P<0.01$) pela suplementação, os coeficientes mudaram de negativos no controle para positivos em animais suplementados. O balanço de N no rúmen apresentou o mesmo comportamento. Na média, dos três dias do ciclo de suplementação, a degradabilidade *in situ* da fibra em detergente neutro potencialmente digestível (FDNpd) também não foi afetada ($P\geq0.26$) pelos tratamentos. Contudo, foi detectada ($P<0.02$) uma interação entre os dias do ciclo de suplementação e os tratamentos. O grupo controle e os animais suplementados diariamente apresentaram taxa de degradabilidade constante ao longo dos três dias do ciclo de suplementação. Em animais suplementados com proteína e amido a cada três dias, observou-se um desestimulo sobre a taxa de degradação da fibra no dia em que estes animais eram suplementados, o primeiro dia do ciclo de suplementação (dia 1), nos dias subsequentes (dia 2 e dia 3), quando estes animais não eram suplementados, observou-se recuperação na taxa de degradação. Adicionalmente, em animais suplementados com proteína a cada três dias, contrariamente, os maiores valores foram observados no dia 1 e decresceram gradualmente ao longo dos dias 2 e 3, acompanhado o comportamento dos níveis de N-amoniacal ruminal (NAR) observado nestes animais. Na média, a suplementação aumentou ($P<0.01$) os níveis de NAR de 4,2 para 8,7 mg/dL, respectivamente, para o controle e suplementados. Entre animais suplementados, a suplementação infrequente ampliou ($P<0.04$) os níveis de NAR quando comparada a suplementação frequente. A interação entre dias e tratamentos ($P<0.01$) apontou que os níveis de NAR permanecem constantes ao longo dos dias para o controle e animais suplementados diariamente, ao passo que para animais suplementados infrequentemente os níveis de NAR são maiores no dia 1 e então decrescem gradualmente nos dias subsequentes chegando, no dia 3, a valores semelhantes aos observados para o controle. A suplementação não afetou ($P\leq0.21$) o pH e a concentração total de ácidos graxos voláteis no fluido ruminal, mas entre animais suplementados, a suplementação infrequente reduziu ($P<0.02$) a relação acetato:propionato, e a adição de amido ao suplemento reduziu ($P<0.02$) a proporção molar de acetato. A suplementação promoveu um aumento ($P<0.02$) de 90% na retenção de N (balanço de N). Entre animais suplementados, não houve ($P\geq0.43$) diferença no balanço nitrogenado. O fluxo microbiano para o intestino e a eficiência de produção microbiana não diferiram ($P\geq0.18$) entre tratamentos. Em média, animais suplementados apresentaram maior ($P<0.01$) excreção urinária de N (EUN) do que animais não suplementados. A suplementação, em média, também ampliou ($P<0.01$) o N ureico no soro (NUS). Entre animais suplementados, a adição de amido ao suplemento reduziu ($P<0.04$) EUN, mas isto não resultou em maior balanço de N em comparação a animais recebendo apenas proteína. Observou-se interação ($P<0.01$) entre dias e tratamentos para NUS e para as características de excreção urinaria de N. Animais não suplementados e animais suplementados diariamente apresentaram valores constantes para estas variáveis ao longo do ciclo de suplementação. Por outro lado, em animais suplementados infrequentemente mudanças nos níveis de NUS resultaram em um desacoplamento entre os níveis de NUS e NAR ao longo do ciclo de suplementação. Entre os dois primeiros dias do ciclo, enquanto NAR diminuiu, NUS aumentou, atingindo um pico no dia 2. Tal desacoplamento parece ser suportado pelo aumento no salvamento do N-ureico filtrado nos rins, observado no dia 2. Em outras palavras, as modificações observadas em NUS e nas características urinarias parecem apontar o dia após a suplementação, como um dia importante para a economia de N em ruminantes suplementados infrequentemente. A maior retenção de N observada em animais suplementados associou-se à maiores ($P<0.02$) níveis de IGF-1 e insulina nestes animais. Não se observou ($P\geq0.07$) alterações na função hepática acessada através de dosagem da atividade de transaminases hepáticas no sangue. Em suma, animais suplementados com proteína apresentam maior balanço de N do que animais não suplementados, seja a suplementação aplicada diariamente ou a cada três dias. A adição de amido aos suplementos proteicos, apesar de reduzir a excreção urinária de N, não melhora o balanço de N. Ressalta-se que animais recebendo diferentes suplementos aplicaram mecanismos distintos, no que diz respeito as movimentações do N através do rúmen, do sangue e dos rins, mas o balanço de N não diferiu entre animais suplementados.

# Introduction

In developing tropical countries, grasses commonly cultivated for beef cattle production do not usually receive great amounts of nitrogen (N) fertilizers after pasture establishment. Therefore, even in the rainy season (typically summer), it is not common to find grasses with crude protein (CP) content greater than 80-100 g/kg dry matter (DM). In spite of this, grasses grazed during the rainy season may be described as medium- to high-quality forages, as they present greater CP content and are more digestible than forages grazed in the dry season (typically winter, with lower temperature and rainfall). These characteristics allow greater intake and digestibility of high-quality grasses, but they still cannot be considered as a balanced diet, because they maypresent nutritional or metabolic unbalances associated with a relative excess of energy, or a relative deficiency of protein (Detmann et al., 2014a; 2014b). Such unbalancing in the diet impairs cattle growing exclusively under grazing during rainy season of achieving an optimal performance. To overcome such a constraint to production, the ratio of CP intake to digested organic matter intake (CP:DOM) should be enhanced. In order to achieve that, cattle must be fed protein supplements.

On the other hand, under the productive conditions described above, supplementation with high degradable carbohydrates may reduce CP:DOM, increasing dietary unbalance and broadening the metabolic constraints to an adequate voluntary forage intake (Illius and Jessop 1996). However, supplementation exclusive with protein could imply in excessive nitrogen loss and, in some circumstances, even decreases feed intake (Rufino, 2015). In this context, the use of both protein and degradable carbohydrates at an ideal proportion could support a better assimilation of supplemental N in the rumen (Souza, et al., 2010) and N accretion in the body (Franco et al., 2017).

Poppi and McLennan (1995) and Paulino et al. (2008) pointed out that supplementing cattle under grazing during the rainy season may increase by 200 g/d the weight gain when comparing to non-supplemented animals. Consequently, supplementation can decrease the age of slaughtering and subsequently may be used to decrease beef cattle production costs. However, implementing a supplementation program may require great investments and also increase the need for labor force. Infrequent supplementation may help to overcome these challenges as it is an effective way to reduce both costs and labor in beef cattle production (Melton and Riggs, 1964).

Part of the success of an infrequent supplementation program is probably because a fraction of the supplemental N is recycled to the rumen and thus supplements may present a “carry-over effect” sustaining benefits even in the days when the animals are not supplemented. On other hand, according to our arguments previously presented, we may hypothesize that supplemental energy (e.g., starch) has positive effects on assimilation of infrequently supplemented N. Furthermore, if the infrequent supplementation with energy has mechanisms to sustain their possible benefits on N assimilation over the days in which animals are not supplemented, these mechanisms still remain to be understood.

Furthermore, there are other possible metabolic changes which seem to be associated with recycling and N salvage when protein is infrequently supplemented to cattle. Rufino (2015), for instance, pointed out an overtime decoupling between the rumen ammonia and blood ureaconcentration, which seems an attempt of the animal to save a greater proportion of the infrequently supplemented N. Maintaining average urinary-N excretion (UNE) unaltered also appear to be important to the success of infrequent supplementation, since no variation on average excretion has being noted when applying supplementation in alternated days or every three days (Wickersham, et al., 2008;

Atkinson et al., 2010; Rufino, 2015). However, the pattern of urinary characteristics over time (days in which animals have or have not access to supplements) are not commonly described in the literature. Studying the effects of infrequent supplementation over time may bring new evidences of how and why N is retained in infrequently supplemented animals.

Aiming to address some of these questions, we carried out a trial in which heifers fed high-quality tropical forage were subjected to daily or every three days supplementation with protein or protein and starch. A control with no supplementation was also maintained. Then, in order to get answers, we measured the effects of treatments on intake, digestibility, N metabolism, rumen dynamics, urinary excretion characteristics, blood-circulating hormones and metabolites, and liver function.

# Material and Methods

This experiment was carried out at the Department of Animal Science, Universidade Federal de Viçosa, Minas Gerais, Brazil. All practices involving the use of animals were approved by the Institutional Animal Care Committee (Protocol no. 03/2016).

## 1.1 Animals and management

Five ruminally and abomasilly fistulated Nellore heifers, averaging 299±7.5 kg of body weight (BW), were used in a 5 × 5 Latin square design. The heifers were housed in individual stalls (2 × 5 m) with concrete floor and equipped with individual feeders and water dispensers. Heifers had *ad libitum* access to a complete commercial macro/micro mineral mixture (80 g/kg of phosphorus).

The basal diet consisted of a high-quality Tifton hay (*Cynodom* sp., Table 1), which was *ad libitum* fed twice a day at 06:00 and 18:00, allowing approximately 100 g/kg in orts (as fed).

## 1.2 Treatments

The five treatments were: 1) control, without supplementation; 2) daily supplementation of 200 g of CP; 3) daily supplementation of 200 g of CP and 400 g of starch; 4) supplementation of 600 g of CP every 3 days; and 5) supplementation of 600 g of CP and 1200 g of starch every 3 days.

Therefore, over the three-day-supplementation cycle all supplemented animals received on average 200 g CP/d. Additionally, energy-supplemented animals also had access to starch and over each supplementation cycle received 400 g of starch in a daily basis. This ratio of crude protein to starch (1:2) was chosen based on observations described in Franco et al. (2017), who found a better N balance using that ratio in cattle fed tropical forage-based diet. The protein supplement was composed (as fed) by 740 g/kg of soybean meal, 234 g/kg urea, and 26 g/kg ammonium sulfate, and was planned to present 1000 g CP/kg as fed.

The supplemental amount of CP (200 g/d) corresponded to 1/3 of the CP requirements of a Nellore heifer with 300 kg BW and an expected average daily gain of 0.5 kg (Valadares Filho et al., 2016). The total amount of supplements was divided in two equal parts, packed into paper bags, and placed in the rumen of the animals before morning and evening hay feeding.

### 1.3 Experimental procedures and sample collections

The experiment consisted of five experimental periods, which lasted 27 d each, with 15 d for adapting the animals to the treatments (Machado et al., 2016) and 12 d for sample collections. The animals were weighed at the beginning and at the end of each experimental period in order to calculate the average BW and relative voluntary intake.

It is important to note that all evaluations were performed during at least one supplementation cycle (3 d, or 3-d multiples). The 3 days period that characterize each supplementation cycle will be referred hereafter (mainly in results and discussion) as: day 1, day in which both frequently and infrequently supplemented animals had access to supplements; followed by day 2 and day 3, the days in which only frequently supplemented animals had access to supplements.

In each period, voluntary forage intake was quantified from d 16 to d 21 of each experimental period (covering 2 cycles of supplementation). The amount of hay offered from d 16 to d 21 and orts obtained from d 7 to d 22 were taken into account for voluntary intake quantification. Within each day, representative samples of forage and orts were taken and ground in a knife mill to pass through a 2-mm screen sieve. After that, half of each sample was ground again to pass through a 1-mm screen sieve. Samples were then pooled and stored for subsequent chemical analyses.

Total fecal output was measured from d 17 to d 22. Feces were collected immediately after each spontaneous defecation and stored in 35L buckets. At the end of each 24-h collection day (06:00), feces were weighed and manually blended, and an aliquot (50 g/kg) was taken and oven-dried (60°C). Fecal samples were ground and pooled as previously described.

Digesta flow into abomasum was estimated with the double-marker method, using indigestible neutral detergent fiber (iNDF) and Co-EDTA as particulate and fluid markers, respectively. Five g/d of Co-EDTA were diluted in 4 L of water and infused in the rumen continually from d 11 to d 22 in each period using a peristaltic pump (Milan Scientific Equipment, Inc., Colombo, Paraná, Brazil).

Six abomasal samples (1,300 mL per sample) were collected from d 16 to d 22 of each experimental period. Sample collection began after discarding digesta accumulated in the cannula neck. Each sampling occurred at 28-h intervals to represent every 4 hours of a 24-h period in order to account for diurnal variation. Sampling schedule was: d 16 at 06:00, d 17 at 10:00, d 18 at 14:00; d 19 at 18:00, d 20 at 22:00, and d 22 at 02:00. After collection, abomasal samples were divided in two parts: 800 mL were filtered through a nylon filter (100-µm , Sefar Nitex 100/44; Sefar, Thal, Switzerland) for separation of the particle phase from the fluid phase. Fluid and particle phase samples were freeze-dried, ground as previously described and pooled, and analyzed separately in order to estimate abomasal flow. The remaining 500 mL were used to isolate bacteria associated with the fluid phase and bacteria associated with the particle phase, according to the procedures described by Reynal et al. (2005). The bacterial pellets (from solid and liquid phases) were frozen (-80°C), freeze-dried and grounded using a mortar and pestle.

From d 22 to d 24 samples of rumen digesta were taken in the liquid-solid interface of the rumen mat at 06:00, 12:00, 18:00, and 24:00. Samples were filtered through a nylon filter (100-µm, Sefar Nitex 100/44; Sefar, Thal, Switzerland) and assessed regarding pH using a potentiometer. After that, a 20-mL aliquot of ruminal fluid was combined with 5 mL of a metaphosphoric acid solution (250 g/L) and frozen (-20°C) for subsequent analysis of volatile fatty acids (VFA). Another 40-mL aliquot was combined with 1 mL of a $H_2SO_4$ solution (9 M) and frozen (-20°C) for later analysis of ruminal ammonia-N (RAN) concentration.

Simultaneously to the rumen digesta sampling procedures, samples of blood were taken directly from the jugular vein using vacuum tubes with either coagulation accelerator gel (BD Vacutainer®, SST II Advance, Franklin Lakes, NJ, USA) or coagulation inhibitor gel (BD Vacutainer® K2, Franklin Lakes, NJ, USA). Then tubes were promptly centrifuged in order to separate, respectively, serum and plasma.

From d 22 to d 24, the degradation of the forage fiber was also evaluated. Aliquots of the hay (2 mm) were packed in nylon bags (60-µm mesh; Sefar Nitex, Sefar, Switzerland; 20 mg DM/cm²) and incubated in the rumen, in duplicate, for 6, 12, and 24 h counted from the morning supplementation. At the end of each incubation period, the bags were removed from the rumen, washed in tap water, and subjected to neutral detergent fiber (NDF) evaluation.

Also from d 22 to d 24, urine was completely collected using a 2-way Foley catheter (no. 24; Rush Amber, Kamuting, Malaysia) with a 30-mL balloon. At the free end of the catheter, a polyethylene tube was attached through which the urine was conducted to a clean urine collection vessel (20 L). Vessels were maintained all time in polystyrene boxes with ice in order to avoid N losses. After each 24 h of collection (06:00) the vessels were changed and the urine output had the weight and volume measured, and two representative samples were taken: one sample was immediately subjected to a Kjeldahl evaluation of N concentration, and the other one was frozen (-20°C) for subsequent chemical analysis.

The ruminal pool of fiber was measured at 10:00 (4 h after morning feeding) on d 25 and at 06:00 (just before morning feeding) on d 27 of each period (Allen and Linton, 2007). Whole ruminal contents were manually evacuated through the ruminal cannula, placed into a plastic container, weighed, and hand mixed. A sample (50 g/kg) was then taken and the remaining ruminal contents were returned to the rumen. After that, samples were weighted, oven-dried, and grounded as previously described. In order to minimize animal stress, rumen evacuation was not evaluated during the three-day-supplementation cycle.

### 1.4 Laboratory Analyses

Samples of hay, orts, feces, abomasal digesta (liquid and particle phases), and ruminal contents (the samples collected from ruminal evacuation), processed to pass through a 1-mm sieve, were pooled per animal and experimental period, and analyzed with regards DM (dried over-night at 105ºC, method INCT-CA G-003/1), organic matter (OM, complete combustion in a muffle furnace at 600ºC, method INCT-CA M-001/1), CP (Kjeldahl procedure; method INCT-CA N-001/1), and NDFap (NDF analyzed using a heat-stable α-amylase, omitting sodium sulfite, and correcting for contaminant ash and protein; method INCT-CA F-002/1) contents according to the standard analytical procedures of the Brazilian National Institute of Science and Technology in Animal Science (INCT-CA; Detmann et al., 2012). Starch and protein supplement were sampled in each experimental period and also subjected to chemical analyses as described above. Samples of hay, orts, abomasal digesta, and ruminal contents, processed to pass through a 2-mm screen sieve, were evaluated with regards to iNDF content using F57 filter bags (Ankom Technology Corp., Macedon, NY, USA) and a 288-h *in situ* incubation procedure (Valente et al., 2011). Cobalt concentration in abomasal samples (both fractions) was quantified with atomic absorption spectrometry (GBC Avanta Σ atomic absorption spectrophotometer, Scientific Equipment, Braeside, Victoria, Australia) after nitro-perchloric digestion.

Abomasal digesta (both fractions) and the microorganisms isolated from it were analyzed for purine bases (Ushida et al., 1985) and CP (method INCT-CA N-001/1; Detmann et al., 2012) contents. The $N_{RNA}$:$N_{total}$ ratio in microorganisms was used as the marker for estimating microbial production in the rumen.

Samples of rumen fluid and blood were pooled per animal, experimental period, and days of supplementation cycle. The RAN concentration was quantified using the colorimetric technique described by Detmann et al. (2012, method INCT-CA N-006/1). The VFA concentration were analyzed using HPLC (Shimadzu HPLC class VP series, model SPD-10A VP, Shimadzu Corporation, Kyoto, Japan) with a reverse phase column (using a mobile phase of orthophosphoric acid in water, 10 mL/L) and a UV detector at a wavelength of 210 nm.

The urea and creatinine concentrations in urine and blood serum were evaluated using the enzymatic-colorimetric (K056, Bioclin Co, Belo Horizonte, MG, Brazil) and alkaline picrate (K067, Bioclin Co., Belo Horizonte, MG, Brazil) methods, respectively.. Blood concenrations of glucose, total protein , albumin and activities of aspartate transaminase (AST), alanine transaminase (ALT) and γ-glutamyl transpeptidase (GGT) were measured in a autoanalyser (Mindray BS-200E; Shenzhen Mindray Bio-Medical Electronics Co. Ltd., Shenzhen, Guangdong, China) with the respective commercial kits: K082, K031, K040, K048, K0049 and K080, all of them produced by Bioclin Co., Belo

Horizonte, MG, Brazil. Insulin was analyzed by chemiluminescence with ultrasensitive insulin reagent in the Access 2 Immunoassay System (Ref. Number 33410, Beckman Coulter, Brea, CA, USA). Blood IGF-1 (chemiluminesense method, Diasorin®) and amino acids (HPLC, quantitative chromatograph) were analyzed in a commercial laboratory (Hermes Pardini Laboratory, Belo Horizonte, MG, Brazil).

## 1.5 Calculations

The abomasal flow was estimated using iNDF as internal marker of the particle phase and Co-EDTA as external marker of the liquid phase. The iNDF was assumed as an ideal marker of the solid phase, while Co-EDTA was assumed as non-ideal marker of liquid phase. The reconstitution factor of the abomasal digesta was calculated as stated by France and Siddons (1986).

From rumen evacuation, the rates of intake and ruminal passage of NDF, potentially digestible NDF (pdNDF) and iNDF were estimated by the ratio of intake and abomasum flow on the rumen mass, respectively. The degradation rate of pdNDF was obtained as the difference between the rates of intake and passage (Allen and Linton, 2007).

For *in situ* evaluation of pdNDF degradation rate degradability, the non-degraded fraction of NDF was quantified for 6, 12 and 24 h of ruminal incubation of the forage. Data were log-linearized and the degradation rate was obtained within each day of the supplementation cycle as the slope of the regression of non-degraded residues on time.

The N balance (N retention) was calculated by subtracting fecal N excretion and UNE from N intake. For this calculation, it was considered the average UNE across the three-day-supplementation cycle. Rumen N balance was calculated by subtracting N abomasal flow from N intake.

The urea-N filtered in the kidneys *glomerulus* and the urea-N salvage were calculated from the following equations:

$$UNFK = \frac{UEC}{SC} \times SUN \qquad (1),$$

$$Urea - N\ Salvage = 1 - \frac{UUNE}{UNFK} \qquad (2),$$

where UNFK is the urea-N filtered through the kidneys *glomerulus* (g/d), UEC is the urinary excretion of creatinine (g/d), SC is the average concentration of serum creatinine (mg/dL), SUN is the average concentration of serum urea-N (mg/dL), Urea-N salvage is the fraction of the urea-N filtered through kidneys that is reabsorbed from nephron to blood (g/g), and UUNE is the urinary urea-N excretion (g/d).

### 1.6 Statistical analyses

The experiment was carried out and analyzed according to a 5 × 5 Latin square design with five treatments (fixed effect), five animals (random effect), and five experimental periods (random effect). Treatment effects were interpreted according to a 2 × 2 + 1 factorial arrangement (two frequencies, daily *versus* every 3 days; starch supplementation, with or without; and a control treatment). To assure an orthogonal comparison among treatments, the control treatment was compared against all supplemented treatments using one contrast.

The analyses of forage DM intake and variables related to rumen fermentation, blood characteristics, *in situ* degradation rate, and urinary excretion characteristics were performed by considering the days of the supplementation cycle as repeated measures (fixed effect). The choice of the best structure of (co)variance matrix was based on Akaike's information criterion with correction. The degrees of freedom were estimated by the Kenward–Roger method. Statistical analyses were performed using the MIXED procedure of SAS 9.4 (SAS Inst. Inc., Cary, NC) and significances were declared at $P<0.05$.

# Results

## 1.7 Intake and digestibility

Overall, supplementation increased ($P<0.02$) intake of CP and digested OM (DOM), and CP:DOM (Table 2). When frequency of supplementation was decreased (from daily to every 3 days), there was a decrease ($P<0.02$) in forage intake, which implied a decrease in DM, OM, CP, NDFap, iNDF, DOM, and digested NDF intakes. When intake was expressed as a fraction of BW, similar negative effects of infrequent supplementation on intake were observed. The addition of starch to the supplement increased ($P<0.02$) DOM intake and decreased ($P<0.01$) CP:DOM. When intake was expressed as fraction of animal body weight starch addition decreased ($P<0.04$) both NDFap and iNDF intake (Table 2). No effect ($P\geq0.61$) of interaction between frequency of supplementation and starch addition was observed on voluntary intake.

Additionally, to better understand the negative effect of infrequent supplementation on forage intake, the day of the supplementation cycle was add to the statistical model. An interaction between days and treatments was detected ($P<0.01$). On the first day of the supplementation cycle, animals infrequently supplemented (with our without starch supplementation) presented a decreased forage intake ($P<0.01$; Figure 1A), but there were no differences among treatments ($P\geq0.23$) regarding forage intake for the subsequent days of the supplementation cycle.

Supplementation enhanced ($P<0.01$) total digestibility of OM and CP, and also the dietary DOM (Table 3), but did not affect ($P>0.93$) the total NDFap digestibility. Moreover, at rumen and intestines, supplementation only increased ($P<0.03$) the CP digestibility. It must be noticed the ruminal CP digestibility changed from negative (control) to positive with the supplementation. No effect ($P\geq0.12$) of frequency of supplementation was observed on digestibility. The addition of starch to supplements enhanced ($P<0.05$) ruminal and total digestibility of OM, and also the DOM content of the diet ($P<0.01$), but did not affect ($P\geq0.30$) NDFap digestibility at any digestion site.

## 1.8 Ruminal pool and dynamics of fibrous compounds

Rumen evacuation pointed out no effect ($P\geq0.14$) of supplementation, supplementation frequency or starch on rumen pool, intake rate, degradation rate, and passage rate of the fibrous compounds of the diet (Table 4).

## 1.9 Rumen fermentation profile and *in situ* degradation rate of NDF

On average, supplementation increased ($P<0.01$) RAN concentration (4.2 and 8.7 mg/dL, respectively, for control and supplemented animals). Among supplemented animals, on average, feeding supplements infrequently increased ($P<0.04$) RAN (Table 5). However, an interaction between day and treatments was detected ($P<0.01$) for RAN. The control and frequently supplemented animals presented constant ($P\geq0.18$) RAN across the days of the supplementation cycle (Figure 2A). On the other hand, the infrequent supplementation implied in decreasing levels of RAN from day 1 to day 3 of the supplementation cycle ($P<0.01$). Animals infrequently supplemented presented the greatest concentrations of RAN on day 1. On day 3 of the supplementation cycle, RAN concentrations of infrequently supplemented animals reached values close to those ones observed in the control.

There was no alteration ($P\geq0.72$) on pH among treatments and days of the supplementation cycle (Table 5). Total concentration of VFA was not ($P\geq0.21$) affected by either treatments or days of the supplementation cycle, averaging 12.8 mmol/dL. On average, infrequent supplementation enhanced ($P<0.02$) propionate and decreased

($P<0.03$) acetate molar proportion. Infrequently supplemented animals also presented a lower ($P<0.02$) acetate-to-propionate ratio. The addition of starch to supplements decreased ($P<0.02$) the molar proportion of acetate, but did not affect ($P>0.11$) the acetate-to-propionate ratio.

On average of the three-day-supplementation cycle, the *in situ* essay also pointed out no overall differences among treatments ($P\geq0.26$) with regards fiber degradation rate (Table 5). However, an interaction ($P<0.02$) between day of supplementation cycle and treatments was detected (Table 5). Control and frequently supplemented animals presented stable degradation rates of NDF along days of the supplementation cycle ($P\geq0.58$, Figure 1B). However, the infrequent protein supplementation (without starch) caused a higher ($P<0.03$) degradation rate on day 1 followed by successive decreases in the consecutive days 2 and 3, when the animals were not supplemented. On the other hand, the addition of starch to the supplement of infrequently supplemented animals was accompanied by constraints to degradation rate on day 1 followed by an improvement of fiber degradation rate for the two consecutive days ($P<0.01$), when the animals were not supplemented.

### 1.10 Nitrogen retention, ruminal nitrogen balance, and microbial production

On average, supplementation increased total N intake ($P<0.01$) and body N balance ($P<0.02$, Table 6). There was no difference ($P\geq0.09$) among treatments with regard to efficiency of N utilization. Overall, supplementation also increased ($P<0.01$) ruminal N balance (RNB). Control presented a negative value, whereas supplemented animals presented positive values of RNB. Among the supplemented animals, an interaction ($P<0.03$) between frequency of supplementation and starch was detected for RNB. The addition of starch to animals supplemented infrequently did not change RNB

($P>0.05$), but in animals daily supplemented the addition of starch decreased RNB ($P<0.05$, Table 6). There was no effect ($P\geq0.16$) of treatments either on microbial N flow or efficiency of microbial production expressed as g of microbial CP per kg of DOM. However, the assimilation of dietary N into microbial protein was greater ($P<0.01$) for control when compared to supplemented animals.

## 1.11 Serum urea nitrogen and urinary excretion

In general, supplementation increased ($P<0.01$) SUN (7.9 mg/dL for control, and 12.6 mg/dL for supplemented animals), UNE (30 g/d for control, and 54 g/d for supplemented animals), UUNE (14.5 g/d for control and, 28.8 g/d for supplemented animals), the ratio UUNE:UNE (0.48 g/g for control, and 0.54 g/g for supplemented animals), and UNFK (32.3 g/d for control, and 59.0 g/d for supplemented animals, Table 7). Moreover, on average, supplementation decreased ($P<0.04$) urea-N salvage in the kidneys. In general, among supplemented animals, addition of starch to supplements decreased ($P<0.04$) SUN and UNE. However, for all variables showed in Table 7, excepting UUNE:UNE ($P>0.06$), there was an interaction effect ($P<0.01$) between day of the supplementation cycle and treatments.

Animals subjected to control and frequent supplementation kept constant SUN concentrations ($P\geq0.05$) across days of the supplementation cycle. However, animals subjected to infrequent supplementation presented a short peak of SUN on day 2 ($P<0.01$) followed by a large drop on day 3 of the supplementation cycle. This drop on day 3 leaded the SUN concentration of infrequent supplemented animals on last day to a level similar to those ones observed for control (Figure 2C). The UNFK present a pattern of response across days similar to SUN (Figure 2D).

The evaluation of the interaction between days of the supplementation cycle and treatment for UNE showed a pattern across days similar to those one observed for RAN concentrations. Control and frequently supplemented animals presented constant UNE along days (P≥0.12), whereas infrequently supplemented animals presented decreasing amounts of UNE along the three-day supplementation cycle (P<0.01). Therefore, on day 3, infrequently supplemented animals presented UNE almost as low as observed for control (Figure 2B).

### 1.12 Blood characteristics and liver function

On average, supplementation increased (P<0.02) blood IGF-1 and insulin concentrations (Table 8). However, an interaction between days of the supplementation cycle and treatments was detected for blood insulin (P<0.01). Infrequent supplementation provided higher insulin concentrations (P<0.02) on the first day of the supplementation cycle and then they decreased for the two following days (Figure 3). For control and frequently supplemented animals, there was no differences in insulin levels across the days of supplementation cycle (P≥0.06). Liver function was evaluated by dosing AST, ALT and GGT activities in blood serum, but no effects (P≥0.06) were observed for these variables. There were no effects (P≥0.08) on blood glucose, amino acids, and proteins concentrations.

# Discussion

The definition of high-quality forage applied here was proposed taking into account the average quality of the forages commonly available to beef cattle under continuous grazing in the tropics. In this context, the hay utilized presented a high OM digestibility (0.653 g/g), which agrees with its low iNDF content. Forage also presented a CP content (80 g/kg DM) that is considered to be adequate for sustaining fiber degradation in the rumen (Lazzarini et al., 2009).

When compared to control, supplements enhanced CP and DOM intake, but without any effect on forage intake. The absence of alteration on forage intake agrees with other results obtained in the tropics, where impacts of protein supplementation are not expected when forages presented CP content equal or higher than 70-80 g/kg DM (Lazzarini et al., 2016; Rufino et al., 2016; Batista et al., 2017). Therefore, considering a constant forage intake across supplements, the increase in CP and DOM intake is a direct reflex of including high-protein and high digestible supplements to the diet

The most prominent changes in voluntary intake was observed among supplemented animals. Infrequent supplementation decreased forage intake, on average, from 5.26 (daily supplemented) to 4.89 kg/d (infrequently supplemented). Accordingly, there was a decrease in the voluntary intake of several fractions of the diet by infrequently supplemented animals. The negative effect of infrequent supplementation on forage intake was better understood when the interaction between days of the supplementation cycle and treatments was evaluated. The forage intake by infrequently supplement animals was constrained mainly on the first day of the supplementation cycle, when animals had access to supplements. On day 1, the levels of NAR (mg/dL) of infrequently supplemented animals were the highest among all average values observed. In spite of the daily average values in the first day of the supplementation cycle have been found below the RAN concentrations associated with decreases in intake (Detmann et al., 2014a), the RAN concentrations in infrequently supplemented animals were higher than 23 mg/dL six hours after supplementation (data not shown). Such concentrations seem to exceed the capacity of ammonia uptake in the rumen (Detmann et al., 2014b) and have been associated with decreased voluntary intake in the tropics (Detmann et al., 2014a). Ammonia is rapidly transferred to blood and distributed to other corporal fluids reaching liver and neuronal structures where it can act to impair feed intake. Mechanisms related to ammonia hypophagia still not fully understood. Effects of ammonia in terms of neurotoxicity (Visek, 1968; Kertz et al., 1982) or impairment in liver metabolism (Allen et al., 2009) may act together to constraint voluntary feed intake.

This excess of ammonia impairing intake of infrequently fed animals may also help to explain the pattern of SUN concentrations. When a great load of ammonia is being detoxified, the urea synthesis capacity of liver may be overloaded and then part of the ammonia could be temporally captured in perivenous hepatocytes as glutamine (Häussinger et al.,1992) extending (delaying) urea syntheses over time (Allen et al., 2009). According to this delay in urea synthesis, SUN concentrations of infrequently supplemented animals reached a peak on the day after supplementation, which was also observed by Krehbiel et al. (1998) and Rufino (2015).

In addition, beyond ammonia metabolism and neurotoxicity, insulin may have acted on feed intake control of infrequently supplemented animals as well. On the first day of the supplementation cycle, great concentrations of blood insulin were observed in infrequently supplemented animals. According to Allen et al. (2009), such as ammonia, insulin can also favor greater fuel oxidation (i.e., propionate) in the liver. These authors also pointed out that if greater blood concentrations of insulin are accompanied by a greater availability of propionate, hepatic fuel oxidation would be even greater. In spite of interaction between days of the supplementation cycle and treatments has not been observed, on average, infrequent supplementation also enhanced molar proportion of propionate in the rumen.

Prior to the experiment, we hypothesized that liver of infrequently supplemented animals would be challenged by the greater load of ammonia to be detoxified. That is the reason why we examined the liver function. However, there were no alterations on liver function among treatments. To our knowledge, there are three possible explanations for that pattern. First, if great ammonia load from rumen is damaging the liver, there would be a probability of such damage cannot be detected through dosing transaminases activities in blood (Antonelli et al., 2007). Second, a probable liver damage would take a long time to occur and could not be detected through short term evaluations, such as done in our experiment. Third, which we consider to be more plausible, even with high-protein supplements, in spite of negative effects on intake, the infrequent supplementation does not cause significant damages or severe compromises in the liver function.

Considering both supplementation frequencies, on average, animals with access exclusively to protein and animals with access to protein plus starch presented a voluntary forage intake of, respectively, 16.85 and 16.05 g DM/kg BW. In spite of being not significant, that decrease caused by starch supplementation on forage intake was enough to cause a decrease in NDFap and iNDF intake (g/kg BW). Thus, such a pattern brings into evidence a negative effect of starch on voluntary intake of forage, as supplement had very small contents of fiber.

Negative effects of supplementation with highly-fermentable carbohydrates on tropical forage intake are commonly described in the literature (Souza. et al., 2010; Costa et al., 2011a; Figueiras, et al., 2016). When studying multiple high-forage diets with or without supplementation, Egan (1977) suggested that when an optimum range of protein-to-energy ratio in animal metabolism is achieved, intake is optimized. An adequate protein-to-energy ratio reduces constraints to intake, such as excessive heat dissipation or excessive amino acid catabolism (Illius and Jessop 1996; Detmann et al., 2014a). A diet able to supply approximately 210-220 g of CP per kg of DOM would be related to maximum forage intake and maximum supplemental N retention (Poppi and McLennan, 1995; Reis et al., 2016). We may notice that starch supplementation decreased the dietary CP:DOM compared to exclusive protein supplementation. Therefore, we believe that decrease in CP:DOM ratio caused by starch addition to supplements was the main fact that support the negative effect of starch on forage intake.

On the other hand, on average, supplementation enhanced CP:DOM from 129 (control) to 176 g/kg (supplemented). Besides the better protein-to-energy balance in the diet achieved with supplementation, forage intake was not enhanced by supplementation, probably because the adequate CP:DOM range for an optimal voluntary intake (210-220 g CP/kg DOM, Reis et al., 2016) was not achieved in any supplementation scheme here evaluated.

Generally, supplementation enhanced OM digestibility and among supplemented animals adding starch to supplements also caused the same pattern. Starch and the protein ingredients used in the supplement have high content of digestible OM. Considering the absence of effects on NFDap, we can infer that effects on OM digestibility could not be caused by any alteration on forage digestibility. Thus, the high digestibility of the supplements can solely explain the increments in OM digestibility caused by supplementation. We may use the same argument to explain the overall improvement in total CP digestibility caused by supplements. Furthermore, supplementation also increased CP intake. Non-fibrous compounds have their apparent digestion positively associated with intake, since there will be a dilution of the metabolic fecal fraction (Van Soest, 1994).

Rumen dynamics of fibrous compounds obtained by rumen evacuation showed no effects of treatments. However, as we aimed to understand if the pattern of digestion would change during the supplementation cycle, we implemented an *in situ* assay. The results obtained from that assay pointed out an interaction between days of the supplementation cycle and treatments. Animals infrequently supplemented exclusively with protein presented a higher fiber degradation rate on day 1. After that, it decreased in the two following days, when supplement was not offered. This pattern is probably associated to the decreasing levels of RAN throughout the supplementation cycle. There is a positive association between adequate RAN availability and fibrolytic activity (Russell, 2002; Detmann at al., 2009). Besides this positive association, a greater protein input in diets is also accompanied by great growth of fibrolytic microorganisms and great growth of microorganisms positively related to fiber degradation (e.g, fungi, *Ruminococcus albus*; Belanche et al., 2012). However, animals infrequently supplemented with both protein and starch presented a different pattern with regard fiber degradation rate. There was a great constraint to NDF degradation rate on day 1 of the supplementation cycle followed by an improvement in the rate on the two following days, when the animals had no access to supplements. This low degradation rate on day 1 is probably related to the great amount of starch fed at this day. Negative effects of high-degradable carbohydrates on fiber digestion in animals fed tropical forage is known (Souza et al., 2010). Enhancing starch availability in the rumen favors the setting up of amensal relationships between fribrolytic and non-fibrolytic bacteria which compete for common resources. This competition may favor non-fybrolytic bacteria, which present higher growth rate and greater competition capacity for substrates compared to fibrolytic bacteria (Carvalho et al., 2011).

The higher RAN concentrations presented by supplemented animals are associated with the increment in protein intake and also with the high degradability of the supplemental CP. Among supplemented animals there was higher RAN concentrations for infrequent supplementation. Interestingly, besides infrequently supplemented animals have access to supplements only on the first day of the supplementation cycle, just on the third day the values of RAN of these animals were smaller than those of frequently supplemented animals, and even on the third day of the supplementation cycle, RAN was still a bit above control. Rufino (2015) also observed the same pattern when every three-day protein supplementation was applied to cattle fed high-quality forage. Therefore, keeping improved levels of RAN during the supplementation cycle may be a part of the physiological mechanisms to save N by infrequently supplemented animals as stated by Farmer et al. (2004).

To better understand the importance of keeping high levels of RAN, we should attempt to the results of ruminal digestibility of CP and RNB. Both response variables were increased by supplements and changed from negative in control to positive in supplemented animals. Thus, for non-supplemented animals, the flow of N leaving the rumen was greater than N intake, which is an indicative that recycling of N to rumen was playing a prominent role in supplying N to the rumen of non-supplemented animals. This great relative participation of N recycling in animals fed high quality forage is common since in these conditions negative RNB have been observed in several experiments (Costa et al., 2011b; Rufino et al., 2016; Figueiras et al., 2016; Batista et al., 2017), when animals are not supplemented.

The RAN concentration is sustained mainly by dietary CP and urea recycling from blood to rumen. The RAN are commonly inversely related to urea recycling (Reynolds and Kristensen 2008). According to the hypothesis of greater recycling participation in non-supplemented animals, average daily RAN concentration in control (4,2 mg/dL) was 108% lower than the mean value observed for supplemented animals (8,7 mg/dL). This greater participation of recycled N in the RAN pool of non-supplemented animals may decrease utilization efficiency of metabolizable protein. In brief, part of the amino acids absorbed in the intestine and amino acids from other body pools could be used to sustain urea production and recycling. Therefore, those amino acids would not be available to muscle synthesis (Detmann et al, 2014a). Taking this hypothesis into account, supplemental CP would enhance RAN, decrease the relative participation of recycled N in the rumen and enhance the efficiency of use of the digested CP. The fact that protein supplementation of cattle fed high-quality forage commonly results in great N retention, but without improvement in microbial flow to intestines, reinforces that the efficiency of utilization of post ingested protein may play an important role in N retention of supplemented cattle fed high-quality forage (Egan, 1965)

In fact, we did not detect any difference among treatments regarding microbial flow from the rumen. In spite of this, supplementation supported a 90% increase in N retention (8 g/d in control, 15 g/d in supplemented animals). However, differently from other authors who applied protein supplementation to cattle fed high-quality tropical forage (Figueiras et al., 2016; Lazarinni et al., 2016), we did not detected differences between non-supplemented and supplemented animals with regard the efficiency of use of the N ingested (P=0,09). On average, supplemented animals retained 0.15, while control retained 0.11 g N/g N intake.

In spite of no significant effects in the efficiency of N utilization in the animal body, we could state that N status in the animal metabolism (Detmann et al., 2014b) was improved and not affect by the supplementation schemes, since the positive changes in RAN and RNB were followed by increments in N retention of supplemented animals. On the other hand, the positive response of supplementation on N retention was not influenced by the supplemental starch. Therefore, as proposed by Detmann et al. (2014a), our results highlight the nitrogenous compounds as priority to supplementation programs even when applying supplementation to animals grazing high-quality tropical forages. To support our statement, it is important to note that benefits achieved with supplementation in terms of N accretion in the body were positively associated with blood IGF-1 and insulin, which are known to indicate improvements in the body anabolism. Drewnoski et al. (2014) and Rufino (2015) also observed that protein supplementation (frequent or infrequent) increased IGF-1 and insulin, which were associated, respectively, with greater BW gain and N balance.

On average, there was no difference with regards blood insulin among supplemented animals, but for infrequent supplementation it varied across the three-day supplementation cycle. As infrequently supplemented animals received a great amount of supplement on day 1, greater metabolic availability of propionate and then glucose precursors, and protein may help to explain the greater blood insulin observed on that day. Drewnoski et al. (2014) also reported that animals infrequently supplemented presented greater insulin levels on the day of supplementation. However, whereas Drewnoski et al. (2014) found that insulin and glucose presented a positive related pattern, in our experiment there was no differences for blood glucose among treatments.

Despite of the theory of enhancing N retention by reducing the relative role of recycling in sustaining ruminal environment, we emphasize that it may be important to explain performance between supplemented and non-supplemented animals. However, recycling may not be taken as something necessarily undesirable. Actually, among supplemented animals, when frequency of supplementation is decreased, recycling may be important to keep rumen working well over the days when animals are not supplemented (and also to keep an adequate N status in the animal metabolism). In our experiment, recycled N was probably responsible to keep RAN of infrequently supplemented animals always above the levels of control even in the days when animals had no access to supplements.

As fecal excretion of N was not affected by treatments (averaging 27.5 g/d), N retention was a direct function of N intake and UNE. On average, N intake was enhanced by supplementation from 66 g/d (control) to 95 g/d (supplemented), which implied in higher SUN in supplemented animals and then in a greater urinary urea and N excretion. On average, control and supplemented animals excreted respectively, 30 and 53 g N/d. Nonetheless, as discussed before, the efficiency of N utilization was maintained constant among treatments, thus the larger N intake of supplemented animals was accompanied of a larger N retention. Among supplemented animals, adding starch to the supplements reduced UNE, probably because starch also decreased RAN and SUN. However, this decrease in UNE caused by starch was not enough to enhance N retention. On average, animals supplemented exclusively with protein retained 14 g N/d, while animals supplemented with protein and starch retained 16 g N/d.

In spite of supplemented animals present the same N retention, the different supplementation schemes implied on different ways of saving N in the animal metabolism, which can be understood from the evaluation of interactions between treatments and days of the supplementation cycle for SUN and urinary excretion characteristics.

Serum urea nitrogen and urinary excretion characteristics remained constant among days for control and frequently supplemented animals. However, for infrequently supplemented animals, RAN concentrations decreased between day 1 and 2 of the supplementation cycle, while, inversely, SUN increased and achieved a peak on day 2. Rufino (2015) also observed the same pattern and stated that this untypical decoupling between RAN and SUN seem to represent a kind of mechanism to save N. In addition, we noted that this peak in SUN was not accompanied by a peak in UUNE and UNE, another untypical pattern. The peak of SUN on day 2 did result in a peak in UNFK. However, also on day 2, a greater fraction of UFNK was reabsorbed from the nephrons to blood. In other words, infrequently supplemented animals also presented a peak in urea-N salvage on day 2. As urea-N salvage peaked on day 2, the peaks of SUN and UNFK were not converted in a peak of UUNE. In summary, in infrequently supplemented animals, important mechanisms of N saving are set up on the day after supplementation (day 2), since the greater levels of blood urea and the greater load of urea filtered throughout kidneys on this day, untypically, were not converted to greater N excretion.

In our trial, feed animals with protein plus high degradable carbohydrates together did not result in benefits in regard to microbial protein flow to intestines. As animal scientists usually infer about microbial growth in the rumen using data from microbial flow, we may think that microbial growth and metabolism of ruminal microorganisms were fairly constant across treatments. However, microbial growth in the rumen and microbial flow are not necessarily related to each other. For instance, Belanche et al. (2012), evaluating diets with different fiber-to-starch ratios and protein contents, have shown that changes in diet composition may change microorganism's growth in the rumen without affect microbial flow to intestines (the flow was reported in a companion paper by Fanchone et al., 2013). Therefore, in spite of no change in the flow, the oscillations in RAN and RNB across treatments may be related to different microbial growth and metabolism in the rumen. For illustration, the addition of starch to supplements tended (P=0.063) to decrease RAN which may be, in part, a result of greater rumen microbial N uptake and growth.

As we pointed out in the introduction, and also suggested by our data, part of the supplemental N given infrequently is recycled to the rumen and can cause, therefore, a kind of "carry-over effect". On the other hand, when starch is infrequently supplemented it would be reasonable to expect that its effect will be restricted to the day when it is offered to the animals. According to this point of view, among animals infrequently supplemented, the role of starch addition in N saving seems to be restricted mainly to day 1 of the supplementation cycle. Adding starch to supplements of infrequently supplemented animals implied, on day 1, in smaller RAN and UUNE, which was supported by greater urea-N salvage on that day. On day 2 and 3, there were no differences related to starch addition and thus similar values of RAN, UUNE and urea-N salvage were observed for infrequently supplemented animals (with or without starch).

# Conclusion

Animals supplemented with protein have greater nitrogen balance than non-supplemented animals. Starch addition to protein supplements decreases urinary nitrogen excretion, but does not enhance nitrogen accretion in the animal body. In spite of no changes in nitrogen balance between supplemented animals, animals receiving different supplements seems to use distinct mechanisms regarding of nitrogen transactions along rumen, blood, and kidneys. The changes in kidneys metabolism of nitrogen seem to be an important mechanism to keep similar nitrogen retention between animals supplemented daily or every three days.

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# TABLES

**Table 1.** Chemical composition of the Tifton hay, protein supplement and starch

| Item | Tifton hay[1] | Protein supplemet[2] | Starch |
|---|---|---|---|
| Dry matter[3] | 887±1.2 | 908 | 862 |
| Organic matter[4] | 936±1.5 | 954 | 1000 |
| Crude protein[4] | 80±0.5 | 1.133 | 8 |
| Neutral detergent fiber (NDFap)[4,5] | 758±1.3 | 70 | - |
| Indigestible NDF[4] | 190±1.0 | 9 | - |

[1] Mean±standard error. [2] Feed composition (as fed): 740 g/kg of soybean meal, 234 g/kg urea and 26 g/kg ammonium sulfate. This supplement was planned to present 1000 g of crude protein/kg as fed. [3] g/kg as fed. [4] g/kg of dry matter. [5] NDFap, neutral detergent fiber assayed with a heat-stable amylase and corrected for contaminant ash and protein.

**Table 2.** Effects of supplementation, frequency of supplementation and addition of starch to protein supplements on voluntary intake in heifers fed high-quality tropical forage

| | Treatments[1] | | | | | | P value[2] | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Item | Control | P/D | PS/D | P/3D | PS/3D | SEM | C *vs.* Sup. | F | S | F × S |
| | | | Intake, kg/d | | | | | | | |
| DM | 5.23 | 5.56 | 5.67 | 5.13 | 5.37 | 0.289 | 0.194 | 0.014 | 0.199 | 0.626 |
| Forage DM | 5.23 | 5.38 | 5.15 | 4.94 | 4.84 | 0.289 | 0.305 | 0.014 | 0.215 | 0.623 |
| OM | 4.91 | 5.21 | 5.34 | 4.81 | 5.05 | 0.270 | 0.174 | 0.013 | 0.148 | 0.615 |
| CP | 0.414 | 0.627 | 0.611 | 0.591 | 0.588 | 0.0259 | <0.001 | 0.017 | 0.369 | 0.563 |
| NDFap | 3.97 | 4.09 | 3.90 | 3.76 | 3.65 | 0.223 | 0.276 | 0.010 | 0.143 | 0.653 |
| Indigestible NDF (iNDF) | 0.929 | 0.962 | 0.902 | 0.878 | 0.850 | 0.0612 | 0.321 | 0.027 | 0.133 | 0.574 |
| Digested OM (DOM) | 3.20 | 3.48 | 3.65 | 3.24 | 3.48 | 0.161 | 0.011 | 0.019 | 0.017 | 0.651 |
| Digested NDFap (DNDF) | 2.73 | 2.83 | 2.68 | 2.61 | 2.52 | 0.141 | 0.350 | 0.016 | 0.103 | 0.669 |
| | | | g CP intake : kg digested OM intake | | | | | | | |
| CP:DOM | 129 | 180 | 169 | 183 | 170 | 2.2 | <0.001 | 0.212 | <0.001 | 0.734 |
| | | | Intake, g/kg BW | | | | | | | |
| DM | 17,1 | 18,2 | 18,2 | 17,1 | 17,3 | 0.81 | 0.314 | 0.049 | 0.910 | 0.763 |
| Forage DM | 17.1 | 17.7 | 16.5 | 16.4 | 15.6 | 0.81 | 0.299 | 0.049 | 0.052 | 0.761 |
| OM | 16.1 | 17.1 | 17.1 | 16.0 | 16.2 | 0.75 | 0.290 | 0.048 | 0.798 | 0.757 |
| NDFap | 13.0 | 13.4 | 12.5 | 12.5 | 11.7 | 0.62 | 0.275 | 0.036 | 0.032 | 0.781 |
| iNDF | 3.0 | 3.2 | 2.9 | 2.9 | 2.7 | 0.18 | 0.313 | 0.072 | 0.039 | 0.685 |

[1] Control, only hay (without supplementation); P/D, daily protein supplementation (200 g of CP); PS/D, daily protein plus starch supplementation (200 g of CP plus 400 g of starch), P/3D, infrequent protein supplementation (600 g of CP every 3 days); PS/3D, infrequent protein plus starch supplementation (600 g of CP plus 1800 g of starch every 3 days).

[2] C *vs.* Sup., control versus supplemented; F, effect of the frequency of supplementation (frequent/daily *vs* infrequent/every 3 days); S, effect of starch (presence *vs* absence); F × S, effect of interaction between F and S.

**Table 3.** Effects of supplementation, frequency of supplementation and addition of starch to protein supplements on digestibility coefficients in heifers fed high-quality tropical forage

| | Treatments[1] | | | | | | P value[2] | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Item | Control | P/D | PS/D | P/3D | PS/3D | SEM | C *vs*. Sup. | F | S | F × S |
| Total digestibility, g/g | | | | | | | | | | |
| OM | 0.653 | 0.668 | 0.684 | 0.669 | 0.687 | 0.0077 | 0.008 | 0.749 | 0.026 | 0.861 |
| CP | 0.574 | 0.712 | 0.702 | 0.720 | 0.710 | 0.0108 | <.001 | 0.481 | 0.362 | 0.978 |
| NDFap | 0.687 | 0.691 | 0.684 | 0.690 | 0.687 | 0.0084 | 0.934 | 0.854 | 0.468 | 0.760 |
| Digested OM content in the diet, g/kg DM | | | | | | | | | | |
| Dietetic DOM | 613 | 626 | 643 | 627 | 647 | 7.4 | 0.008 | 0.679 | 0.015 | 0.859 |
| Ruminal digestibility[3], g/g | | | | | | | | | | |
| OM | 0.482 | 0.500 | 0.505 | 0.477 | 0.517 | 0.0141 | 0.141 | 0.577 | 0.041 | 0.111 |
| CP | -0.142 | 0.192 | 0.103 | 0.186 | 0.180 | 0.0260 | <.001 | 0.127 | 0.052 | 0.082 |
| NDFap | 0.627 | 0.624 | 0.618 | 0.618 | 0.632 | 0.0095 | 0.670 | 0.678 | 0.678 | 0.259 |
| Intestinal digestibility[3], g/g | | | | | | | | | | |
| OM | 0.331 | 0.335 | 0.360 | 0.364 | 0.350 | 0.0198 | 0.168 | 0.478 | 0.684 | 0.151 |
| CP | 0.627 | 0.643 | 0.667 | 0.655 | 0.645 | 0.0112 | 0.024 | 0.588 | 0.464 | 0.074 |
| NDFap | 0.162 | 0.175 | 0.173 | 0.185 | 0.150 | 0.0232 | 0.654 | 0.703 | 0.299 | 0.352 |

[1] Control, only hay (without supplementation); P/D, daily protein supplementation (200 g of CP); PS/D, daily protein plus starch supplementation (200 g of CP plus 400 g of starch), P/3D, infrequent protein supplementation (600 g of CP every 3 days); PS/3D, infrequent protein plus starch supplementation (600 g of CP plus 1800 g of starch every 3 days).

[2] C *vs.* Sup., control versus supplemented; F, effect of the frequency of supplementation (frequent/daily *vs* infrequent/every 3 days); S, effect of starch (presence *vs* absence); F × S, effect of interaction between F and S.

[3] As a fraction of the total arriving in the compartment of digestion.

**Table 4.** Effects of supplementation, frequency of supplementation and addition of starch to protein supplements on rumen pool and rates of intake, passage, and degradation of fibrous compounds accessed by rumen evacuation in heifers fed high-quality tropical forage

| | Treatments[1] | | | | | | P value[2] | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Item | Control | P/D | PS/D | P/3D | PS/3D | SEM | C *vs*. Sup. | F | S | F × S |
| | | | Rumen pool, g/kg BW | | | | | | | |
| NDF | 11.51 | 11.15 | 10.96 | 10.98 | 10.76 | 0.641 | 0.344 | 0.708 | 0.691 | 0.976 |
| Potential digestible NDF | 4.53 | 4.46 | 4.15 | 4.17 | 4.05 | 0.344 | 0.256 | 0.432 | 0.397 | 0.708 |
| Indigestible NDF | 6.99 | 6.69 | 6.81 | 6.81 | 6.71 | 0.434 | 0.500 | 0.978 | 0.973 | 0.720 |
| | | | Rates, /h | | | | | | | |
| | | | NDF | | | | | | | |
| Intake | 0.0477 | 0.0505 | 0.0472 | 0.0473 | 0.0454 | 0.00330 | 0.980 | 0.377 | 0.369 | 0.803 |
| Passage | 0.0178 | 0.0191 | 0.0181 | 0.0181 | 0.0167 | 0.00142 | 0.900 | 0.332 | 0.356 | 0.869 |
| | | | Potential digestible NDF | | | | | | | |
| Intake | 0.0924 | 0.0969 | 0.0980 | 0.0973 | 0.0937 | 0.00816 | 0.590 | 0.779 | 0.850 | 0.724 |
| Degradation | 0.0787 | 0.0837 | 0.0822 | 0.0825 | 0.0791 | 0.00730 | 0.630 | 0.715 | 0.680 | 0.865 |
| Passage | 0.0137 | 0.0132 | 0.0157 | 0.0148 | 0.0146 | 0.00131 | 0.497 | 0.835 | 0.349 | 0.268 |
| | | | Indigestible NDF | | | | | | | |
| Passage | 0.0186 | 0.0199 | 0.0173 | 0.0178 | 0.0169 | 0.00158 | 0.606 | 0.288 | 0.141 | 0.475 |

[1] Control, only hay (without supplementation); P/D, daily protein supplementation (200 g of CP); PS/D, daily protein plus starch supplementation (200 g of CP plus 400 g of starch), P/3D, infrequent protein supplementation (600 g of CP every 3 days); PS/3D, infrequent protein plus starch supplementation (600 g of CP plus 1800 g of starch every 3 days).

[2] C *vs*. Sup., control versus supplemented; F, effect of the frequency of supplementation (frequent/daily *vs* infrequent/every 3 days); S, effect of starch (presence *vs* absence); F × S, effect of interaction between F and S.

**Table 5.** Effects of supplementation, frequency of supplementation and addition of starch to protein supplements on ruminal ammonia N (RAN), rumen pH, volatile fat acid concentration in the rumen, and degradation rate of forage NDF in heifers fed-high quality tropical forage

| | Treatments[1] | | | | | | P value[2] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Item | Control | P/D | PS/D | P/3D | PS/3D | SEM | C *vs.* S | F | S | F × E | T × D |
| RAN, mg/dL | 4.20 | 8.85 | 6.60 | 10.43 | 9.11 | 1.038 | <0.001 | 0.037 | 0.063 | 0.606 | 0.003 |
| pH | 6.87 | 6.83 | 6.76 | 6.89 | 6.81 | 0.118 | 0.726 | 0.649 | 0.557 | 0.966 | 0.823 |
| VFA, mmol/dL | | | | | | | | | | | |
| Total[3] | 12.00 | 12.53 | 13.45 | 12.58 | 13.23 | 1.065 | 0.216 | 0.899 | 0.254 | 0.829 | 0.647 |
| VFA, mmol/mmol | | | | | | | | | | | |
| Acetate (A) | 0.807 | 0.813 | 0.803 | 0.803 | 0.789 | 0.0093 | 0.409 | 0.023 | 0.016 | 0.707 | 0.522 |
| Propionate (P) | 0.119 | 0.113 | 0.122 | 0.127 | 0.131 | 0.0078 | 0.323 | 0.014 | 0.156 | 0,530 | 0.607 |
| Butyrate | 0.073 | 0.073 | 0.073 | 0.071 | 0.077 | 0.0043 | 0.999 | 0.683 | 0.266 | 0.283 | 0.937 |
| A:P | 7.07 | 7.81 | 6.78 | 6.41 | 6.17 | 0.606 | 0.528 | 0.014 | 0.116 | 0.319 | 0.599 |
| *In situ* degradation rate of pdNDF, /h | | | | | | | | | | | |
| | 0.0271 | 0.0261 | 0.0252 | 0.0274 | 0.0275 | 0.00191 | 0.762 | 0.260 | 0.787 | 0.319 | 0.013 |

[1] Control, only hay (without supplementation); P/D, daily protein supplementation (200 g of CP); PS/D, daily protein plus starch supplementation (200 g of CP plus 400 g of starch), P/3D, infrequent protein supplementation (600 g of CP every 3 days); PS/3D, infrequent protein plus starch supplementation (600 g of CP plus 1800 g of starch every 3 days).

[2] C *vs.* Sup., control versus supplemented; F, effect of the frequency of supplementation (frequent/daily *vs* infrequent/every 3 days); S, effect of starch (presence *vs* absence); F × S, effect of interaction between F and S; T × D, effect of interaction between treatments and day of the supplementation cycle.

**Table 6.** Effects of supplementation, frequency of supplementation and addition of starch to protein supplements on nitrogen intake and fecal excretion, nitrogen retention in animal body, ruminal balance of nitrogen, and microbial nitrogen production in the rumen in heifers fed high-quality tropical forage

| | Treatments[1] | | | | | | P value[2] | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Item | Control | P/D | PS/D | P/3D | PS/3D | SEM | C *vs.* Sup. | F | S | F × S |
| N intake, g/d | | | | | | | | | | |
| Hay | 66.2 | 67.6 | 63.4 | 60.6 | 60.0 | 4.43 | 0.112 | 0.010 | 0.188 | 0.316 |
| Supplement | 0,0 | 33,0 | 33,4 | 33,0 | 33,2 | - | - | - | - | - |
| Total | 66.2 | 100.6 | 96.8 | 93.6 | 93.2 | 4.48 | <.001 | 0.010 | 0.256 | 0.353 |
| N excretion, g/d | | | | | | | | | | |
| Fecal | 28.0 | 29.0 | 29.2 | 26.4 | 27.2 | 1.84 | 0.968 | 0.061 | 0.661 | 0.792 |
| N retention | | | | | | | | | | |
| g/d | 8.0 | 14.2 | 16.1 | 14.6 | 16.6 | 3.86 | 0.015 | 0.869 | 0.433 | 0.973 |
| g/g of N intake | 0.108 | 0.138 | 0.158 | 0.152 | 0.171 | 0.0404 | 0.090 | 0.571 | 0.418 | 0.989 |
| g/ g of N digested | 0.182 | 0.192 | 0.228 | 0.210 | 0.240 | 0.0609 | 0.345 | 0.661 | 0.343 | 0.930 |
| Ruminal N balance | | | | | | | | | | |
| g/d | -8.9 | 19.1 | 10.0 | 17.2 | 17.0 | 2.21 | <.001 | 0.161 | 0.020 | 0.024 |
| Microbial N nitrogen production | | | | | | | | | | |
| g/d | 65.6 | 67.7 | 74.6 | 66.4 | 65.1 | 5.74 | 0.512 | 0.188 | 0.489 | 0.302 |
| g/g N intake | 0.98 | 0.67 | 0.76 | 0.71 | 0.70 | 0.041 | <.001 | 0.712 | 0.346 | 0.207 |
| g microbial CP/g DOM | 127 | 122 | 129 | 132 | 118 | 7.2 | 0.814 | 0.988 | 0.661 | 0.162 |

[1] Control, only hay (without supplementation); P/D, daily protein supplementation (200 g of CP); PS/D, daily protein plus starch supplementation (200 g of CP plus 400 g of starch), P/3D, infrequent protein supplementation (600 g of CP every 3 days); PS/3D, infrequent protein plus starch supplementation (600 g of CP plus 1800 g of starch every 3 days).

[2] C *vs.* Sup., control versus supplemented; F, effect of the frequency of supplementation (frequent/daily *vs* infrequent/every 3 days); S, effect of starch (presence *vs* absence); F × S, effect of interaction between F and S.

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**Table 7.** Effects of supplementation, frequency of supplementation and addition of starch to protein supplements on serum urea nitrogen, urinary excretion characteristics, and kidneys function in heifers fed high-quality tropical forage

| | Treatments[1] | | | | | | P value[2] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Item | Control | P/D | PS/D | P/3D | PS/3D | SEM | C *vs.* Sup. | F | S | F × S | T × D |
| | Serum urea N | | | | | | | | | | |
| mg/dL | 7.9 | 14.3 | 11.5 | 12.4 | 12.0 | 0.99 | <.0001 | 0.271 | 0.026 | 0.0792 | <0.001 |
| | Urinary excretion g/d | | | | | | | | | | |
| Urinary nitrogen excretion (UNE) | 30.3 | 57.2 | 51.6 | 52.6 | 50.3 | 2.52 | <0.001 | 0.104 | 0.036 | 0.355 | <0.001 |
| Urinary urea nitrogen excretion (UUNE) | 14.5 | 29.7 | 28.7 | 29.4 | 27.4 | 1.70 | <0.001 | 0.609 | 0.353 | 0.765 | <0.001 |
| UUNE:UNE | 0.48 | 0.52 | 0.56 | 0.55 | 0.54 | 0.027 | 0.009 | 0.860 | 0.661 | 0.215 | 0.069 |
| | Kidneys function | | | | | | | | | | |
| Urea nitrogen filtered through kidneys, g/d | 32.3 | 65.5 | 56.6 | 57.9 | 56.1 | 3.92 | <0.001 | 0.206 | 0.106 | 0.273 | <0.001 |
| Nitrogen salvage, g/g | 0.54 | 0.54 | 0.50 | 0.48 | 0.50 | 0.032 | 0.036 | 0.042 | 0.620 | 0.049 | <0.001 |

[1] Control, only hay (without supplementation); P/D, daily protein supplementation (200 g of CP); PS/D, daily protein plus starch supplementation (200 g of CP plus 400 g of starch), P/3D, infrequent protein supplementation (600 g of CP every 3 days); PS/3D, infrequent protein plus starch supplementation (600 g of CP plus 1800 g of starch every 3 days).

[2] C *vs.* Sup., control versus supplemented; F, effect of the frequency of supplementation (frequent/daily *vs* infrequent/every 3 days); S, effect of starch (presence *vs* absence); F × S, effect of interaction between F and S; T × D, effect of interaction between treatments and day of the supplementation cycle.

**Table 8.** Effects of supplementation, frequency of supplementation and addition of starch to protein supplements on blood characteristics and liver function in heifers fed high-quality tropical forage

| | Treatments[1] | | | | | | P value[2] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Item | Control | P/D | PS/D | P/3D | PS/3D | SEM | C *vs.* S | F | S | F × S | T × D |
| Blood characteristics | | | | | | | | | | | |
| Glucose, mg/dL | 68.7 | 68.6 | 68.5 | 69.4 | 71.96 | 2.54 | 0.525 | 0.086 | 0.307 | 0.290 | 0.420 |
| IGF-1, ng/mL | 222 | 264 | 231 | 274 | 273 | 32.0 | 0.019 | 0.063 | 0.204 | 0.251 | 0.150 |
| Amino acids., nmol/mL | 1433 | 1387 | 1461 | 1438 | 1467 | 66.7 | 0.930 | 0.556 | 0.296 | 0.652 | 0.521 |
| Total protein, g/dL | 7.30 | 7.18 | 7.40 | 7.26 | 7.30 | 0.191 | 0.903 | 0.931 | 0.343 | 0.534 | 0.661 |
| Albumin, g/dL | 2.76 | 2.78 | 2.73 | 2.74 | 2.80 | 0.184 | 0.965 | 0.812 | 0.867 | 0.172 | 0.350 |
| Globulin, g/dL | 4.54 | 4.40 | 4.67 | 4.52 | 4.51 | 0.301 | 0.927 | 0.899 | 0.450 | 0.402 | 0.811 |
| Insulin, µIU/mL | 1.97 | 2.40 | 2.84 | 2.84 | 2.79 | 0.368 | 0.003 | 0.326 | 0.333 | 0.218 | 0.002 |
| Liver function | | | | | | | | | | | |
| AST, U/L | 56.9 | 57.0 | 58.9 | 53.3 | 58.2 | 5.53 | 0.985 | 0.476 | 0.285 | 0.633 | 0.631 |
| ALT, U/L | 15.7 | 17.2 | 17.0 | 14.7 | 16.7 | 2.25 | 0.446 | 0.078 | 0.237 | 0.146 | 0.062 |
| GGT, U/L | 16.1 | 15.2 | 14.4 | 15.3 | 15.9 | 1.91 | 0.214 | 0.233 | 0.913 | 0.293 | 0.311 |

[1] Control, only hay (without supplementation); P/D, daily protein supplementation (200 g of CP); PS/D, daily protein plus starch supplementation (200 g of CP plus 400 g of starch), P/3D, infrequent protein supplementation (600 g of CP every 3 days); PS/3D, infrequent protein plus starch supplementation (600 g of CP plus 1800 g of starch every 3 days).

[2] C *vs.* Sup., control versus supplemented; F, effect of the frequency of supplementation (frequent/daily *vs* infrequent/every 3 days); S, effect of starch (presence *vs* absence); F × S, effect of interaction between F and S; T × D, effect of interaction between treatments and day of the supplementation cycle.

# FIGURES

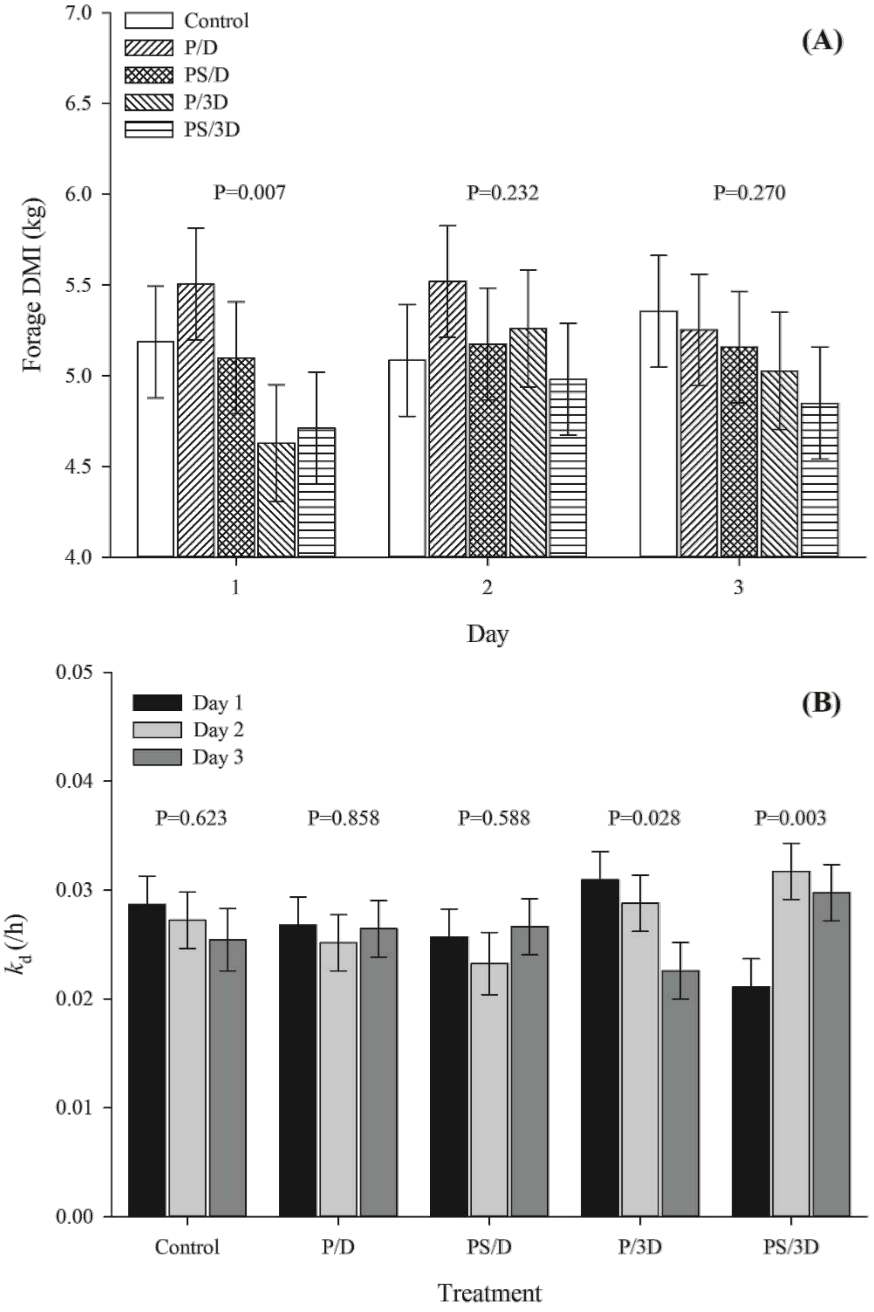

**Figure 1.** Evaluation of the interaction effect between treatments and days of the supplementation cycle on forage DM intake (A) and *in situ* degradation rate of forage NDF (kd, B). Control, only forage; P/D, daily protein supplementation; PS/D, daily protein plus starch supplementation, P/3D, infrequent protein supplementation; PS/3D, infrequent protein plus starch supplementation. Day1, day in which both frequently and infrequently supplemented animals had access to supplements; Day 2 and Day3, days in which only frequently supplemented animals had access to supplements.

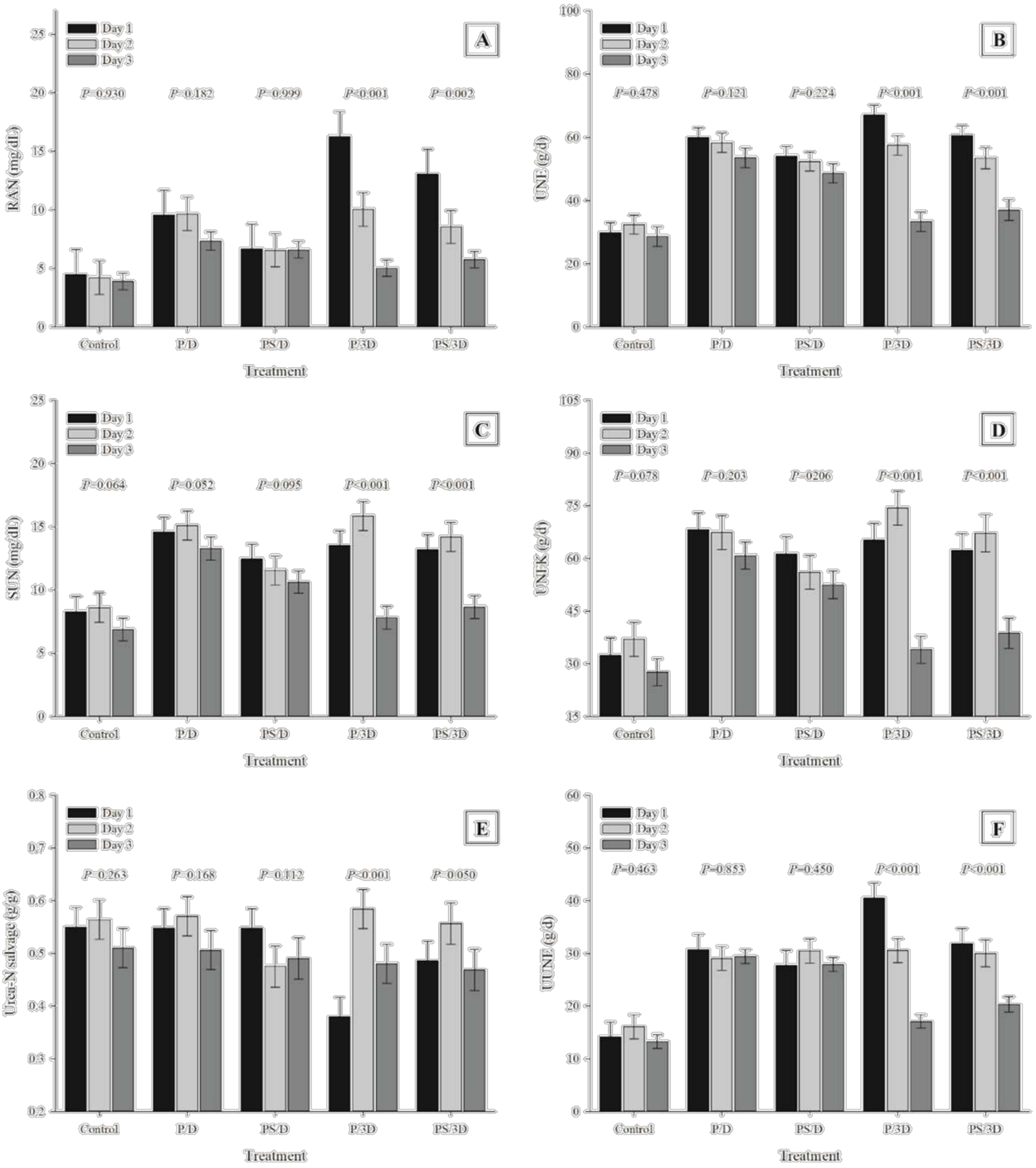

**Figure 2.** Evaluation of the interaction effect between treatments and days of the supplementation cycle on rumen ammonia nitrogen (RAN, A), urinary nitrogen excretion (UNE, B), serum urea nitrogen (SUN, C), urea nitrogen filtered through kidneys (UNFK, D), urea nitrogen salvage (E) and urinary urea nitrogen excretion (UUNE, F). Control, only forage; P/D, daily protein supplementation; PS/D, daily protein plus starch supplementation; P/3D, infrequent protein supplementation; PS/3D, infrequent protein plus starch supplementation. Day1, day in which both frequently and infrequently supplemented animals had access to supplements; Day 2 and Day3, days in which only frequently supplemented animals had access to supplements.

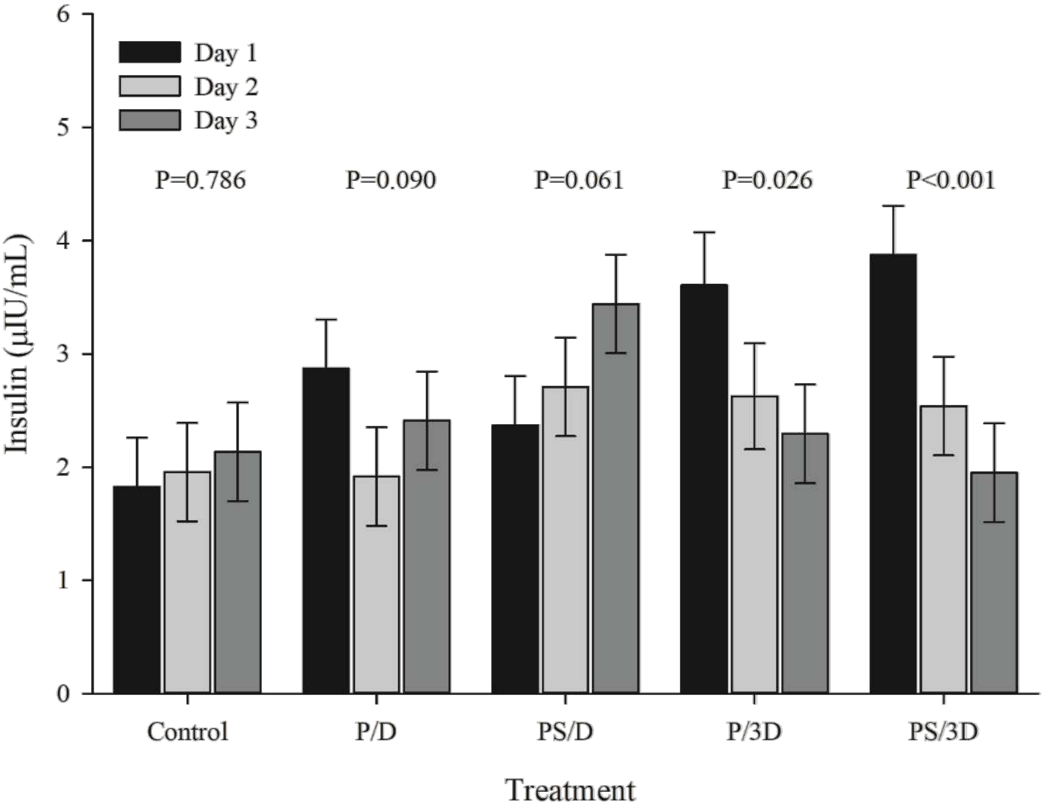

**Figure 3.** Evaluation of the interaction effect between treatments and days of the supplementation cycle on serum insulin. Control, only forage; P/D, daily protein supplementation; PS/D, daily protein plus starch supplementation; P/3D, infrequent protein supplementation; PS/3D, infrequent protein plus starch supplementation. Day1, day in which both frequently and infrequently supplemented animals had access to supplements; Day 2 and Day3, days in which only frequently supplemented animals had access to supplements.