

ALEX LOPES DA SILVA

**PREDICTION OF STARTER FEED INTAKE OF PREWEANED DAIRY
CALVES AND EFFECTS OF RUMEN UNDEGRADABLE PROTEIN ON
PERFORMANCE AND DIGESTIVE CHARACTERISTICS OF DAIRY
HOLSTEIN HEIFERS**

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

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APROVED: February 23, 2017.

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DEDICATION

For my parents, who have always done the possible and sometimes tried to do the impossible in favor of my dreams, at times leaving aside their own dreams.

For Fernanda, for all love and affection, for stay on my side in every single moment.

I love you!!!

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BIOGRAPHY

ALEX LOPES DA SILVA, son of Jairo Lopes da Silva and Maria Aparecida da Silva, was born in Barbacena, Minas Gerais - Brazil on March 08, 1986.

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On February of 2017, he submitted his thesis to the thesis committee to obtain the Doctor Science degree in Animal Science.

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ABSTRACT

SILVA, Alex Lopes da, D.Sc., Universidade Federal de Viçosa, February, 2017. **Prediction of starter feed intake of preweaned dairy calves and effects of rumen undegradable protein on performance and digestive characteristics of dairy holstein heifers.** Adviser: Marcos Inácio Marcondes. Co-advisers: Edenio Detmann and Luciana Navajas Rennó.

This work was developed from three studies. Therefore, the objective of the first study was to identify variables that influence starter feed intake (SFI), and to develop equations to predict SFI in milk-fed dairy calves up to 64 days of age. The database was composed of individual data of 189 calves from 8 experiments, totaling 6,426 daily observations of intake. The information collected from the studies were: birth body weight (birth BW; kg), SFI (kg/day), fluid milk or milk replacer intake (MI; L/day), sex (male or female), breed (Holstein or Holstein×Gyr crossbred), and age (days). A correlation between SFI and the quantitative variables MI, birth BW, metabolic birth BW (birth BW^{0.75}), and age was established. Subsequently, data were graphed and based on a visual appraisal of the pattern of the data; an exponential function was chosen. The data were evaluated using a meta-analysis approach to estimate fixed and random effects of the experiments using nonlinear mixed coefficients statistical models. Cross-validation was used to estimate the accuracy and precision of the developed equations using the mean square error of prediction (MSEP), concordance correlation coefficient, which was decomposed into accuracy (C_b) and precision (ρ) parameters, and the coefficient of determination (r²) as a proxy for precision. In addition, a bootstrap analysis was used to estimate the bias associated with each parameter of the developed equation structure. A negative correlation between SFI and MI was observed (r = -0.388), but age had a positive correlation with SFI (r=0.66). No effect of liquid feed source (milk or milk replacer) was observed in developing the equation. However, 2 equations, significantly different for all parameters, were fit to predict SFI for calves that consume less than 5 (SFI_{<5}) or more than 5 (SFI_{>5}) L/day of milk or milk replacer: $SFI_{<5} = 0.1839_{\pm 0.0581} \times MI \times \exp^{((-0.0040_{\pm 0.0011} \times MI + 0.0333_{\pm 0.0021}) \times (A - (6.0332_{\pm 0.3583} \times MI + 0.8302_{\pm 0.5092})) - (0.12 \times MI))}$; $CC_{>5} = 0.1225_{\pm 0.0005} \times MI \times \exp^{((-0.0015_{\pm 0.0001} \times MI + 0.0217_{\pm 0.0006}) \times (A - (1.9508_{\pm 0.1710} \times MI + 3.5382_{\pm 1.3140})) - (0.12 \times MI))}$; where SFI_{<5} and SFI_{>5} = starter feed intake prediction for calves that consume less than 5 and more than 5 L/day of milk or milk replacer, respectively (kg/d); MI = milk or milk replacer intake (L/d) and A = age (days). These equations had high accuracy (C_b of 0.97 and 0.95, respectively) and the random errors of MSEP were 99.8 and 99.9% for SFI_{<5} and SFI_{>5} equations, respectively. Small biases were observed with the bootstrap analyses

for all estimated parameters. The equations' precision was moderate, with r^2 values of 0.61 and 0.52 and ρ values of 0.78 and 0.72 for $SFI_{<5}$ and $SFI_{>5}$, respectively. In conclusion, the use of milk or milk replacer as liquid feed did not affect SFI, or development of SFI over time, which increased exponentially with calf age. Starter feed intake was negatively affected by MI and, and for this reason, different equations are necessary to predict calf SFI according to MI. The second study aimed to evaluate the influence of different levels of rumen undegradable protein (RUP) on intake, performance, carcass characteristics, N balance, mammary gland development and hormonal status of Holstein heifers at different physiological stage (PS). Sixteen prepubertal (PRE) heifers with 106 ± 7.6 kg of initial body weight and aged 4 ± 0.46 months old, as well as, 16 pubertal (PUB) heifers with 224 ± 7.9 kg of initial body weight and aged 12 ± 0.45 months old were used. The experiment was carried out during 84 days following a complete randomized design, in a 4×2 factorial arrangement, with four levels of RUP in dietary protein (38, 44, 51, and 57%), and animals at two PS (prepubertal or pubertal). Between days 36 and 40 and between days 78 and 82 the animals were subjected to digestibility trials with collections of feeds, orts and spot collections of feces and urine. At days 0 (immediately before the beginning of the experiment) and 83 body ultrasound images were taken at the rib area. At days 0, 21, 42, 63 and 84 images of the mammary gland were taken via ultrasound. Finally, at days 0 and 84 blood samples were taken to estimate serum concentration of progesterone, estrogen, IGF-I and insulin. The PS affected the apparent digestibility of DM, and PRE heifers presented lower values. The PRE heifers also presented a preferential consumption for neutral detergent fiber corrected for ash and protein (NDFap) and sorting activity against CP greater than PUB heifers. The average daily gain (ADG) and N retention were affected by PS, and PRE heifers presented an ADG of 505 g/d, while PUB heifers presented an average value of 905 g/d. In addition, these variables were affected by RUP levels, where greater values were obtained for treatments with 51% of RUP. The analysis of pixels in mammary gland images pointed effects of PS, and PRE heifers presented greater pixels values. The initial and final measurements of serum progesterone demonstrated that PRE and PUB heifers kept their PS during the experiment. The IGF-I concentration was only affected by PS, and PUB heifers presented greater values. In addition, insulin was not affected by PS, but presented a tendency to be greater at treatments with 51% of RUP. In conclusion, the dietary level of 51% of RUP is responsible to increase the performance of PRE and PUB Holstein heifers. In addition, PRE heifers have lower sorting ability, what affected, negatively, their digestibility and performance. Finally, these animals are more likely to

have greater fat proportion in the mammary gland, even under moderate rates of growth. The third study aimed to evaluate the influence of increasing levels of RUP on intake, total and partial digestibilities, rumen kinetics and characteristics, as well the N use of dairy Holstein heifers. Eight rumen-cannulated Holstein heifers, with an average initial body weight of 276 ± 8.3 were used in a double 4×4 Latin Square design with four levels of RUP in the total dietary protein, as follows: 38% of RUP (38RUP); 44% of RUP (44RUP); 51% of RUP (51RUP) and 57% of RUP (57RUP). The experiment was carried out during 84 days subdivided into 4 experimental periods of 21 d (14 d for adaptation + 7 d for collections). Eight spot collections of feces, urine, ruminal content and omasal digesta were performed with 9 hours interval between each collection, as follows: on 15th d samples were collected at 0600 h and 1500 h; on 16th day samples were collected at 0000 h, 0900 h and 1800 h; on 17th d samples were collected at 0300 h, 1200 h and 2100 h. On 19th d a complete rumen evacuation was performed, 4 hours after the morning feeding and on 21st d immediately before morning feeding. Daily intake of DM, organic matter (OM), CP, NDFap and potential digestible NDFap (pdNDFap) were not affected by RUP levels. In addition, the relative intake (g/kg of body weight) of DM, CP and NDFap, as well as the ruminal outflow of DM, OM, CP, NDFap and pdNDFap were not affected by RUP levels. Total digestibility of DM, OM, CP, NDFap and pdNDFap, as well as ruminal and intestinal digestibilities of DM, OM, NDFap and pdNDFap were not affected by RUP levels. However, ruminal and intestinal digestibilities of CP presented a tendency to decrease according to RUP supply increase. Effects of RUP levels were not observed on the ingestion, passage and digestion rates, as well on the total volatile fatty acid concentration. Treatment 38RUP presented greater concentration of rumen ammonia nitrogen in relation to the other treatments. The urinary N excretion presented a tendency to decrease according to RUP supply increase. In addition, retained N presented a tendency to increase according to RUP levels and greater values were observed for treatments 51RUP and 57RUP. Microbial crude protein (CP_{mic}) synthesis, microbial efficiency and the efficiency of use of N for microbial synthesis, decrease as the supplied RUP increases. Flow of RUP increased, while the amounts of rumen degradable protein decrease according to RUP supply increase. In addition, the flow of metabolizable protein presented a tendency to increase and greater values were observed for treatments 51RUP and 57RUP. In conclusion, the intestinal digestibility of CP is negatively affected by RUP levels, what is due to the reduction in the CP_{mic} flow, which, probably, has greater intestinal digestibility than protein of feedstuffs. The urinary N excretion decrease according to RUP supply increase, what is due to the decrease in ruminal N losses as

ammonia and the increase in N recycling. The reduction in the urinary N excretion allied to increases in the flow of metabolizable protein, which are promoted by increases in the flow of RUP to the small intestine, are determinant to promote increases in the retained N, which occurred for treatments 51RUP and 57RUP.

RESUMO

SILVA, Alex Lopes da, D.Sc., Universidade Federal de Viçosa, fevereiro de 2017. **Predição do consumo de concentrado de bezerros leiteiros em aleitamento e efeitos da proteína não-degradável no rúmen sobre o desempenho e características digestivas de novilhas leiteiras holandesas.** Orientador: Marcos Inácio Marcondes. Coorientadores: Edenio Detmann e Luciana Navajas Rennó.

Este trabalho foi desenvolvido a partir de três estudos. Assim, o objetivo do primeiro estudo foi identificar variáveis que afetam o consumo de concentrado (CC), e desenvolver equações para prever o CC de bezerros leiteiros até os 64 dias de vida. O banco de dados foi composto pelos dados individuais de 189 bezerros de 8 experimentos, totalizando 6.426 observações diárias de consumo. A informação coletada dos estudos foram: peso corporal ao nascimento (PCi; kg), CC (kg/dia), consumo de leite ou sucedâneo (CL; L/dia), sexo (macho ou fêmea), raça (Holandês ou mestiço Holandês×Gir) e idade (dias). A correlação entre o CC e as variáveis quantitativas CL, PCi, PCi metabólico (PCi^{0,75}) e idade foi estabelecida. Posteriormente, foi realizada uma avaliação gráfica do comportamento padrão dos dados; e um modelo exponencial foi escolhido. Os dados foram avaliados usando a técnica de meta-análise para estimar os efeitos fixos e os efeitos aleatórios associados aos experimentos através de modelos mistos não-lineares. A técnica de validação cruzada foi utilizada para estimar a acurácia e precisão das equações desenvolvidas, com base no quadrado médio do erro de predição (QMEP), coeficiente de correlação de concordância, que foi decomposto em acurácia (C_b) e precisão (ρ). De forma adicional, uma análise tipo “bootstrap” foi utilizada para estimar o viés associado a cada parâmetro das equações desenvolvidas. Uma correlação negativa entre CC e CL foi observada (r= -0,388), no entanto, idade apresentou uma correlação positiva com CC (r= 0,66). Não foi observado efeito do tipo de alimento líquido utilizado (leite ou sucedâneo) no desenvolvimento da equação. No entanto, foram ajustadas 2 equações, significativamente diferente para todos os parâmetros, para prever o CC para bezerros consumindo menos que 5 (CC_{<5}) ou mais que 5 (CC_{>5}) litros/dia de leite ou sucedâneo: $CC_{<5} = 0,1839_{\pm 0,0581} \times CL \times \exp^{((-0,0040_{\pm 0,0011} \times CL + 0,0333_{\pm 0,0021}) \times (I - (6,0332_{\pm 0,3583} \times CL + 0,8302_{\pm 0,5092})))} - (0,12 \times CL)$; $CC_{>5} = 0,1225_{\pm 0,0005} \times CL \times \exp^{((-0,0015_{\pm 0,0001} \times CL + 0,0217_{\pm 0,0006}) \times (I - (1,9508_{\pm 0,1710} \times CL + 3,5382_{\pm 1,3140})))} - (0,12 \times CL)$; onde CC_{<5} e CC_{>5} = consumo predito de concentrado para bezerros que consomem menos de 5 ou mais de 5 litros/dia de leite ou sucedâneo, respectivamente (kg/dia); CL = consumo de leite ou sucedâneo (L/dia) e I = idade (dias). Estas equações apresentaram alta acurácia (C_b de 0,97 e 0,95 para CC_{<5} e CC_{>5}, respectivamente) e o erro aleatório do QMEP foi de 99,8 e 99,9% para CC_{<5} e CC_{>5},

respectivamente. A análise “bootstrap” indicou um baixo viés para todos os parâmetros estimados em ambas as equações. A precisão das equações foi moderada, com valores de r^2 de 0,61 e 0,52 e valores de ρ de 0,78 e 0,72 para $CC_{<5}$ e $CC_{>5}$, respectivamente. Conclui-se que a utilização de leite ou sucedâneo como alimento líquido não afeta o CC, ou o desenvolvimento do CC ao longo do tempo, o qual aumenta exponencialmente de acordo com a idade do bezerro. O CC foi negativamente afetado pelo CL e, por esta razão, diferentes equações são necessárias para estimar o CC de acordo com o CL. O segundo estudo objetivou avaliar a influência de diferentes níveis de proteína não-degradável no rúmen (PNDR) sobre o consumo, desempenho, características de carcaça, balaço de N, desenvolvimento da glândula mamária e o status hormonal de novilhas Holandesas em diferentes estágios fisiológicos (EF). Foram utilizadas 16 novilhas pré-púberes (PRE), com peso corporal inicial de $106 \pm 7,6$ kg e $4 \pm 0,46$ meses de idade, bem como 16 novilhas púberes (PUB), com peso corporal inicial de $224 \pm 7,9$ kg e $12 \pm 0,45$ meses de idade. O experimento teve uma duração de 84 dias e foi conduzido segundo um delineamento inteiramente casualizado, segundo um esquema fatorial 4×2 , com 4 níveis de PNDR na proteína total da dieta ((38, 44, 51 e 57%) e animais em duas idades diferentes (pré-púberes e púberes). Entre os dias 36 e 40 e os dias 78 e 82 os animais foram submetidos à ensaios de digestibilidade com coleta de alimentos, sobras e coletas “spot” de fezes e urina. No dia 0 (imediatamente antes do início do experimento) e dia 83 foram tomadas imagens de ultrassom na área lombar dos animais. Nos dias 0, 21, 42, 63 e 84 foram tomadas imagens de ultrassom da glândula mamaria. E, por fim, nos dias 0 e 84 foram tomadas amostras de sangue para estimar as concentrações séricas de progesterona, estrógeno, IGF-I e insulina. O EF afetou a digestibilidade aparente da MS, e novilhas PRE apresentaram menores valores. Novilhas PRE também apresentaram consumo preferencial por fibra em detergente neutro corrigida para cinzas e proteína (FDNcp) e atividade de seleção contra a proteína bruta (PB) maior que novilhas PUB. O ganho médio diário (GMD) e a retenção de N foram afetados pelo EF, e novilhas PRE apresentaram um GMD de 505 g/dia, enquanto novilhas PUB apresentaram um valor médio de 905 g/dia. Adicionalmente, estas variáveis foram afetadas pelo nível de PNDR, e maiores valores foram obtidos para o tratamento com 51% de PNDR. A análise de pixels na glândula mamária apontou efeito do EF, e novilhas PRE apresentaram maiores valores de pixel. As mensurações iniciais de progesterona sérica demonstraram que as novilhas PRE e PUB mantiveram seu EF durante o experimento. A concentração de IGF-I foi afetada somente pelo EF, e novilhas PUB apresentaram maiores valores. A concentração de insulina não foi afetada pelo EF, mas apresentou uma tendência de ser maior para os

tratamentos com 51% de PNDR. Conclui-se que o nível dietético de 51% de PNDR é responsável por aumentar o desempenho de novilhas PRE e PUB. Adicionalmente, novilhas PRE tem menor habilidade de seleção, o que afeta, negativamente, a sua digestibilidade e desempenho. Finalmente, estes animais são mais propensos a ter uma maior proporção de gordura na glândula mamária, mesmo sob moderadas taxas de crescimento. O terceiro estudo foi conduzido com o objetivo de avaliar de níveis crescentes de PNDR sobre o consumo, as digestibilidades parciais e total, a cinética e as características ruminais, bem como a utilização do N de novilhas leiteiras holandesas. Foram utilizadas 8 novilhas holandesas fistuladas no rúmen, com peso corporal inicial de $276 \pm 8,3$, em delineamento em quadrado latino 4×4 duplo, com quatro níveis de PNDR na proteína dietética, como segue: 38% de PNDR (38PNDR); 44% de PNDR (44PNDR); 51% de PNDR (51PNDR) e 57% de PNDR (57PNDR). O experimento teve duração de 84 dias, subdivididos em 4 períodos experimentais de 21 dias (14 dias de adaptação + 7 dias de coletas). Foram realizadas 8 coletas “spot” de fezes, urina, conteúdo ruminal e digesta omasal, com intervalo de 8 horas entre cada coleta, como segue: no 15º dia as amostras foram coletadas as 0600h e 1500h; no 16º dia as amostras foram coletadas as 0000h, 0900h e 1800h; e no 17º dia as amostras foram coletadas as 0300h, 1200h e 2100h. No 19º dia foi realizado esvaziamento completo do rúmen 4 horas após a alimentação matutina e no 21º dia o esvaziamento foi realizado imediatamente antes da alimentação matutina. O consumo diário de MS, matéria orgânica (MO), PB, FDNcp e FDNcp potencialmente digestível (pdFDNcp) não foi afetado pelos níveis de PNDR. Adicionalmente, o consumo relativo (g/kg de peso corporal) de MS, PB e FDNcp, assim como o fluxo ruminal de MS, MO, PB, FDNcp e pdFDNcp não foram afetados pelos níveis de PNDR. A digestibilidade total da MS, MO, PB, FDNcp e pdFDNcp, bem como as digestibilidades ruminal e intestinal da MS, MO, FDNcp e pdFDNcp não foram afetados pelos níveis de PNDR. No entanto, as digestibilidades ruminal e intestinal da PB apresentaram uma tendência de decréscimo de acordo com o aumento nos níveis de PNDR. Não foram observados efeitos dos níveis de PNDR sobre as taxas de ingestão, passagem e digestão, assim como sobre a concentração de ácidos graxos voláteis. O tratamento 38PNDR apresentou maior concentração de nitrogênio amoniacal ruminal em relação aos outros tratamentos. A excreção de nitrogênio urinário apresentou tendência de queda de acordo com o aumento nos níveis de PNDR. Adicionalmente, o nitrogênio retido apresentou tendência de aumento de acordo com o aumento nos níveis de PNDR e foi maior para os tratamentos 51PNDR e 57PNDR. A síntese de proteína bruta microbiana, a eficiência de síntese de proteína microbiana (PBmic) e a eficiência de uso

do nitrogênio para síntese de proteína microbiana diminuíram de acordo com o aumento nos níveis de PNDR na dieta. O fluxo de PNDR aumentou, enquanto a quantidade de proteína degradável no rúmen decresceu de acordo com o aumento nos níveis de PNDR. Adicionalmente, o fluxo de proteína metabolizável apresentou tendência de aumento de acordo com o aumento nos níveis de PNDR e maiores valores foram observados para os tratamentos 51PNDR e 57PNDR. Conclui-se que a digestibilidade intestinal da PB é negativamente afetada pelos níveis de PNDR, o que é proporcionado pela redução no fluxo de PBmic, sendo que esta, provavelmente, digestibilidade intestinal da proteína maior que os alimentos. A excreção urinária de N diminui à medida que a PNDR dietética aumenta, o que é proporcionado pela queda nas perdas ruminais de nitrogênio na forma de amônia e pelo aumento na reciclagem de nitrogênio para o rúmen. A redução na excreção urinária de nitrogênio, aliada ao aumento no fluxo de proteína metabolizável, o qual foi promovida pelo aumento no fluxo de PNDR ao intestino delgado, são determinantes para promover aumentos na retenção de nitrogênio, o que ocorreu nos tratamentos 51PNDR e 57PNDR.

GENERAL INTRODUCTION

Systems of ruminant production, especially of dairy cattle production, are very complex and composed by animals of different categories. In a general form, these categories can be divided into productive animals that are represented by lactating cows and non-productive animals, which are represented, mainly, by calves and heifers. Despite calves and heifers are responsible for increases in production costs, these animals represent the maintenance and renovation of herds and practices to improve the performance and growth of these animals are necessary, aiming to reduce their impact on the farm costs.

Regarding calves rearing, starter feed intake (SFI) has a great importance to increase the performance, once that is responsible to increase the availability of nutrients for maintenance and production (Silva et al., 2015). Thus, an accurate prediction of SFI is necessary to establish a correct relationship between intake and nutrients requirements aiming to develop balanced diets, avoiding under- or over-feeding (Roseler et al., 1997; NRC, 2001).

Mathematical models (Tedeschi and Fox, 2009; Tedeschi et al., 2013) have been developed to predict dry matter intake for animals of all categories, such as lactating cows and growing heifers (Hayirli et al., 2003; Souza et al., 2014; Krizsan et al., 2014). However, no models have been developed to estimate dry matter intake, or more properly, SFI of preweaned dairy calves. Therefore, to have an accurate prediction of SFI is important to assist with the management of dairy farms, to improve the determination of weaning age, and to control the composition of the starter feed that would allow meeting calves' requirements for energy and nutrients.

Concerning the rearing of heifers, one of the big challenges is to adequate nutritional strategies to target high growth rates that allow reducing age at first calving

and, consequently, reduce the impact of heifers on farm costs (Piantoni et al., 2012; Albino et al., 2015; Geiger et al., 2016). In this respect, one of the strategies that can be used is to increase the metabolizable protein (MP) availability, which can be understood as an amino acids mixture that is absorbed in the small gut, and that can be used for muscle and mammary gland development, among other functions (Cervieri et al., 2001; Boye et al., 2012).

Although the main source of amino acids in the MP is the microbial crude protein (CP_{mic}) because of his good amino acids profile, which has great equilibrium with milk and muscle proteins (Santos et al., 1998; Ma et al., 2010; Mikolayunas et al., 2011). Once that the microbial N requirements have been met through rumen degradable protein, supplying of rumen undegradable protein (RUP) may increase the flow of MP and, consequently, improve animal performance and N retention (Tomlinson et al., 1997; Mezzomo et al., 2011). Besides the increase on the MP flow, RUP supply also may cause changes in the ruminal metabolism, with alterations on CP_{mic} synthesis, N availability, fiber degradation and, even on N recycling (Reynal et al., 2005; Batista et al., 2016).

However, studies of RUP utilization on dairy heifers' diets are still scarce and conflicting on their results. Studies using fish or blood meals, presented improves on performance and N retention (Bethard et al., 1997; Zanton et al., 2007), while studies that used corn gluten meal, reports absence of effects (Ribeiro et al., 2005; Oliveira et al., 2008). This conflict of results is, probably, due to the amino acids profile of the RUP sources, once that corn gluten meal is generally poor in the lysine content (Santos et al., 2011). However, to the best of our knowledge there are no studies using by-pass soybean meal as RUP source on heifers' diets, which could provide beneficial results because of its good amino acids profile (Santos et al., 1998). In addition, there is a lack of studies that report the influence of protein degradability on dairy heifers diets (Lascano et al., 2016) and, mainly, the effect of RUP supplementation on the digestive parameters of

Holstein dairy heifers feeding diets with high proportions of forage, that is typically used for this category (Zanton et al., 2007; Lascano and Heinrichs, 2009).

Therefore, given the exposed above about the specific necessities of information about calves and heifers, this study was carried out aiming to:

- 1) Identify variables that influence SFI, and to develop predictive equations for SFI of preweaned dairy calves up to 64 days of age;
- 2) Evaluate the influence of increasing levels of RUP, from soybean meal, on intake, performance, N balance, carcass characteristics, mammary gland development and hormonal status, as well total and partial digestibilities, rumen kinetics and characteristics of dairy Holstein heifers.

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PAPER 1

*Under review in the Plos One

Development of Equations, Based on Milk Intake, to Predict Starter Feed Intake of Preweaned Dairy Calves

Predicting Starter Feed Intake of Dairy Calves

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Abstract

A multi-study analysis was conducted to identify variables that influence starter feed intake (SFI), and to develop equations to predict SFI in milk-fed dairy calves up to 64 days of age. The database was composed of individual data of 189 calves from 8 experiments, totaling 6,426 daily observations of intake. The information collected from the studies were: birth body weight (birth BW; kg), SFI (kg/day), fluid milk or milk replacer intake (MI; L/day), sex (male or female), breed (Holstein or Holstein×Gyr crossbred), and age (days). A correlation between SFI and the quantitative variables MI, birth BW, metabolic birth BW (birth BW^{0.75}), and age was established. Subsequently, data were graphed and based on a visual appraisal of the pattern of the data; an exponential function was chosen. The data were evaluated using a meta-analysis approach to estimate fixed and random effects of the experiments using nonlinear mixed coefficients statistical models. Cross-validation was used to estimate the accuracy and precision of the developed equations using the mean square error of prediction (MSEP), concordance correlation coefficient, which was decomposed into accuracy (C_b) and precision (ρ) parameters, and the coefficient of determination (r²) as a proxy for precision. In addition, a bootstrap analysis was used to estimate the bias associated with each parameter of the developed equation structure. A negative correlation between SFI and MI was observed (r = -0.388), but age had a positive correlation with SFI (r = 0.66). No effect of liquid feed source (milk or milk replacer) was observed in developing the equation. However, 2 equations, significantly different for all parameters, were fit to predict SFI for calves that consume less than 5 (SFI_{<5}) or more than 5 (SFI_{>5}) L/day of milk or milk replacer: SFI_{<5} = 0.1839_{±0.0581} × MI × exp^{((-0.0040_{±0.0011} × MI + 0.0333_{±0.0021}) × (A - (6.0332_{±0.3583} × MI + 0.8302_{±0.5092}))) - (0.12 × MI); CC_{>5} = 0.1225_{±0.0005} × MI × exp^{((-0.0015_{±0.0001} × MI + 0.0217_{±0.0006}) × (A - (1.9508_{±0.1710} × MI + 3.5382_{±1.3140}))) - (0.12 × MI); where SFI_{<5} and SFI_{>5} = starter feed intake prediction for calves that consume less than 5 and more than 5 L/day of milk or milk replacer,}}

respectively; MI = milk or milk replacer intake (L/d) and A = age (days). These equations had high accuracy (C_b of 0.97 and 0.95, respectively) and the random errors of MSEP were 99.8 and 99.9% for $SFI_{<5}$ and $SFI_{>5}$ equations, respectively. Small biases were observed with the bootstrap analyses for all estimated parameters. The equations' precision was moderate, with r^2 values of 0.61 and 0.52 and ρ values of 0.78 and 0.72 for $SFI_{<5}$ and $SFI_{>5}$, respectively. In conclusion, the use of milk or milk replacer as liquid feed did not affect SFI, or development of SFI over time, which increased exponentially with calf age. Starter feed intake was negatively affected by MI and, for this reason, different equations are necessary to predict calf SFI according to MI.

Introduction

Feed intake is the most important variable influencing animal performance because it determines the amount of nutrients ingested and their availability for maintenance and production [1]. Thus, an accurate prediction of feed intake is necessary to develop balanced diets, avoiding under- or over-feeding, and to improve nutrients use and animal performance [1,2].

Mathematical models [3,4] and empirical equations [1,5] have been developed to predict DMI for animals of all categories, such as lactating cows and growing heifers, and a series of management and environmental conditions have also been evaluated [6–8]. However, to our knowledge, no models or equations have been developed to estimate DMI, or more properly, starter feed intake (SFI) of preweaned dairy calves. This is due to the great variability presented by SFI, which causes constraints to develop models or equations.

Solid feed intake is important for promoting ruminal development and adaptation to future solid diets, and it is also used as a criterion for weaning [9]. It also influences body composition and, consequently, nutrient requirements [10,11]. Thus, an accurate

prediction of SFI is important to assist with the management of dairy farms, to improve the determination of weaning age, and to control the composition of the starter feed that would allow meeting calves' requirements for energy and nutrients. Thus, the objective of this study was to identify variables that influence SFI, and to develop predictive equations for SFI of preweaned dairy calves up to 64 days of age.

Material and Methods

Our study was composed of compiling data from studies previously carried out. Thus, no animal care and use protocol was needed; however, all individual studies followed local guidelines for animal care and use.

Developmental Database

Data used to develop predictive equations for SFI, were obtained from individual preweaned dairy calves ($n = 188$) from 8 experiments, totaling 6,426 daily observations of intake. Among these experiments, five studies were carried out at the Universidade Federal de Viçosa (Viçosa, Minas Gerais, Brazil) and three at the University of Guelph, Kemptville Campus (Kemptville, Ontario, Canada) (Table 1 and 2). Information that was collected from the studies included information on birth BW (kg), SFI (kg/day), milk or milk replacer intake (**MI**; L/d), sex (male or female), breed (Holstein or Holstein×Gyr crossbred), and age (days) (Tables 1 - 3).

The milk replacer was mixed at ratio greater than the DM content of raw milk (Table 2). Therefore, a correction of the amounts of milk replacer consumed was performed to values equivalent to the average DM concentration of milk, that was 120 g kg^{-1} of DM (e.g. 6 L of milk replacer mixed at 150 g/L is equivalent to 8 L with 120 g/L or 12% of DM).

Table 1. Summary of the database used to develop and evaluate models to predict starter feed intake of preweaned dairy calves.

Table 2. Summary of liquid and solid feed composition and the range of liquid and starter feed intake of the database used to develop and evaluate models to predict starter feed intake of preweaned dairy calves.

Table 3. Descriptive statistics of the data used (n=6,426) to develop and evaluate models to predict starter feed intake of preweaned dairy calves.

Statistical Analysis

Pearson's coefficients of correlation were determined for the association between SFI and relevant quantitative variables (e.g., MI, birth BW, metabolic birth BW (birth BW^{0.75}), and age) to determine which variables had more influence on SFI. Subsequently, a preliminary graphical appraisal was carried out to identify the pattern of the data; and as a result, an exponential function was chosen, which is in agreement with previous studies [18,19].

Because the data were comprised of observations from different studies, it was necessary to quantify the variance associated with the studies. Therefore, each experiment was considered as a random sample for a large population [20] and the inclusion of experimental effects in the exponential model required the estimation of fixed effects, as well as random effects associated with the experiments, as described by Vyas and Erdman [21]. The PROC NLMIXED of SAS [22] was used to fit the following exponential function:

$$Y_{ij} = \beta_0 \times MI \times \exp^{((\beta_1 \times MI + \beta_2) \times (A - (\beta_3 \times MI + \beta_4)))} - (0.12 \times MI),$$

where Y_{ij} = SFI (kg/d) for the i individual observations (ranging from 1 to 6,426) and j experiments (ranging from 1 to 8); MI = milk or milk replacer intake (L/d); A = age (d); $0.12 \times MI$ = adjust factor.

The effects of the liquid feed source (milk or milk replacer) as well as MI were tested using the “ESTIMATE” statement in PROC NLMIXED, and parameter coefficients were considered different when $P < 0.05$. The effects of sex and breed were not tested because of the low number of female and crossbred calves. Observations with studentized residuals greater than $|2.5|$ were considered “outliers” and excluded from the database. For all statistical procedures, a significance level of 5% was adopted.

Sensitivity Analysis

To evaluate the adequacy of the chosen variables, a cross-validation technique [23] was performed with 2,000 simulations using the nonlinear least squares function of R [24] and the packages “boot” and “mass”. For each simulation, the original database was randomly divided into two new subsets with, approximately, the same size. The first subset (training subset) was used to obtain the equations and the second subset (testing subset) was used to test the equations to obtain the adequacy statistics. After each simulation, the dataset was reorganized, and all processes were repeated 2,000 times and the average of the adequacy statistics were computed.

The cross-validation results were used to estimate the accuracy and precision of developed empirical equation through the mean square error of prediction (MSEP), concordance correlation coefficient (CCC), and the coefficient of determination (r^2). The MSEP was decomposed in 3 main sources of variation: (1) mean bias, that represents a central tendency of deviation; (2) systematic bias, that represents the deviation of the slope from 1; and (3) random error, that represents the variation around the linear regression and that is not explained by the regression [25]. The CCC was used to assess, simultaneously, model accuracy and precision and was decomposed into correlation coefficient estimate (ρ) that estimates model precision and bias correction factor (C_b) that indicates the accuracy. Values of CCC, ρ , and C_b ranging from 0 to 1, where values close to 1 indicate precise and/or accurate models [25,26].

In addition, a bootstrap analysis was carried out to evaluate the equations adequacy by estimating the bias associated with each parameter estimate. The bootstrap analysis consisted of building a pseudo-database by replicating and resampling the original database n times and summarizing the outcome [27]. We performed 2,000 simulations to estimate the bias based on independent observations. The bootstrap analysis was also carried out using the software R [24] and the package “boot” [28].

Results and Discussion

No significant correlation between SFI and birth BW or birth $BW^{0.75}$ was observed (Table 4). However, a negative correlation between SFI and MI was observed, which suggested that lower SFI occurs when greater amounts of milk are offered to the calves [15,29]. In contrast, age was highly and positively correlated with SFI (Table 4), which is mainly related to gastrointestinal tract development, which increases SFI capacity with age and size [10]. Independent of MI, SFI during the first weeks of life was low due to the low intake capacity of the calves. After the third week of life, the development of the gastrointestinal tract allows for greater SFI [10]. The negative relationship between SFI and MI (Table 4) suggested that lesser SFI was observed when greater amounts of milk were offered, even after the first weeks of life (Hill et al., 2010).

Table 4. Pearson’s coefficient of correlation of dependent variables and starter feed intake of preweaned dairy calves.

Besides MI and age, we expected the impact of growth (ADG) in the empirical equation to predict SFI, as ADG is linked to feed intake. Other empirical equations indicate that ADG is associated with DMI of growing heifers and finishing rams [31,32]. However, MI and ADG were highly correlated ($r=0.961$; Fig 1), which allowed the use of only MI in the SFI predictive equation. In addition, it is well reported that composition

of liquid feed, mainly the percentage of fat, has a negative effect on SFI, which is due, basically, to changes on energy intake [33,34]. The same pattern was observed with the database of this study, where fat and crude protein intake presented a negative correlation with SFI (Table 4). However, these variables presented high correlation with MI, with values of 0.84 and 0.97 for fat and crude protein intake, respectively. Therefore, fat and crude protein intake were driven, basically, by the amount of liquid feed ingested, and as these variables are highly correlated, only MI intake was kept in the model.

Fig 1. Pearson’s correlation between average daily gain and daily fluid milk intake for preweaned dairy calves (n = 174).

Effects of liquid feed source (milk or milk replacer) were not observed ($P>0.05$) for any of the equation parameters (Fig 2). It was not possible to fit a single equation to predict SFI for all scenarios, mainly due to the difference in SFI patterns over time for calves that consumed high or low amounts of milk (Fig 3).

Fig 2. Average daily starter feed intake by experiments for preweaned dairy calves that received milk (n=208) or milk replacer (n=155).

Fig 3. Average daily starter feed intake by experiments for preweaned dairy calves that consumed less (n=208) or more (n=201) than 5 L/d of milk or milk replacer.

Therefore, two equations were fitted to predict SFI for calves that receive less than 5 or more than 5 L/d of milk, as follows (Estimate Parameter \pm SEM):

$$SFI_{<5} = 0.1839_{\pm 0.0581} \times MI \times \exp^{((-0.0040_{\pm 0.0011} \times MI + 0.0333_{\pm 0.0021}) \times (A - (6.0332_{\pm 0.3583} \times MI + 0.8302_{\pm 0.5092})))} - (0.12 \times MI);$$

$$SFI_{>5} = 0.1225_{\pm 0.0005} \times MI \times \exp^{((-0.0015_{\pm 0.0001} \times MI + 0.0217_{\pm 0.0006}) \times (A - (1.9508_{\pm 0.1710} \times MI + 3.5382_{\pm 1.3140})))} - (0.12 \times MI);$$

where $SFI_{<5}$ and $SFI_{>5}$ = starter feed intake prediction for calves that receive less than 5 and more than 5 L/d of milk or milk replacer, respectively; MI = milk or milk replacer intake (L/d) and A = age (d).

The cross-validation indicated a very high accuracy for both equations through the C_b values of 0.97 and 0.95 for $SFI_{<5}$ and $SFI_{>5}$ equations, respectively (Table 5). In addition, the partition of MSEP indicated a low error of prediction directly associated with the fixed variables (Table 5) because the majority of the error of prediction was associated with random errors (99.8 and 99.9% for $SFI_{<5}$ and $SFI_{>5}$ equations, respectively).

Table 5. Adequacy measures of the predict starter feed intake equations estimated by the cross-validation technique.

In agreement with the cross-validation results, small biases were observed with the bootstrap analyses (Table 6), indicating that variables were consistent and sufficient to predict SFI, for both equations [35]. The equations presented a moderate precision with R^2 values of 0.61 and 0.52, and ρ values of 0.78 and 0.72, for $SFI_{<5}$ and $SFI_{>5}$, respectively, that yielded moderate to high CCC values (Table 5). The high accuracy demonstrated by the equations represents their adequacy, since that accuracy is the most important measure of goodness of fit, as it represents the model's ability to predict true values [25].

Table 6. Estimated parameters of the predict starter feed intake equations and their respective biases estimated by the bootstrap analysis.

Conclusions

In conclusion, the use of milk or milk replacer as liquid feed did not differentially affect SFI in dairy calves up to 64 d of age. Neither did the type of milk influence the pattern of SFI over time, which exponentially increased with calf age. Starter feed intake was negatively associated with MI; for this reason, different equations were fit to predict calf SFI consuming low (less than 5 L/d) or high (more than 5 L/d) MI. Overall, the proposed equations were suitable to predict SFI for milk-fed dairy calves, presenting moderate precision and very high accuracy.

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Table 1. Summary of the database used to develop and evaluate models to predict starter feed intake of preweaned dairy calves.

Author/Year	n ¹	PE ²	Liquid feed type	Sex	Breed
Silva et al. (2015)	18	15 to 64	Milk	Male	Crossbred
Rodrigues et al. (2016)	23	4 to 55	Milk	Male	Holstein
Marcondes et al. (2016)	20	6 to 60	Milk	Female	Holstein
Unpublished data	17	4 to 60	Milk	Male	Holstein
Jolomba (2015)	32	5 to 53	Milk replacer	Male	Crossbred
Miller-Cushon et al. (2013a)	20	8 to 42	Milk replacer	Male	Holstein
Miller-Cushon et al. (2013b)	10	8 to 56	Milk replacer	Male	Holstein
Overvest et al. (2016)	48	15 to 38	Milk replacer	Male	Holstein

¹number of calves

²period, in days of life, under evaluation (Not all calves had the intake evaluated during all period)

Table 2. Summary of liquid and solid feed composition and the range of liquid and starter feed intake of the database used to develop and evaluate models to predict starter feed intake of preweaned dairy calves.

Author/Year	Composition, g/kg							RM, g/L	Intake			
	Liquid Feed			Starter Feed					Liquid, L/d		Starter, kg/d	
	DM	CP	EE	DM	CP	EE	NDF		Min	Max	Min	Max
Silva et al. (2015)	114	256	285	899	191	25	131	-	2	8	0	1.33
Rodrigues et al. (2016)	124	239	257	862	193	15	133	-	2	8	0	1.12
Marcondes et al. (2016)	120	263	282	884	190	23	135	-	2.8	4.9	0	1.07
Unpublished data	123	258	276	893	194	19	129	-	3.1	4.1	0	1.44
Jolomba (2015)	960	200	160	890	200	30	150	160;180;200	8	12	0	1.47
Miller-Cushon et al. (2013a)	950	220	180	957	192	39	173	150	6.2	14.2	0	1.02
Miller-Cushon et al. (2013b)	950	220	180	904	211	38	151	150	3.7	10	0	1.28
Overvest et al. (2016)	950	260	160	903	209	38	147	150	7.3	14.1	0	0.43

DM=dry matter (as fed basis); CP=crude protein (dry matter basis); EE=ether extract (dry matter basis); NDF=neutral fiber detergent (dry matter basis);RM=rate of mixing; Min=minimum; Max=maximum.

Table 3. Descriptive statistics of the data used (n=6,426) to develop and evaluate models to predict starter feed intake of preweaned dairy calves.

Variables	BW _b ¹ (kg)	Age (days)	SFI ² (kg/day)	MI ³ (L/day)
Minimum	22.5	1	0.00	2.00
Maximum	51.5	64	1.778	14.25
Mean	36.3	30.5	0.236	7.07
Median	35.0	30	0.128	6.00
Mode	35.0	23	0.020	6.00
SEM ⁴	0.075	0.188	0.003	0.052

¹Birth body weight; ²Starter feed intake; ³Milk or milk replacer intake; ⁴Standard error of mean.

Table 4. Pearson's coefficient of correlation of dependent variables and starter feed intake of preweaned dairy calves.

Variables	Starter Feed Intake (kg/d)	
	Coefficient of correlation	P
Milk intake (L/d)	- 0.388	<0.001
Initial body weight (kg)	0.014	0.301
Initial metabolic body weight (kg)	0.015	0.238
Age (days)	0.660	<0.001
Fat intake (g/d) ¹	-0.337	<0.001
Crude protein intake (g/d) ¹	-0.364	<0.001

¹Intake computed just from milk/milk replacer.

Table 5. Adequacy measures of the predict starter feed intake equations estimated by the cross-validation technique.

Item ¹	SFI _{<5} ²	SFI _{>5} ²
MSEP, kg×kg	0.027	0.020
Partition of MSEP, %		
Mean Bias	0.08	0.03
Systematic bias	0.12	0.07
Random Error	99.8	99.9
CCC, ranging from 0 to 1	0.76	0.68
ρ	0.78	0.72
C _b	0.97	0.95
Coefficient of determination	0.61	0.52

¹MSEP=mean square error of prediction; CCC=concordance correlation coefficient; ρ=correlation coefficient estimate; C_b=bias correction factor; ²SFI_{<5} and SFI_{>5}=starter feed intake prediction for calves that receive less than 5 and more than 5 L/d of milk or milk replacer, respectively.

Table 6. Estimated parameters of the predict starter feed intake equations and their respective biases estimated by the bootstrap analysis.

Parameter ¹	SFI _{<5} ²		SFI _{>5} ²	
	Estimate _{±SEM}	Bias	Estimate _{±SEM}	Bias
β ₀	0.1839 _{±0.0581}	0.001378	0.1255 _{±0.0005}	0.000015
β ₁	-0.0040 _{±0.0011}	0.000005	-0.0015 _{±0.0001}	-0.000003
β ₂	0.0333 _{±0.0021}	-0.000039	0.0217 _{±0.0006}	0.000026
β ₃	6.0332 _{±0.3583}	0.023892	1.9508 _{±0.1710}	0.004645
β ₄	0.8302 _{±0.5092}	0.011961	-3.5382 _{±1.3140}	-0.016449

¹β₀; β₁; β₂; β₃ and β₄=estimated parameters of the predictive equations; ²SFI_{<5} and SFI_{>5} = starter feed intake prediction for calves that receive less than 5 and more than 5 L/d of milk or milk replacer, respectively.

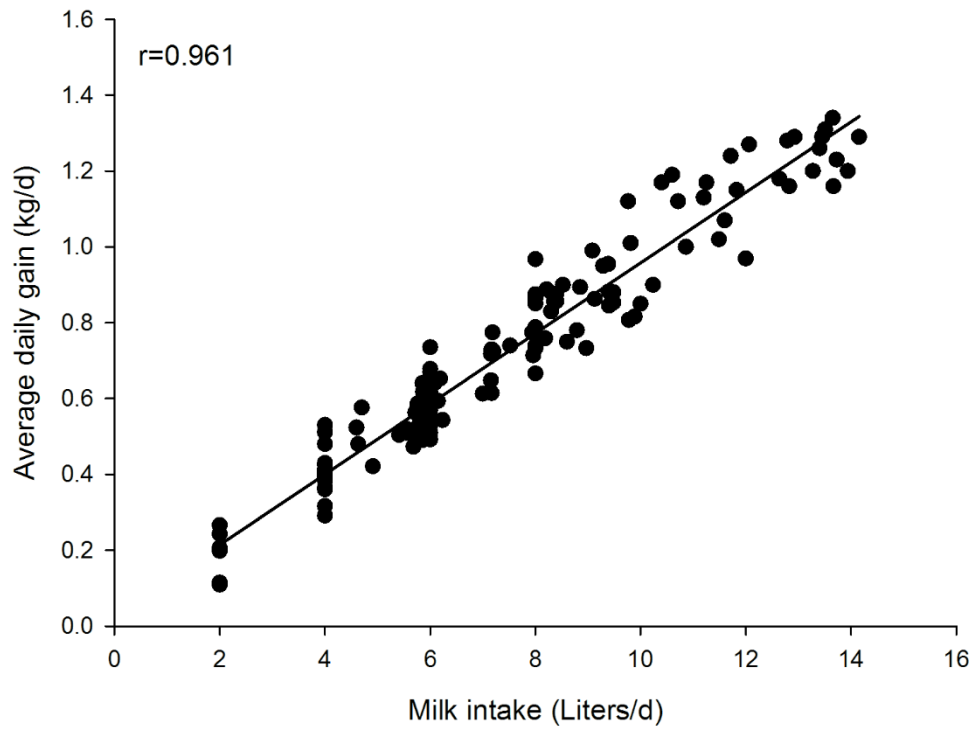


Fig 1. Pearson's correlation between average daily gain and daily fluid milk intake for preweaned dairy calves (n = 174).

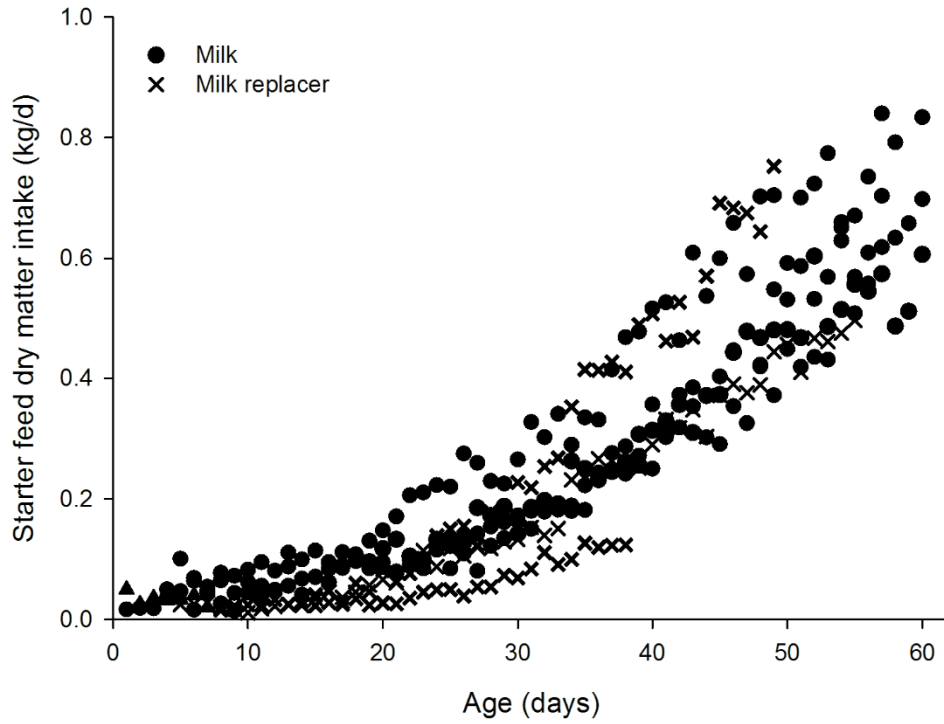


Fig 2. Average daily starter feed intake by experiments for preweaned dairy calves that received milk (n=208) or milk replacer (n=155).

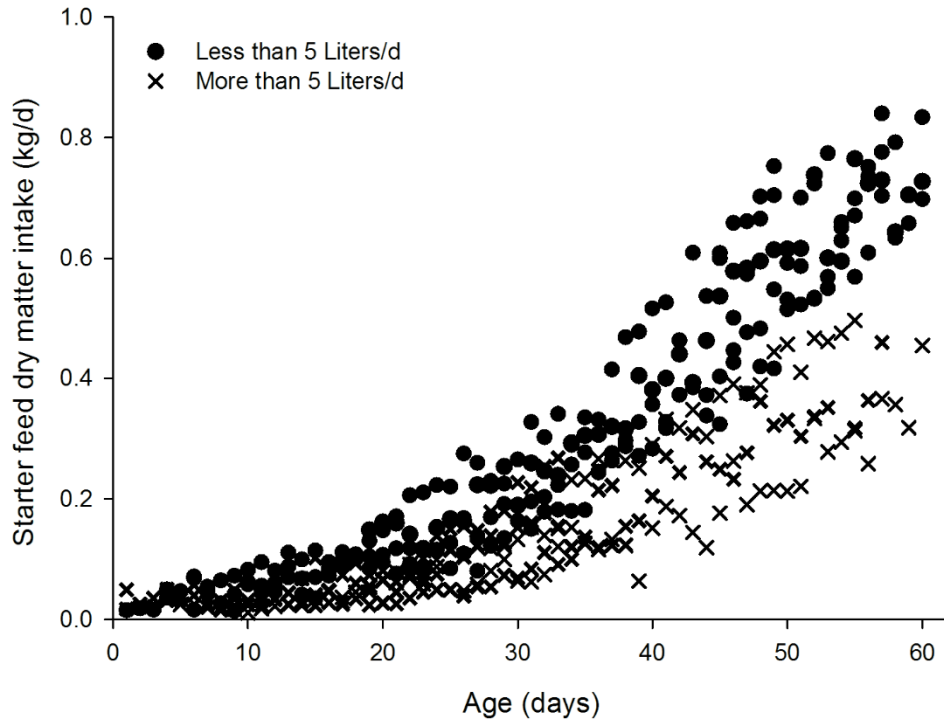


Fig 3. Average daily starter feed intake by experiments for preweaned dairy calves that consumed less (n=208) or more (n=201) than 5 L/d of milk or milk replacer.

PAPER 2

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Interpretive summary: Effects of rumen undegradable protein on intake, performance and mammary gland development in prepubertal and pubertal dairy heifers. By Silva et al. Protein evaluation experiments with simultaneous use of prepubertal and pubertal heifers are scarce. Therefore, this study aimed to examine the utilization of rumen undegradable protein (38, 44, 51 and 57% of dietary CP) for heifers before or after puberty. A level of 51% of rumen undegradable protein improved weight gain, feed efficiency, and nitrogen retention of both prepubertal and pubertal heifers. The proportion of fat in the mammary gland was greater for prepubertal heifers, even at moderate growth rates.

RUMEN UNDEGRADABLE PROTEIN FOR DAIRY HEIFERS

Effects of rumen undegradable protein on intake, performance and mammary gland development in prepubertal and pubertal dairy heifers

Abstract: The objective was to evaluate the influence of different levels of rumen undegradable protein (**RUP**) on intake, performance, carcass characteristics, N balance, mammary gland development and hormonal status of Holstein heifers at different physiological stage (**PS**). Sixteen prepubertal (**PRE**) heifers aged 4.3 ± 0.46 months (mean \pm SEM) with an initial body weight of 106 ± 7.6 kg, and 16 pubertal (**PUB**) heifers aged 12.6 ± 0.45 months with an initial body weight of 224 ± 7.9 kg were used. The experiment was carried out during 84 days following a completely randomized design, in a 4×2 factorial arrangement, with four levels of RUP (38, 44, 51, and 57% of dietary protein), and heifers at two PS (prepubertal or pubertal). Between days 36 and 40 and between days 78 and 82, animals were subjected to digestibility trials with collections of feeds, orts and spot collections of feces and urine. At days 0 (immediately before the beginning of the experiment) and 83, body ultrasound images were taken at the rib and rump areas. At days 0, 21, 42, 63 and 84, images of the mammary gland were taken via ultrasound. Finally, at days 0 and 84, blood samples were taken to estimate serum concentration of progesterone, estrogen, IGF-1 and insulin. No interaction between PS and level of RUP occurred for any trait. Digestibility of DM, OM and NDFap was lower for PRE compared with PUB heifers. Sorting against NDFap and for CP was more pronounced for PUB compared with PRE heifers. Daily gain and N retention were smaller for PRE heifers (505 and 12.5 g/d, respectively) than for PUB heifers (905 and 25.9 g/d, respectively). In addition, daily gain and N retention was greater with 51% RUP compared with 38, 44 and 57% RUP in dietary protein. Mammary ultrasonography indicated no effects on mammary gland composition of rumen undegradable protein level, whereas PRE heifers had greater pixels values than PUB heifers, indicating relatively higher amounts of fat as opposed to protein in mammary gland of PRE compared with PUB heifers. The plasma IGF-1 concentration was only affected by PS, and PRE heifers had greater values IGF-1 concentrations than PUB heifers. Plasma insulin was not

affected by PS, but tended to be greater at treatments with 51% of RUP. In conclusion, the dietary level of 51% of RUP increases body weight, daily gain, feed efficiency, and N retention independent of physiological stage. In addition, PRE heifers have lower sorting ability, associated with a reduced intake, digestibility and retention. Finally, PRE heifers have greater fat content in the mammary gland, even at moderate rates of growth.

Key words: growth, prepubertal, pubertal

INTRODUCTION

The rearing of heifers is an essential element in dairy production systems, because these animals represent the maintenance and renovation of herds. However, replacement heifers represent a large proportion of total costs of milk production on dairy farms, and practices are necessary to increase the rate of growth from birth to first calving, in order to reduce the impact of heifers on farm costs (Sejrsen and Purup, 1997; Geiger et al., 2016). As the reproductive characteristics are correlated to BW, high growth rates have been used as strategy to reduce age at first calving (NRC, 2001; Piantoni et al., 2012; Albino et al., 2015).

One of the strategies to increase ADG of growing heifers is to increase the RUP content in the diet (Tomlinson et al., 1997). However, studies of RUP utilization on dairy heifers' diets are still scarce and results are conflicting. The use of fish or blood meals improved performance and N retention (Bethard et al., 1997; Zanton et al., 2007), while with corn gluten meal no effects on performance were reported (Ribeiro et al., 2005; Oliveira et al., 2008). To the best of our knowledge there are no studies using by-pass soybean meal as RUP source on heifers' diets, although soybean meal is globally a widely used feed ingredient for cattle and easy to treat to improve RUP content.

Besides improving ADG, another persistent concern is the mammary gland development of dairy heifers. High growth rates at key periods of development are negatively associated with mammary development (Geiger et al., 2016). Most of studies

have proposed that the mammary gland development is more pronounced during the prepubertal phase (Whitlock et al., 2002; Daniels et al., 2009; Piantoni et al., 2012), and for this reason this phase is more sensitive to changes in the mammary gland composition (Sinha and Tucker, 1969). However, inadequate management may negatively affect mammary gland development even during pubertal phase (Sejrsen et al., 1986; Purup et al., 2000; Rowson et al., 2012).

Although there is a large number of studies on dairy heifer's performance and mammary gland development, experiments with simultaneous use of prepubertal (**PRE**) and pubertal (**PUB**) heifers are still scarce and little information on this subject have been generate since the study of Sejrsen et al. (1982).

Therefore, we hypothesized that increasing amounts of RUP from soybean meal improve intake, performance and mammary gland development; and that the response is different for PRE and PUB Holstein dairy heifers. Furthermore, the aim of the present study was to evaluate the influence of increasing levels of RUP from soybean meal on intake, performance, N balance, carcass characteristics, mammary gland development and hormonal status of Holstein dairy heifers before or after puberty.

MATERIAL AND METHODS

The experiment was approved by the Ethics Commission on the Use of Farm Animals of Universidade Federal de Viçosa (Viçosa, MG, Brazil), under protocol no. 039/2015.

Animals, Treatments and Management

Thirty-two Holstein heifers were subdivided into 2 groups according to their physiological stage (**PS**). PRE heifers (initial BW 106 ± 7.6 kg, aged 4.3 ± 0.46 months, serum progesterone concentration 0.24 ± 0.023 ng/mL) and PUB heifers (initial BW 224 ± 7.9 kg, aged 12.6 ± 0.45 months, serum progesterone concentration 1.04 ± 0.012 ng/mL).

The experiment was carried out following a complete randomized design, in a 4×2 factorial arrangement, with four levels of RUP in the total dietary protein (intended levels 38, 44, 51, and 57%), and heifers at two different PS (prepubertal or pubertal). Thus, 8 treatments, with 4 replicates each, were formed: 38% of RUP for prepubertal heifers (**38PRE**); 44% of RUP for prepubertal heifers (**44PRE**); 51% of RUP for prepubertal heifers (**51PRE**); 57% of RUP for prepubertal heifers (**57PRE**); 38% of RUP for pubertal heifers (**38PUB**); 44% of RUP for pubertal heifers (**44PUB**); 51% of RUP for pubertal heifers (**51PUB**); 57% of RUP for pubertal heifers (**57PUB**).

The animals were housed in individual stalls with an area of 10 m² and provided with bunkers and drinkers. Heifers received a corn silage and concentrate diet (70:30 on DM basis; Table 1). The diets were composed for an estimated ADG of 1.0 kg/d, according to the recommendations of NRC (2001). All animals had ad libitum access to feed, which was supplied at 0700 and 1500 h.

Experimental Period, Collections and Sampling

The experiment was carried out during a period of 84 d and was preceded by adaptation period of 15 d to adapt animals to diets, facilities and management. Two digestibility trials were carried out from 36 to 40 d (first digestibility trial) and from 78 to 82 d (second digestibility trial), with measurements of intake and sampling of offered feeds, orts, feces and urine. In each digestibility trial, 8 spot collections of feces and urine were made with a 15h interval between each collection, according to the following scheme: on day 36 or 78, samples were collected at 0600 h and 2100 h; on day 37 or 79 samples were collected at 1200 h; on day 38 or 80, samples were collected at 0300 h and 1800 h; on day 39 or 81, samples were collected at 0900 h and on day 40 or 82 samples were collected at 0000 h and 1500 h. At each collection, approximately 200 g of feces were sampled, by manual collection directly in the rectum. In addition, approximately 50 mL of urine was sampled by stimulated urination, which was immediately frozen (-20°

C) to prevent N losses. At the end of each digestibility trial, samples of feeds, orts and feces were pooled and stored at -20° C. To pool the urine samples, 10 mL of pure urine was diluted into 40 mL of sulfuric acid (0.036N) and stored at -20° C to prevent purine derivate degradation (Valadares et al., 1999).

At d 0 (immediately before the beginning of the experiment) and d 83 ultrasound images were taken at the rib area, at the Longissimus dorsi muscle, between the 12th and 13th rib. The images were taken using an ultrasound (Aloka SSD-500V, Aloka Co., Ltda., Tokio, Japan) equipped with a linear transducer of 18 cm, operating at a frequency of 3.5 MHz. To obtain images, a standoff was used to guarantee the contact between transducer and animals' body; vegetable oil was used as a couplant to provide adequate acoustic contact between the transducer and standoff, as well as between these and animals' skin. Images were captured and recorded for further analysis.

At d 0, 21, 42, 63 and 84 images of the mammary gland were taken via ultrasound (Mindray DP2200), equipped with a micro-convex transducer, operating at a frequency of 6 MHz (for details, see Albino et al., 2016). Images were taken of each mammary quarter in a standardized position, with an inclination of 45° in relation to teat insertion, and saved in bitmap (BMP) format, a technique described by Nishimura et al. (2011) and adapted by Albino et al. (2016).

At d 0 and d 84 blood samples were taken through venipuncture on the jugular vein, into vacuum tubes of 10 mL containing separator gel and coagulation activator. Subsequently, the tubes were placed into a styrofoam box containing ice and transferred to the lab where samples were centrifuged at 3,000 × g for 20 min to obtain blood serum. Five samples of blood serum of each animal were stored in Eppendorf tubes and stored at -20° C for further analysis.

Laboratory Analysis and Calculations

Samples of feeds, orts and feces were oven-dried (55° C) and, subsequently, ground to 2 mm and 1 mm in a knife mill (Detmann et al., 2012). Samples ground to 1 mm were used to analyze the content of DM (AOAC International, 2005; method 934.01), CP (AOAC International, 2005; method 990.13), ash (AOAC International, 2005; method 942.05), NDF corrected for ash and protein contents (**NDFap**) (Detmann et al., 2012; methods INCT-CA F-002/1; N-004/1 and M-002/1) and gross energy (measured using a dynamic calorimeter (IKA, C 5001)). In addition, samples ground to 2 mm were used to determine the indigestible NDF, which was used as internal marker to estimate the DM fecal excretion. Briefly, samples of feeds, orts and feces were incubated into the rumen of a cow, using non-woven textile (100 g/m²) bags for 240 h, and NDF was estimated in the post-incubation material (Valente et al., 2011).

The composition of feeds and orts was used to estimate the intake of animals, while feces composition together with intake was used to estimate diet digestibility. The metabolizable energy content was calculated based on an average efficiency of use of digestible energy of 0.82 (NRC, 2001). In addition, the content of NDFap and CP of offered feed and orts were used to assess the sorting behavior of the animals, which was calculated as the relation between the actual intake and the predicted intake of each fraction (Leonardi and Armentano, 2003). Values equal to 100% indicate no sorting activity, while values under 100% indicate refusal (sorting against) and values greater than 100% indicate preferential intake (sorting for).

The urine samples were analyzed for the content of N (AOAC International, 2005; method 990.13), and creatinine was measured using the colorimetric endpoint method, with the use of picrate and acidifier (Labtest Diagnóstica S.A. Lagoa Santa, Minas Gerais, Brazil). In addition, the concentration of uric acid and allantoin in urine was determined according to Fujihara et al. (1987) and Chen and Gomes (1992), respectively. The total

daily urinary excretion was estimated using the daily creatinine excretion as proposed by Chizzotti et al. (2008) for Holstein heifers. The ruminal microbial CP (**CP_{mic}**) synthesis was estimated as a function of absorbed purines, which was calculated from the excretion of the purine derivatives uric acid and allantoin according to the equations proposed by Prates et al. (2012), for Holstein heifers.

The daily intake of RDP was considered equal to CP_{mic} production (Batista et al., 2016b), while the flow of true digestible microbial crude protein was calculated as follows:

$$MP_{mic} = CP_{mic} \times 0.64 \quad \text{Eq. 1}$$

where: MP_{mic}= metabolizable protein from microorganisms (g/d); CP_{mic}=microbial crude protein (g/d); 0.64=conversion factor of microbial crude protein to true digestible microbial crude protein, which considers that the microbial crude protein has 80% of true protein and 80% of digestibility (NRC, 2001).

After that, the digestible RUP intake was estimated as the difference between the CP and RDP intakes, and the daily intake of metabolizable protein was estimated by the sum of metabolizable protein from microorganisms and metabolizable protein from feedstuffs, which was calculated as proposed by NRC (2001), as follows:

$$MP_f = (CPI - RDPI) \times 0.8 \quad \text{Eq. 2}$$

where: MP_f=metabolizable protein from feedstuffs (g/d); CPI=daily intake of crude protein (g/d); RDPI=daily intake of rumen degradable protein (g/d); 0.8=coefficient of digestibility of rumen undegradable protein.

Body ultrasound images were processed using the software BioSoft Toolbox[®] II for Beef (Biotronics Inc., Ames, Iowa, USA). To assess the carcass characteristics, rib eye area and subcutaneous fat thickness at the rib area were measured.

Mammary gland ultrasound images were evaluated for pixels brightness as described by Albino et al. (2015), using the software ImageJ[®] (NIH, Bethesda, MD,

USA), calibrated for a pixels scale of 100 pixels/cm using the straight tracer tool. After the calibration, 3 squares with a fixed area of 16 mm² each were randomly collected near the ductal structures of each image. From the pixels values of the 3 squares of each image, an averaged value for each mammary quarter was determined, and after that, an average value was computed for the mammary gland of each animal. All images were evaluated in an 8 bit format, where each pixel was represented on a scale of 256 thousands shades of gray (0=black and 256=white), according to the brightness. Structures composed mainly by fat tissue have a high ability to reflect sound waves and present greater pixel values, whereas parenchymal tissue has a limited ability to reflect sound waves and present lower pixel values (Albino et al., 2015).

Blood serum samples were evaluated for concentrations of progesterone, estrogen, insulin and IGF-1 by chemiluminescence immunoassay (Immulite 1000; Siemens Medical Solutions Diagnostics, Los Angeles, USA). The sensitivity of this assay was 0.1 ng/mL, 20 pg/mL, 0.1 μ UI/mL and 25 ng/mL for progesterone, estrogen, insulin and IGF-1, respectively.

In situ degradability trial

Two Holstein heifers (BW \pm SEM 325 \pm 20.6 kg) fitted with a ruminal cannula were used to estimate RDP and RUP of feedstuffs through an in situ ruminal trial. These animals were individually housed and were fed twice a day with the treatment 38PUB diet. Nylon bags with an inner size of 15 \times 8 cm and pore size of 50 μ m (Sefar Nitex, Thal, Switzerland) were filled with approximately 5 g of either corn silage, corn meal, soybean meal, by-pass soybean meal, or wheat bran, all previously ground at 2 mm. Three bags of each feedstuff were incubated in each animal for 16 h (Calsamiglia and Stern, 1995; Paz et al., 2014). After this time, bags were removed from the rumen, washed in running water and oven-dried (55 $^{\circ}$ C). The residuals were analyzed for DM and CP contents, following techniques described above. The RDP content (g/kg DM) of the feed ingredients was

estimated as the amount of CP (g/kg DM) that disappeared from the nylon bag after incubation, and RUP (g/kg DM) was estimated as CP – RDP.

Statistical analysis

The data was analyzed following a completely randomized design in a 4×2 factorial scheme using the MIXED procedure of SAS (SAS Institute Inc, 2008). The data from the digestibility trials and pixel values from the mammary gland images were included as repeated measures in the experimental model. The variance components, compound symmetry, heterogeneous compound symmetry, heterogeneous first-order autoregressive, and unstructured matrices of (co)variance were tested. The matrix selection was based on the Corrected Akaike's Information Criterion, and was chosen the heterogeneous compound symmetry.

The pixel values from the mammary gland images did not followed a normal distribution and a logarithmic transformation was applied to this data. The initial measurements of carcass characteristics and serum hormone concentrations, as well the initial BW were included as co-variables in the experimental model. For all analysis, the DIFF option was included at the LSMEANS command to provide multiple comparisons and differences were declared when $P < 0.05$ and tendency for $0.05 < P < 0.10$.

RESULTS

Interaction effects between period of collection (digestibility trial or taken of mammary gland images) and levels of RUP and/or PS were not observed ($P > 0.05$) for none of the variables tested in this study (data not shown). In addition, interaction effects between RUP levels and PS were not observed for none of variables (Tables 2, 3 and 4; Figures 1, 2 and 3).

Intake, Digestibility and Sorting Behavior

The voluntary intake was greater at the second digestibility trial when compared to the first digestibility trial ($P < 0.05$), and was not affected by RUP levels ($P > 0.05$; Table

2). In addition, PUB heifers showed greater ($P=0.001$) voluntary intake than PRE heifers, except for the relative DMI (g/kg of BW), which was not affected by PS ($P<0.637$), neither by RUP levels ($P=0.268$) or period of collection ($P=0.131$; Table 2).

The apparent digestibility was not affected by period of collection, as well as by RUP levels ($P>0.05$; Table 2). However, it was observed effect ($P=0.001$) of PS on the apparent digestibility of DM, OM and NDFap, where PRE heifers presented lower digestibility than PUB heifers (Table 2), with average DM digestibility of 672 and 708 g/kg, respectively. On the other hand, it was found a tendency ($P=0.062$) of greater apparent digestibility of CP for PRE heifers (Table 2).

In a general form, all animals presented sorting activity against NDFap dietary content and sorting activity in favor to CP dietary content. Sorting behavior was only affected by PS, and PRE heifers showed a tendency ($P=0.065$) of lower sorting activity against NDFap than PUB heifers (Figure 1A). At same time, PRE heifers also presented ($P=0.031$) a lower selection capacity of CP than PUB heifers (Figure 1B).

Performance and Carcass Characteristics

Feed efficiency (FE) and ADG were affected ($P<0.05$) by RUP levels, and the treatments with 51% of RUP presented greater values (Table 3). The animals' performance was also affected ($P=0.001$) by PS, and the ADG was greater for PUB than PRE heifers, where PUB heifers had an ADG 79% greater than PRE, with average values of 905 and 505 g/d, respectively. In addition, PRE heifers presented better FE than PUB heifers, with averages of 0.156 and 0.139 g/kg, respectively (Table 3).

The carcass characteristics were only affected ($P=0.001$) by PS where PUB heifers presented greater average values for all measurements (Table 3). However, the measurements relative to the BW, did not pointed effect of RUP levels or PS (Table 3).

Nitrogen Balance, Microbial Synthesis and Protein Flow

The N intake demonstrated a tendency ($P=0.051$) to be greater in the second digestibility trial (123.6 and 137.8 g/d for first and second digestibility trials, respectively (Table 4). The urinary N excretion showed a tendency ($P=0.081$) to be lower in treatments with high RUP levels ($P=0.081$; Table 4). Fecal and urinary excretion of N were lower for PRE heifers ($P=0.001$), and the retained N followed the same pattern of ADG, where greater values were observed for treatments with 51% of RUP and also for PUB when compared to PRE heifers.

The synthesis of microbial CP presented a tendency ($P=0.087$) to be greater for animals fed 51% of RUP and was greater for PUB heifers (Table 4). The microbial efficiency was not affected ($P=0.183$) by RUP levels or PS ($P=0.196$; Table 4). The efficiency of use of N for microbial synthesis was lower ($P=0.019$) for PRE heifers and was not affected ($P=0.206$) by RUP levels (Table 4). The flow of digestible RUP and metabolizable protein was greater ($P=0.001$) for PUB heifers and presented a tendency ($P=0.075$ and $P=0.083$, respectively) to be greater for treatments with 51 of RUP.

Mammary Gland Development and Hormonal Status

The analysis of pixels values of the mammary gland images did not pointed effects of period of collection ($P=0.372$) and RUP levels ($P=0.282$; Figure 2). However, effects ($P=0.035$) of PS were observed where PRE presented greater pixels values than PUB heifers, with average values of 2.089 and 2.074 log of pixels/square, respectively.

The initial measurements of serum progesterone demonstrated a concentration of 0.24 and 1.03 ng/mL for PRE and PUB heifers, respectively (data not shown). The final progesterone was not affected by RUP levels ($P=0.775$) and was greater for PUB when compared to PRE heifers ($P=0.001$), with average values of 1.39 and 0.58 ng/mL, respectively (Figure 3A). The estrogen was not affected ($P>0.05$) by RUP levels or PS; Figure 3B). The IGF-1 was only affected ($P=0.001$) by PS and PUB heifers presented

greater values than PRE, with average values of 296.5 and 148.4 ng/mL, respectively. In addition, serum concentration of insulin was not affected ($P=0.550$) by PS, but presented a tendency ($P=0.077$) to be greater in treatments with 51% of RUP.

DISCUSSION

The initial and final measurements of progesterone demonstrated that all animals kept their PS during all experiment, once animals with progesterone equal or greater than 1 ng/mL are considered as pubertal (Cooke and Arthington, 2009). Thus, there was no changes on PS of the heifers along the experiment, what could have generated confounding effects.

As observed by Jiao et al. (2014) and confirmed by our study, young heifers have lower digestibility of NDFap, which can be linked to a lower ruminal microbial activity of these animals. As shown in Table 4, the use of consumed N for microbial synthesis was lower for PRE when compared to PUB heifers, what reveals some ruminal inefficiency of these animals. Thus, with a less microbial synthesis, a lower fiber digestibility has occurred, which contributed to a lower DM digestibility of PRE in relation to PUB heifers.

The behavior of PUB heifers to select feeds against NDFap content and in favor to CP content may indicate that this ability is intrinsically bound to their experience throughout their lives, preferences, and post-ingestive feedback, in addition to an attempt to optimize the ingestion of nutrients and energy (Provenza and Balph, 1987). On the other hand, PRE heifers are under a selection-learning process because they are young, and because, still in the transition stage (between 2 and 3 months old), they receive the forage and concentrate feeds separately. Therefore, they are not fit to consume the diet in total mixed ration form, which would make the selection activity more difficult.

The feed selection inability of PRE heifers result in a greater NDFap selection (Figure 1), which allied to a lower digestibility of NDFap and consequently of the DM

for these animals, resulted in a lower ME intake (g/kg of DM; Table 2). As the performance is directly affected by energy intake (Brown et al., 2005), this fact resulted in lower ADG and N retention of PRE heifers (Tables 3 and 4). Overall, sorting behavior and digestibility results reveal the importance of animal ability and/or rumen microbial to digest diet components (mainly NDFap) and highlight the importance of correctly balance diets to optimize animals' performance (Zanton and Heinrichs, 2016).

The better performance presented by animals fed 51% of RUP, with greater ADG, N retention and FE, is a reflex of the tendency of increase in the flows of digestible RUP and metabolizable protein (Table 4), which may increase the amino acids flow to small intestine (Tomlinson et al., 1997). In addition, RUP supplementation is recognized as a glucogenic source and responsible for increasing serum insulin concentration (Figure 3D; Wiley et al., 1991; Waterman et al., 2006). Thus, the best performance for animals fed 51% of RUP may also be linked to a greater insulin sensitivity caused by this level of RUP inclusion (Waterman et al., 2014).

In addition, the increasing in RUP levels was also responsible for a tendency to decrease urinary N excretion (Table 4), as observed by Batista et al. (2016a) when moved the site of supplementation from rumen to abomasum. This fact may be due to a greater equilibrium between ruminal protein degradation and microbial protein synthesis, resulting in lower ruminal ammonia production and, by consequence, lower urinary N excretion (Archibeque et al., 2007; Hristov, 2013).

The absence of effects of RUP levels on carcass characteristics and mammary gland development must to be interpreted as a positive result, once the level of 51% of RUP was capable of improving performance indexes not followed by negative effects on body composition, i.e., increase fat deposition (Table 3), neither on the mammary gland composition (Figure 2; Capuco et al., 2004; Moallem et al., 2004).

On the other hand, the greater values of pixel per square at the mammary gland images presented by PRE heifers, suggest that these animals had greater deposition of fat at the mammary gland than PUB heifers (Figure 2). This result is in line with other studies (Sejrsen et al., 1982; Purup et al., 2000; Meyer et al., 2006), which suggest that changes in the mammary gland development are more pronounced in PRE heifers, once these animals are in the allometric phase of the mammary gland growth (Sinha and Tucker, 1969). In addition, it is widely affirmed that negative effects (fat deposition) on mammary gland development are more pronounced when animals are submitted to nutritional plans that promote high rates of performance (Whitlock et al., 2002; Piantoni et al., 2012). However, our results suggest that proportion of fat at the mammary gland is greater for PRE when compared to PUB heifers, even when PRE heifers are under moderate ADG (Figure 2; Table 3).

The greater fat proportion at mammary gland is in opposition to the idea that animals under moderate rates of growth have greater proportion of protein in the body gain (Bartlett et al., 2006). However, this result appears to be linked to the lower levels of IGF-1 of PRE heifers (Figure 3C), which can be a result of the lower ME intake (g/kg of DM; Table 2) of these animals, once that IGF-1 concentration is directly affected by restrictions in feed intake (McGuire et al., 1992; Hornick et al., 2000). As the IGF-1 is an important factor for mammary gland development, because of his effect on ductal growth (Purup et al., 2000; Berryhill et al., 2015) and has a positive relationship with body protein deposition (Anderson et al., 1988), the lower IGF-1 concentrations can be negatively affected the mammary gland development of PRE heifers.

CONCLUSIONS

The dietary level of 51% of RUP at total dietary CP appears to be the adequate amount of RUP to be used on diets of growing heifers, once it was responsible for optimizing performance indexes, without negative impacts on carcass and mammary

gland growth. Prepubertal heifers have lower sorting ability, what affects negatively their digestibility and performance. Finally, these animals are more likely to have greater proportion of fat in the mammary gland, even under moderate rates of growth, what highlights the importance of new researches to better understand the relationship between performance and mammary gland development in dairy heifers.

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Table 1. Dietary ingredient and chemical composition of diets for dairy heifers fed different amounts of rumen undegradable protein (RUP) (38, 44, 51 and 57% of dietary protein) before (PRE) or after (PUB) puberty.

Items	Treatments							
	38PRE	44PRE	51PRE	57PRE	38PUB	44PUB	51PUB	57PUB
Diet composition, g/kg of DM								
Corn silage	700	700	700	700	700	700	700	700
Corn meal	26	26	26	26	87	87	87	87
Soybean meal	218	145	73	-	151	100	51	-
By-pass soybean meal	-	73	145	218	-	51	100	151
Wheat bran	32	32	32	32	48	48	48	48
Urea	4	4	4	4	2	2	2	2
Minerals	20	20	20	20	12	12	12	12
Chemical composition								
Crude protein, g/kg of DM	154.1	154.8	155.6	156.3	129.9	130.7	131.6	132.4
RDP ¹ , g/kg of DM	95.0	85.1	75.4	69.4	80.2	73.6	65.4	59.2
RUP ² , g/kg of DM	59.1	69.7	80.2	86.9	49.7	57.1	66.2	73.2
NDFap ³ , g/kg of DM	381.4	382.1	382.9	383.7	383.3	383.7	384.2	384.6
GE ⁴ , Mcal/kg of DM	4.01	4.00	3.99	3.99	4.02	4.01	4.00	4.00
RUP/CP ⁵ , g/kg of CP	383.7	450.1	515.5	556.0	382.7	437.1	503.0	552.8

¹Rumen degradable protein; ²Rumen undegradable protein; ³Neutral detergent fiber corrected for ash and protein; ⁴Gross energy; ⁵Amount of RUP in total CP (calculated using values obtained in the in situ trial).

Table 2. Intake and apparent digestibility of dairy heifers fed different amounts of rumen undegradable protein (RUP) (38, 44, 51 and 57% of dietary protein) before (PRE) or after (PUB) puberty.

Items ¹	Treatments								SEM ²	P-Value ³			
	38PRE	44PRE	51PRE	57PRE	38PUB	44PUB	51PUB	57PUB		RUP	PS	PRD	RUP×PS
Intake													
DM, kg/d	3.74	3.21	3.86	3.02	6.65	6.11	7.26	6.70	0.456	0.273	<0.001	0.023	0.788
OM, kg/d	3.48	2.99	3.59	2.81	6.29	5.76	6.84	6.31	0.430	0.280	<0.001	0.026	0.796
CP, kg/d	0.58	0.54	0.63	0.52	0.93	0.92	1.03	0.96	0.067	0.198	<0.001	0.051	0.767
NDFap, kg/d	1.35	1.17	1.38	1.07	2.41	2.22	2.64	2.42	0.171	0.304	<0.001	0.012	0.775
ME, Mcal/d	8.66	7.39	9.12	6.94	16.84	15.44	18.13	16.76	1.243	0.345	<0.001	0.014	0.884
DM, g/kg BW	28.7	24.4	26.4	26.2	26.7	25.8	25.9	25.6	1.21	0.268	0.637	0.131	0.654
ME, Mcal/kg DM	2.28	2.29	2.36	2.31	2.53	2.52	2.49	2.50	0.051	0.976	<0.001	0.254	0.728
Digestibility, g/kg													
DM	667	667	679	675	707	715	702	707	11.8	0.979	<0.001	0.305	0.743
OM	685	692	701	698	725	732	718	724	10.6	0.927	<0.001	0.595	0.677
CP	722	718	723	710	711	702	701	694	11.4	0.647	0.062	0.300	0.976
NDFap	564	590	584	591	614	633	608	622	13.7	0.368	0.001	0.763	0.798

¹DM=dry matter; OM=organic matter; CP=crude protein; NDFap=neutral detergent fiber corrected for ash and protein; ME=metabolizable energy. ²Standard error of mean.

³RUP=effect of RUP levels; PS=effect of physiological stag; PRD=effect of period of collection; RUP×PS=interaction effect between RUP levels and physiological stage.

Table 3. Performance and body composition of dairy heifers fed different amounts of rumen undegradable protein (RUP) (38, 44, 51 and 57% of dietary protein) before (PRE) or after (PUB) puberty.

Items ¹	Treatments								SEM ²	P-Value ³		
	38PRE	44PRE	51PRE	57PRE	38PUB	44PUB	51PUB	57PUB		RUP	PS	RUP×PS
Performance												
SBWi, kg	98.0	113.7	116.6	96.5	213.2	217.0	236.7	229.7	15.13	0.616	<0.001	0.799
SBWf, kg	139.5 ^B	154.2 ^B	172.0 ^A	128.7 ^B	285.0 ^B	287.2 ^B	326.2 ^A	302.5 ^B	17.35	0.001	<0.001	0.745
ADG, g/d	494 ^B	482 ^B	658 ^A	384 ^B	854 ^B	836 ^B	1,065 ^A	866 ^B	50.5	<0.001	<0.001	0.570
FE, g/kg	144 ^B	159 ^B	183 ^A	138 ^B	133 ^B	136 ^B	152 ^A	135 ^B	9.7	0.024	0.025	0.541
Carcass characteristics												
REA, mm ²	26.3	23.0	28.2	29.5	40.5	35.7	35.8	39.2	2.34	0.233	<0.001	0.531
SFT, mm	0.81	1.29	1.17	0.93	2.41	2.34	2.49	2.42	0.164	0.486	<0.001	0.407
REA, mm ² /100 kg BW	16.7	15.0	16.3	16.5	15.4	14.3	13.8	15.5	0.78	0.155	0.163	0.624
SFT, mm/100 kg BW	0.57	0.88	0.76	0.67	0.78	0.74	0.76	0.76	0.091	0.510	0.589	0.291

¹SBWi=shrunk initial body weight; SBWf=shrunk final body weight; ADG=average daily gain; FE=feed efficiency; REA=rib eye area; STF=subcutaneous fat thickness.. ²Standard error of mean. ³RUP=effect of RUP levels; PS=effect of physiological stage; RUP×PS=interaction effect between RUP levels and physiological stage.

Different letters indicate differences between RUP levels at 5% of probability by Fisher's LSD.

Table 4. Nitrogen balance, microbial synthesis and protein flow for dairy heifers fed different amounts of rumen undegradable protein (RUP) (38, 44, 51 and 57% of dietary protein) before (PRE) or after (PUB) puberty.

Items ¹	Treatments								SEM ²	P-Value ³			
	38PRE	44PRE	51PRE	57PRE	38PUB	44PUB	51PUB	57PUB		RUP	PS	PRD	RUP×PS
Nitrogen balance, g/d													
Intake	93.6	85.7	101.4	83.7	148.8	147.2	165.6	153.6	9.56	0.198	<0.001	0.051	0.767
Fecal	16.2	16.2	21.8	18.3	38.5	38.8	46.2	44.9	3.35	0.371	<0.001	0.145	0.936
Urinary	61.2	57.0	58.4	51.2	87.6	85.7	86.5	83.1	5.94	0.081	<0.001	0.758	0.987
Retained	16.2 ^B	12.5 ^B	21.2 ^A	14.2 ^B	22.7 ^B	22.7 ^B	32.9 ^A	25.6 ^B	3.36	0.023	<0.001	0.772	0.935
RN/NI	0.17	0.15	0.21	0.17	0.15	0.15	0.20	0.17	0.05	0.885	0.889	0.165	0.887
Microbial synthesis													
CPmic, g/d	319	282	335	268	555	526	605	542	34.6	0.087	<0.001	0.285	0.986
MicEf, g/kg	126	124	128	125	133	134	138	132	3.96	0.183	0.196	0.232	0.373
Nmic/NI, g/g	0.54	0.52	0.53	0.51	0.59	0.57	0.58	0.57	0.079	0.206	0.019	0.106	0.281
Protein flow, g/d													
RUPd	213	203	239	204	300	315	344	335	20.8	0.075	<0.001	0.595	0.966
MP	417	383	451	376	655	651	731	682	41.8	0.083	<0.001	0.268	0.984

¹RN/NI=relation between retained N and N intake; CPmic=microbial crude protein; MicEf=microbial efficiency (g of CPmic/kg of digestible organic matter intake); Nmic/NI=relation between microbial N and N intake; RUPd=digestible RUP; MP=metabolizable protein. ²Standard error of mean. ³RUP=effect of RUP levels; PS=effect of physiological stage; PRD=effect of period of collection; RUP×PS=interaction effect between RUP levels and physiological stage.

Different letters indicate differences between RUP levels at 5% of probability by Fisher's LSD.

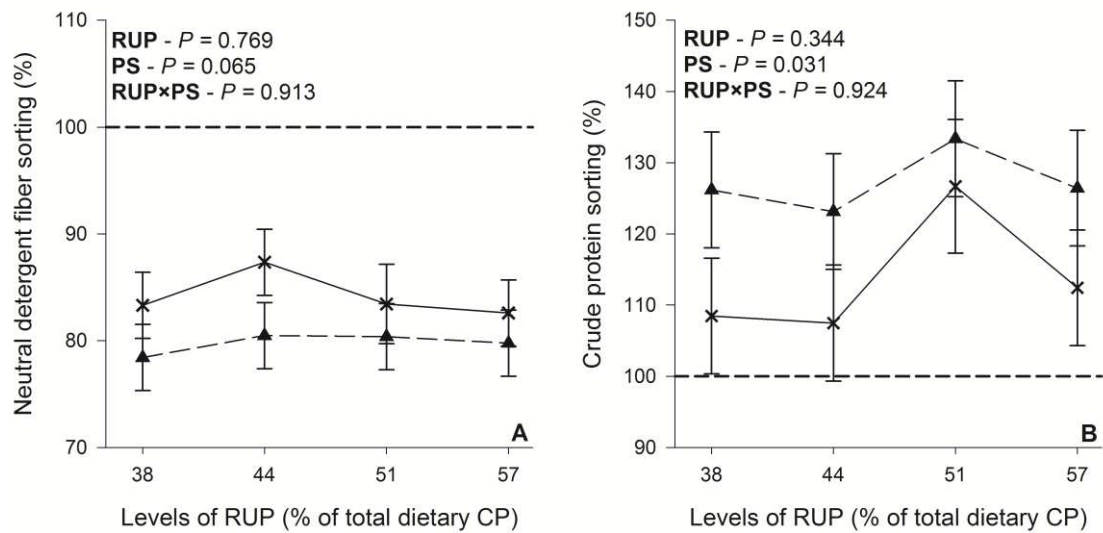


Figure 1. Sorting activity of dairy heifers fed different amounts of rumen undegradable protein (RUP) (38, 44, 51 and 57% of dietary CP) before (—x—) or after (—▲—) puberty, for A) neutral detergent fiber, and B) crude protein. Sorting values equal to 100% indicate no sorting, <100% indicate selective refusals (sorting against), and >100% indicate preferential consumption (sorting for). RUP=effect of RUP levels; PS=effect of physiological stage; RUP×PS=interaction effect between RUP levels and physiological stage.

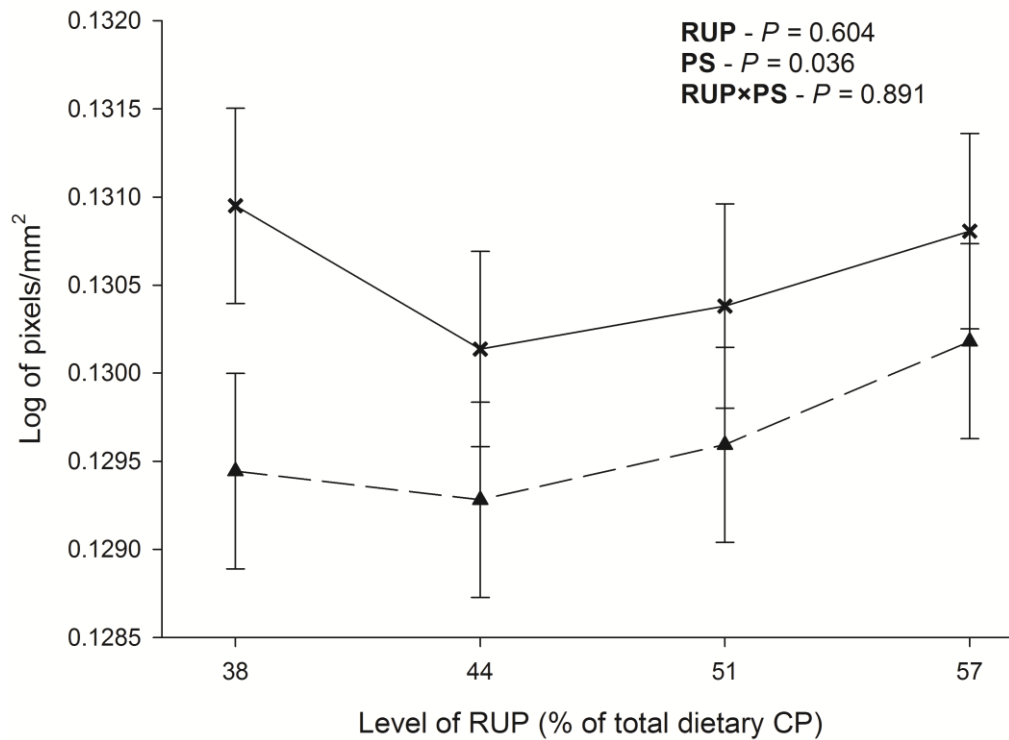


Figure 2. Pattern of pixel values at the mammary gland of dairy heifers feeding different amounts of rumen undegradable protein (RUP) (38, 44, 51 and 57% of dietary crude protein) before (—×—) or after (—▲—) puberty.

RUP=effect of RUP levels; PS=effect of physiological stage; RUP×PS=interaction effect between RUP levels and physiological stage.

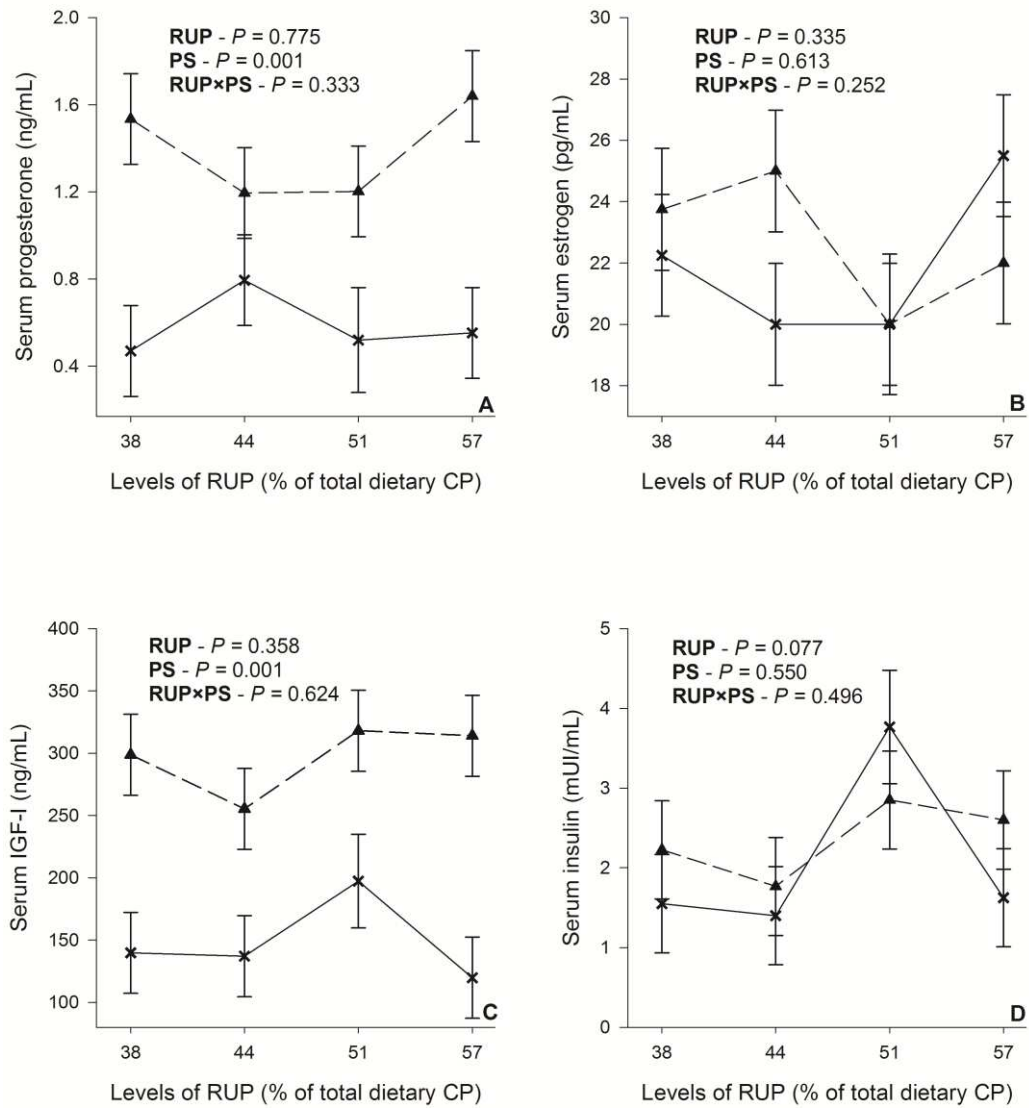


Figure 3. Serum levels of A) progesterone, B) estrogen, C) IGF-1, and D) insulin for dairy heifers fed different amounts of rumen undegradable protein (RUP) (38, 44, 51 and 57% of dietary crude protein) before (—x—) or after (—▲—) puberty.

RUP=effect of RUP levels; PS=effect of physiological stage; RUP×PS=interaction effect between RUP levels and physiological stage.

PAPER 3

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Interpretive summary: Effects of rumen undegradable protein on intake, digestibilities and rumen kinetics and characteristics of dairy heifers. By Silva et al. Information about the effects of rumen undegradable protein supplementation on the digestive parameters of Holstein dairy heifers are scarce. Therefore, this study aimed to evaluate the utilization rumen undegradable protein (38, 44, 51 and 57% of dietary CP) on intake, digestive parameters and the nitrogen use of dairy Holstein heifers. The urinary nitrogen excretion decrease according rumen undegradable protein supply increase. The inclusion of rumen undegradable protein at levels of 51 and 57% is capable to increase nitrogen retention.

DIGESTIVE PARAMETERS OF DAIRY HEIFERS

Effects of rumen undegradable protein on intake, digestibilities and rumen kinetics and characteristics of dairy heifers

Abstract: The aim was to evaluate the influence of increasing levels of rumen undegradable protein (**RUP**) on intake, total and partial digestibilities, rumen kinetics and characteristics, as well as N use of dairy Holstein heifers. Eight rumen-cannulated Holstein heifers, with an average initial body weight of 276 ± 8.3 (Mean \pm SEM) were used in a double 4 \times 4 Latin Square designed with four levels of RUP in the total dietary protein, as follows: 38% of RUP (**38RUP**); 44% of RUP (**44RUP**); 51% of RUP (**51RUP**) and 57% of RUP (**57RUP**). The experiment was carried out during 84 days subdivided into 4 periods of 21 d (14 d for adaptation + 7 d for collections). Eight spot collections of feces, urine, ruminal content and omasal digesta were performed with 9 h interval, as follows: on 15th d samples were collected at 0600 h and 1500 h; on 16th day samples were collected at 0000 h, 0900 h and 1800 h; on 17th d samples were collected at 0300 h, 1200 h and 2100 h. Two complete rumen evacuation were as performed: one on 19th d 4 h after the morning feeding and another on 21st d immediately before morning feeding. Total (kg/d) and relative (g/kg of body weight) intake of dry matter, crude protein (**CP**), neutral detergent fiber corrected for ash and protein were not affected by RUP levels. Ruminal and intestinal digestibilities of CP presented a tendency to decrease according to RUP increase. Effects of RUP levels were not observed on the ingestion, passage and digestion rates, as well on the total volatile fatty acid concentration. Urinary N excretion presented a tendency to decrease according to RUP increase. In addition, retained N presented a tendency to increase according to RUP levels and greater values were observed for treatments 51RUP and 57RUP. Microbial crude protein synthesis, microbial efficiency and the efficiency of use of N for microbial synthesis, decrease as RUP increased. Flow of RUP increased, while rumen degradable protein decreased with RUP increase. In addition, the flow of metabolizable protein presented a tendency to increase and greater values were observed for 51RUP and 57RUP. In conclusion, urinary N excretion decrease according to RUP increase, which allied to increases in the flow of metabolizable protein,

which are promoted by increases in the flow of RUP to the small intestine, were responsible to promote increases in the retained N occurred for treatments 51RUP and 57RUP.

Key words: nitrogen balance, rumen ammonia nitrogen, RUP

INTRODUCTION

Rumen degradable protein is determinant to microbial crude protein (**CP_{mic}**) synthesis, which is the main source of amino acids in the metabolizable protein (**MP**) for ruminants because of his good amino acids profile, once it meets amino acid profile of milk and muscle proteins (Santos et al., 1998; Ma et al., 2010; Mikolayunas et al., 2011).

However, once the microbial N requirements is met through RDP, supplying of RUP may increase the flow of MP and, consequently, improve animal performance and N retention (Tomlinson et al., 1997; Mezzomo et al., 2011). Besides the increase on MP flow, the supply of RUP also may cause changes in ruminal metabolism, with alterations on CP_{mic} synthesis, N availability, fiber degradation, and N recycling (Reynal et al., 2005; Batista et al., 2016b).

The effects of RUP supplementation on rumen kinetics, protein availability and N utilization are well reported for growing beef cattle and lactating dairy cows (Ipharraguerre and Clark, 2005; Pina et al., 2009; Mezzomo et al., 2011; Rufino et al., 2016; Batista et al., 2016b). However, there is a lack of studies reporting the influence of protein degradability on dairy heifers performance (Lascano et al., 2016) and, mainly, the effect of RUP supplementation on digestive parameters of Holstein dairy heifers feeding diets with high proportions of forage, that is typically used for this category (Zanton et al., 2007; Lascano and Heinrichs, 2009).

Therefore, we hypothesized that increasing RUP may alter rumen kinetics and fermentation characteristics, as well increase MP flow and improve N balance of Holstein dairy heifers. Aligned to the current need of information described above, we designed

this study aiming to evaluate the influence of increasing levels of RUP on intake, total and partial digestibilities, rumen kinetics and characteristics, as well the N use of dairy Holstein heifers.

MATERIAL AND METHODS

The experiment and all surgical and care procedures were approved by the Ethics Commission on the Use of Farm Animals of Universidade Federal de Viçosa (Viçosa, MG, Brazil), under protocol no. 039/2015.

Animals, Treatments and Management

Eight rumen-cannulated Holstein heifers, with an average initial BW of 276 ± 8.3 (Mean \pm SEM) were used in a double 4 \times 4 Latin Square design with four levels of RUP in the total dietary protein, as follows: 38% of RUP (**38RUP**); 44% of RUP (**44RUP**); 51% of RUP (**51RUP**) and 57% of RUP (**57RUP**).

The heifers were housed in individual stalls with area of 10 m², provided of bunkers and drinkers. Corn silage was used as forage source, and the forage:concentrate ratio was 70:30, on dry matter basis. The diets were composed to target an average daily gain of 1.0 kg/d, according to the recommendations of NRC (2001), and animals had ad libitum access to feed, which was supplied at 0700 and 1500 h (Table 1).

Experimental Period, Collections and Sampling

The experiment was carried out during 84 days subdivided into 4 experimental periods of 21 days each. The first 14 days of each period were used to treatment adaptation (Machado et al., 2016) and the last 7 days were used for collections. Prior to the experiment, the animals were subjected to a period of 15 d for adaptation to facilities, management and diets.

From 12th to 17th d of each period, ruminal infusions of 5 g/d of the complex Co-EDTA (555 mg/d of Co) (Udén et al., 1980) were performed 4 times daily at 0000 h, 0600 h, 1200 h and 1800 h.

The feeds supplied from 15th to 17th d as well as orts obtained from 16th to 19th d were sampled, pooled by heifer and stored at -20° C. In the same days, 8 spots collections of feces, urine, ruminal content and omasal digesta were performed with 9 h interval between each collection, as follows: on 15th d samples were collected at 0600 h and 1500 h; on 16th d samples were collected at 0000 h, 0900 h and 1800 h; on 17th d samples were collected at 0300 h, 1200 h and 2100 h.

In each collection time, approximately 200 g of feces were sampled, by manual collection directly in the rectum, pooled by heifer and stored at -20° C. In addition, approximately 50 mL of urine were sampled by stimulated urination. To pool the urine samples, 10 mL of pure urine were diluted into 40 mL of Sulfuric Acid (0.036N) and stored at -20° C to prevent the purine derivate degradation (Valadares et al., 1999).

Ruminal content was collected manually, filtered using 4 layers of gauze and used to evaluate pH and rumen ammonia nitrogen (**RAN**) and VFA concentrations. The pH was measured immediately after collection using a digital potentiometer (TEC-3P-MP; Tecnal, Piracicaba, SP, Brazil). Thereafter, an aliquot of 40 mL of rumen fluid was fixed using 1 mL of sulfuric acid (500 mL/L) for further measurement of RAN. Another aliquot of 20 mL was fixed using 5 mL of metaphosphoric acid (250 g/L), pooled by heifers and stored at -20° C for further analysis of VFA.

Before rumen sampling, omasal digesta collection was performed as described by Huhtanen et al. (1997) and following the adaptations described by Mariz et al. (2013). In each collection time, approximately 600 mL of omasal digesta were collected and immediately filtered using 100-µm nylon filter with 44% of pores (Sefar Nitex 100/44, Sefar, Thal, Switzerland) to generate particle and fluid phase samples. At the end of each period, particle and fluid samples were pooled by heifer and stored at -20° C for further analysis.

A complete rumen evacuation was performed on 19th d, 4 hours after the morning feeding, and on 21st d, immediately before morning feeding. After total rumen evacuation, the digesta was weighed and filtered to obtain solid and liquid phases, which were, individually, weighed, sampled and stored at - 20° C. Thereafter, the digesta was reconstituted and returned to the rumen of the respective animals.

Laboratory Analysis and Calculations

Samples of feeds, Orts, feces and rumen evacuation were partially dehydrated in a force-ventilated oven (55° C) for 72h. While samples of omasal digesta (fluid and particle phases), in turn, were partially dehydrated by lyophilization. In addition, all samples were ground to 2 mm and 1 mm in a knife mill (Detmann et al., 2012). After partially dehydrated and ground, samples of rumen evacuation and omasal digesta were pooled by heifer and experimental period, based on dry weight.

Samples ground to 1 mm were used to analyze DM (AOAC International, 2005; method 934.01), CP (AOAC International, 2005; method 990.13), ash (AOAC International, 2005; method 942.05), NDF corrected for ash and protein contents (**NDFap**) (Detmann et al., 2012; methods INCT-CA F-002/1; N-004/1 and M-002/1). Omasal digesta samples were also analyzed for Cobalt concentration by atomic absorption spectrophotometer (Spctr-800; Varian spectrometer, Harbor City, CA) as described by Kimura and Miller (1957). In addition, samples ground to 2 mm were incubated into the rumen of a cow for 240 h to determine the indigestible NDF (**iNDF**) (Casali et al., 2008; Valente et al., 2011). The potential degradable NDFap (**pdNDFap**) was calculated as the difference between NDFap and iNDF.

The DM fecal excretion was estimated using iNDF as internal marker. This marker was also used in combination with Co-EDTA in a system of double marker to estimate the rumen outflow of DM and nutrients. The iNDF was used as marker of particle phase and Co-EDTA as marker of liquid phase, and their concentration in the different omasal

digesta phases was used to calculate the reconstitution factors (France and Siddons, 1986; Rotta et al., 2014).

The urine samples were analyzed for their N content (AOAC International, 2005; method 990.13), as well as, uric acid and allantoin concentrations, according to Fujihara et al. (1987) and Chen and Gomes (1992), respectively. In addition, creatinine concentration was measured using the colorimetric endpoint method, using picrate and acidifier (Labtest Diagnóstica S.A. Lagoa Santa, Minas Gerais, Brazil).

The total daily urinary excretion was estimated using the daily creatinine excretion as proposed by Chizzotti et al. (2008) for Holstein heifers. The ruminal microbial CP synthesis was estimated as a function of absorbed purines, which was calculated from the sum of uric acid and allantoin excretions, as proposed by Chen and Gomes (1992) and using equations to estimate the absorbed purines and microbial nitrogen proposed by Prates et al. (2012), for Holstein heifers.

The RUP flow was determined using the following equation:

$$\text{RUP} = \text{CP}_{\text{RO}} - \text{CP}_{\text{mic}} \quad \text{Eq. 1}$$

where: RUP=flow of rumen undegradable protein (kg/d); CP_{RO} =rumen outflow of crude protein (kg/d); CP_{mic} =microbial crude protein synthesis (kg/d).

The rumen degradable protein was calculated as follows:

$$\text{RDP} = \text{CPI} - \text{RUP} \quad \text{Eq. 2}$$

where: RDP=rumen degradable protein (kg/d); CPI=crude protein intake (kg/d); RUP=flow of rumen undegradable protein (kg/d).

The flow of metabolizable protein was estimated by the sum of true digestible microbial protein and digestible RUP (NRC, 2001), as follows:

$$\text{MP} = (\text{CP}_{\text{mic}} \times 0.64) + (\text{RUP} \times 0.80) \quad \text{Eq. 3}$$

where: MP=metabolizable protein (kg/d); CP_{mic} =microbial crude protein synthesis (kg/d); RUP=flow of rumen undegradable protein (kg/d).

The data of rumen evacuation was used to calculate the rates of ingestion, passage and digestion, according to Allen and Linto (2007). The rate of digestion (k_d ; %/h) of each fraction was calculated as the difference between the rates of intake (k_i) and passage (k_p), and these rates were calculated as presented in equations 4 and 5, respectively.

$$k_i = \left(\frac{\text{Intake}}{\text{Rumen pool}} \right) \times 100 \quad \text{Eq. 4}$$

where: k_i =rate of intake (%/h) and intake and rumen pool are given in kg/h and kg, respectively.

$$k_p = \left(\frac{\text{Rumen outflow}}{\text{Rumen pool}} \right) \times 100 \quad \text{Eq. 5}$$

where: k_p =rate of passage (%/h) and rumen outflow and rumen pool are given in kg/h and kg, respectively.

The RAN concentration was determined using a colorimetric method (Detmann et al., 2012, method INCT-CA N-006-1). In addition, the VFA concentration was determined using HPLC (Dionex Ultimate 3000, Dionex Corporation, Sunnyvale, CA, USA) following technique described by Siegfried et al. (1984).

Statistical analysis

The data was analyzed following a Latin square design using the MIXED procedure of SAS (SAS Institute Inc, 2008), as follows:

$$Y_{ijkl} = \mu + RUP_i + LS_j + (RUP \times LS)_{ij} + A_{(j)k} + P_{(j)l} + \varepsilon_{ijkl}$$

where: Y_{ijkl} =dependent variable; μ =overall mean; RUP_i =fixed effect of RUP levels; LS_j =random effect of Latin square; $RUP \times LS_{ij}$ =random effect of interaction between RUP levels and Latin Square; $A_{(j)k}$ =random effect of animal within Latin square; $P_{(j)l}$ =random effect of period within Latin square and ε_{ijkl} =random error.

The data from different times of sampling for RAN and pH were included as repeated measures in the experimental model. The variance components, compound symmetry, heterogeneous compound symmetry, heterogeneous first-order

autoregressive, and unstructured matrices of (co)variance were tested. The matrix selection was based on the Corrected Akaike Information Criterion, and the heterogeneous compound symmetry was chosen. For all analysis, the DIFF option was included in the LSMEANS command to provide multiple comparisons and differences were declared when $P < 0.05$ and tendency for $0.05 < P < 0.10$.

RESULTS

Intake, Ruminant Outflow and Digestibility

Total intake were not affected ($P > 0.05$) by RUP levels and presented average values of 6.76, 6.28, 0.95, 2.54, 1.73 kg/d for DM, organic matter (OM), CP, NDFap and pdNDFap, respectively (Table 2). In addition, effects were not observed ($P > 0.05$) on the relative intake (g/kg of BW), with average values of 22.94, 3.22 and 8.60 for DM, CP and NDFap, respectively (Table 2). The ruminal outflow was not affected by RUP levels ($P > 0.05$), and presented average values of 4.50, 3.73, 0.90, 1.54 and 0.69 kg/d for DM, OM, CP, NDFap and pdNDFap, respectively (Table 2).

The total digestibility was not affected ($P > 0.05$) by RUP levels and presented average values of 699, 722, 691, 529, 778 g/kg for DM, OM, CP, NDFap and pdNDFap, respectively. Ruminal digestibility of DM, OM, NDFap and pdNDFap were not affected ($P > 0.05$) by RUP levels, presenting average values of 337, 410, 409 and 606, respectively. However, ruminal digestibility of CP presented a tendency ($P = 0.061$) to decrease according to RUP increase in diet (Table 2). In addition, intestinal digestibility of CP also presented a tendency ($P = 0.088$) to decrease when RUP increased. Intestinal digestibility of DM, OM, NDFap and pdNDFap were not affected ($P > 0.05$) by RUP levels and presented average values of 547, 529, 204 and 422 g/kg, for, respectively (Table 2).

Rumen Kinetics and Characteristics

Effects of RUP levels were not observed ($P > 0.05$) on ingestion rate of DM, CP, NDFap, iNDF and pdNDFap, with average values of 7.2, 6.9, 4.3, 2.6 and 6.5%/h,

respectively (Table 4). The passage rate was also not affected by treatments and presented average values of 4.8, 6.5, 2.6, 2.6 and 2.6%/h for DM, CP, NDFap, iNDF and pdNDFap, respectively. In addition, digestion rate was also not affected by RUP levels, presenting average values of 2.4, 1.7 and 4.0%/h for DM, NDFap and pdNDFap, respectively (Table 4).

There were no effects ($P=0.341$; Table 5) of RUP levels on total VFA concentration, which averaged 68.1 mmol/L. RUP levels also did not affect ($P>0.05$) the proportion of each VFA, and the relationship between acetate and propionate, with an average of 4.06 ($P=0.719$; Table 5).

On the other hand, 38RUP presented greater RAN concentration with average value of 22.1 mg/dL, while 44RUP, 51RUP and 57RUP, averaged 18.5, 18.7 and 17.6 mg/dL, respectively. The RAN concentration was also affected by hour of sampling ($P=0.001$), presenting peaks after morning (0700 h) and afternoon (1500 h) feed, with the greatest value observed at 0900 h, which was the first sampling time after morning feed (Figure 1A).

The rumen pH was not affected by RUP levels and treatments 38RUP, 44RUP, 51RUP and 57RUP presented average values of 6.18, 6.23, 6.22 and 6.34, respectively. On the other hand, rumen pH was affected by hour of sampling ($P=0.001$), with lowest values observed after morning and afternoon feed (Figure 1B).

Nitrogen Balance and Microbial Protein Synthesis

The RUP levels did not affect ($P>0.05$) N intake and fecal N excretion, which averaged 151.9 and 46.8 g/d, respectively (Table 6). However, the N urinary excretion presented a tendency ($P=0.077$) to decrease when RUP increased (Table 6). Thus, the retained N presented a tendency ($P=0.087$) to increase according to the RUP levels, and 51RUP and 57RUP presented greater values when compared 44RUP and 38RUP (Table 6). In addition, the efficiency of N utilization (N retained/ N intake) was affected by RUP

levels ($P=0.035$) and treatments 51RUP and 57RUP presented greater values when compared to other treatments (Table 6).

Rumen undegradable protein levels affected CPmic synthesis ($P=0.039$), as well as, the efficiency of microbial crude protein synthesis (**EMS**; $P=0.015$) and the efficiency of use of N for microbial synthesis ($P=0.007$), and all values decreased as RUP supply increased (Table 6). In addition, the ratio between CP intake and digestible OM matter intake (**CP/DOMI**) was lower ($P=0.031$) for 57RUP when compared to other treatments (Table 6). Furthermore, RUP levels affected ($P=0.046$) the flow of RUP and presented a tendency ($P=0.091$) to increase the flow of MP, with greater values for 51RUP and 57RUP (Table 6). On the other hand, the amount of RDP decreased ($P=0.022$) as RUP increased (Table 6).

DISCUSSION

Absence of effects of RUP supply on voluntary feed intake (Bethard et al., 1997; Oliveira et al., 2008) as well as on the apparent digestibility (Zanton et al., 2007; Silva et al., 2017 in a companion paper) has been already observed, what is agreement with the results of this study. Although there were no effects on total digestibility of CP (Table 3), there was a tendency to decrease the ruminal digestibility of CP when the RUP supply increased. As ruminal metabolism of CP involves degradation of RDP, recycling of N and CPmic synthesis, and these effects happen at the same time in the ruminal environment (Batista et al., 2016b), the ruminal digestibility of CP does not represent the real ruminal degradation of CP, but the amount of N that was not incorporated in the CPmic or escaped as RUP, which likely lost as ammonia (Arroyo et al., 2011). In this context, increasing RUP supply decreased the amounts of RDP (Table 6) and, for consequence, was likely responsible for decreasing the amounts of N lost as ammonia (Table 3), and RAN concentration (Figure 1A). The decrease in ruminal losses of ammonia (Table 3) were responsible for a tendency to decrease urinary N excretion

(Table 6), as was observed by us in a companion paper (Silva et al., 2017) and by Batista et al. (2016a). Consequently, there was an increase in N available for anabolism, represented by an increase in the retained N (Table 6).

In addition to the ruminal digestibility, intestinal digestibility of CP also decreased when the RUP supply increased, what it is probably linked to the proportion of CPmic and RUP that achieved small intestine (Table 6). The CPmic represented 64, 61, 57 and 52% of the total protein that achieved the intestine for 38RUP, 44RUP, 51RUP and 57RUP, respectively. Although, RUP and CPmic were assumed, in the pass, to have the same 80% of intestinal digestibility of protein (NRC, 2000, 2001), such equivalence was not observed according to our results, once when CPmic proportion decreased, intestinal digestibility of CP also decreased (Table 3), showing that RUP of this experiment, despite the good quality of the feedstuffs used, has a lower intestinal digestibility than CPmic. Once by-pass soybean meal and soluble soybean meal are considered to have similar intestinal digestibility (Harstad and Prestløkken, 2000; Akbarian et al., 2014), the decrease in the intestinal digestibility appears to be due to proportion between CPmic and RUP in the total protein in the intestine and not derived from the increased amounts of by-pass soybean meal (Table 1).

The observed decrease on CPmic synthesis, as well as on the EMS and the efficiency of use of N for microbial synthesis, usually occur due to factors such as decrease in ruminal N and energy availabilities or a lack of synchrony between N and energy for microbial metabolism (Nocek and Russell, 1988; Cecava et al., 1990; Broderick et al., 2010). Ruminal N availability was not altered across treatments, as can be observed by the positive values of ruminal digestibility of CP, which represents that all treatments proportionated a positive rumen N balance (Table 6). However, CP/DOMI ratio, that represents the synchronism between energy and protein, presented lower value

for treatment 57RUP, which was the treatment with lower values for CP_{mic} synthesis, EMS and efficiency of use of N for microbial synthesis (Table 6).

Despite the decrease in CP_{mic} synthesis and EMS (Table 6), the ruminal fermentation was kept in normal conditions, once there were no negative effects on VFA concentration (Table 5), DM and fiber digestibilities (Table 3), and ruminal rates of digestion and passage of DM, CP and fiber (Table 4). Thus, the inclusion of RUP had no deleterious effects on rumen dynamics, once RAN concentration (Figure 1A) was maintained at sufficient levels to support fibrolytic activity (7.1 mg/dL; Broderick et al., 2010) and to maximize voluntary fiber intake (15 mg/dL; Detmann et al., 2014b), even in treatments with greater RUP levels. In addition, the availability of true rumen degradable protein, that are preferentially used by amylolytic bacteria, were not negatively affected by RUP levels, once the concentration of isoacids and valerate remained unaltered across treatments (Table 5; Lascano and Heinrichs, 2009).

The flow of RUP increased according to RUP supply, which contributed to increase the MP flow, even with the decrease observed in CP_{mic} synthesis (Table 6). The increase in MP flow, allied to the decrease in urinary N excretion contributed to increase the available N for anabolism (Tomlinson et al., 1997; Rufino et al., 2016). Therefore, resulting in increases on growth rates and N retention, as observed in the companion paper (Silva et al., 2017), and on the retained N of this study, which was greater for treatments 51RUP and 57RUP that were the treatments that presented greater RUP and MP flows (Table 6).

Overall, Bethard et al. (1997) reported a increment in the feed efficiency of growing dairy heifers (± 140 kg of initial BW) feeding diets with 50% of RUP when compared to 38% of RUP. In addition, Tomlinson et al. (1997), reported a linear improvement in ADG of growing dairy heifers (± 220 kg of initial BW) when used 38, 43, 50 and 55% of RUP. Ipharraguerre and Clark (2005), reported in their meta-analysis that the best results on

milk yield of dairy cows were achieved using greater RUP levels. Therefore, merging these literature reports it is possible to understand that the results presented in this study, as well as, in the companion paper (Silva et al., 2017) are in agreement with previous studies. Furthermore, these results support the use of greater RUP levels than those regularly recommended in the past years by NRC (2001) – which is based in the beef cattle version (NRC, 2000) – that have recommended close to 30% of RUP to sustain an ADG of 1 kg/d. Moreover, new recommendations of beef NRC (2016) suggest an increment up to 40% in the RUP requirements for beef cattle, thus the necessity of increasing RUP in diets for growing dairy heifers is known by current researchers, however the lack studies have been braking the use of higher RUP levels by nutritionists. Therefore, the results of this study and those of the companion paper (Silva et al., 2017) have reported important new insights on use of RUP in dairy heifers; however, this topic is still scarce in the literature and more studies are necessary.

In summary, the dietary level of RUP until 57% of dietary protein does not have any effect on the voluntary feed intake, as well as in the rates of digestion and passage of DM, CP and fiber. Intestinal digestibility of CP is negatively affected by RUP levels, due to the reduction in CP_{mic} flow, which, probably, has greater intestinal digestibility than protein of feedstuffs. The N urinary excretion decrease when RUP supply increase, due to a decrease in ruminal N losses as ammonia and an increase in N recycling. The reduction in N urinary excretion allied to increases in the flow of metabolizable protein, which are promoted by increases in the flow of RUP to the small intestine, are determinant to promote increases in retained N, which occurred when dietary protein has between 51 and 57% of RUP. Therefore, based on the results of this study and those of the companion paper (Silva et al., 2017) we recommend the use of RUP as 51% of dietary protein for growing dairy heifers.

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Table 1. Dietary ingredient and chemical composition of diets for dairy heifers fed different amounts of rumen undegradable protein (RUP) (38, 44, 51 and 57% of dietary protein).

Items	Treatments			
	38RUP	44RUP	51RUP	57RUP
Diets' composition, g/kg of DM				
Corn silage	700	700	700	700
Corn meal	110	110	110	110
Soluble soybean meal	147	97	50	0
By-pass soybean meal	0	50	97	147
Wheat bran	32	32	32	32
Urea	3	3	3	3
Minerals	8	8	8	8
Chemical composition				
Crude protein, g/kg of DM	129.9	130.7	131.6	132.4
RDP ¹ , g/kg of DM	80.2	73.6	65.4	59.2
RUP ² , g/kg of DM	49.7	57.1	66.2	73.2
NDF ³ , g/kg of DM	383.3	383.8	384.2	384.6
RUP/CP ⁴ , g/kg of CP	382.7	437.1	503.0	552.8

¹/rumen degradable protein.

²/rumen undegradable protein.

³/neutral detergent fiber corrected for ash and protein.

⁴/amount of RUP at total CP.

Amounts of RDP and RUP of feedstuffs were calculated using values obtained in the in situ trial, which is described in the companion paper (Silva et al., 2017).

Table 2. Intake and ruminal outflow of dairy heifers fed different amounts of rumen undegradable protein (RUP) (38, 44, 51 and 57% of dietary protein).

Items ¹	Treatments				SEM ²	P-Value
	38RUP	44RUP	51RUP	57RUP		
Intake, kg/d						
DM	6.81	6.69	6.78	6.75	0.360	0.913
OM	6.33	6.22	6.30	6.27	0.338	0.921
CP	0.95	0.94	0.96	0.95	0.071	0.951
NDFap	2.54	2.52	2.56	2.55	0.171	0.982
pdNDFap	1.72	1.71	1.75	1.75	0.191	0.865
Intake, g/kg of body weight						
DM	22.83	23.03	22.65	23.24	2.016	0.508
CP	3.17	3.24	3.20	3.28	0.227	0.236
NDFap	8.52	8.67	8.58	8.61	0.917	0.969
Ruminal outflow, kg/d						
DM	4.61	4.47	4.50	4.45	0.384	0.755
OM	3.78	3.77	3.71	3.67	0.319	0.809
CP	0.89	0.89	0.90	0.90	0.074	0.923
NDFap	1.52	1.56	1.53	1.55	0.183	0.962
pdNDFap	0.67	0.73	0.69	0.68	0.105	0.870

¹/DM=dry matter; OM=organic matter; CP=crude protein; NDFap=neutral detergent fiber corrected for ash and protein; pdNDFap= potential digestible neutral detergent fiber corrected for ash and protein.

²/Standard error of mean.

Different letters indicate differences between RUP levels at 5% of probability by Least Square Difference of Fisher.

Table 3. Total, ruminal and intestinal digestibility of dairy heifers fed different amounts of rumen undegradable protein (RUP) (38, 44, 51 and 57% of dietary protein).

Items ¹	Treatments				SEM ²	P-Value
	38RUP	44RUP	51RUP	57RUP		
Total digestibility, g/kg						
DM	698	699	696	701	9.5	0.209
OM	712	716	724	734	15.3	0.324
CP	684	697	684	698	7.5	0.196
NDFap	513	523	530	549	14.5	0.124
pdNDFap	762	773	777	801	18.6	0.271
Ruminal digestibility, g/kg of intake						
DM	322	332	335	360	22.2	0.197
OM	414	393	409	424	11.9	0.103
CP	96	92	55	53	11.7	0.061
NDFap	403	401	406	425	26.7	0.791
pdNDFap	608	573	609	632	31.6	0.404
Intestinal digestibility, g/kg of rumen outflow						
DM	553	549	542	544	14.6	0.864
OM	516	532	533	533	23.8	0.832
CP	679	683	668	649	8.8	0.088
NDFap	181	217	207	210	29.7	0.797
pdNDFap	386	433	427	442	51.7	0.857

¹/DM=dry matter; OM=organic matter; CP=crude protein; NDFap=neutral detergent fiber corrected for ash and protein; pdNDFap= potential digestible neutral detergent fiber corrected for ash and protein.

²/Standard error of mean.

Table 4. Rumen pool and rates of ingestion, passage and digestion of dairy heifers fed different amounts of rumen undegradable protein (RUP) (38, 44, 51 and 57% of dietary protein).

Items ¹	Treatments				SEM ²	P-Value
	38RUP	44RUP	51RUP	57RUP		
Rumen pool, kg						
DM	3.99	3.78	4.01	4.03	0.359	0.494
CP	0.55	0.57	0.60	0.62	0.036	0.172
NDF	2.54	2.36	2.58	2.53	0.265	0.359
NDFi	1.42	1.30	1.36	1.35	0.180	0.703
pdNDFap	1.12	1.05	1.23	1.18	0.107	0.217
Ingestion rate, %/h						
DM	7.12	7.46	7.09	7.20	0.402	0.701
CP	7.09	6.86	6.81	6.73	0.485	0.791
NDF	4.19	4.50	4.15	4.47	0.336	0.357
NDFi	2.42	2.70	2.55	2.65	0.237	0.587
pdNDFap	6.60	6.86	5.96	6.71	0.667	0.378
Passage rate, %/h						
DM	4.83	4.97	4.71	4.63	0.358	0.673
CP	6.88	6.46	6.39	6.37	0.499	0.439
NDF	2.51	2.74	2.48	2.59	0.263	0.588
NDFi	2.46	2.76	2.63	2.73	0.262	0.583
pdNDFap	2.64	2.83	2.34	2.52	0.375	0.634
Digestion rate, %/h						
DM	2.28	2.49	2.37	2.57	0.181	0.247
NDF	1.68	1.76	1.67	1.88	0.174	0.435
pdNDFap	3.96	4.03	3.62	4.19	0.445	0.363

¹/DM=dry matter; CP=crude protein; NDFap=neutral detergent fiber corrected for ash and protein; NDFi=indigestible neutral detergent fiber; pdNDFap=potential degradable neutral detergent fiber corrected for ash and protein.

²/Standard error of mean.

Table 5. Volatile fatty acids concentration of dairy heifers fed different amounts of rumen undegradable protein (RUP) (38, 44, 51 and 57% of dietary protein).

Items ¹	Treatments				SEM ²	P-Value
	38RUP	44RUP	51RUP	57RUP		
Lactate, mmol/L	0.65	0.61	0.71	0.62	0.069	0.724
Total VFA, mmol/L	63.5	70.7	68.7	69.5	3.10	0.341
VFA, mol/100 mol						
Acetate	66.7	66.9	67.5	67.3	0.59	0.553
Propionate	16.8	16.6	16.4	16.6	0.36	0.809
Butyrate	9.8	9.9	9.6	10.0	0.31	0.212
Isobutyrate	1.1	1.0	1.1	1.0	0.07	0.905
Valerate	2.5	2.5	2.5	2.3	0.17	0.651
Isovalerate	3.0	3.1	3.2	2.7	0.20	0.337
A/P, mol/mol	4.01	4.05	4.13	4.06	0.121	0.719

¹VFA=volatile fatty acids; A/P=relation between acetate and propionate.

²Standard error of mean.

Table 6. Nitrogen balance, microbial protein synthesis and protein flow of dairy heifers fed different increasing amounts of rumen undegradable protein (RUP) (38, 44, 51 and 57% of dietary protein).

Items ¹	Treatments				SEM ²	P-Value
	38RUP	44RUP	51RUP	57RUP		
Nitrogen balance						
Intake, g/d	152.6	150.4	154.2	150.3	12.46	0.951
Fecal, g/d	47.4	45.5	46.5	47.6	4.52	0.342
Urinary, g/d	92.3	92.4	85.7	81.2	6.13	0.077
Retained, g/d	12.9	12.5	22.0	21.7	7.54	0.087
NR/NI, g/g	0.08 ^B	0.10 ^B	0.14 ^A	0.14 ^A	0.046	0.035
Microbial synthesis						
CPmic, g/d	568 ^A	541 ^{AB}	519 ^{BC}	477 ^C	41.8	0.039
EMS, g/kg	127 ^A	121 ^A	115 ^{AB}	104 ^B	9.9	0.015
Nmic/NI, g/g	0.61 ^A	0.58 ^{AB}	0.54 ^{BC}	0.51 ^C	0.043	0.007
CPI/DOMI, g/kg	211.6 ^A	211.1 ^A	211.3 ^A	204.1 ^B	2.82	0.031
Protein flow, g/d						
RUP	322 ^B	349 ^B	391 ^A	433 ^A	34.7	0.046
RDP	624 ^A	591 ^{AB}	568 ^B	513 ^C	36.8	0.022
MP	621	625	645	652	14.5	0.091

¹/NR/NI=relation between nitrogen retained and nitrogen intake; CPmic=microbial crude protein; EMS=efficiency of microbial crude protein synthesis (g of CPmic/kg of digestible organic matter intake); Nmic/NI=relation between microbial N synthesis and N intake; CPI/DOMI=relation between crude protein intake and digestible organic matter intake; RUP=rumen undegradable protein; RDP=rumen degradable protein; MP=metabolizable protein.

²/Standard error of mean.

Different letters indicate differences between RUP levels at 5% of probability by Least Square Difference of Fisher.

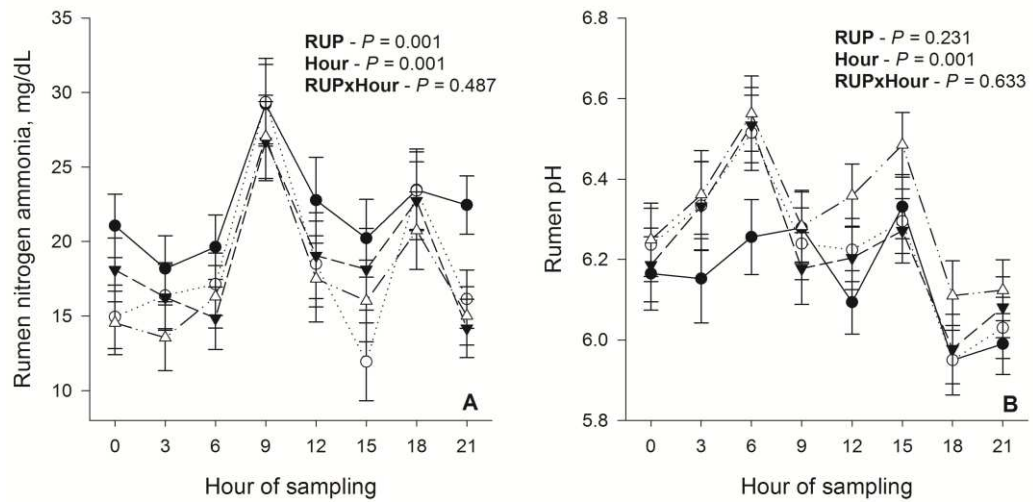


Figure 1. Rumen ammonia nitrogen concentration (A) and rumen pH (B), according to hour of sampling of Holstein heifers fed 38 (—●—); 44 (.....○.....); 51 (---▼---) or 57% (—△—) of rumen undegradable protein (RUP) in total dietary protein.

GENERAL CONCLUSIONS

In conclusion, the use of milk or milk replacer as liquid feed did not differentially affect SFI of dairy calves up to 64 d of age. Neither did the type of milk influence the pattern of SFI over time, which exponentially increased with calf age. Starter feed intake was negatively associated with MI; for this reason, different equations were fit to predict calf SFI consuming low (less than 5 L/d) or high (more than 5 L/d) MI. Overall, the proposed equations were suitable to predict SFI for milk-fed dairy calves, presenting moderate precision and very high accuracy.

Regarding the utilization of RUP for dairy heifers, the dietary level of 51% of RUP at total dietary CP appears to be the adequate amount of RUP to be used on diets of growing heifers, once it was responsible for optimizing performance indexes, without negative impacts on carcass and mammary gland growth. In addition, the dietary level of RUP until 57% of dietary protein does not have any effect on the voluntary feed intake, as well as in the rates of digestion and passage of DM, CP and fiber. Intestinal digestibility of CP is negatively affected by RUP levels, due to the reduction in CP_{mic} flow, which, probably, has greater intestinal digestibility than protein of feedstuffs. The N urinary excretion decrease when RUP supply increase, due to a decrease in ruminal N losses as ammonia and an increase in N recycling. The reduction in N urinary excretion allied to increases in the flow of metabolizable protein, which are promoted by increases in the flow of RUP to the small intestine, are determinant to promote increases in retained N.