MARCELO MESSIAS DUARTE CASTRO

METABOLIC RESPONSES, PERFORMANCE, MAMMARY GLAND DEVELOPMENT AND GENE EXPRESSION IN LIVER AND MUSCLE OF HOLSTEIN × GYR CROSSBRED HEIFERS GRAZING INTENSIVELY-MANAGED *BRACHIARIA DECUMBENS* SUPPLEMENTED WITH VARIED CRUDE PROTEIN AND ASSOCIATION OF HOUSING AND MANAGEMENT PRACTICES WITH MILK YIELD, MILK COMPOSITION, AND FATTY ACID PROFILE, PREDICTED USING FOURIER-TRANSFORM MID-INFRARED SPECTROSCOPY, IN FARMS WITH AUTOMATED MILKING SYSTEMS

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

Adviser: Marcos Inácio Marcondes

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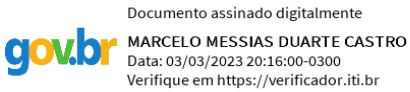

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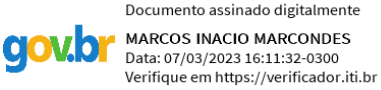

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Adviser

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BIOGRAPHY

Marcelo Messias Duarte Castro, son of Sebastião Rocha Castro and Vera Lúcia Lopes Duarte Rocha, was born in Estevão de Araújo, Araponga, Minas Gerais – Brazil on September 04, 1989.

In February 2010, he started his undergraduate studies in the Animal science department at Universidade Federal de Viçosa and obtained a Bachelor of Science degree in Animal Science in January 2015.

In March of 2015, he started the Master Science program in Animal Science at Universidade Federal de Viçosa with major in Ruminant Production and Nutrition under the supervision of Prof. Marcos Marcondes. He obtained the Master's Science degree in Animal Science in February of 2017.

In March of 2017, he started his Ph.D. at the Animal Science department with a major in Ruminant Production and Nutrition at the Universidade Federal de Viçosa under the supervision of Prof. Marcos Marcondes. In 2020, he had the opportunity to work on research projects in partnership with the University of Guelph – Canada, under the supervision of Prof. Trevor DeVries. On February 22, 2022, he will present his Ph.D. thesis to obtain the Ph.D. Science degree in Animal Science.

ABSTRACT

CASTRO, Marcelo Messias Duarte, D.Sc., Universidade Federal de Viçosa, February, 2022. **Metabolic responses, performance, mammary gland development and gene expression in liver and muscle of Holstein × Gyr crossbred heifers grazing intensively-managed *brachiaria decumbens* supplemented with varied crude protein levels and association of housing and management practices with milk yield, milk composition, and fatty acid profile, predicted using fourier-transform mid-infrared spectroscopy, in farms with automated milking systems.** Adviser: Marcos Inácio Marcondes.

The objectives of the first and second study were to evaluate the effect of crude protein (CP) supplementation on the metabolic characteristics, performance, muscle, and mammary gland development and expression of genes involved in the urea cycle, and muscle tissue development of Holstein × Gyr crossbreed heifers grazing *Brachiaria decumbens* throughout the year. Thirty-eight heifers were randomly assigned to four treatments: three protein supplements (SUP) fed at 5g/kg of body weight, plus a control group. The supplement CP levels were 12, 24, and 36%. The experimental period was divided into four seasons: rainy, dry, rainy-dry transition, and dry-rainy transition. The data were analyzed using PROC GLIMMIXED of the SAS with repeated measures. SUP animals had a greater intake of dry matter, metabolizable energy, and metabolizable protein. Furthermore, SUP animals had a greater average daily gain, rib eye area and fat thickness than non-supplemented animals. Among SUP animals, we observed a quadratic response to ADG, with the highest level in S24. No supplementation effects were detected on mammary gland development. In muscle, we observed greater expression of AMPK in non-supplemented animals than SUP animals. No differences were observed for mTOR. We observed greater urea excretion and retention coefficient in SUP animals than non-supplemented animals. In this sense, we also observed greater gene expression of CPS, ASL, and ARG in SUP animals than non-supplemented, and among SUP animals, the supplement CP linearly affected CPS expression. We observed a positive linear effect of urea excretion, nitrogen intake, nitrogen retention, and retention coefficient among SUP animals. In conclusion, SUP animals had greater intake, performance than non-supplemented animals, with S24% demonstrating the best results. The third study aimed to describe the FA profile, as predicted using Fourier transform mid-infrared (FTIR) spectroscopy, of bulk tank milk from automated milking system (AMS) farms and to assess the association of management and housing factors with the bulk tank milk composition and FA profile of those AMS farms. The data used were collected from 124 commercial Canadian Holstein dairy farms. Information regarding individual cow milk yield (kg/d), days in milk (DIM), parity, and

the number of milking cows were automatically collected by the AMS units on each farm. Multivariable regression models were used to associate herd-level housing factors and management practices with milk production, composition, and FA profile. Milk yield was positively associated with using a robot feed pusher (+2.1 kg/d) and the use of deep bedding (+2.6 kg/d). The use of a robot feed pusher, deep bedding, and greater stall raking frequency were positively associated with greater yield (kg/d) of de novo FA, mixed FA, preformed FA, and de novo + mixed FA. Greater frequency of PMR delivery (>2x/d vs. 1 and 2 x/d) was positively associated with a greater proportion (g/100 g of FA) of de novo, mixed, and de novo + mixed FA and negatively associated with the proportion of preformed FA. Overall, these associations indicate that bulk tank FA profile can be used to monitor and adjust management and housing in AMS farms.

Keywords: Nitrogen metabolism. Pasture. Robotic milking. Season.

RESUMO

CASTRO, Marcelo Messias Duarte, D.Sc., Universidade Federal de Viçosa, fevereiro de 2022. **Respostas metabólicas, desempenho, desenvolvimento da glândula mamária e expressão gênica no fígado e no músculo de novilhas mestiças Holandês × Gir em pastejo de *Braquiária decumbens* manejadas intensivamente suplementadas com níveis variados de proteína bruta e associação das práticas de manejo e instalações com a produção de leite, composição do leite e perfil ácido graxo, preditos usando espectroscopia de infravermelho por transformação de Fourier (FTIR), em fazendas com sistemas de ordenha robotizados.** Orientador: Marcos Inácio Marcondes.

Os objetivos do primeiro e segundo estudo foram avaliar o efeito da suplementação de proteína bruta (PB) sobre as características metabólicas, desempenho, desenvolvimento muscular, glândula mamária e expressão de genes envolvidos no ciclo da ureia e no desenvolvimento do tecido muscular de novilhas Holandês×Gir em pastejo Braquiária decumbens ao longo ano. Trinta e oito novilhas foram distribuídas aleatoriamente em quatro tratamentos: três suplementos proteicos (SUP), alimentadas com 5g/kg de peso corporal, mais um grupo controle. Os níveis de PB avaliados foram 12, 24 e 36%. O período experimental foi dividido em quatro estações: águas, seca, transição águas-seca e transição seca-águas. Os dados foram analisados pelo PROC GLIMMIXED do SAS com medidas repetidas. SUP tiveram maior ingestão de matéria seca, energia e proteína metabolizável. Entre os animais SUP, observamos uma resposta linear positiva para consumo de proteína metabolizável. SUP apresentaram maior ganho médio diário (GMD), área de olho de lombo e espessura de gordura que os não suplementados. Entre os animais SUP observamos uma resposta quadrática para o GMD, com maior nível em S24. Foi observado maior atividade da AMPK nos animais não suplementados que nos SUP. Observamos maior excreção de ureia, coeficiente de retenção e maior expressão das enzimas CPS, ASL e ARG em animais SUP que em animais não suplementados e entre os SUP observamos resposta linear positiva para CPS, excreção de ureia e coeficiente de retenção entre os animais SUP. Em conclusão, os animais SUP tiveram maior consumo, desempenho que os animais não suplementados, com o tratamento S24 demonstrando os melhores resultados. O terceiro estudo objetivou descrever o perfil de ácidos graxos (AG) usando espectroscopia de infravermelho por transformação de Fourier, do leite do tanque em fazendas com sistema de ordenha automatizados (AMS) e avaliar a associação de fatores de manejo e instalações com a composição do leite e o perfil de AG. Os dados utilizados foram coletados em 124 fazendas canadenses de gado leiteiro da raça Holandesa. As informações de produção de leite por vaca (kg/d), dias em lactação, ordem de parto e número de vacas ordenhadas foram

coletadas automaticamente pelo AMS. Modelos de regressão multivariada foram usados para associar fatores de instalações e manejo com produção de leite, composição e perfil de AG. A produção de leite foi positivamente associada ao uso de robô para empurrar a dieta para o cocho (+2,1 kg/d) e o uso de cama sobreposta (+2,6 kg/d). O uso do robô para empurrar a dieta, o uso da cama sobreposta e a maior frequência de limpeza das baias foram associados positivamente com maior produção (kg/d) de AG de novo, AG misto e AG pré-formado. Maior frequência de fornecimento da dieta parcial (>2x/d vs. 1 e 2 x/d) foi positivamente associada a uma maior proporção de AG (g/100 g de FA) de novo e misto e negativamente associado a proporção de AG pré-formados. Assim, essas associações indicam que o perfil de AG do leite do pode ser usado para monitorar e ajustar o manejo e as instalações em fazendas com AMS.

Palavras-chave: Estação do ano. Metabolismo de nitrogênio. Ordenha robotizada. Pasto.

SUMMARY

General introduction

Replacement heifer rearing has a high cost of production for dairy farmers (Salte et al., 2020) due to expenses related to land, labor, and primarily feed, representing 64% of the total cost to raise a replacement heifer (Gabler et al., 2000). According to Akins and Hagedorn (2015), the total cost to raise a dairy heifer from weaning to freshening was $2,510 in 2015, 3.3% greater than the value in 2013 ($2,427). Of note, Boulton et al. (2017) reported a reduction in the mean total cost of 17.1% for reducing the age at first calving (AFC) from 26 to 23 months and a substantial increase of 25.2% when AFC raged from 26 to ≥ 30 months. Additionally, a more recent study, evaluating a heifer cost simulation model (Hawkins et al., 2020), obtained costs for three different housing types, including confinement: $2,100.57, dry-lot: $ 1,737.26, and pasture: $1,423.94. Thus, a pasture-based system is a promising alternative to reduce production costs and improve profit rates (Aguirre-Villegas et al., 2017 and Lowe et al., 2016). Nevertheless, balancing diets for grazing animals is challenging and nutritional constraints can frequently limit intake, digestibility, and consequently, animal performance (Figueiras et al., 2016). Thus, an adequate supplementation strategy is necessary to increase animal performance and production efficiency.

In this sense, crude protein **(CP)** supplementation strategies for grazing cattle have been studied (Lazzarini et al., 2009; Detmann et al., 2014; Batista et al., 2016; da Silva-Marques et al., 2018; Machado et al., 2019). However, designing the best supplementation strategy and pasture management for grazing dairy heifers in tropics conditions is not a simple task, since the pasture may have high variability in production and composition throughout the year. For example, during the dry season, pasture has low dry matter (**DM**) production and is of low nutritional quality, mainly due to low **CP** level and low fiber digestibility in tropical conditions (Detmann et al., 2014 and Sampaio et al., 2010). During seasons when pasture has low quality, the CP is the first limiting for synthesis of enzymes involved in the fiber degradation process

(Detmann et al., 2014). On the other hand, pasture has high DM production and better nutritive value during the rainy season compared to the dry season. However, there is still an unbalance of nutrients in that pasture (da Silva-Marques et al., 2018) since exclusive pasture diets rarely provide an adequate balance of all nutrients, limiting animal performance.

Due to high pasture variability both qualitatively and quantitatively throughout the year, different CP supplementation strategies must be provided. However, it is necessary to understand the nitrogen metabolism, urea cycle, and their connection with pasture composition throughout the year to ensure optimal animal performance and efficiency of nitrogen use. However, there is an absence of studies regarding CP supplementation for grazing dairy heifers across the year, since most of the studies with grazing animals were carried out with beef cattle and evaluated only one season of the year, dry or rainy (da Silva-Marques et al., 2018; Figueiras et al., 2016). Therefore, the evaluation of responses of dairy grazing heifers to supplementation throughout the year (rainy, rainy-dry transition, dry and dry-rainy transition) is of utmost importance to define best supplementation strategies for each season to improve the performance, efficiency of nitrogen use, and to reduce the age at first calving in dairy heifers.

Moreover, understanding the factors that influence milk components is very important because milk is commonly marketed using a component pricing system, which defines the milk price based on the fat, protein, and other solids composition of the milk (Bailey et al., 2005). Thus, bulk tank fatty acids (FA) composition has become of increased interest, primarily due to its high correlation with bulk tank milk fat and protein content (Barbano et al., 2014) and due to the ease and speed of prediction the FA composition using Fourier transform mid-infrared **(FTIR)** spectroscopy**.** Moreover, bulk tank FA composition provides information on herd nutritional status since management practices, nutrition, and facilities are the primary factors that affect milk FA composition (Palmquist et al., 1993).

The use of automatic milking systems **(AMS)** is continually increasing worldwide. For example, in Canada, ~11 % of dairy herds enrolled in a milk recording program use AMS (Lactanet, 2019). With the adoption of AMS, not only are milking procedures changed, but also various aspects of dairy farm management, including facilities and nutritional management (Svennersten-Sjaunja and Pettersson, 2008). As a result, there is potential for these practices to be associated with milk FA content. While researchers have previously investigated the effect of AMS use on free-fatty acid content of milk (Klungel et al., 2000; De Marchi et al., 2017), to our knowledge, there is no research to date on the association of management and housing with bulk tank milk FA content in AMS farms.

Therefore, this thesis was made up of three chapters, where the objectives were to (Chapter 1 and 2) evaluate the effect of increasing CP supplementation on the metabolic characteristics, performance, genes expression of enzymes involved in the urea cycle and muscle tissue growth, mammary gland, and muscle development of Holstein × Gyr crossbreed heifers grazing intensively managed *Brachiaria decumbens* throughout the year. The objective of the third chapter was to describe the FA profile, as predicted using FTIR, of bulk tank milk from AMS farms and assess the association of management and housing factors with bulk tank milk composition and FA profile in AMS farms.

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Chapter 1

Metabolic responses and performance of Holstein × Gyr heifers grazing *Brachiaria decumbens* supplemented with varied crude protein levels. *By Castro et al., 2021.* Studies evaluating increasing supplement crude protein (CP) and their interactions with season are scarce for grazing crossbred dairy heifers. We aimed to evaluate the effect of providing increasing supplement CP (Control, 12, 24, and 36% of CP) for Holstein × Gyr crossbred heifers grazing *Brachiaria decumbens* across seasons (rainy, dry, transition rainy-dry, and dry-rainy). The CP supplementation improved metabolism and performance indexes, and the supplementation with 24% of CP demonstrated the best metabolism and performance results.

Metabolic responses and performance of Holstein × Gyr heifers grazing *Brachiaria decumbens* supplemented with varied crude protein levels

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Abstract

Studies evaluating the effects of increasing supplement CP and their interactions with season are scarce for Holstein × Gyr crossbreed heifers grazing. We aimed to evaluate the effect of supplemental CP on the nutritional characteristics and performance of Holstein × Gyr crossbreed heifers grazing intensively-managed *Brachiaria decumbens* throughout the year. Thirty-eight heifers were randomly assigned to four treatments: three protein supplements (SUP) composed of soybean meal and ground corn fed at 5g/kg of BW, plus a control group (CON). The supplements had 12, 24 and 36% of CP for treatments **S12** (n=9), **S24** (n=10), and **S36** (n=9), respectively. The experiment latest one year, subdivided into four seasons: rainy, dry, rainy-dry transition (RDT), and dry-rainy transition (DRT). Feces and pasture samples were collected for 4 days in each season, using chromium oxide, titanium dioxide, and indigestible neutral detergent fiber (NDF) to estimate fecal excretion, supplement, and pasture intake, respectively. The data were analyzed using PROC GLIMMIXED of the SAS with repeated measures. No effects of supplementation were detected on pasture and NDF intake. However, SUP animals had a greater intake of DM, metabolizable energy, and metabolizable protein. A positive linear response on metabolizable protein intake was observed among SUP animals. We observed an interaction between treatment and season for all digestibility variables, with SUP animals having greater digestibility of OM in dry and DRT. There was a positive linear response in CP digestibility among SUP animals during all seasons, and in OM digestibility in RDT, dry, and rainy seasons. For NDF digestibility, we observed a positive linear response in RDT and rainy seasons and a quadratic response during the dry season. Furthermore, SUP animals had greater ADG than non-supplemented animals, and among SUP animals, there was a quadratic response to ADG, with the greatest gain observed in S24. We observed greater urea excretion, nitrogen intake, nitrogen retention, and retention coefficient in SUP animals than in non-supplemented animals. Non-supplemented animals had greater microbial efficiency than SUP animals. We observed a positive linear effect of supplemental

CP level on urea excretion, nitrogen intake, nitrogen retention, and retention coefficient among SUP animals. Supplemental CP did not affect microbial protein production and efficiency. We observed an interaction between treatment and season for blood glucose, with SUP animals having greater glucose concentration in all seasons than non-supplemented animals. Additionally, we observed a quadratic response among SUP animals only during RDT and dry season, with the greatest glucose concentration in S24. SUP animals had greater blood concentrations of urea and IGF-1. Moreover, there was a negative linear response in triglycerides and a positive linear response in urea. In conclusion, SUP animals had greater intake, digestibility, and performance than non-supplemented animals, with the 24% CP supplement demonstrating the best metabolic responses and performance.

Keywords: dairy heifers, grazing, seasons, protein supplementation

Introduction

In the dairy industry, the replacement heifers represent the future lactating cows. However, the cost of production for raising heifers is high (Salte et al., 2020). In this sense, a pasture-based system is an alternative to reduce production costs and improve profitability (Tozer et al., 2003; Lowe et al., 2016). Nevertheless, balancing diets for grazing animals is challenging and nutritional constraints can frequently limit intake, digestibility, and, consequently, animal performance (Figueiras et al., 2016). Supplementation of CP for grazing beef cattle has been previously studied to increase animal performance and production (Lazzarini et al., 2009; Detmann et al., 2014; Batista et al., 2016).

Designing the best supplementation strategy and pasture management for grazing dairy heifers in tropical conditions is not a simple task since the pasture may have high variability in production and composition throughout the year. For example, pasture has low DM production during the dry season and is of lower nutritional quality, mainly due to low CP level and low fiber digestibility in tropical conditions (Detmann et al., 2014; Sampaio et al., 2010). During seasons when pasture has low quality, the CP represents the first limiting for the synthesis of enzymes involved in the fiber degradation process (Detmann et al., 2014). On the other hand, pasture has high DM production and better nutritive value during the rainy season compared to the dry season. However, there may still be an imbalance of nutrients in the pasture, mainly with surplus energy in relation to protein (da Silva-Marques et al., 2018). Thus, supplementation with adequate CP content is needed.

Due to high variability in production and pasture composition throughout the year, different CP supplementation strategies must be provided for grazing dairy heifers in each season to ensure optimal animal performance and reduce the age at first calving (Macdonald et al., 2005). However, most studies with grazing animals were carried out with beef cattle, lacking studies regarding supplementation strategies for Holstein × Gyr crossbreed dairy heifers grazing. In other words, animal nutrient requirement differences can be attributed to several

factors, such as environmental conditions and feeding management; however, the breed is one of the main reasons for response variability (Castro et al., 2020). Thus, supplementation strategies developed for beef cattle may not be adequate to ensure optimal performance and achieve the target age at first parity of Holstein × Gyr crossbreed dairy heifers grazing due to differences in nutrient requirements. In this sense, the evaluation of responses of grazing Holstein × Gyr dairy heifers to supplementation throughout the year is of utmost importance to define the best supplementation strategies for each season (rainy, rainy-dry transition, dry and dry-rainy transition).

We hypothesize that there is an interaction between CP level in the supplement and season of the year for Holstein × Gyr grazing dairy heifers grazing intensively-managed *Brachiaria decumbens*, where high CP levels in the supplement composed of soybean meal and corn ground would promote a greater performance during the dry season. Therefore, the objective was to evaluate the effect of increasing CP supplementation on the metabolic characteristics and performance of Holstein × Gyr crossbreed heifers grazing intensively-managed *Brachiaria decumbens* throughout the year.

Materials and methods

The experiment was carried out at the Department of Animal Science of the Universidade Federal de Viçosa (Viçosa, Minas Gerais, Brazil; 20º45' S and 42º52'). Data for temperature and rainfall throughout the experimental period are presented in Figure 1. All animal handling and procedures were approved by the ethics committee for animal use at Universidade Federal de Viçosa under protocol #041/2017. The number of animals for this study was limited to that allowed by the ethics committee for animal use at Federal University Viçosa. The sample size was sufficient to detect a 20% difference (from control treatment) in the outcome variables (15% coefficient of variation), with 95% confidence at 90% power (WinPepi version 11.65; Abramson, 2011).

Treatments and Experimental Design

Thirty-eight crossbred heifers (½ Holstein × ½ Gyr), with average initial BW of 172.5 ± 11.15 kg (mean ± SE) and 8.2 ± 0.54 mo of age, were used. Initially, all heifers were treated for ectoparasites and endoparasites (Ivomec, Paulina, Sao Paulo, Brazil). The heifers were randomly assigned to 1 of 4 treatments: three supplements (SUP) had increasing levels of CP composed of soybean meal and corn ground (Table 2), plus a control group (**CON**; n=10) in which the animals received only mineral mixture *ad libitum*. The supplements had 12, 24 and 36% of CP for treatments **S12** (n=9), **S24** (n=10) and **S36** (n=9), respectively (Table 2). Every day, at 1000 h, all heifers were moved from the pasture to a management area (250 meters distance), and supplements were fed to each group (treatment) separately. However, we estimated the individual supplement intake using TiO_2 as the external marker, following Titgemeyer et al. (2001) methodology. Thus, the experimental unit was the animal. The supplement was supplied at 5 g/kg of BW per day with a feeder bunk space of 50 cm per animal, and no orts were observed. The amount of supplement (5g/kg of BW) was chosen to meet the energy requirements of crossbreed dairy heifers, with an ADG of 0.5 kg/d grazing *Brachiaria decumbens* according to recommendations of NRC (2001). All heifers were weighed every 15 d to adjust supplement supply. The animals of the control treatment (non-supplemented) were also moved to the management area, but no supplementation was provided. We spent 1 h with this management per day, and the animals returned to paddocks afterward.

The heifers were managed in a rotational grazing system, with 27 paddocks, each 1700 m^2, of *Brachiaria decumbens* fertilized with 120 kg of N and 60 kg of K_2O per hectare per year. An additional area on the side of the main area was used during the dry season, with 23 paddocks, 1700 m^2 each. All paddocks had free access to the resting area with shade (3 m^2/animal), water, and mineral mix *ad libitum*. All heifers were kept in the same paddock, using

one paddock per day, except during the dry season, when we used two paddocks per day (using the additional area described above) due to low pasture DM production in that season.

The experiment lasted from January 7 (2018) to January 22 (2019), subdivided into four seasons, plus an adaptation period of 30 days. The adaptation period lasted from January 7 to February 6, and all heifers were fed the same supplement containing 18% CP (Table 2) at 5 g/kg of BW. The first season lasted from February 7 to April 24 (76 d), which was named the rainy-dry transition (RDT) season. The second season lasted from April 25 to July 27 (94 d), which was named the dry season. The third season lasted from July 28 to November 5 (101 d), and it was named the dry-rainy transition (DRT) season. Finally, the fourth season lasted from November 6 to January 22 (78 d), which was named the rainy season.

The last 14 d of each season were used to collect samples (collection period) of pasture, feces, urine, body measures, body weight, and blood samples (Figure 1). D 1 to 8 of the collection period were used to estimate digestibility, pasture intake, and supplement intake. From d 9 to 11, body measurements and body weight were recorded. Animals were weighed for three consecutive days (always at 0800 h) each period to avoid any scale or filling effects. On d 14, blood samples were collected.

Herbage measurements, production, and composition

The pasture was managed under intermittent stocking. Pre-grazing herbage accumulation was estimated in each paddock on d 5 to 8 of each collection period, following the methodology described by Machado et al. (2019). Briefly, two 1.0 × 1.5 m (width × length) exclusion cages were placed in representative areas (based on height and morphological structure) immediately before beginning a new grazing cycle in each paddock. After that, pasture samples were collected at the actual post-grazing canopy height by clipping the area within the exclusion cages.

The pasture production was 1785, 1080, 1778, and 3092 kg of DM/ha, with average CP values (g/kg) of 108, 77.8, 86.8, and 100 for the RDT, dry, DRT, and rainy seasons, respectively (Table 1). Moreover, pasture allowance was 8.00, 9.67, 8.45, and 14.4 kg of DM/animal per day for the RDT, dry, DRT, and rainy seasons, respectively (Table 1).

Feces and urine sampling

Between days 1 and 8 of each collection period, 10 g of dioxide of titanium (TiO_2) by kg of DM of the supplement was mixed in the supplement to estimate the supplement intake of each animal (Titgemeyer et al., 2001). On the same days, 15 g of chromium oxide (Cr_2O_3) was infused by the esophagic probe in each animal to estimate fecal excretion (Detmann et al., 2001). On d 5 to 8 of each experimental collection period, feces and urine samples were collected at 0600, 1000, 1200, and 1800 h, respectively. Approximately 300 g of feces were sampled directly from the rectum. The same procedures of drying and grinding done with forage samples were applied to fecal samples. In addition, urine samples were taken by vulva stimulation, and 50 mL of urine was sampled. To pool the urine, 10 mL of pure urine was diluted into 40 mL of sulfuric acid (0.036N) and stored at − 20ºC, to prevent purine derivatives degradation(Valadares et al., 1999)(Valadares et al., 1999).

Performance and Body Measurements

The heifers were weighed at the beginning of the experimental period and on days 9, 10, and 11 of each experimental collection period, always before supplementation, to calculate the **ADG** per period. In addition, thoracic circumference, body length, rump length, rump width, withers height, and rump height were measured using a hipometer on the same days of the weighing in each experimental period.

Chemical Analyses

Pasture and feces samples were oven-dried at 55º C for 72 h and then ground in a Wiley mill (TECNAL. Piracicaba. São Paulo. Brazil) with 2 mm and 1 mm using a knife mill (Detmann et al., 2012). Samples of feces, pasture, corn, and soybean meal ground at 1 mm were

analyzed for DM (AOAC, 1990; method 930.15), OM (AOAC, 1990; method 924.05), CP (AOAC, 1990; method 984.13), EE (AOAC, 1990; method 920.39 and NDF (Detmann *et al.* 2012; method G-002/1). The samples ground at 2 mm were analyzed for undigestible NDF (uNDF). They were also incubated into the rumen of a ruminally-fistulated cow for 288 h, using non-woven textile bags (100 g/m^2), and NDF was analyzed from the post-incubation material (Valente et al., 2011).

The non-fiber carbohydrates (**NFC**, g/kg) contents in the pasture, supplement, and feces were calculated as proposed by NRC (2001).

$$NFC = OM - (NDF + CP + EE)$$

NFC = non-fiber carbohydrate (g/kg); NDF = neutral detergent fiber (g/kg) CP = crude protein (g/kg); OM = organic matter (g/kg) and EE = ether extract (g/kg).

Additionally, fecal samples were analyzed for chromic oxide (method INCT-CA M-005/1) and titanium dioxide (method INCT-CA M-007/1) according to Detmann *et al*. (2012).

Fecal DM excretion was estimated based on the ratio between the amount of the indicator provided (Cr_2O_3) and its concentration in feces (Detmann et al., 2001). Next, individual supplement intake was estimated using TiO_2 as the external marker (Titgemeyer et al., 2001), using the equation:

$$DMSI = \frac{(FE \times MCF)}{MCS}$$

DMSI = dry matter supplement intake (kg/day); FE = fecal excretion (kg/day); MCF = marker concentration in the animal feces (kg/kg), MCS = marker concentration in the supplement (kg/kg).

Individual pasture intake was estimated using uNDF as the internal marker (Detmann et al., 2001), using the equation:

$$DMPI = \frac{(FE \times CMF) - (CMS \times DMSI)}{CIF}$$

DMPI= dry matter pasture intake (kg/day); CMF = concentration of marker (uNDF) in feces (kg/kg); CIF = concentration of indicator(uNDF) in the forage (kg/kg); DMSI = dry matter supplement intake (kg/day); FE = fecal excretion (kg/day) and CMS = concentration of the marker(uNDF) in the supplement (kg/kg).

The urine samples were analyzed for N (AOAC International, 2005; method 990.13), and creatinine was measured using the colorimetric endpoint method using picrate and acidifier (Labtest Diagnostica S. A. Lagoa Santa, Minas Gerais, Brazil). In addition, the concentrations of uric acid and allantoin in the urine were determined according to Fujihara et al. (1987) and Chen and Gomes (1992), respectively. Total daily urinary excretion was estimated using the daily creatinine excretion, as proposed by (Chizzotti et al., 2008) for Holstein cattle. Ruminal microbial CP 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).

Metabolizable protein (MP) intake was calculated as true digestible microbial protein synthesis plus digestible rumen undegraded protein. The true fraction and digestibility of microbial protein were considered 80% (NRC, 2001). Rumen-undegradable protein intake was estimated as the difference between CP intake and rumen-degradable protein intake, and its intestinal digestibility adopted for rumen-undegradable protein (RUP) was 80% (NRC, 2001).

Blood Sample and Analysis

Blood samples were collected on the 13th day of each collection period by puncturing the jugular vein. We used 10 mL vacutainer tubes containing separator gel and clot activator (silica) for serum collection and 5 mL tubes containing sodium fluoride for plasma collection. The tubes were kept on ice until centrifugation (3,000 × *g* at 4ºC for 20 min). Then, the serum and plasma were separately pipetted into Eppendorf tubes and stored (-20ºC) until analysis.

The serum sample was used to analyze blood urea nitrogen (BUN), total protein, albumin, total cholesterol, triglycerides, insulin and IGF-1, and the plasma sample was used for

glucose analysis. Concentrations of BUN, glucose, total protein, albumin, total cholesterol, and triglycerides were measured by biochemical multi-analyzer (HumanStar 300; Human GmbH, Wiesbaden, DEU) with Bioclin kits. In addition, analyses of insulin and IGF-I were performed using chemiluminescence immunoassay (Immulite 1000; Siemens Medical Solutions Diagnostics, Los Angeles, USA).

Statistical Analysis

The response variables were analyzed using the PROC GLIMMIX procedure of SAS (Statistical Analysis System University Edition). For all performance variables (BW and body measurements), measurements at d 0 were tested as covariates separately for each outcome variable and subsequently removed from the models as they were all non-significant ($P > 0.05$). The response variables were analyzed as a completely randomized design, and the season was included as a repeated measure in the model as follows:

$$Y_{ijk} = \mu + T_i + \delta_{ij} + S_k + (T \times S)_{ik} + \varepsilon_{ijk}$$

Where: Y_{ijk}= observation ijk; μ= the overall mean; T_i = fixed effect of treatment i; δ_{ij} = random error with mean 0 and variance σ_δ^2, the variance between animals within the treatment and it is equal to the covariance between repeated measurements within animals; S_k= fixed effect of season k; $T \times P_{ik}$= fixed effect of interaction between the treatment i and season k; and ε_{ijk}= random error with the mean 0 and variance σ^2, the variance between measurements within animals. Fifteen variance-covariance structures were tested for each response variable. Then, we used the variance-covariance structure that provided the best fit based on the lowest Akaike information criterion. The variance components was the variance-covariance structure used. The observations with externally studentized residuals greater than |2.5| were first checked to make sure they were not a recording error. After checking and evaluating that the information was not a recording error, the observations were considered outliers and consequently excluded from the dataset. The analysis of possible outliers was performed only once for each outcome

variable in order not to create erroneous results. After the outlier analysis, one animal was removed from the database as an outlier. The least-square means were considered different when $P \leq 0.05$, and the tendency was used when $0.05 < P < 0.100$

Least square means (treatment effect) were compared by the following orthogonal contrasts: 1) effect of supplementation (non-supplemented animals-CON vs. S12 + S24 +S36) supplemented animals-SUP); 2) linear effect of supplement CP level; and 3) quadratic effect of supplement CP level. Those same contrasts were evaluated within each season in case of significant interactions.

Results

Intake and Digestibility

Supplementation did not affect (Table 4) the intakes of pasture (kg/d and g/kg of BW), NDF, and uNDF (kg/day; $P > 0.050$). Animals with SUP had greater intakes (Table 4) of DM (kg/d and g/kg BW; $P < 0.015$), CP, and OM (kg/d; $P < 0.050$), metabolizable energy (Mcal/d; $P < 0.001$), and metabolizable protein (g/d; $P < 0.001$). Among SUP animal, there was no effect (Table 4) of CP level ($P > 0.050$) on intakes of pasture. DM (kg/d or g/kg BW), NDF, uNDF, OM (kg/d), and metabolizable energy (Mcal/d). Among SUP animals, we observed a linear positive response (Table 4) for CP (kg/d – $P < 0.001$), RUP (g/d; $P = 0.005$) and metabolizable protein intake (g/d; $P < 0.001$).

Moreover, for the relationship between CP and digestible OM (CP:DOM) intake, there was an interaction between treatment and season ($P = 0.010$; Table 4), in which the SUP animals had greater CP:DOM intake than non-supplemented animals in all seasons (Figure 2), but among SUP animals, there was a positive linear response of CP level in all seasons (Figure 2).

We observed an interaction between treatment and season ($P < 0.05$) for all digestibility coefficients (CP, NDF, and OM; Table 4). Supplemented animals had greater apparent digestibility of OM and CP in dry and DRT seasons ($P < 0.05$; Figure 3). However,

non-supplemented animals had greater NDF digestibility in the rainy season (Figure 3). There was a positive linear response of CP level in CP digestibility among SUP animals during all periods ($P < 0.050$; Figure 3). There was a positive linear response to supplemental CP for OM digestibility in the RDT, dry, and rainy seasons ($P < 0.05$; Figure 3). For NDF digestibility, we observed a positive linear response of CP supplement in the RDT and rainy seasons, and a convex quadratic response during the dry season ($P< 0.05$; Figure 3)

Performance and body measurements

The SUP animals had greater final BW, withers height, thoracic perimeter, body length, and rump height than non-supplemented animals ($P < 0.05$; Table 5). However, there was no detected difference in final BW and body measurements among SUP animals.

When evaluating relative performance, SUP animals had greater ADG, withers height gain, and thoracic perimeter than non-supplemented animals ($P < 0.050$; Table 6). In addition, among SUP animals, there was a quadratic response ($P = 0.040$) to ADG (Table 6), which was the greatest ADG observed in treatment S24 (Table 6).

Urine and Nitrogen Balance

We observed a greater urea excretion (mg/d), nitrogen intake (g/d), nitrogen retention (g/d) and retention coefficient (g/kg) in SUP animals when compared with non-supplemented animals ($P < 0.05$; Table 7). Non-supplemented animals had greater microbial efficiency in g/kg of digestible OM and g/ kg of CPI ($P < 0.050$; Table 7) than SUP animals.

Among SUP animals, we observed a linear positive effect ($P < 0.001$) of CP supplementation on urea excretion, nitrogen intake, nitrogen retention, and retention coefficient (Table 7). However, the supplemental CP did not affect ($P > 0.050$) microbial protein production (g/d), microbial efficiency (g/kg of OM digestible), and g/ kg of CPI (Table 7).

Despite the observed results, microbial protein synthesis results should be carefully evaluated. Although functional, estimating microbial protein synthesis using purine derivates

has intrinsic limitations. According to Hristov et al. (2019), the principal concern with purines is related to unequal purine-to-total N ratios in protozoal and bacterial pools coupled with the need to assume that dietary purines are completely degraded in the rumen (Smith and Mcallan, 1970; Mcallan and Smith, 1973). Furthermore, recent studies have shown that dietary purines can contribute between 13 and 33% of the purine flow in the duodenum of cattle, which would overestimate microbial protein synthesis (Pérez et al., 1997; Vicente et al., 2004; Hristov et al., 2005). Thus, Hristov et al. (2019) mentioned that overall, calculating absolute changes in MPS based on the urinary purine derivatives is not advisable; however, for a controlled experimental setting (which is the case of this study), differences in excretion of total purine derivatives in urine could indicate differences in microbial protein synthesis. Thus, although the absolute microbial protein synthesis value observed in this study should not be used as a reference, considering that purine derivates are the most practical method to determine microbial protein synthesis in non-cannulated grazing animals, the variations among treatments could still render objective interpretations.

Blood

We observed an interaction between treatment and season ($P = 0.021$) for blood glucose (Table 8), where SUP animals had greater glucose concentration ($P < 0.05$) in all seasons than non-supplemented animals (Figure 4). Additionally, we observed a quadratic response among SUP animals only during the RDT and dry seasons, with the greatest glucose concentration observed for S24 (Figure 4). No differences ($P > 0.05$). among SUP animals were detected in the other seasons

Supplemented animals had greater blood concentrations ($P > 0.05$) of urea and IGF-1 (Table 8) than non-supplemented animals. Among SUP animals, there was a negative linear response to CP supplementation in triglycerides ($P = 0.003$) and positive linear response ($P < 0.001$) to urea (Table 8). For cholesterol, triglycerides, total protein, albumin globulin, and

insulin, there were no detected differences ($P > 0.05$) between SUP and non-supplemented animals (Table 8). In addition, we did not observe differences ($P > 0.050$) in blood cholesterol, total protein, albumin, globulin, IGF-1, and insulin among SUP animals (Table 8).

Discussion

Pasture Measurements

In this study, the high pasture availability (g/kg of BW) during the dry season was due to the utilization of two paddocks per day because of the low DM production per hectare during that season. Paulino et al. (2004) suggested offering at least 40 to 50 g/kg BW of potentially digestible DM (pdDM) to promote maximum performance of grazing animals (in studies with beef animals). The present study's pasture availability was 39.58, 42.34, 29.79, and 44.04 g/kg of BW during the RDT, dry, DRT, and rainy seasons, respectively. The values observed in the present study are within the range suggested by Paulino et al. (2004), given that researchers estimated pasture availability by cutting sampling forage at the ground level, while we determined pasture availability by sampling the grazing stratum (Machado et al., 2019). Thus, we assume that our animals had no limitation of pasture intake during all evaluated seasons.

Intake and Digestibility

Previous studies have described the benefits of CP supplementation to pasture-fed cattle on DMI since it improves the rumen environment by providing substrate (carbon, peptides, and amino acids) for the growth of fibrolytic bacteria, increasing fiber digestibility and, consequently, pasture intake (Souza et al., 2010; Franco et al., 2017). However, other studies have also highlighted that N supplementation mainly stimulates pasture intake when the forage CP level is less than 70 g/kg DM of pasture (Lazzarini et al., 2009). In our study, the lowest pasture CP level observed was 77.89 g/kg of DM (during the dry season, Table 3), thus explaining why there was no difference in pasture intake among the treatments (Table 4). In this sense, it can be concluded that the supplementation level used in this study (5 g/ kg of BW)

had no beneficial effect on pasture intake, but also did not cause a substitutive effect on pasture intake.

Despite no differences in pasture intake, the supplementation promoted greater DMI (kg/d and g/kg of BW). Consequently, non-supplemented animals had lower metabolizable energy (Mcal/d) and protein (g/d) intakes than SUP animals. Regardless of treatments, all our diets were high in NDF due to the high concentration of pasture NDF (average 629.3 g/kg), which likely affected the intake limit (Allen, 1996). However, we highlight that there were no differences in NDF intake between SUP animals and non-supplemented animals and among SUP animals with increasing levels of CP (Table 4). Moreover, despite the large numerical difference (no statistical analyse) in the levels of EE and NFC among supplement (Table 2) due to ingredients used in its formulation (soybean meal and ground corn), when we simulated the diets in the NRC 2001 program, these differences were not enough to ensure a difference in energy intake among SUP animals, as demonstrated in Table 4.

The relationship between CP and DOM is an indicator of the protein:energy dietary ratio and might be linked to the metabolic effects of protein on intake. The synchronism between protein and energy may promote the maximum microbial production and reduce N losses via ammonia and energy of carbohydrates, promoting digestion improvements, mainly NDF digestibility (Neto et al., 2007); hence, increasing the availability of metabolizable energy and protein for animal growth. Thus, the CP:DOM (gCP/kgDOM) works as a regulating parameter, directly influencing voluntary DMI (Poppi and McLennan, 1995; Detmann et al., 2014). In the present study, the CP:DOM ratios observed were 208.2, 202.2, 243.3, and 271.1 g of CP/kg DOM for CON, S12, S24, and S36, respectively. Non-supplemented animals had a lower CP:DOM ratio than SUP animals in all seasons (Figure 2), with an average value (208.2 g/kg DOM) lower than that suggested for optimizing DMI which could explain once again the lower DMI in non-supplemented animals than SUP animals. Among SUP animals, we observed a

trend of quadratic response for DMI, where the highest DMI was observed for S24. Dietary CP might explain this quadratic response in CP:DOM ratios in SUP animals, which showed a linear response in all seasons (Figure 2). The lowest DMI observed in S12 and S36 than S24 may be explained by different reasons. First, the low CP:DOM ratio in S12 was below the minimum suggested by Detmann et al. (2014) and Poppi and McLennan (1995) for maximum DMI (210 g/kg). Second, the S36 treatment likely had excess CP relative to the energy available. This excess of N in relation to energy (CP:DOM) has consequences on animal metabolism, leading to greater heat production (Poppi and McLennan, 1995), deficiency of ATP in liver metabolism due to a high rate of the utilization urea cycle (Visek, 1984), and animal indisposition due to excess ammonia in the blood (Detmann et al., 2014). Indeed, our S36 animals had greater urea excretion, which indicates that some of those metabolic pathways were in play in those animals. Additionally, a quadratic response in intake due to increased CP supplementation was also observed in other studies (Sampaio et al., 2010; Detmann et al., 2004). Corroborating with the results presented in this study, we simulated the experimental diets in a formulation program (NRC, 2001), and observed that S36 had greater metabolizable protein available for gain than metabolizable energy available for gain. Similar values were observed for S24. Lastly, greater metabolizable energy available for gain, compared to metabolizable protein, was observed for S12.

The digestibility coefficient of nutrients can be influenced by factors such as pasture quality, level intake, chemical compositions of the feed, and others. CP supplementation improved overall nutrient digestibility in our study, mainly in the seasons where pasture showed the worst nutritional values (Figure 3). This is supported by the greater digestibility coefficient of the SUP animals for DM and OM in the dry, DRT, and rainy seasons, which had a poorer quality pasture (Table 1). Corroborating these results, SUP animals had greater CP digestibility in the dry and DRT seasons. Further, we observed a positive linear effect among the SUP

animals in almost all seasons for DM, OM, NDF, and CP, except for DM, OM, and NDF in the DRT season and NDF in the dry season.

The benefits of supplementation were increased in seasons where forage had the lowest CP content (dry, TDR, and rainy), indicating the benefits of CP supplementation for grazing cattle. Previous studies have shown that the benefits of CP supplementation are increased when forage has low CP content (Figueiras et al., 2016; da Silva-Marques et al., 2018), mainly increasing nutrient intake and digestibility coefficients (Kang et al., 2015; McGuire et al., 2013). In our case, the improvement in digestibility is caused by the increase in the supply of essential substrates (N and starch), minimizing the deficiencies of nutrients required by ruminal microorganisms. Nitrogen is likely the most important nutrient in this case since it is involved in the synthesis of enzymes linked to the degradation of fiber in low-quality forages (Detmann et al., 2014; Souza et al., 2010).

Performance and Body Measurements

We observed that the animals SUP had greater ADG, withers height, and thoracic perimeter gain (mm/d) than non-supplemented animals. Consequently, SUP animals had greater final BW weight, final thoracic perimeter, final body length, final withers, and rump height. In general, SUP animals had greater nutrient intake and digestibility. Consequently, they had greater metabolizable energy and protein intake, which resulted in better performance (Poppi and McLennan, 1995; Paulino et al., 2008).

We observed a quadratic effect for final body weight, final thoracic perimeter, and ADG in SUP animals, with S24 animals having the best performance. The lowest performance was observed in the S12 and S36 animals and might be explained by deficiency and excess of N in those treatments, respectively. Previous studies carried out with supplementation of cattle in pasture also have observed quadratic responses to N supplementation (Detmann et al., 2004). As mentioned before, in a tropical pasture system, the first limiting nutrient is N (Detmann et

al., 2014; Leng, 1990), which might have limited microbial growth and subsequent intake, digestibility, and performance (Leng, 1990; Paulino et al., 2008) of the S12 animals. Hence, we observed the lowest dietary CP, MP intake, CP:DOM ratio, and digestibility in S12 animals compared to only SUP animals.

The decline in the performance of S36 animals may be related to the excess of dietary CP since there is a need to excrete it as urea via urine, and this process increases heat production (Martin and Blaxter, 1965). Additionally, increased ammonia level in the liver due to excessive-high dietary CP is linked to decreased levels of NADPH, NADP, and NADH, due to competition for ATP between the urea cycle and other gluconeogenic pathways (NRC, 2001), thus damaging energetic metabolism in the body (Chalupa et al., 1970) and ATP synthesis in the liver and other tissues. Moreover, there was a decrease in DMI in the S36 animals in our study. High N diets usually involve increased heat production due to increased urea cycling. This could lead to extra energy used for dissipation of this additional heat increase and augmented heat stress, impacting animals' performance (Dettmann et al., 2004).

Urine and Blood

The greater N intake in SUP animals promoted a greater blood urea concentration and, consequently, greater urea excretion (mg/d) than in non-supplemented animals. Moreover, SUP animals were more efficient than non-supplemented animals regarding N metabolism since they had greater retained nitrogen and retention coefficient. Once again, these results highlighted the beneficial effects of N supplementation, which improved N status in the body and N components balance between rumen and bloodstream, consequently improving animal performance (Lazzarini et al., 2016). Furthermore, we observed higher glucose and IGF-1 in SUP animals than in non-supplemented animals. Therefore, the benefits of N supplementation go beyond their effects on intake and digestibility, such as improved energy and nitrogen status, which resulted in greater levels of IGF-1 and glucose.

A positive linear response of supplemental CP was detected for N intake, retained N, and retention coefficient among SUP animals. Furthermore, Detmann et al. (2014) observed a positive relationship between CP supplementation and N retention. Thereby, the supplementation with N allowed for greater availability of N for all metabolic and physiological processes in the body. Nitrogen supplementation causes a negative impact on the breakdown rate of myofibrillar protein and positively affects N and anabolic hormone concentrations in the blood (e.g., IGF-1; Franco, 2017; Batista et al., 2016)). These responses resulted in increased nitrogen retention in the animal's body.

Interestingly, microbial protein production estimates (g/d) were not different between SUP and non-supplemented animals. However, SUP animals had lower microbial efficiency in g/kg of digestible OM and g/kg of CPI. Thus, we observed an increase in the efficiency of non-supplemented animals because of a severe deficiency in rumen N or ammonia (Cabral et al., 2014). Thus, non-supplemented animals were highly dependent on recycled N to keep microbial activity in the rumen environment (Detmann et al., 2014), increasing their efficiency per N intake unit. Furthermore, in this situation, the animals decrease their urinary N excretion and increase the recycling of dietary N within the rumen (Hennessy et al., 1983). Lastly, animals may mobilize protein from tissue in N deficient diet to keep the amount of recycled N (NRC, 2001), with a consequent flow of N to the abomasum greater than N intake (Detmann et al. 2014).

Curiously, we did not observe an effect of supplemental CP on microbial protein production in g/d, microbial efficiency in g/kg of digestible OM and g/kg of CPI among the SUP animals. Similar results were observed by Lazzarini et al. (2016) and Souza et al. (2010), highlighting that increasing N content alone is not enough to ensure an increase in microbial protein synthesis. In our study, despite the similar energy level in the diets (TDN – Table 2)

among SUP animals, the increase in the CP:DOM ratio (Table 4) was not enought to ensure an increase in microbial protein synthesis.

As mentioned previously, SUP animals had greater glucose blood concentration than non-supplemented animals. The difference observed would mostly be due to greater intake and digestibility of nutrients in SUP than in non-supplemented animals, which increased glucogenic precursors in the blood. Our concentrates were rich in corn, which would stimulate propionate production in the rumen. Propionate is then transported to the liver and converted into glucose, increasing gluconeogenic precursors' availability (Young, 1977). Moreover, N supplementation improves N status in the body, decreasing the requirements of amino acid catabolism for urea synthesis in the liver (Parker et al., 1995). In this sense, N supplementation could decrease the use of gluconeogenic amino acids for urea synthesis, enhancing its availability for glucose synthesis and contributing to raised glucose content in the body in SUP animals. Lastly, we observed a quadratic effect among SUP animals during RDT and dry season, with the greatest concentration in S24 animals, resulting from greater intake of DM and OM and, consequently, greater intake of gluconeogenic precursors.

The IGF-1 is an endocrine regulator of muscle growth in cattle, with independent action and promotes important links between growth hormone and the metabolic growth process, mainly in skeletal muscle (Pell and Bates, 1990; Lobley, 1992). The IGF-1 concentrations are usually raised in response to diet, with a more prominent response to dietary protein concentration than energy concentration (Pell and Bates, 1990). Furthermore, SUP animals had greater IGF-1 serum concentration than non-supplemented animals. Similar results were obtained by Franco et al. (2017) and Rufino (2015). Interestingly, the increase in IGF-1 was also linked to an increase in glucose concentration, which may have accelerated the anabolic stimulus for body protein synthesis, justifying the greater N retained and retention coefficient in SUP animals, as mentioned previously.

Among SUP animals, we observed a linear negative response for blood triglycerides concentration with the increase in supplement CP. Blood triglycerides in growing heifers usually originate from body reserves or diet mobilization. In the present study, the SUP animals were in a positive energy balance. In this sense, the range in blood triglyceride concentrations observed can only be explained by diet composition variation or the proportion of each ingredient (ground corn and soybean meal) used in the supplement composition. The supplements with higher CP had a greater proportion of soybean meal. This switch likely caused a slight decrease in supplemental energy density, which may have caused the observed decrease in blood triglycerides.

Conclusion

The supplementation of grazing dairy heifers improved DMI, OMI, ratio CP:OM, energy, and protein metabolizable intake. Moreover, supplementation increased the digestibility coefficient of DM and OM in the dry, DRT, and rainy seasons and CP in the dry and DRT seasons. In addition, the CP supplementation resulted in greater levels of glucose and IGF-1 and improved N balance. Consequently, SUP animals had greater performance than non-supplemented animals. Among SUP animals, we observed the best results in animals supplemented with 24% CP in the supplement, which had the greatest ADG, DM, and OM intake and glucose concentration in the RDT and dry seasons. Therefore, the supplementation with 5 g/kg of BW of supplement composed of soybean meal and ground corn for Holstein × Gyr crossbreed heifers grazing intensively managed *Brachiaria decumbens* throughout the year promoted greater levels of intake and digestibility, improved nitrogen balance, and animal performance, and 24% of CP showed the best results among supplemented animals. However, we emphasize that further research should be carried out with grazing animals throughout the seasons to validate the observed results.

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Table 1: Production characteristics and chemical composition of pasture *Brachiaria decumbens* throughout experimental periods

Item	Season			
	RDT[1]	Dry[2]	DRT[1]	Rainy[1]
Accumulated pasture (kg DM/ha per cycle)	1785	1080	1778	3092
Accumulated pasture (kg DM/paddock per cycle)	300	180	320	550
Pasture allowance (kg DM/animal per day)	8.00	9.67	8.45	14.4
Body weight (kg)	202	233	283	326
Forage pasture (g DM of herbage/kg BW)	39.6	41.4	29.8	44.0
Chemical composition (g/kg)				
Dry matter	173	283	255	226
Crude protein	108	77.8	86.8	100
Neutral detergent fiber	598	619	624	664
Undigestible neutral detergent fiber	143	200	191	173
Ether extract	40.2	32.7	37.2	38.5
Organic matter	900	920	924	923
Non-fiber carbohydrates	153	190	175	120

[1]during RDT, DRT and rainy season we used one paddock/day.

[2]during the dry season we used two paddocks/day.

RDT = rainy dry transition- February 7 to April 24.

Dry - April 25 to July 27.

DRT = dry rainy transition - July 28 to November 5.

Rainy - November 6 to January 22.

Table 2: Proportion of ingredients and chemical composition of supplement for crossbred Holstein × Gyr heifers supplemented with different levels of crude protein throughout the year

Item	Adaptation[1]	Supplement[2]		
		S12	S24	S36
Ingredients, g/ kg of diet DM				
Ground corn	751	909	593	277
Soybean meal	249	91	407	723
Chemical composition, g/kg of diet DM				
Dry matter	909	907	912	918
Crude Protein	174	116	232	348
Neutral detergent fiber	116	104	128	150
Indigestible neutral detergent fiber	14.0	14.6	13.4	12.2
Ether extract	53.3	56.4	50.2	43.9
Non-fiber carbohydrate	618	694	543	395
Organic Matter	962	971	953	936
Total digestible nutrients[3]	815	844	787	789

[1]Supplemented provided to all heifers across the adaptation period;

[2]S12= supplement formulated to contain 12% crude protein; S24= supplement formulated to contain 24% crude protein and S36 = supplement formulated to contain 36% crude protein;

[3]Total digestible nutrients were calculated using equation from NRC (2001).

Table 3: Crude protein (CP) level in diet (g/kg of dry matter) throughout the experiment period for crossbred Holstein × Gyr heifers non-supplemented (CON) or supplemented with different levels of crude protein throughout the year

Season	Treatments[1]			
	CON	S12	S24	S36
[2]RDT	108	109	125	144
[3]Dry	78.0	84.5	106	128
[4]DRT	86.8	92.8	114	136
[5]Rainy	100	103	121	140

[1]CON = Control; S12 = supplement with 12% of CP; S24 = supplement with 24% of CP; S36 = supplement with 36% of CP;

[2]RDT = rainy dry transition- February 7 to April 24.

[3]Dry - April 25 to July 27.

[4]DRT = dry rainy transition - July 28 to November 5.

[5]Rainy - November 6 to January 22.

Table 4: Least squares means for intake and apparent digestibility of nutrients of crossbred Holstein × Gyr heifers non-supplemented (CON) or supplemented with different levels of crude protein throughout the year

Item	Treatments[3]				SEM	*P*-value				
	CON	S12	S24	S36		S	T × S[4]	SUP[5]	L[6]	Q[7]
Intake										
Supplement (kg/d)	--	1.17	1.21	1.09	--	--	--	--	--	--
Pasture (kg/d)	5.89	5.79	6.07	5.27	0.245	0.001	0.449	0.532	0.162	0.082
Dry matter (kg/d)	5.89	6.96	7.29	6.36	0.262	0.001	0.237	0.002	0.128	0.062
Pasture (g/kg of BW)	23.4	21.9	21.9	21.6	0.912	0.001	0.807	0.133	0.877	0.863
Dry matter (g/kg of BW)	23.4	26.1	26.4	26.1	0.977	0.001	0.721	0.015	0.983	0.852
Crude protein (kg/d)	0.56	0.68	0.85	0.88	0.030	0.001	0.117	0.001	0.001	0.060
Neutral detergent fiber (kg/d)	3.71	3.64	3.85	3.37	0.150	0.001	0.324	0.619	0.228	0.072
Indigestible neutral detergent fiber (kg/d)	1.03	1.05	1.10	0.94	0.042	0.001	0.506	0.997	0.111	0.055
Organic matter (kg/d)	5.40	6.35	6.62	5.76	0.237	0.001	0.309	0.003	0.103	0.062
Metabolizable energy (Mcal/d)	9.96	12.2	13.0	12.5	0.565	0.001	0.332	0.001	0.722	0.353
Rumen degradable protein (g/d)	392	388	337	423	32.4	0.001	0.999	0.798	0.471	0.089
Rumen undegradable protein (g/d)	233	326	492	475	36.7	0.001	0.398	0.001	0.005	0.051
Metabolizable protein (g/d)	437	508	609	651	26.1	0.001	0.274	0.001	0.001	0.364
CP: DOM (g CP/kg DOM)[1,2,5]	208	202	243	271	3.47	0.001	0.010	0.001	0.001	0.135
Apparent digestibility										

Dry matter (g/kg)[2]	457	499	499	539	5.96	0.001	0.001	0.001	0.001	0.009
Neutral detergent fiber (g/kg) [6]	582	552	564	584	5.38	0.001	0.001	0.010	0.001	0.507
Crude protein (g/kg) [2]	410	403	502	628	10.9	0.001	0.001	0.001	0.001	0.357
Organic matter (g/kg) [2]	502	533	540	574	5.47	0.001	0.005	0.001	0.001	0.049

[1]DOM = digestible organic matter (apparent DOM was estimated in the total tract);

[2]CP:DOM = ratio between CP and DOM contents;

[3]CON = Control; S12 = supplement with 12% of CP; S24 = supplement with 24% of CP; S36 = supplement with 36% of CP;

[4]S = season effect; T × S = interaction between season and treatment; SUP = supplemented *vs* non-supplemented; L = linear effect among supplemented animals; and Q = quadratic effect among supplemented animals;

[5]The interaction T × S for CP:DOM ratio is described in Figure 2;

[6]The interaction T × S for apparent digestibility of DM, neutral detergent fiber, CP and organic matter are described in Figure 3.

Table 5: Final body measurements and final body weight of crossbred Holstein × Gyr heifers non-supplemented or supplemented with different levels of crude protein throughout the year

Item	Treatments[1]				SEM	*P* – value[2]		
	CON	S12	S24	S36		SUP	L	Q
Final BW (kg)	307	338	345	316	0.828	0.008	0.060	0.068
Final withers height (cm)	118	121	123	121	0.893	0.001	0.923	0.071
Final thoracic perimeter (cm)	157	164	165	161	1.41	0.001	0.168	0.206
Final body length (cm)	131	134	138	137	1.49	0.006	0.461	0.246
Final rump width (cm)	43.8	44.7	46.1	44.0	0.924	0.293	0.611	0.143
Final rump length (cm)	45.2	46.8	46.5	45.6	0.754	0.199	0.262	0.776
Final rump height (cm)	123	125	127	125	0.926	0.044	0.719	0.084

[1]CON = Control; S12 = supplement with 12% of CP; S24 = supplement with 24% of CP; S36 = supplement with 36% of CP;

[2]SUP = supplemented *vs* non-supplemented; L = linear effect among supplemented animals; Q = quadratic effect among supplemented animals.

Table 6: Average daily gain (ADG) and body measurements gain (mm/day) of crossbred Holstein × Gyr heifres non-supplemented (CON) or supplemented with different levels of crude protein throughout the year

Item	Treatments[2]				SEM	*P*-value[3]				
	CON	S12	S24	S36		S	T × S	SUP	L	Q
ADG (kg/d)[1]	0.39	0.46	0.49	0.42	0.020	0.001	0.713	0.006	0.192	0.040
Withers height gain	0.41	0.51	0.57	0.51	0.033	0.001	0.567	0.003	0.890	0.178
Thoracic perimeter gain	0.73	0.92	0.95	0.85	0.044	0.266	0.671	0.001	0.284	0.251
Body length gain	0.49	0.60	0.70	0.66	0.074	0.001	0.115	0.063	0.602	0.439
Rump length gain	0.27	0.32	0.30	0.27	0.027	0.001	0.182	0.474	0.276	0.833
Rump width gain	0.28	0.31	0.35	0.30	0.026	0.002	0.282	0.157	0.555	0.136
Rump height gain	0.39	0.41	0.49	0.42	0.029	0.001	0.217	0.225	0.820	0.025

[1]ADG = average daily gain;

[2]CON = Control; S12 = supplement with 12% of CP; S24 = supplement with 24% of CP; S36 = supplement with 36% of CP;

[3]S = season effect; T × S = interaction between season and treatment; SUP = supplemented vs non-supplemented; L = linear effect among supplemented animals; and Q = quadratic effect among supplemented animals.

Table 7: Urine and nitrogen balance of crossbred Holstein × Gyr non-supplemented (CON) heifers or supplemented with different levels of crude protein throughout the year

Item	Treatments[1]				SEM	*P*-value[2]				
	CON	S12	S24	S36		S	T × S	SUP	L	Q
Urea (mg/d)	236	237	375	583	19.5	0.001	0.696	0.001	0.001	0.130
Microbial protein (g/d)[3]	392	388	337	423	32.4	0.003	0.999	0.798	0.472	0.089
Microbial efficiency (g/kg DOM)[4]	177	124	104	146	14.0	0.001	0.314	0.002	0.292	0.082
Microbial efficiency (g/ kg CPI)	849	635	433	542	61.0	0.001	0.108	0.001	0.309	0.057
Nitrogen intake (g/d)	90.8	108	136	140	4.80	0.001	0.136	0.001	0.001	0.060
Nitrogen retained (g/d)	11.8	13.8	28.5	38.2	3.57	0.001	0.240	0.001	0.001	0.551
Retention coefficient (%)	4.57	8.64	18.5	23.0	2.17	0.001	0.062	0.001	0.001	0.329

[1]CON = Control; S12 = supplement with 12% of CP; S24 = supplement with 24% of CP; S36 = supplement with 36% of CP;

[2]S = season effect; T × S = interaction between season and treatment; SUP = supplemented vs non-supplemented; L = linear effect among supplemented animals; and Q = quadratic effect among supplemented animals;

[3]Microbial protein was estimated as a function of absorbed purines, which was calculated from the excretion of the purine derivatives (uric acid and allantoin);

[4]Apparent DOM was estimated in total tract.

Table 8: Blood parameters of crossbred Holstein × Gyr heifers non-supplemented (CON) or supplemented with different levels of crude protein throughout the year

Item	Treatments[1]				SEM	*P* – value[2]				
	CON	S12	S24	S36		S	T × S	SUP	L	Q
Glucose (mg/dL)[1]	57.9	62.7	66.7	60.5	1.14	0.001	0.021	0.001	0.179	0.001
Cholesterol (mg/dL)	96.5	95.8	93.2	88.1	2.62	0.015	0.906	0.184	0.054	0.702
Triglycerides (mg/dL)	28.4	30.6	29.8	25.9	1.10	0.001	0.832	0.763	0.003	0.233
Total Protein (mg/dL)	7.20	7.44	7.43	7.45	0.121	0.001	0.132	0.087	0.893	0.954
Albumin(mg/dL)	2.98	3.02	2.97	3.02	0.056	0.001	0.545	0.729	0.969	0.455
Globulin (mg/dL)	4.22	4.44	4.46	4.43	0.112	0.005	0.222	0.078	0.096	0.840
Urea (mg/dL)	15.0	16.4	19.5	27.6	1.09	0.001	0.275	0.001	0.001	0.056
IGF-1(mg/dL)	163	183	208	201	9.33	0.001	0.948	0.001	0.181	0.138
Insulin (ng/mL)	1.55	1.04	2.21	2.12	0.465	0.302	0.385	0.637	0.106	0.250

[1]CON = Control; S12 = supplement with 12% of CP; S24 = supplement with 24% of CP; S36 = supplement with 36% of CP;

[2]S = season effect; T × S = interaction between season and treatment; SUP = supplemented vs non-supplemented; L = linear effect among supplemented animals; and Q = quadratic effect among supplemented animals;

[3]The interaction T × S for glucose concentration is described in Figure 4.

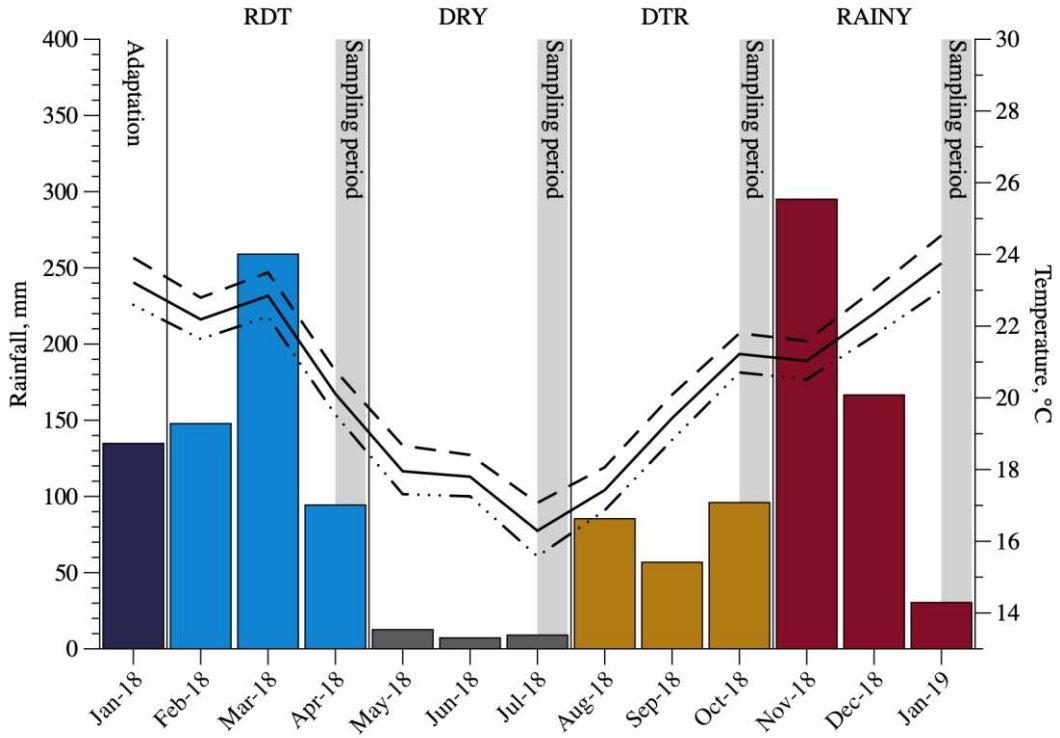

Figure 1: rainfall (mm), minimum, average and maximum (˚C) temperatures throughout seasons, rainy dry transition (RDT), dry, dry rainy transition (DRT) and rainy. Source: Department of Agricultural Engineering – Universidade Federal de Viçosa.

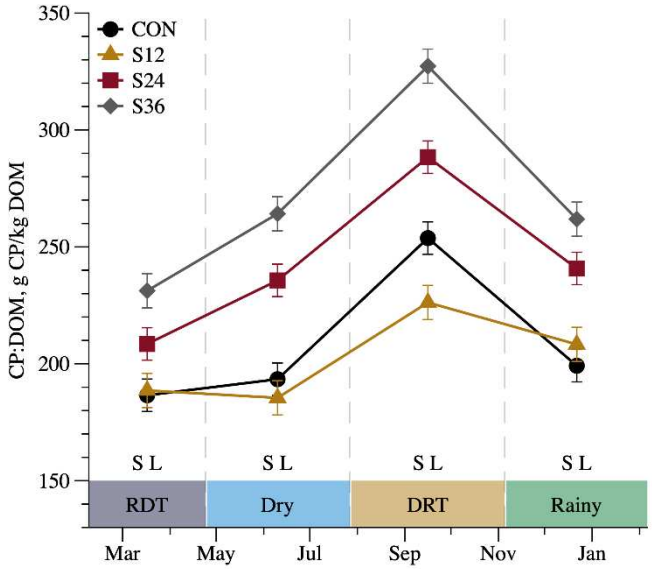

Figure 2: Interaction between treatment and season (P = 0.010) for ratio between crude protein and digestible organic matter (CP:DOM) in g CP/kg DOM of crossbred Holstein × Gyr heifers non-supplemented (CON) or supplemented with 12 (S12), 24 (S24) or 36% (S36) of CP in supplement throughout seasons, rainy dry transition (RDT - February 7 to April 24.), dry (April 25 to July 27), dry rainy transition (DRT - July 28 to November 5) and rainy (November 6 to January 22). S = supplemented vs non-supplemented effect (P < 0.050) and L = linear effect among supplemented animals (P < 0.050).

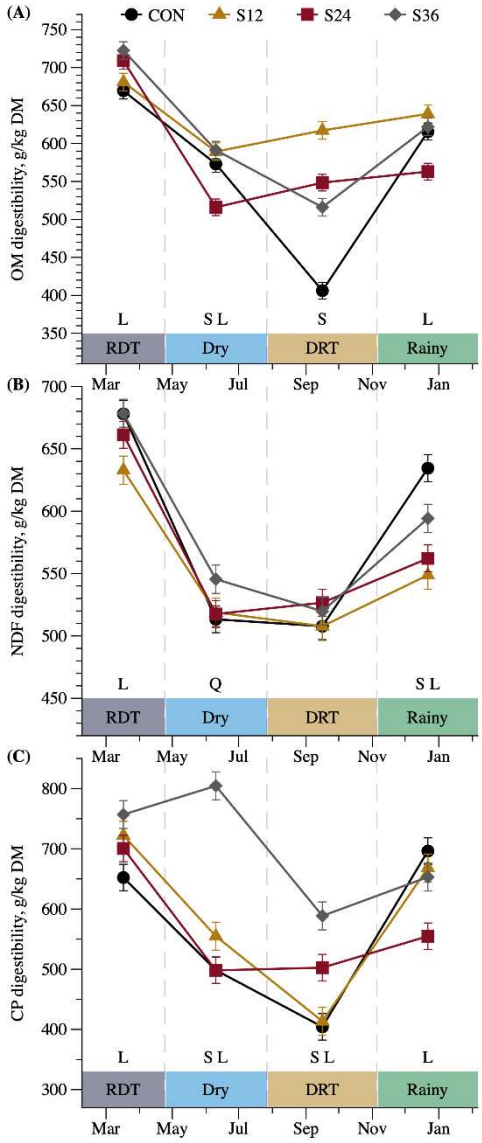

Figure 3: Interaction between treatment and season for apparent digestible coefficient of crossbred Holstein × Gyr heifers of organic matter(P = 0.005; OM- A), neutral detergent fiber (P < 0.001; NDF-B) and crude protein (P < 0.001; CP) throughout seasons, rainy dry transition (RDT - February 7 to April 24.), dry (April 25 to July 27), dry rainy transition (DRT - July 28 to November 5) and rainy (November 6 to January 22)in function of treatments control (CON), supplement with 12 % of CP (S12), supplement with 24 % of CP (S24) and supplement with 36 % of CP (S36). S = supplemented vs non-supplemented (P < 0.050), L = linear effect among supplemented animals (P < 0.050) and Q = quadratic effect among supplemented animals (P < 0.050).

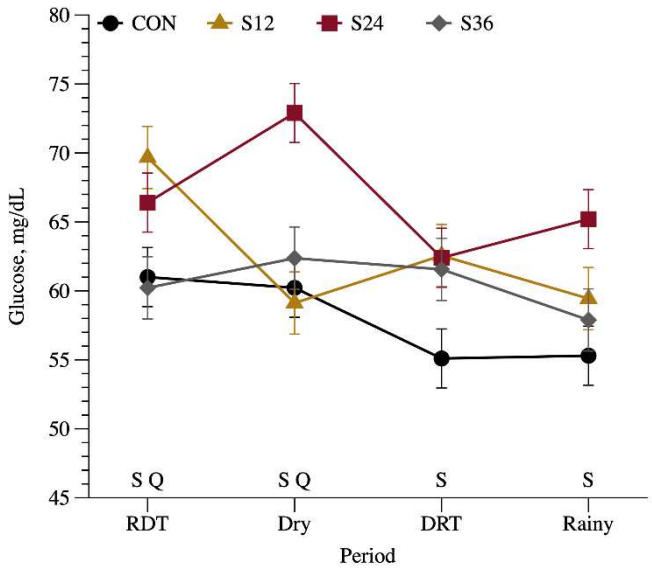

Figure 4: Interaction between treatment and season (P = 0.021) for glucose concentration (mg/dL) of crossbred Holstein × Gyr heifers non-supplemented (CON) or supplemented with 12 (S12), 24 (S24) or 36% (S36) of crude protein in supplement throughout seasons, rainy dry transition (RDT - February 7 to April 24.), dry (April 25 to July 27), dry rainy transition (DRT - July 28 to November 5) and rainy (November 6 to January 22). S = supplemented vs non-supplemented (P < 0.050) and Q = quadratic effect among supplemented animals (P < 0.050).

Chapter 2

Expression of enzymes involved in the urea cycle, and muscle tissue and mammary gland developments of crossbred Holstein × Gyr heifers in a rotational grazing system supplemented with increasing crude protein levels

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Abstract

Studies evaluating the crude protein (CP) supplementation strategies across the year for grazing cattle and its association with the enzymes involved in the urea cycle and muscle and mammary gland developments are scarce. Thus, we aimed to evaluate the effect of supplementation with different levels of CP on expression of genes involved in the urea cycle, and muscle and mammary gland development of Holstein × Gyr crossbreed heifers grazing intensively managed *Brachiaria decumbens* throughout the year. Thirty-eight heifers with average initial BW of 172.5 ± 11.15 kg (mean ± SE) and 8.2 ± 0.54 mo of age were randomly assigned to 1 of 4 treatments: 3 protein supplements (SUP) fed at 5g/kg of body weight, plus a control group (CON - non-supplemented animals). The supplement CP levels evaluated were: 12, 24, and 36%. The experimental period was divided into 4 seasons: rainy, dry, rainy-dry transition (RDT), and dry-rainy transition (DRT). On the penultimate day of each experimental period, ultrasound images of the carcass and mammary gland were taken. Five animals from each treatment were randomly chosen on the last day of each experimental period, and liver and muscle tissue biopsies were performed. The target genes expression evaluated in the current study were the mammalian target of rapamycin (mTOR) and adenosine monophosphate-activated protein kinase (AMPK) in the muscle samples. Carbamoyl phosphate synthetase (CPS), ornithine transcarbamylase (OTC), argininosuccinate synthetase (ASS), arginosuccinate lyase (ASL), and arginase (ARG) were evaluated in the liver samples. Data were analyzed using PROC GLIMMIX of the SAS with repeated measures. We observed a greater rib eye area (cm^2) and fat thickness (mm) in SUP animals than non-supplemented animals. However, we did not observe differences among SUP levels for both variables. No effects of supplementation were detected on mammary gland development. Nevertheless, seasonal effects were observed, where the RDT and dry season had the most and least accumulated fat in the mammary gland, respectively. In muscle, we observed greater expression of AMPK in non-supplemented animals than SUP animals. On the other hand, no differences were observed in gene expression

between SUP and non-supplemented animals and among SUP animals for mTOR. Season affected both AMPK and mTOR, heifers had a greater AMPK gene expression in rainy than RDT. For mTOR, we observed greater gene expression in RDT and DRT than rainy. No differences were observed among RDT, dry and DRT, and betwenn dry and rainy seasons for mTOR. We observed greater gene expression of CPS, ASL and ARG in SUP animals than non-supplemented animals. Among SUP animals, supplement CP linearly affected CPS. In conclusion, the supplementations strategy did not affect mammary gland development and mTOR expression in muscle tissue. However, we observed a season effect on mammary gland development and AMPK and mTOR expression. The CP supplementation increased the rib eye area and fat thickness, directly affecting AMPK expression in the muscle. Moreover, the CP supplementation increased urea cycle enzyme expression, indicating greater urea production in the liver.

keywords: biopsy, liver, nitrogen metabolism, pasture, season

Introduction

Crude protein supplementation strategies for grazing cattle have been focused, because exclusive pasture rarely provides a balanced diet, which limits animal performance (Detmann et al., 2014; Batista et al., 2016; da Silva-Marques et al., 2018). Pasture varies qualitatively and quantitatively throughout the year, where during the rainy season, tropical forages have higher quality (Detmann et al., 2014a), and during the dry season, the pasture quality drops, because of its high fiber and low CP contents (Sampaio et al., 2010). Thus, understanding nitrogen (N) metabolism and its connection with pasture composition throughout the year is necessary to plan the best supplementation strategy for grazing animals. Different supplementation strategies in each season will be essential to improve animal performance and efficiency of N use.

Under normal nutritional conditions, ammonia absorbed in the rumen is transported to the liver, where it enters the ornithine cycle and is converted into urea. Urea recycled may be directed to the gastrointestinal tract, mainly to the rumen, and can be reused as a nitrogen source for microbial protein, or may be excreted in the urine when CP is provided in excess (Lapierre and Lobley, 2001). Thus, excess of urea excreted in the urine is one of the main factors contributing to the low efficiency of N use, which negatively impacts the environment (Dijkstra et al., 2013).

The process of converting ammonia into urea in the ornithine cycle is performed in five steps by five key enzymes (Takagi et al., 2008). First, the cycle needs carbamoyl phosphate synthetase **(CPS)** and ornithine transcarbamylase **(OTC),** located in the mitochondrial matrix; then, the cycle needs argininosuccinate synthetase **(ASS)**, arginosuccinate lyase **(ASL)**, and arginase **(ARG),** located in the cytosol (Takiguchi and Mori, 1995). Despite the crucial importance of these five enzymes in the urea cycle, the literature lacks studies evaluating their expression and association with supplementation strategy and pasture quality across the year.

In general, CP supplementation promotes greater performance and, consequently, high body gain and fat deposition rates in grazing cattle (Guerra et al., 2016; Machado et al., 2019; Machado et al., 2020). On the other hand, previous studies have demonstrated that dairy heifers submitted to a high-performance diet, without an adequate nutritional balance between energy and protein may cause damages to the development of the mammary gland due to excessive fat deposition (Weller et al., 2016; Albino et al., 2017b). However, there are limited studies evaluating CP supplementation strategies, while monitoring body and mammary gland development in grazing dairy heifers.

We hypothesized that the expression of the genes involved in the urea cycle might be affected by the interaction between CP supplementation strategy and season of the year for grazing dairy heifers, where high CP supplement levels would promote a greater expression of genes involved in the urea cycle, mainly during seasons where pasture has high CP content. Moreover, we predicted that CP supplementation would promote a greater activity of genes involved in tissue synthesis, impacting muscle and mammary gland lean tissue gain. Therefore, we aimed to evaluate the effect of increasing CP supplementation on the genes expression of enzymes involved in the urea cycle and muscle tissue growth, mammary gland, and muscle development of Holstein × Gyr crossbreed heifers grazing intensively managed *Brachiaria decumbens* throughout the year.

Materials and methods

The experiment was carried out in the Department of Animal Science of the Universidade Federal de Viçosa (Viçosa, Minas Gerais, Brazil; 20º45’ S and 42º52’). All animal handling and procedures were approved by the ethics committee for animal use at Universidade Federal de Viçosa, under protocol # 041/2017. More information regarding the management practices in this present study can be accessed in (Castro et al., 2022).

Treatments and Experimental design

Thirty-eight crossbred heifers (½ Holstein × ½ Gyr), with average initial BW of 172.5 ± 11.15 kg (mean ± SE) and 8.2 ± 0.54 mo of age, were used. Initially, all heifers were treated for ectoparasites and endoparasites (Ivomec, Paulina, Sao Paulo, Brazil). The heifers were randomly assigned to 1 of 4 treatments: three supplements (SUP) had increasing levels of CP composed of soybean meal and corn ground, plus a control group (CON; n=10) in which the animals received only mineral mixture ad libitum. The supplements had 12, 24 and 36% of CP for treatments S12 (n=9), S24 (n=10) and S36 (n=9), respectively, respectively and fed at 5 g/ kg of BW.

The experiment lasted from January 7, (2018) to January 22, (2019). The experimental period was divided into 4 periods, plus an adaptation period of 30 d. The adaptation period lasted from January 7 to February 6, where all heifers were fed the same supplement containing 18% CP at 5 g/kg of BW. The first season lasted from February 7 to April 24 (76 d), which was named the rainy-dry transition (RDT) season. The second season lasted from April 25 to July 27 (94 d), which was named the dry season. The third season lasted from July 28 to November 5 (101 d), and it was named the dry-rainy transition (DRT) season. Finally, the fourth season lasted from November 6 to January 22 (78 d), which was named the rainy season.

The heifers were managed in a rotational grazing system, with 27 paddocks (1700 m^2 each), of *Brachiaria decumbens* fertilized with 120 kg of N and 60 kg of K_2O per hectare per year. An additional area on the side of the main area was used during the dry season, with 23 paddocks (1700 m^2 each). All paddocks had free access to a resting area with shade (3 m^2/animal), water, and *ad libitum* mineral mix. All heifers were kept in the same paddock, accessing one paddock per day, except during the dry season, when accessed two paddocks per day (using the additional area described above) due to low pasture DM production in that season.

Every day, at 1000 h, all heifers were moved from the pasture to a management area (250 meters distance), and supplements were fed to each group (treatment) separately. However, we estimated the individual supplement intake using TiO_2 as the external marker, following Titgemeyer et al. (2001) methodology. Thus, the experimental unit was the animal. The supplement was supplied at 5 g/kg of BW per day with a feeder bunk space of 50 cm per animal, and no orts were observed. The amount of supplement (5g/kg of BW) was chosen to meet the energy requirements of crossbreed dairy heifers, with an ADG of 0.5 kg/d grazing *Brachiaria decumbens* according to recommendations of NRC (2001). All heifers were weighed every 15 d to adjust supplement supply. The animals of the control treatment (non-supplemented) were also moved to the management area, but no supplementation was provided. We spent 1 h with this management per day, and the animals returned to paddocks afterward.

The last 14 d of each experimental period were used to collect samples (collection period) of pasture, feces, urine, body measures, weighing animals, liver, and muscle tissue biopsy and blood samples. Days 1 to 8 of the collection period were used to estimate digestibility, pasture intake, and supplemented intake. From day 9 to 11, we took body measurements and weighed the animals. On the 12 d, ultrasound images of the carcass and mammary gland were taken. On d 13, liver and muscle tissue biopsies were performed, and on d 14, blood samples were collected. Information regarding analysis and results of intake, digestibility, performance, and blood paraments can be observed in Castro et al. (2022). This study focused on ultrasound analyses of the carcass and mammary gland and liver and muscle biopsies.

Carcass Characteristics and Mammary Gland Development

Ultrasound images of the carcass and the mammary gland were taken on d 0 of the experiment and d 12 of each experimental period collect. For the carcass characteristics, measurements of the *gluteus medius* and the *biceps femoris* muscle intercessions were taken by scanning between the 12^{th} and 13^{th} ribs and the rump in the P8 region. We used an 18-cm linear

array ultrasound instrument (Aloka SSD-500V, Aloka Co., Ltd., Tokyo, Japan) operated at a frequency of 3.5 MHz. A standoff (Aloka long standoff guide-beef, Aloka Co., Ltd. Tokyo, Japan) and vegetable oil were used for adequate acoustic contact between the transducer, the standoff, and the animals' skin. Ultrasound images were recorded and later analyzed for backfat thickness and loin depth using the BioSoft Toolbox® II for 200 Beef (Biotronics Inc., Ames, Iowa, USA) software.

Mammary gland ultrasound images were taken using a micro convex transducer (Mindray DP2200, Shenzhen, China), operating at 6 MHz (Albino et al., 2017). Images were taken of each mammary quarter in a standardized position, with an inclination of 45° in relation to teat insertion, and recorded in bitmap format, a technique described by Albino et al. (2016). Mammary gland ultrasound images were evaluated for pixel values in an 8-bit format as defined by Albino et al. (2016) using the software ImageJ (National Institutes of Health, Bethesda, MD). The pixel value was estimated according to the brightness on a scale of 256 shades of gray (0 = black and 256 = white). Before the analysis, the software was calibrated for a pixel scale of 100 pixels/cm using the straight tracer tool. The pixel value of each mammary quarter was obtained as the mean from 3 squares (16 mm^2 each) randomly collected near the ductal structures from each image of each quarter. Subsequently, the pixel value of the whole mammary gland was obtained as an average value of each mammary quarter.

Liver and Muscle Tissue Collection

Five animals from each treatment were randomly chosen for gene expression analysis in the muscle and liver tissue, and collections were performed at the end of each experimental period, as mentioned above. The same animals were used for these analyses in all periods.

The liver tissue samples were taken via needle biopsy (Tru-Cut biopsy needle; Care Fusion Corporation, San Diego, CA, USA) 4 h before supplement was fed, following the procedure described by Mølgaard et al. (2012). An incision was performed between the 11^{th} and 12^{th} ribs from the right hepatic lobe following the procedure that described by Miranda et

al. (2010). Muscle tissues samples were made on the left side at the 13th rib, at three-fifths of the distance from the medial to the lateral edge of the *Longissimus dorsi* muscle. After collection, liver (110 mg of tissue) and muscle (1.50 cm^3) samples were placed into cryotubes, frozen, and stored in liquid nitrogen at −196°C until processing and further analyses.

Gene Expression Analyses

Relative quantification real-time PCR (qRT-PCR) was performed in duplicate on an ABI Prism 7300 Sequence Detection System thermocycler (Applied Biosystems, Foster City, CA, USA) using GoTaq qPCR Master Mix (Promega) Corporation, Madison, USA) following the manufacturer's instructions.

The amplification efficiency of each gene was calculated by constructing a cDNA serial dilution curve at concentrations of 25, 75, and 225 ng cDNA and concentrations of 100, 200, and 400 ng primer per reaction. The reactions were considered efficient when the amplification efficiency of the target gene and the control gene were approximately equal, with a tolerance of 5% variation in relation to the control gene. Amplification conditions for all systems were performed with an initial step at 95°C for 2 min, following the second step of 40 denaturation cycles at 95˚C for 15 sec and a final extension at 60˚C for 60 sec.

The expression for each target gene for each heifer was determined by subtracting the Ct value for the geometric mean of the control genes from the target gene Ct (target gene Ct – Ct endogenous reference), where Ct reflects the PCR cycle number at which the fluorescence generated crosses an arbitrary threshold. Then, the gene relative expression values were estimated using the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001).

Target genes evaluated in the current study were the mammalian target of rapamycin (mTOR) and adenosine monophosphate-activated protein kinase (AMPK) in muscle tissue. The genes associated with the urea cycle in the liver were: carbamoyl phosphate synthetase (CPS), ornithine transcarbamylase (OTC), argininosuccinate synthetase (ASS), arginosuccinate lyase

(ASL), and arginase (ARG). The primer pairs for each target and internal control genes are listed in Table 2.

Statistical Analysis

The response variables were analyzed using the PROC GLIMMIX procedure of SAS (Statistical Analysis System version 9.4. For all variables, measurements at d 0 (ultrasound carcass and mammary gland) were tested as covariates and removed from the models as they were all non-significant (P > 0.05). The variables were analyzed as a completely randomized design, with period included as a repeated measure in the model as follows:

$$Y_{ijk} = \mu + T_i + \delta_{ij} + P_k + (T \times P)_{ik} + \varepsilon_{ijk}$$

Where: Y_{ijk} = observation ijk; μ = the overall mean; T_i = fixed effect of treatment i; δ_{ij} = random error with mean 0 and variance σ_δ^2, the variance between animals within the treatment and it is equal to the covariance between repeated measurements within animals; P_k = fixed effect of period k; $T \times P_{ik}$ = fixed effect of interaction between treatment i and period k; and ε_{ijk}= random error with the mean 0 and variance σ^2, the variance between measurements within animals. Fifteen variance-covariance structures were tested for each response variable. Thus, we used the variance-covariance structure that provided the best fit based on the lower Akaike information criterion. The variance components were the most common variance-covariance structure used. Observations with externally studentized residuals higher than |2.5| were first checked to ensure they were not a recording error. After checking and evaluating that the data has not a recording error, these observations were considered outliers and consequently excluded from the dataset. The analysis of possible outliers were performed only once for each outcome variable, in order not to create erroneous results. In the end, we observed only one animal as an outlier. Least-square means were considered different when $P \leq 0.05$, and tendency was declared when $0.05 < P < 0.100$.

The pixel values from the analysis of ultrasound images did not follow a normal distribution, and we had to use logarithmic transformation to normalize the data for analysis. However, the pixel data results are shown in untransformed format to make visualization easier.

Means were compared by the following orthogonal contrasts: 1) effect of supplementation (non-supplemented animals-CON vs. supplemented animals-SUP); 2) linear effect of supplement CP level in supplemented animals; and 3) quadratic effect of supplement CP level in supplemented animals.

Results

We observed a greater rib eye area (cm^2) in SUP animals than non-supplemented animals ($P = 0.001$, Figure 1). However, we did not observe differences among SUP animals ($P = 0.166$). When we evaluated the effect of season, the greatest rib eye area was observed in the rainy season, followed by DRT, dry, and DRT ($P = 0.001$; Figure 1).

We observed greater fat thickness (mm) in SUP animals than non-supplemented animals ($P = 0.009$; Figure 2). Among SUP animals, no differences were detected ($P = 0.442$). However, a seasonal effect was detected ($P = 0.001$), in the rainy season heifers had greater fat thickness (mm) than other seasons ($P = 0.001$, Figure 2).

Mammary gland development was not affected by treatment ($P > 0.267$; Figure 3). However, seasonal effects were observed, in the RDT and dry season heifers had the greatest and lowest amount of fat accumulated in the mammary gland, respectively (Figure 3).

The results of relative mRNA abundance of AMPK and mTOR in muscle tissue, and CPS, OTC, ASS, ASC, and ARG in liver tissue from crossbred Holstein × Gyr heifers supplemented with increasing supplement CP levels are shown in Table 2. In the muscle, we observed greater gene expression of AMPK in non-supplemented animals than SUP animals ($P = 0.002$; Table 2). No differences were observed among SUP animals for AMPK ($P > 0.186$; Table 2). We detected no differences in gene expression between SUP and non-supplemented animals and among SUP animals for mTOR ($P > 0.305$; Table 2). However, seasonal effects

were observed for AMPK and mTOR, as demonstrated in Table 3. For AMPK, a difference was observed between the RDT and rainy seasons, whereby heifers had greater gene expression of AMPK in the rainy season compared to the RDT ($P = 0.05$; Table 3). No differences were observed for AMPK expression among RDT, dry and DRT and among the dry, DRT and rainy ($P > 0.050$, Table 3). For mTOR, we observed greater gene expression of mTOR in the RDT and DRT seasons than in the rainy season ($P = 0.002$; Table 3). No differences were observed for mTOR expression among the RDT, dry and DRT seasons, and among the dry, and rainy seasons ($P > 0.050$, Table 3).

In the liver, we observed greater gene expression of CPS, ASL and ARG in SUP animals than in non-supplemented animals ($P < 0.028$; Table 2). On the other hand, we detected no differences in gene expression between the SUP and non-supplemented animals for OTC and ASS ($P > 0.256$; Table 2). Among SUP animals, differences were observed only for CPS, in which we detected a positive linear response ($P = 0.022$; Table 2). In other words, as the level of CP supplementation increased, the expression of CPS increased. In addition, no seasonal effects were observed for all enzymes evaluated in liver tissue ($P > 0.424$; Table 2), which were: CPS, OTC, ASS, ASL and ARG.

Discussion

An adequate CP supplementation strategy is necessary to increase animal performance and production efficiency; however in several studies researches have associated ADG and balance between energy and protein with mammary gland development (Sejrsen and Purup, 1997; Albino et al., 2015). In this study, we did not observe any effect of CP supplementation strategy on parenchymal mammary tissues growth; nevertheless, we observed that the mammary gland could remodel itself across seasons as there was an evident season effect. The season effect was mainly due to the availability of nutrients, where during the RDT season the pasture had greater availability of forage, as demonstrated in our companion study (Castro et al., 2022) and, consequently, a greater amount of fat was deposited in the mammary gland. On

the other hand, pasture availability decreased quantitatively and qualitatively during the dry season, reducing heifer performance and the amount of fat in the mammary gland. In other words, the mammary gland can remodel itself across the year depending on performance and nutrient availability/intake in each season, accumulating more or less fat in the mammary gland. In agreement with these results, Albino et al. (2015) observed that parenchymal mammary tissues could be influenced by ADG and different proportions of the metabolizable energy and the protein intake. Interestingly, in our study greater amount of fat in the mammary gland was observed during RDT season, followed by the rainy and dry seasons, following the same sequence of animal performance across seasons, in agreement with Albino et al. (2015).

The AMPK is a sensor of peripheral energy balance, and its activation is critical to maintain energy balance in the body. In this sense, when cellular energy is low, AMPK is activated, and the processes that spend ATP are inhibited, such as protein synthesis (Appuhamy et al., 2014). Moreover, when AMPK is activated, there is an increase in ATP production processes such as fatty acid oxidation and glucose uptake (Mukherjee et al., 2008). Conversely, the mTOR is responsible for increasing the cell energy availability; thus, it could be seen as a sensor for nutrient availability, stimulating the body's anabolic processes and protein proliferation (Lie et al., 2019). As such, these pathways have been reported as the main drivers regulating energy balance in muscle (Smith et al., 2013). In agreement this, we detected a greater AMPK expression in non-supplemented animals than SUP animals. These results clearly indicate that the central AMPK signal pathway was inhibited in SUP animals, which promoted greater lipid and muscle synthesis in these animals. These results are confirmed by greater intake, performance, IGF-1, and glucose levels in the SUP animals, as observed in our companion paper (Castro et al., 2022).

These results could also be confirmed by the analysis of the rib eye area and fat thickness, since the rib eye area is an indication of carcass muscularity and meat yield (Scholz

et al., 2015). In fact, we observed a greater rib eye area and fat thickness in SUP animals than non-supplemented animals. Similar to our results, Underwood et al. (2008) observed lower AMPK activity in animals with high intramuscular fat, confirming that the activation of AMPK inhibits adipogenesis (Giri et al., 2006). Thereby, AMPK activity directly interferes with cellular proliferation, depending on its activation or not. Hence, the lower expression of AMPK in the SUP animals is likely linked to the greater rib eye area and fat thickness observed in these animals (Figure 3).

On the other hand, these differences were insufficient to support the same response in mTOR expression, where no differences were detected between SUP and non-supplemented animals and among SUP animals. Nevertheless, researchers have previously reported a negative association between mTOR and AMPK (Appuhamy et al., 2014) since they are regulators of cell growth, which are regulated by the availability of nutrients (Lie et al., 2019). Curiously we observed a seasonal effect in AMPK and mTOR (Table 3) gene expression. Briefly, the greatest expression of AMPK and the lowest expression of mTOR were detected during the rainy season. These results are consistent with Appuhamy et al. (2014), who reported an inverse relationship between mTOR and AMPK phosphorylation.

Ruminants can recycle urea in the liver, which helps prevent excess N from becoming toxic to the animal. Urea recycled in the liver is released into the blood and can be excreted in the urine or re-enter the digestive tract by diffusion in saliva or across the rumen wall (Huntington and Archibeque, 2000). The urea cycle can be divided into 5 steps and 5 different enzymes play a role in urea synthesis from ammonia (Nelson and Cox, 2002). Briefly, 1) the carbamoyl phosphate is formed from ammonia and bicarbonate (CPS catalyzes this reaction); 2) OTC catalyzes the reaction between ornithine and carbamoyl phosphate forming citrulline; 3) the second group amino from aspartate is added to citrulline by condensation reaction to form arginosuccinate (this reaction occurs in the cytosol and is catalyzed by the ASS); 4)

Arginosuccinate is broken down into arginine and fumarate by ASL; and 5) arginine is broken down into urea and ornithine by ARG.

Given the importance of understanding protein supplementation, the urea cycle, and the enzymes involved in this process, previous research has been conducted to evaluate these to improve the efficiency of N use (Waldo, 1968; Sun et al., 2016; de Moura et al., 2020). The current study evaluated the 5 enzymes involved in this process, and we observed differences in gene expressions between SUP and non-supplemented animals for CPS, ASL, and ARG. Moreover, we observed a positive linear response for CPS among SUP animals.

Previous studies have demonstrated that CPS is considered a rate-limiting step within the urea cycle (Takagi et al., 2008) because it is responsible for converting ammonia into carbamoyl phosphate (Visek, 2009). Moreover, CPS is the first enzyme in the pathway for urea synthesis, and its activity is an essential step for ammonia detoxification by ruminants (Meijer et al., 1985). Thus, the CPS activity results align with the treatment differences in CP intake between SUP and non-supplemented animals and among SUP animals. In agreement with our results, Takagi et al.(2008) reported that an increase in urea concentration in blood was accompanied by increased CPS activity in Holstein calves after weaning (6 weeks of age).

Other researchers have also reported a positive correlation between urea cycle enzymes and urea synthesis rates, CPS and ASL included (Rattenbury et al., 1980). Payne and Morris (1969) also observed greater activity of urea cycle enzymes in sheep fed a high protein diet compared to those fed a low-protein diet. Therefore, the greater expression of CPS, ASL, and ARG in the SUP animals than non-supplemented animals is likely linked to the increased nitrogen intake, which would lead to greater urea cycle enzyme expression and, consequently, urea production (Castro et al., 2022).

Surprisingly, differences in gene expression for OTC and ASS between SUP and non-supplemented animals and among SUP animals were not observed. Given the differences

observed for other urea cycle enzymes, as cited previously, it is not clear why we observed these results for OTC and ASS. This may, however suggest that the level of supplementation was not extreme enough to alter the expression of these genes involved in the urea cycle. However, we emphasize that further research must be carried out with grazing cattle to clarify these results.

Conclusion

The supplementations of CP to pasture fed heifers did not affect mammary gland development. However, the results indicate that the mammary gland could remodel itself based on the availability of pasture (energy and protein) across the year. On the other hand, the CP supplementation increased the rib eye area and fat thickness. Further, the supplementation directly affected the AMPK expression, where a greater AMPK expression was observed in non-supplemented animals than SUP animals; thus, confirming the regulatory effects of AMPK on cell growth, which are controlled by the availability of nutrients. Lastly, CP supplementation stimulated the expression of certain urea cycle enzymes (CPS, ASL, and ARG), and a positive linear response to protein supplementation was observed for CPS. Overall, these results indicate that greater CP intake led to increased urea cycle enzymes expression and promoted a greater urea production in the liver.

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Table 1. Gene names, accession numbers, and primers sequences.

Gene symbol	Accession number	Primer sequence (5´- 3´)
mTOR	XM_002694043.6	Forward:AAGGAGAAGGAACGGACA
		Reverse:CCAGCACACGAGGTAAATAG
AMPK	NM_001109802.2	Forward:AGTTGCCTACCACCTCAT
		Reverse:GTGGTGATCGTCGAGAAAC
CPS	I3750D07	Forward:CTGACTGACCCTTCCTACA
	I3751A07	Reverse:CCGAACTCATCCACTTCATC
OTC	I3750D08	Forward:AATGGGCTGTCGGATTTG
	I3750D09	Reverse:GGAGTGGAGGATGTTATTCC
ASS	I3750D10	Forward:TTCAGGGGCCAGGTGTA
	I3750D11	Reverse:ATCAACCGGCTCGTAGT
ASL	I3751A03	Forward:ACTGGTGTCATCTCTACCC
	I3751A04	Reverse:TTTCGGACCAGGTAGTAGG
ARG	I3751A05	Forward:GGTGGCAGAAGTCAAGAAG
	I3751A06	Reverse:CACCCAAATGACACAGAGG
18S	NR_036642.1	Forward:GCCGCTAGAGGTGAAATTCT
		Reverse:TCGGAACTACGACGGTATCT

mTOR = mammalian target of rapamycin; AMPK =adenosine monophosphate activated protein kinase; CPS = carbarbamoyl phosphate synthetase;OTC = ornithine transcarbamylase; ASS = argininosuccinate synthetase; ASL = arginosuccinate lyase, ARG = arginase and *18S* = ribosomal RNA. Accession number in GenBank (http://www.ncbi.nlm.nih.gov).

Table 2. Gene expression of AMPK and mTOR in muscle tissue, and CPS, OTC, ASS, ASC, and ARG in liver tissue from crossbred Holstein × Gyr heifers supplemented with different crude protein levels in supplement throughout the experiment period. Gene expression in LSM± $2^{-\Delta Ct} \times 10000$

Item	Treatments				SEM	*P-value*				
	CON	S12	S24	S36		S	T x S	SUP	L	Q
AMPK	0.055	0.041	0.047	0.039	0.004	0.050	0.764	0.002	0.664	0.186
mTOR	0.024	0.028	0.025	0.028	0.002	0.002	0.974	0.438	0.938	0.305
CPS	0.005	0.006	0.012	0.014	0.003	0.726	0.114	0.028	0.022	0.403
OTC	0.166	0.138	0.124	0.156	0.017	0.918	0.349	0.256	0.526	0.337
ASS	0.574	0.739	0.616	0.590	0.157	0.921	0.312	0.661	0.484	0.819
ASL	1.715	7.004	8.134	5.124	1.794	0.562	0.082	<0.001	0.399	0.355
ARG	15.789	59.096	82.680	25.940	19.271	0.424	0.056	0.004	0.088	0.081

mTOR = mammalian target of rapamycin; AMPK =adenosine monophosphate activated protein kinase; CPS = carbamoyl phosphate synthetase; OTC = ornithine transcarbamylase; ASS = argininosuccinate synthetase; ASL = arginosuccinate lyase, ARG = arginase.

CON = control; S12 = supplement with 12% of CP; S24 = supplement with 24% of CPS36= supplement with 36% of CP.

SEM = square error mean, S= season effect, T × S = interaction between season and treatment, SUP = supplemented *vs* non-supplemented, L = linear effect among supplemented animals.and Q = quadratic effect among supplemented animals.

Table 3. Gene expression of AMPK and Mtor in muscle tissue in LSM± $2^{-\Delta Ct} \times 10000$ from throughout the experiment season

Item	Seasons				*SEM*	*P-value*
	RDT	Dry	DRT	Rainy		
AMPK	0.038^{B}	0.045^{AB}	0.043^{AB}	0.055^{A}	0.004	0.050
Mtor	0.030^{A}	0.024^{AB}	0.032^{A}	0.021^{B}	0.002	0.002

mTOR = mammalian target of rapamycin; AMPK =adenosine monophosphate activated protein kinase; RDT = rainy dry transition, DRT = dry rainy transition and SEM = square error mean.

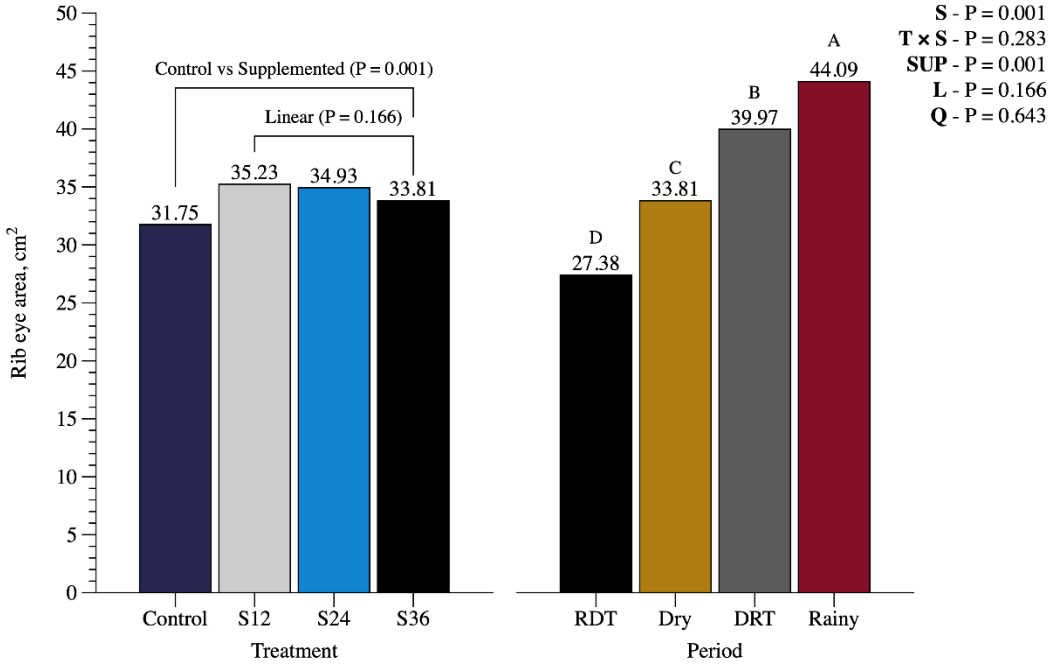

Figure 1: Rib eye area (cm^2) of crossbred Holstein × Gyr heifers non-supplemented (Control) or supplemented with 12 (S12), 24 (S24) or 36% (S36) of crude protein in supplement throughout experimental periods, rainy-dry transition (RDT), dry, dry-rainy transition (DRT) and rainy.; S = season; T × S = interaction between treatment and season; SUP = supplemented vs. non-supplemented; L= linear effect among supplemented animals and Q = quadratic effect among supplemented animals.

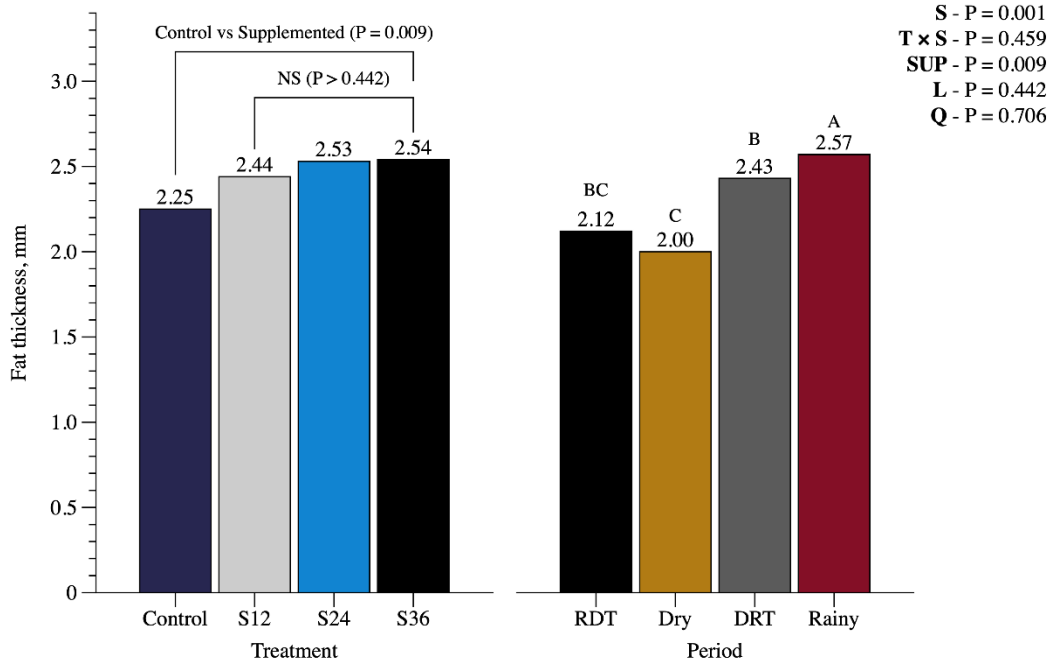

Figure 2: Fat thickness (mm) of crossbred Holstein × Gyr heifers non-supplemented (Control) or supplemented with 12 (S12), 24 (S24) or 36% (S36) of crude protein in supplement throughout experimental periods, rainy-dry transition (RDT), dry, dry-rainy transition (DRT) and rainy. S = season; T × S = interaction between treatment and season; SUP = supplemented vs. non-supplemented; L= linear effect among supplemented animals and Q = quadratic effect among supplemented animals.

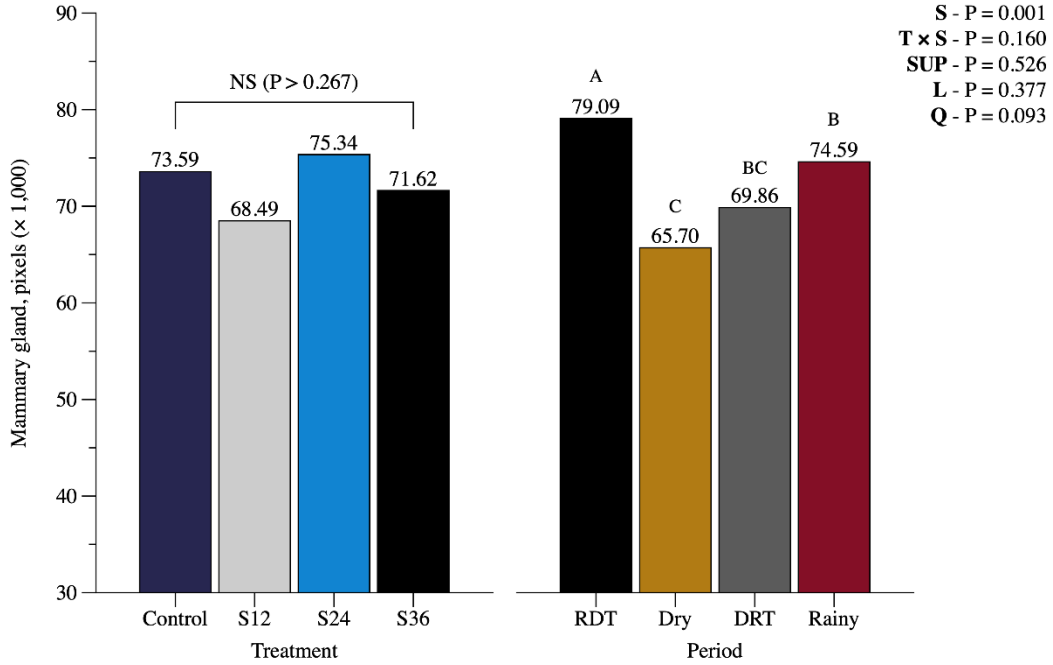

Figure 3: Mammary gland pixels' pattern of crossbred Holstein × Gyr heifers non-supplemented (Control) or supplemented with 12 (S12), 24 (S24) or 36% (S36) of crude protein in supplement throughout experimental periods, rainy-dry transition (RDT), dry, dry-rainy transition (DRT) and rainy. S = season; T × S = interaction between treatment and season; SUP = supplemented vs. non-supplemented; L= linear effect among supplemented animals and Q = quadratic effect among supplemented animals.

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		Reverse:GTGGTGATCGTCGAGAAAC
CPS	I3750D07	Forward:CTGACTGACCCTTCCTACA
	I3751A07	Reverse:CCGAACTCATCCACTTCATC
OTC	I3750D08	Forward:AATGGGCTGTCGGATTTG
	I3750D09	Reverse:GGAGTGGAGGATGTTATTCC
ASS	I3750D10	Forward:TTCAGGGGCCAGGTGTA
	I3750D11	Reverse:ATCAACCGGCTCGTAGT
ASL	I3751A03	Forward:ACTGGTGTCATCTCTACCC
	I3751A04	Reverse:TTTCGGACCAGGTAGTAGG
ARG	I3751A05	Forward:GGTGGCAGAAGTCAAGAAG
	I3751A06	Reverse:CACCCAAATGACACAGAGG
18S	NR_036642.1	Forward:GCCGCTAGAGGTGAAATTCT
		Reverse:TCGGAACTACGACGGTATCT

mTOR = mammalian target of rapamycin; AMPK =adenosine monophosphate activated protein kinase; CPS = carbarbamoyl phosphate synthetase;OTC = ornithine transcarbamylase; ASS = argininosuccinate synthetase; ASL = arginosuccinate lyase, ARG = arginase and *18S* = ribosomal RNA. Accession number in GenBank (http://www.ncbi.nlm.nih.gov).

Table 2. Gene expression of AMPK and mTOR in muscle tissue, and CPS, OTC, ASS, ASC, and ARG in liver tissue from crossbred Holstein × Gyr heifers supplemented with different crude protein levels in supplement throughout the experiment period. Gene expression in LSM± $2^{-\Delta Ct} \times 10000$

Item	Treatments				SEM	*P-value*				
	CON	S12	S24	S36		S	T x S	SUP	L	Q
AMPK	0.055	0.041	0.047	0.039	0.004	0.050	0.764	0.002	0.664	0.186
mTOR	0.024	0.028	0.025	0.028	0.002	0.002	0.974	0.438	0.938	0.305
CPS	0.005	0.006	0.012	0.014	0.003	0.726	0.114	0.028	0.022	0.403
OTC	0.166	0.138	0.124	0.156	0.017	0.918	0.349	0.256	0.526	0.337
ASS	0.574	0.739	0.616	0.590	0.157	0.921	0.312	0.661	0.484	0.819
ASL	1.715	7.004	8.134	5.124	1.794	0.562	0.082	<0.001	0.399	0.355
ARG	15.789	59.096	82.680	25.940	19.271	0.424	0.056	0.004	0.088	0.081

mTOR = mammalian target of rapamycin; AMPK =adenosine monophosphate activated protein kinase; CPS = carbamoyl phosphate synthetase; OTC = ornithine transcarbamylase; ASS = argininosuccinate synthetase; ASL = arginosuccinate lyase, ARG = arginase.

CON = control; S12 = supplement with 12% of CP; S24 = supplement with 24% of CPS36= supplement with 36% of CP.

SEM = square error mean, S= season effect, T × S = interaction between season and treatment, SUP = supplemented *vs* non-supplemented, L = linear effect among supplemented animals.and Q = quadratic effect among supplemented animals.

Table 3. Gene expression of AMPK and Mtor in muscle tissue in LSM± $2^{-\Delta Ct} \times 10000$ from throughout the experiment season

Item	Seasons				*SEM*	*P-value*
	RDT	Dry	DRT	Rainy		
AMPK	0.038^{B}	0.045^{AB}	0.043^{AB}	0.055^{A}	0.004	0.050
Mtor	0.030^{A}	0.024^{AB}	0.032^{A}	0.021^{B}	0.002	0.002

mTOR = mammalian target of rapamycin; AMPK =adenosine monophosphate activated protein kinase; RDT = rainy dry transition, DRT = dry rainy transition and SEM = square error mean.

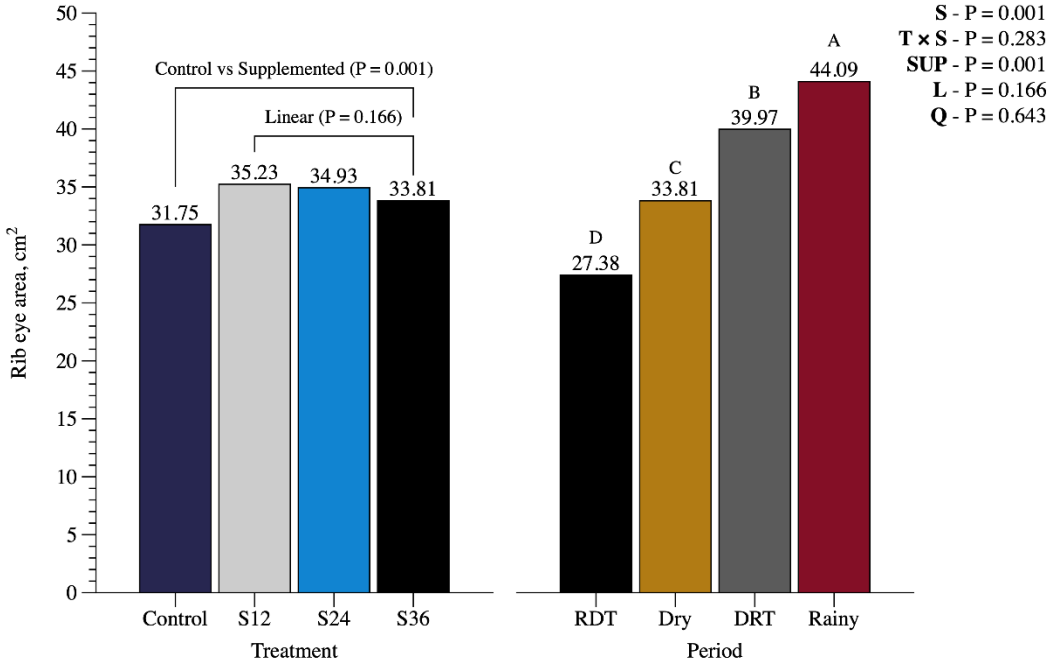

Figure 1: Rib eye area (cm^2) of crossbred Holstein × Gyr heifers non-supplemented (Control) or supplemented with 12 (S12), 24 (S24) or 36% (S36) of crude protein in supplement throughout experimental periods, rainy-dry transition (RDT), dry, dry-rainy transition (DRT) and rainy.; S = season; T × S = interaction between treatment and season; SUP = supplemented vs. non-supplemented; L= linear effect among supplemented animals and Q = quadratic effect among supplemented animals.

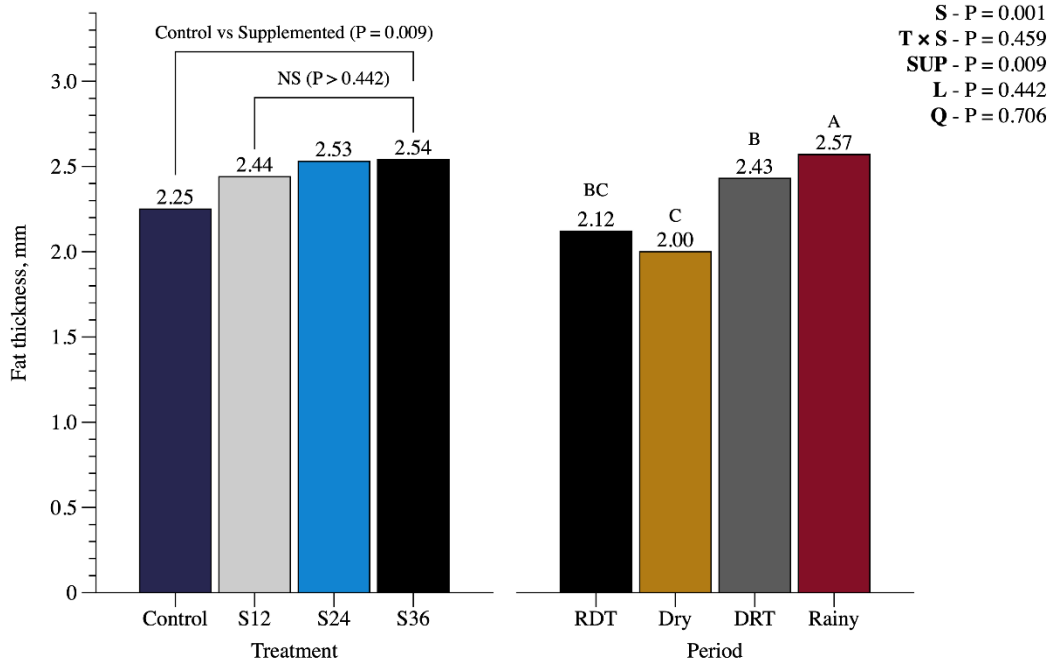

Figure 2: Fat thickness (mm) of crossbred Holstein × Gyr heifers non-supplemented (Control) or supplemented with 12 (S12), 24 (S24) or 36% (S36) of crude protein in supplement throughout experimental periods, rainy-dry transition (RDT), dry, dry-rainy transition (DRT) and rainy. S = season; T × S = interaction between treatment and season; SUP = supplemented vs. non-supplemented; L= linear effect among supplemented animals and Q = quadratic effect among supplemented animals.

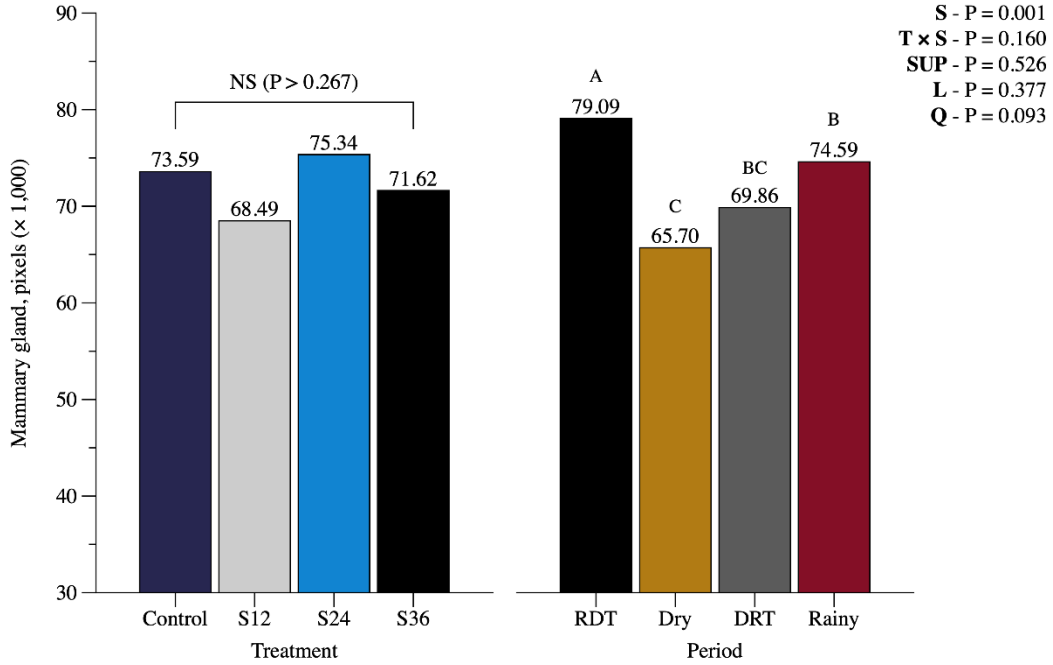

Figure 3: Mammary gland pixels' pattern of crossbred Holstein × Gyr heifers non-supplemented (Control) or supplemented with 12 (S12), 24 (S24) or 36% (S36) of crude protein in supplement throughout experimental periods, rainy-dry transition (RDT), dry, dry-rainy transition (DRT) and rainy. S = season; T × S = interaction between treatment and season; SUP = supplemented vs. non-supplemented; L= linear effect among supplemented animals and Q = quadratic effect among supplemented animals.

Chapper 3

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Association of housing and management practices with milk yield, milk composition, and fatty acid profile, predicted using Fourier-transform mid-infrared spectroscopy, in farms with automated milking systems

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Abstract

Milk fatty acid (FA) profile can be divided into: 1) de novo (C4-C14) that are synthesized in the mammary gland; 2) preformed (≥C18) that are absorbed from blood and originate from mobilized adipose tissues or dietary fat; and 3) mixed (C16), which has both origins. Our objectives were to describe the FA profile, as predicted using Fourier transform mid-infrared (FTIR) spectroscopy, of bulk tank milk from automated milking system (AMS) farms and to assess the association of management and housing factors with the bulk tank milk composition and FA profile of those AMS farms. The data used were collected from 124 commercial Canadian Holstein dairy farms with AMS, located in the provinces of Ontario (n = 68) and Quebec (n = 56). The farms were visited once from April to September 2019, and information were collected on barn design and herd management practices. Information regarding individual cow milk yield (kg/d), days in milk (DIM), parity, and the number of milking cows were automatically collected by the AMS units on each farm. These data were extracted for the entire period that the bulk tank milk samples were monitored, from April 2019 to April 2020 in Quebec and from August 2019 to May 2020 in Ontario. Across herds, milk yield averaged 35.8±0.4 kg/d, with 3.97±0.01 % fat and 3.09±0.01 % protein, while FA profile averaged 26.2±0.1, 33.1±0.1, and 40.7±0.2 g/100 g of FA for de novo, mixed, and preformed, respectively. FA yield averaged 0.34±0.01, 0.44±0.01, and 0.54±0.01 kg/d for de novo, mixed, and preformed, respectively. Multivariable regression models were used to associate herd-level housing factors and management practices with milk production, composition, and FA profile. Milk yield was positively associated with using a robot feed pusher (+2.1 kg/d) and the use of deep bedding (+2.6 kg/d). The use of a robot feed pusher, deep bedding, and greater stall raking frequency were positively associated with greater yield (kg/d) of de novo FA, mixed FA, preformed FA, and de novo + mixed FA. Use of deep bedding was negatively associated with concentration of fat, de novo FA, mixed FA, and de novo + mixed FA, expressed in g/100 g (%) of milk. A wider lying alley width (≥ 305 cm) was associated with a greater concentration

(g/100 g of milk) of de novo and de novo + mixed FA. Greater frequency of PMR delivery (>2x/d vs. 1 and 2 x/d) was positively associated with a greater proportion (g/100 g of FA) of de novo, mixed, and de novo + mixed FA and negatively associated with the proportion of preformed FA. Overall, these associations indicate that bulk tank FA profile can be used as a tool to monitor and adjust management and housing in AMS farms.

Keywords: de novo, fat, robotic milking

Introduction

Understanding the factors that influence milk components is very important because milk is commonly marketed using a component pricing system, which defines the milk price based on the fat, protein, and other solids composition of the milk (Bailey et al., 2005). Bovine milk fat comprises 95 to 98% of triglycerides, which are formed through linkages of a glycerol backbone and 3 fatty acids (FA - Pegolo et al., 2016). The milk FA profile can be divided into 3 different origins: de novo, mixed and preformed (Barbano, 2017). De novo (C4 to C14) and a fraction of the mixed FA (C16) are synthesized in the mammary gland from acetate and butyrate (Woolpert et al., 2017). Mixed FA can also enter the mammary gland cells as preformed FA taken up from the circulatory system (Ungerfeld et al., 2019). Preformed FA (≥ C18) are absorbed from the bloodstream and can originate from the mobilization of adipose tissues or dietary fat (Palmquist et al., 1993).

Bulk tank FA composition has become of increased interest, primarily due to its high correlation with bulk tank milk fat and protein content (Barbano et al., 2014) and due to the ease and speed of prediction the FA composition using Fourier transform mid-infrared **(FTIR)** spectroscopy**.** Moreover, bulk tank FA composition provides information on herd nutritional status since management practices, nutrition, and facilities are the primary factors that affect milk FA composition (Palmquist et al., 1993). Woolpert et al. (2016; 2017) observed that farms with greater bulk tank fat and de novo FA content had greater feed bunk space, lower stall stocking density, and fed a TMR at least twice per day. Moreover, diets with high PUFA and

non-fibrous carbohydrate content (Harvatine and Bauman, 2011), lesser levels of physically effective NDF (Woolpert et al., 2016), and with fat supplementation (Harvatine et al., 2009) may result in lesser milk fat content and alter FA composition (Stoffel et al., 2015).

The use of automatic milking system **(AMS)** is continually increasing worldwide. For example, in Canada, ~11 % of dairy herds enrolled in a milk recording program use AMS (Lactanet, 2019). With the adoption of AMS, not only are milking procedures changed, but also various aspects of dairy farm management, including facilities and nutritional management (Svennersten-Sjaunja and Pettersson, 2008). As a result, there is potential for these practices to be associated with milk FA content. While researchers have previously investigated the effect of AMS use on free-fatty acid content of milk (Klungel et al., 2000; De Marchi et al., 2017), to our knowledge, there is no research to date on the association of management and housing with bulk tank milk FA content in AMS farms.

Thus, our objectives were to: 1) describe the FA profile, as predicted using FTIR, of bulk tank milk from AMS farms and 2) assess the association of management and housing factors with bulk tank milk composition and FA profile in AMS farms. We hypothesized that management practices and facilities that improve cow comfort and promote favorable feeding behavior, such as greater feed bunk and water space, greater feed delivery and push-up frequency, lesser stall stocking density and cows per robot, in dairy farms with AMS, would be associated with greater milk components and de novo FA content in bulk tank milk.

Materials and methods

The data used in this study were collected from 124 commercial Canadian dairy farms with AMS, located in the provinces of Ontario (n = 68) and Quebec (n = 56). These farms were a subset of those participating in a national study of AMS farms from across Canada (Matson et al., 2021); detailed information on farm recruitment and study design are provided in that study. In brief, the national study's inclusion requirements included: (1) using an AMS to milk

their cows and, (2) participating in DHI milk recording. Additionally, our current study required farms to: (1) reside in the province of Ontario or Quebec (due to availability of bulk tank FTIR predicted FA data for farms in those provinces) and (2) have herds composed of more than 90% Holstein cattle. Researchers contacted all farmers through Lactanet Canada (Sainte-Anne-de-Bellevue, Quebec) to request participation in the study. Subsequent email communications encouraging participation in the study were sent through equipment dealers. Animal use, data collection, and study design were approved by the University of Guelph Animal Care Committee (AUP#3963) and Research Ethics Board (REB#19-01-012), and animal use complied with the guidelines of the Canadian Council on Animal Care (2009). The number of farms available for this study was limited to that recruited for the Matson et al. (2021) study, with our additional selection criteria. The sample size of 124 herds was determined sufficient to detect a 9% difference in the primary outcome variables (bulk tank FAs) for various predictors, with 95% confidence at 80% power (WinPepi version 11.65; Abramson, 2011).

Farms were visited once between April and September 2019, during which management and housing information were collected. As described by Matson et al. (2021), a survey with questions regarding management and housing was applied on-farm by trained research personal, who surveyed the farm owner or manager. The following management practices were recorded: cow hoof trimming frequency, foot bathing frequency, alley cleaning frequency, stall raking frequency, PMR mixing frequency, PMR delivery frequency, PMR feed push up frequency, target PMR feeding level (% of refusals), cow traffic system (free-flow or guided), and number of groups of lactating cows. Housing information collected were: ventilation system (ceiling fans, panel fans, natural and tunnel), feed push-up type (human or robot), feed alley surface (concrete, plastic, ceramic, fiberglass, stainless steel, rubber, epoxy, wood, and others), flooring type (grooved concrete, smooth concrete, concrete with rubber mats, slatted flooring and others), stall base (deep bedding and mattress), and bedding composition (organic

or inorganic). Moreover, the information regarding free-stall stocking (cows/stall), feed alley width (cm), feed bunk space per cow (cm/cow), linear water space per cow (cm/cow), lying alley width (cm), stall width (cm), neck rail height (cm), and length of stall (cm) were measured by research personal. Stocking densities (number of cows per stall, cm of feed bunk space per cow, and cm of linear water space per cow) were calculated, on a monthly basis across the data collection period, using the average total number of milking cows in that month, as recorded automatically from the daily AMS data. A follow-up, online survey was conducted with the farm owners or managers, between May and August 2020, to verify that no changes in recorded housing and management practices occurred over the data collection period.

After collecting the information some variables were separated into groups and analyzed as categorical due to the frequency of observation and distribution (Table 1). Other variables were kept as continuous variables (cows per robot, linear feed bunk per cow, stall stocking density, linear water space per cow, neck rail height, and stall length; Table 2).

Milk yield, milk composition, and milk fatty acid analysis

Information regarding individual cow milk yield (kg/d), DIM, parity, and the number of milking cows were automatically collected by the AMS units on each farm. These data were extracted for the entire period of time that the bulk tank milk samples were monitored.

Data from bulk tank milk samples were collected for every farm milk shipment from April 2019 to April 2020 in the Quebec farms (average = 15.4 ± 0.18 shipments per month) and from August 2019 to May 2020 in Ontario farms (average = 13.6 ± 0.41 shipments per month).

Bulk tank milk samples were analyzed for true protein, fat, lactose, total solids and FA composition in g/ 100 g of milk (Table 3). Milk samples from the province of Quebec were analyzed by **FTIR** spectrophotometer using two different kind of instruments: MilkoScan™ FT 7 and MilkoScan^{TM} FT^{+} (Foss, Hillerød, Denmark) at the Lactanet laboratory (Sainte-Anne-de-Bellevue, Quebec). Milk samples from the province of Ontario were analyzed by FTIR spectrophotometer using MilkoScan™ FT^{+} (Foss, Hillerød, Denmark) at the University of

Guelph, Laboratory Services Division (Guelph, ON, Canada). Calibration for the FTIR for milk components prediction (fat, true protein, lactose, and total solids) was done following the reference method (IDF, 1991).

Milk FA composition, including FA groups (de novo, mixed, and preformed) was predicted by FTIR (as described by Schwarz et al., 2018) with the same instruments previously cited for prediction of milk components. More details on these FA groups and their composition obtained with FTIR predictions, including the reference analyses, are described by Schwarz et al. (2021). Standardization of the instruments were conducted daily according to that described by Winning (2014). Calibrations of the instruments were performed once a month using a reference set of 14 local milk samples that were analyzed by GC, according to the methods described by Gervais et al. (2009). Concentrations of de novo (C4:0, C6:0, C8:0, C10:0, C12:0, C14:0, and C14:1), mixed (C16:0 and C16:1), and preformed (C15:0, C17:0, C18:0, C18:1, C18:2, C18:3, C20:0, C20:2, C20:4, C22:0, and C24:0) FA were predicted in g/100 g of milk (concentration) and converted to proportion in g/100 g of total FA and g/d based on total herd milk production for each sampling time frame (Table 3).

Statistical Analysis

The statistical analyses were conducted with SAS (version 9.4, SAS Institute Inc., Cary, NC) using farm as the experimental unit. All data were averaged across time on a per farm basis and were checked for normality using the UNIVARIATE procedure of SAS. To verify that the bulk tank FA followed a normal biological response, the CORR procedure of the SAS was used to evaluate the correlations among bulk tank FA composition (g/100 g of milk, g/100 g of FA and kg/d), fat (g/100 g of milk and kg/d), and milk yield (kg/d).

The MIXED procedure of SAS was used to model associations between the various explanatory variables (Table 1 and Table 2) and outcome variables, which included: milk yield (kg/d), fat (kg/d and g/100 g of milk), and de novo, mixed and preformed FA yield (kg/d), concentration (g/100 g of milk) and proportion (g/100 g of FA). These models of the association

of management and housing factors with milk FA profile (concentration and proportion) and the yield of milk, fat, and FA were constructed in a 4-step procedure. In the first step, explanatory variables were analyzed individually for each outcome variable using univariable models, with region (Ontario and Quebec) as a random effect in the model and degrees of freedom estimated using the Kenward-Roger option in the MODEL statement. Those variables significant at $P < 0.25$ were considered for inclusion in the multivariable models (Dohoo et al., 2009). In the second step, all kept variables, for each outcome variable, were tested for correlation. Pearson correlation was estimated using the CORR procedure of SAS (version 9.4), and variables were declared correlated if $r > 0.6$. Next, the associations between categorical variables were analyzed using a Chi-Square test, using the FREQ procedure, and associations were declared if $P < 0.1$. In cases of correlation, for both continuous and categorical variables, the most biologically plausible variable, based on biological knowledge and previous research, was chosen to be kept for the multivariable model (Dohoo et al., 2009). In the third step, a multivariable model was fit using manual backward elimination until only variables with a $P \leq 0.1$ remained in the final model. Once again during this procedure, region (Ontario and Quebec) was treated as a random effect, with the degrees of freedom estimated using the Kenward-Roger option in the MODEL statement. Lastly, in the fourth step, the model fit was visually evaluated and confirmed for each outcome variable based on residual normality histograms produced through the RESIDUAL option in the model statement.

Results and discussion

Housing and management practices

The categorical housing and management variables are described in Table 1. Farms with free cow traffic to the AMS were predominant (88.7%), with only 11.3 % of farms being guided systems. The number of lactating cow groups of the surveyed farms were categorized into farms with their lactating cows in more than one group (23.4%) and farms with all their lactating cows

in one group (76.6%), indicating that, within the studied AMS farms, using only one group was predominant for lactating cows. We observed that more than half of the surveyed farms (55.3%) mixed the partial mixed ration (PMR) < 2 times per day, and 44.7% of farms mixed the PMR ≥ 2 times per day. Furthermore, 33.9% of surveyed farms fed PMR once per day, 33.1% fed PMR twice per day, and 33.1% fed PMR > 2 times per day. Of the surveyed farms, 81.8% used a robot feed pusher, while only 18.2% of farms pushed feed manually (i.e., human-operated machine or by hand). It was observed that 46.7% of farms fed lactation cows for < 5% of refusals and 55.3% for ≥ 5% of refusals. The majority of surveyed farms (73.4%) cleaned out the feed bunk at least once per day. Regarding stall raking frequency, 42.2% raked stalls ≥ 3 and 57.8% raked ≤ 2 times per day. The majority of farms (82.3%) hoof trimmed their cows > 2 times per year, and 17.7% of farms trimmed ≤ 2 times per year. Further, 27.4% of farms used a footbath ≥ 2 times per week, 47.6% < 2 times per week, and 25% did not use footbath. In this study, 30.7% of surveyed farms used tunnel ventilation system, while 69.4% used some other kind of system, such as ceiling fans, panel fans, or natural ventilation. Feed alley width was categorized as ≤ 426 cm (49.6% of farms) and > 426 cm (50.4% of farms). Lying alley width was categorized as < 305 cm (41.4% of farms) and ≥ 305 cm (58.6% of farms) and stall width was categorized as > 117 cm (42.5% of farms) and ≤ 117 cm (57.5% of farms). The surface of the feeding alley, where the feed is located, was categorized as concrete (33.3%), ceramic (39.2%), and other (plastic, fiberglass, stainless steel, rubber, epoxy, or wood; 27.5%). Flooring type was categorized based on the predominant flooring type within the barn, with 30.9% of farms using rubber matting and 69.1% using other types, such as grooved concrete, smooth concrete, and slatted flooring (Table 1). From surveyed farms, 51.8% had deep bedding, and 48.2% had a mattress stall base (Table 1). Inorganic (sand) bedding was used in 30.7% of farms, while organic bedding was used on 69.3% of the studied farms (Table 1).

Descriptive statistics of herd information for the continuous variables are described in Table 2. Mean milk yield, herd parity, DIM, and cows per robot were 35.8 kg/d, 2.4 lactations, 167.7 DIM, and 45.9 cows/AMS, respectively. The mean feed bunk and linear water space were 65.5 and 7.9 cm/cow, respectively. Stall stocking density (cows/lying stalls × 100) averaged 92%, and average neck rail height was 124.2 cm, measured from the base of the stall to the middle of the neck rail. Stall length averaged 249.3 cm, as measured from the front of the stall structure to the back of the curb.

Milk composition, fatty acid composition, and correlation among them

Descriptive statistics of milk composition (g/100 g of milk), FA concentration (g/100 g of milk), FA proportion (g/100 g FA), and FA yield (kg/d) are presented in Table 3. These were similar to those reported in a study of commercial freestall and tiestall farms with high de novo FA content by Woolpert et al. (2017) for de novo concentration (g/100 g of milk: 0.97 vs. 0.99), FA proportion (g/100 g of FA: 26.2 vs. 25.9), and FA yield (kg/ d: 0.34 vs. 0.31) and fat concentration (g/100 g of milk: 3.97 vs. 3.98) and fat yield (kg/d: 1.42 vs. 1.27). Our numbers are also similar to those from another study of commercial freestall and tiestall farms with high de novo FA content by Woolpert et al. (2016) for de novo concentration (0.97 vs. 1.1 g/100 g of milk) and proportion (26.2 vs. 25.6 g/100 g of FA). However, Woolpert et al. (2016) reported values slightly greater for fat concentration (3.97 vs. 4.33 %); however, they reported lesser yields (kg/d) for de novo (0.34 vs. 0.26) and fat (1.42 vs. 1.1). These differences are likely due to differences in milk yield, as cows in our study herds had much greater milk yields (35.8. vs 26.3 kg/d), and breed, given that breed affects milk FA composition (Soyeurt et al., 2006) and in the study of Woolpert et al. (2016) only 33.7% of their total farms characterized as high de novo were Holstein herds.

Correlations among bulk tank FA composition, fat, and milk yield are described in Table 4. A positive correlation detected between fat and de novo FA concentration (g/100 g of milk) was detected ($r = 0.77$), in agreement with previous studies (Barbano et al., 2014; Woolpert et

al., 2016). There was also an association between mixed FA and fat concentration (g/100 g of milk; r = 0.83; Table 4). Moreover, positive correlations were observed between fat concentration (g/100 g of milk) with de novo FA (r = 0.23), and mixed FA (r = 0.23) proportion (g/100 g FA). There was a negative correlation of milk yield (kg/d) with fat, de novo FA, and mixed FA concentration (g/100 g of milk). This indicates a dilution effect, whereas milk yield increases, the concentration on a milk basis (g/100 g of milk) of fat, mixed FA, and de novo FA decreases. The opposite occurs when milk production decreases (Harvatine and Allen, 2005; Machado et al., 2017). Strong positive correlations were also detected among yield (kg/d) of milk, fat, de novo FA, mixed FA, and preformed FA (Table 4). Thus, when milk yield increases, the yield (kg/d) of all these milk components also increases (Harvatine and Allen, 2005; Machado et al., 2017). This emphasizes that we must be careful when evaluating only the bulk tank milk FA concentration (g /100 g of milk), because variations in FA content may be related to other factors that lead to changes in milk yield and consequently change FA concentration. Hence, this emphasizes that we must verify the FA concentration in g/100 g of milk, but we need also to check the FA composition expressed in other ways (i.e.: proportion [g/ 100 g of FA] and yield [kg/d]) for better decision making. It should be highlighted that many of these variables, which we correlated, are not completely independent (i.e. one may be a derivative of the other). However, these correlations were not performed to (nor do not) imply causation, but rather to demonstrate that these variables follow normal, expected biological responses in these herds and also to help explain associations detected in the multivariable models.

We detected a correlation between preformed FA concentration (g/100 g of milk) and milk fat concentration (g/100 g of milk - r = 0.55; Table 4), which is similar to the results observed by Barbano et al. (2014). On the other hand, preformed FA proportion (g /100 g of FA) were negatively correlated with milk fat concentration (g/100 g of milk; -0.26; Table 4), which differed from results observed for de novo and mixed as described above. Moreover, we

observed a negative correlation among de novo FA and mixed FA proportion (g/100 g FA) and concentration (g/100 g milk), with preformed FA proportion (g/100 g of FA). These correlations reflect the different origins of de novo, mixed, and preformed FA, whereby an increase in the proportion of de novo is a resultant of a greater proportion of FA synthesized in the mammary gland from acetate and butyrate (Palmquist et al., 1993; Urrutia and Harvatine, 2017)). On the other hand, a greater proportion of preformed FA would result from increased fat intake or increased lipid mobilization (Palmquist et al., 1993).

Factors associated with yield (kg/d) of milk, fat, de novo, mixed, and preformed FA

The final multivariable models of factors associated with yield (kg/d) of milk, fat, de novo FA, mixed FA, preformed FA, and de novo + mixed FA are in Table 5. In this study, milk yield was positively associated with using a robot feed pusher (+2.1 kg/d) and the use of deep bedding (+2.6 kg/d). As demonstrated in the parent study to the current one, milk yield was associated with greater feed push-up frequency and use of sand bedding (Matson et al. 2021), which were associated with the use of a robot feed pusher type and deep bedding, respectively. Researchers have previously highlighted the benefits of increased feed push-up frequency in AMS farms (Siewert et al., 2018) due to improved feed access, decreased sorting, and less time searching for feed and more time spent lying down (Deming et al., 2013; King et al., 2016), which all may have positive impacts on milk yield. The use of deep bedding has been associated with lesser lameness prevalence in AMS farms (Salfer et al., 2018) and with greater lying duration (Gomez and Cook, 2010; King et al., 2016). As such, greater milk yield associated with deep bedding may result from greater cow comfort, increased lying time, and reduced lameness prevalence. Given that greater milk yield is associated with a greater yield of fat and various FAs (Table 4), the use of a robot feed pusher and deep bedding were also then associated with yield (kg/d) of fat, de novo FA, mixed FA, preformed FA and de novo + mixed FA (Table 5).

Interestingly, greater stall raking frequency was positively associated with greater yield (kg/d) of fat, de novo FA, mixed FA, preformed FA, and de novo + mixed FA (Table 5). An increase in stall raking frequency may be associated with greater stall use and, subsequently, increased lying time (Drissler et al., 2005). These improvements to cow comfort may affect milk component yield, as predicted by Grant (2015). Moreover, greater stall raking frequency may be associated with improved stall cleanliness, directly associated with udder hygiene (DeVries et al., 2012). This, in turn, may be associated with fewer intramammary infections (Ruud et al., 2010) and, consequently, with greater milk component yield (Gill et al., 1990; Hippen et al., 2007).

In addition, feed alley surface tended to be associated with yield (kg/d) of preformed FA (Table 5). Farms with a feed alley composed of concrete were associated with lesser preformed FA yield (P=0.045) than those with other feed alley surfaces (plastic, fiberglass, stainless steel, rubber, epoxy and wood). It is possible that a surface that is easier to clean and maintain may contribute to improving feed quality, less feed sorting, and greater DMI, which may consequently increase milk components. To our knowledge, there are no studies in which the effects of feed alley surface have been evaluated; thus, future studies should assess how feed alley surface affects feed quality and cow behavior and performance.

Factors associated with fat, de novo, mixed and preformed in g/ 100 g of milk

The final multivariable models of factors associated with fat, de novo FA, mixed FA, preformed FA, and de novo + mixed FA, concentration in g/100 g (%) of milk are described in Table 6. Use of deep bedding as stall base was negatively associated with the percentage of fat, de novo FA, mixed FA, and de novo + mixed FA (Table 6). Given that the yield (kg/d) of those variables, plus milk itself, were positively associated with the use of deep bedding (Table 5), it is likely that these negative associations with the percentage of fat, de novo FA, mixed FA, and de novo + mixed FA (Table 6) are due to the dilution of these with greater milk yield, as demonstrated in Table 4.

A wider lying alley width (≥ 305 cm) was associated with greater concentration (g/100 g of milk) of de novo and de novo + mixed FA when compared with narrow lying alleys widths (< 305 cm) (Table 6). This association may be linked to the effects of lying alley width on cow hygiene and milk quality, given that greater SCC has been associated with altered milk composition, primarily significantly lesser de novo FA synthesis (Turini et al., 2020). A wider lying alley width could help spread manure, resulting in a smaller amount of manure per unit of flooring space, thus contributing to less risk of cows becoming dirty (DeVries et al., 2012). As mentioned, this may be associated with fewer intramammary infections (Ruud et al., 2010). In support of this, Matson et al. (2021) reported that wider alley widths were associated with lower SCC in AMS farms.

Preformed FA concentration (g/100 g of milk) was negatively associated with neck rail height (Table 6). Further, greater preformed FA concentration was associated with farms with wider feed alleys (> 426 cm) as compared to those with narrower feed alleys (≤ 426 cm; Table 6). Surprisingly, we expected the opposite of these associations due to knowing that higher neck rail placement and narrower feed alleys could result in a greater risk of dirtier cows, as indicated by Fregonesi et al. (2009) and Matson et al. (2021), respectively. As discussed above, dirtier cows could lead to greater intramammary infection risk, and consequently, reduce de novo FA and increase preformed FA content. Thus, it is not clear why we observed these opposite associations, suggesting there were factors other than SCC driving this association with preformed FA content. Therefore, further research is needed to investigate these associations.

Factors associated with de novo, mixed and preformed in g/ 100 g of fatty acids

The factors associated with FA proportion (expressed in g/ 100 g of FA) are found in Table 7. The frequency of feed (PMR) delivery was positively associated with greater de novo, mixed, and de novo + mixed FA proportion and negatively associated with proportion of preformed FA (Table 7). This is in agreement with Woolpert et al. (2017), who reported a tendency for high de novo FA concentration herds to be more likely to feed cows twice

compared to once per day. Researchers have previously demonstrated that feeding cows more frequently encourage cows to eat more times per day, reduce meal size, and decrease sorting against long particles (Sova et al., 2013; DeVries, 2019). These behaviors help maintain a more stable ruminal pH (Macmillan et al., 2017), which improves the ruminal environment and favors the synthesis of de novo FA, as observed by Fukumori et al. (2021), and, consequently, milk fat content. These findings are corroborated with the results of Sova et al. (2013), Rottman et al. (2014), and Macmillan et al. (2017), who all reported greater milk fat content with greater feed delivery frequency.

The negative association of preformed FA proportion (g/ 100 g of FA) with greater feed delivery frequency (Table 7) reflects the strong negative correlation (Table 4) observed between de novo and preformed FA proportion (g/ 100 g of FA - r = -0.88) and mixed FA and preformed FA proportion (in g/ 100 g of FA - r = -0.90). Therefore, this indicates that factors that contribute to greater de novo and mixed FA proportion contribute to lesser preformed FA proportion, or vice versa, as demonstrated by Lock and Souza (2018).

Conclusions

This study documented the bulk tank milk FA composition of AMS herds, as predicted by FTIR. Moreover, we associated housing and management practices with that bulk tank FA composition. The use of robot feed pusher and deep bedding were associated with a greater yield of milk, milk fat, de novo FA, mixed FA, de novo + mixed FA, and preformed FA. Additionally, greater stall raking frequency was associated with the yield of milk fat, de novo FA, mixed FA, de novo + mixed FA, and preformed FA. Further, the use of concrete as feed alley surface was associated with a lesser yield of preformed FA. Given negative associations of milk yield with concentrations (g/ 100 g milk) of milk fat, de novo FA, mixed FA, de novo + mixed FA, and preformed FA, the concentration of these all were negatively associated with the use of deep bedding. When expressed in g/ 100 g of FA, the frequency of feed (PMR)

delivery was positively associated with greater proportion of de novo, mixed, and de novo + mixed FA and negatively associated with proportion of preformed FA. These associations indicate that bulk tank FA composition can be used as a tool to monitor and adjust management and housing in AMS farms.

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Table 2. Categorical housing and management variables for herds in the provinces of Ontario (n = 68) and Quebec (n = 56), in Canada, using automated milking systems

Variable	Categories	n	% of total farms
Management			
Cows traffic system	Free	110	88.7
	Guided	14	11.3
Stall raking frequency (#/d)	≤ 2	67	57.8
	≥ 3	49	42.2
Cow hoof trimming frequency (#/yr)	≤ 2	22	17.7
	>2	102	82.3
Footbath frequency (#/mo)	No foot bath	31	25.0
	< 2 times per week	59	47.6
	≥ 2 times per week	34	27.4
Mix PMR (#/d)	< 2	68	55.3
	≥ 2	55	44.7
PMR delivery frequency (#/d)	1	42	33.8
	2	41	33.1
	≥ 2	41	33.1
Target refusal level (% orts)	< 5	56	46.7
	≥ 5	64	53.3
Feed bunk cleaning frequency (#/day)	< 1	33	26.6
	≥ 1	91	73.4
Lactating cow groups	1	95	76.6
	>1	29	23.4
Housing			
Ventilation system	Tunnel	38	30.6
	Others	86	69.4
Feed push type	Robot	90	81.8
	Human	20	18.2
Feed alley width (cm)	≤ 426	61	49.6
	> 426	62	50.4
Lying alley width (cm)	<305	46	41.4
	≥305	65	58.6
Stall width (cm)	≤ 117	65	57.5
	> 117	48	42.5
Feed alley surface	Concrete	40	33.3
	Ceramic	47	39.1
	Others	33	27.5
Flooring type	Rubber matt	38	30.9
	Others	85	69.1
Stall base	Mattress	55	48.3
	Deep bedding	59	51.7
Bedding composition	Organic	86	69.4
	Inorganic	38	30.6

Table 3. Descriptive statistics of the continuous variables for herds in provinces of Ontario (n = 68) and Quebec (n = 56), in Canada, using automated milking systems

Variable	n	Mean	SD	Minimum	Maximum
Lactating herd size	124	100.0	81.1	39.9	595.8
Days in milk	124	167.7	15.1	116.5	229.1
Parity	124	2.4	0.3	1.7	3.1
Cows per robot	124	45.9	9.0	16.3	61.3
Feed bunk space (cm/cow)	121	65.5	23.6	8.9	167.7
Cows per stall	112	0.9	0.2	0.49	1.3
Water linear space (cm/cow)	123	7.9	3.5	1.7	23.0
Neck rail height (cm)	112	124.2	7.6	101.3	143.5
Length of stall (cm)	109	249.3	16.3	212	285.7

Table 4. Descriptive statistics of milk yield, milk components, and fatty acid composition (as predicted by FTIR) for herds (n = 124) in the provinces of Ontario (n = 68) and Quebec (n = 56), in Canada, using automated milking systems

Item	Mean	SD	Minimum	Maximum
Concentration (g/100 g of milk)				
Fat	3.97	0.15	3.67	4.44
True protein	3.09	0.08	2.90	3.41
Lactose	4.60	0.04	4.49	4.70
Total solids	12.80	0.20	12.27	13.42
Urea	11.25	1.56	7.40	15.05
C14	0.39	0.03	0.33	0.46
C16	1.16	0.06	1.02	1.36
C18	0.40	0.02	0.33	0.48
C18:1	0.89	0.06	0.77	1.04
De novo[1]	0.97	0.06	2.91	1.12
Mixed[2]	1.22	0.07	1.34	1.41
Preformed[3]	1.50	0.08	1.34	1.81
Proportion (g/100 g of fatty acids)				
De Novo	26.22	1.01	23.60	28.50
Mixed	33.07	1.11	30.15	35.91
Preformed	40.70	1.89	36.65	45.55
Yield (kg/d)				
Milk	35.85	4.27	24.99	47.48
Fat	1.42	0.15	0.99	1.86
True protein	1.11	0.12	0.76	1.46
De Novo	0.34	0.04	0.24	0.44
Mixed	0.44	0.05	0.32	0.56
Preformed	0.54	0.06	0.35	0.72

[1]De novo fatty acids = C4:0, C6:0, C8:0, C10:0, C12:0, C14:0, and C14:1.
[2]Mixed fatty acids = C16:0 and C16:1.
[3]Preformed fatty acids = C15:0, C17:0, C18:0, C18:1, C18:2, C18:3, C20:0, C20:2, C20:4, C22:0, and C24:0.

Table 4. Correlation coefficient (r) among bulk tank fatty acids (FA) and milk fat and yield.

Item	Unit	Fat	De Novo	Mixed	Preformed	De Novo	Mixed	Preformed	Milk	Fat	De Novo	Mixed	Preformed
		g/100 g of milk				g/100 g of FA			kg/d				
Fat	g/100 g of milk	1.00	0.77*	0.83*	0.55*	0.23*	0.23*	-0.26*	-0.52*	-0.25*	-0.14	-0.14	-0.30*
De Novo			1.00	0.86*	0.02	0.79*	0.48*	-0.71*	-0.47*	-0.27*	0.06	-0.06	-0.48*
Mixed				1.00	0.03	0.55*	0.72*	-0.71*	-0.47*	-0.24*	-0.01	0.02	-0.47*
Preformed					1.00	-0.54*	-0.63*	0.66*	-0.25*	-0.10	-0.28*	-0.27*	0.18*
De Novo	g/100 g of FA					1.00	0.59*	-0.88*	-0.21*	-0.16	0.24*	0.07	-0.45*
Mixed							1.00	-0.90*	-0.15	-0.08	0.14	0.23*	-0.42*
Preformed								1.00	0.20*	0.13	-0.21*	-0.17*	0.49*
Milk	kg/d								1.00	0.96*	0.85*	0.87*	0.91*
Fat										1.00	0.92*	0.95*	0.92*
De Novo											1.00	0.95*	0.74*
Mixed												1.00	0.76*
Preformed													1.00

* $P < 0.05$

Table 5. Multivariable linear model of the factors associated with milk, fat, and fatty acids (FA) yield (kg/d) in farms using automated milking systems

Outcome	Variable	Estimate	SE	*P*
Milk, kg/d				
	Intercept	34.90	1.69	0.004
	Feed pusher type			0.035
	Human	-2.07	0.967	
	Robot	Ref[1]	-	
	Stall base			0.001
	Deep bedding	2.64	0.774	
	Mattress	Ref[1]	-	
Fat, kg/d				
	Intercept	1.43	0.037	<0.001
	Feed pusher type			0.020
	Human	-0.08	0.033	
	Robot	Ref[1]	-	
	Stall base			0.006
	Deep bedding	0.075	0.027	
	Mattress	Ref[1]	-	
	Stall raking frequency/d			0.019
	≤ 2	-0.067	0.027	
	≥ 3	Ref[1]	-	
De novo FA, kg/d				
	Intercept	0.35	0.007	<0.001
	Feed pusher type			0.040
	Human	-0.018	0.009	
	Robot	Ref[1]	-	
	Stall base			0.020
	Deep bedding	0.016	0.007	
	Mattress	Ref[1]	-	
	Stall raking frequency/d			0.024
	≤ 2	-0.016	0.007	
	≥ 3	Ref[1]	-	
Mixed FA, kg/d				
	Intercept	0.44	0.009	<0.001
	Feed pusher type			0.034
	Human	-0.022	0.010	
	Robot	Ref[1]	-	
	Stall base			0.015
	Deep bedding	0.021	0.008	
	Mattress	Ref[1]	-	
	Stall raking frequency/d			0.048
	≤ 2	-0.018	0.009	
	≥ 3	Ref[1]	-	
Preformed FA, kg/d				
	Intercept	0.56	0.021	<0.001

	Feed pusher type			0.011
	Human	-0.039	0.015	
	Robot	Ref[1]	-	
	Stall base			0.031
	Deep bedding	0.027	0.012	
	Mattress	Ref[1]	-	
	Stall raking frequency/d			0.044
	≤ 2	-0.026	0.012	
	≥ 3	Ref[1]	-	
	Feed alley surface			0.094
	Ceramic	-0.007	0.016	
	Concrete	-0.032	0.015	
	Others	Ref[1]	-	
De Novo + Mixed FA, kg/d				
	Intercept	0.79	0.014	<0.001
	Feed pusher type			0.034
	Human	-0.040	0.018	
	Robot	Ref[1]	-	
	Stall base			0.013
	Deep bedding	0.038	0.015	
	Mattress	Ref[1]	-	
	Stall raking frequency/d			0.027
	≤ 2	-0.033	0.015	
	≥ 3	Ref[1]	-	

[1]Ref = referent category

Table 6. Multivariable linear model of the factors associated with fat and fatty acid (FA) concentration (g/ 100 g of milk) in farms using automated milking systems

Outcome	Variable	Estimate	SE	*P*
Fat				
	Intercept	4.00	0.044	0.003
	Stall base			0.057
	Deep bedding	-0.051	0.026	
	Mattress	Ref[1]	-	
De novo FA				
	Intercept	0.99	0.034	0.017
	Stall base			0.057
	Deep bedding	-0.019	0.009	
	Mattress	Ref[1]	-	
	Lying alley width			0.015
	< 305 cm	-0.024	0.009	
	≥ 305 cm	Ref[1]	-	
Mixed FA				
	Intercept	1.23	0.027	0.010
	Stall base			0.071
	Deep bedding	-0.023	0.012	
	Mattress	Ref[1]	-	
Preformed FA				
	Intercept	1.78	0.129	<0.001
	Feed alley width			0.084
	≤ 426 cm	-0.029	0.016	
	> 426 cm	Ref[1]	-	
	Neck rail height (cm)	-0.002	0.001	0.043
De Novo + Mixed FA				
	Intercept	2.23	0.060	0.011
	Stall base			0.035
	Deep bedding	-0.046	0.021	
	Mattress	Ref[1]	-	
	Lying alley width			0.039
	< 305 cm	-0.045	-0.022	
	≥ 305 cm	Ref[1]	-	

[1]Ref = referent category

Table 7. Multivariable linear model of the factors associated with fatty acid (FA) proportion (g/ 100 g of fatty acids) in farms using automated milking systems

Outcome	Variable	Estimate	SE	*P*
De novo FA				
	Intercept	26.53	0.367	0.004
	PMR delivery frequency/d			0.055
	1	-0.33	0.224	
	2	-0.52	0.212	
	>2	Ref[1]	-	
Mixed FA				
	Intercept	33.39	0.167	<0.001
	PMR delivery frequency/d			0.033
	1	-0.35	0.237	
	2	-0.63	0.240	
	>2	Ref[1]	-	
Preformed FA				
	Intercept	40.01	0.388	<0.001
	PMR delivery frequency/d			0.089
	1	0.82	0.477	
	2	1.24	0.434	
	>2	Ref[1]	-	
De Novo + Mixed FA				
	Intercept	59.99	0.388	<0.001
	PMR delivery frequency/d			0.020
	1	-0.82	0.477	
	2	-1.24	0.434	
	>2	Ref[1]	-	

[1]Ref = referent category