DAVID ESTEBAN CONTRERAS MARQUEZ

STRATEGIC SUPPLEMENTATION OF GRAZING BEEF COWS DURING GESTATION: EFFECTS ON POSTPARTUM REPRODUCTIVE TRAITS OF THE DAMS AND SKELETAL MUSCLE DEVELOPMENT OF THE PROGENY

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

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BIOGRAPHY

David Esteban Contreras Marquez, son of Tomas Segundo Contreras (R.I.P) and Enith Marquez Castañeda, was born in Caucasia /Antioquia-Colombia on December 25, 1986.

He started an undergraduate degree in Animal Science at the Universidad de Cordoba in 2005 and obtained a Bachelor of Science in Animal Science in 2010.

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ABSTRACT

MARQUEZ, David Contreras, D.Sc., Universidade Federal de Viçosa, February, 2017. **Strategic supplementation of grazing beef cows during gestation: effects on postpartum reproductive traits of the dams and skeletal muscle development of the progeny**. Adviser: Mário Fonseca Paulino. Co-advisers: Marcio de Sousa Duarte and Luciana Navajas Rennó.

The present study was carried out with the objective of determining the effect of supplementation of Nelore matrices maintained in grazing during the different gestation periods on postpartum fertility and progeny performance up to 8 months old (weaning). Thus, 27 Nelore matrices were used, divided into three groups (n = 9 for each group) as follows: matrices not supplemented during gestation (UNS), matrices supplemented from 30 to 180 days of gestation (MID) and supplemented matrices of 181 to 281 days of gestation (LATE). The percentage composition of the supplements provided for the matrices was: ground corn (26.25%), wheat bran (26.25%) and soybean meal (47.5%). The supplement was formulated to contain 30% CP. 150 kg of supplement were provided per animal during each supplementation period (1 and 1.5 kg / day for cows in the MID and LATE groups, respectively), accompanied by a mineral blend offered ad libitum. UNS matrices received only mineral mixture ad libitum during gestation. Blood samples were taken to determine the concentrations of NEFA, BHBA (β-hydroxybutyrate) and glucose at days 30, 105, 180, 230 and 281 of gestation of the matrices. After delivery on days 10, 18 and 30, blood samples were taken to determine the progesterone concentration (P4), as well as ultrasonographic assessments to determine the size, absence of fluid in the uterine horns and presence of ovarian follicles and bodies luteous. Fixed-time artificial insemination (FTAI) was performed in all matings at 45 days postpartum and twenty-eight days after insemination, a diagnosis was made to determine the percentage of gestation. After birth, all progenies were supplemented for eight months until weaning, with the same supplement in equal amounts. The percentage composition of the supplement was: ground corn grain (30%), wheat bran (30%), soybean meal (35%) and molasses (5%). The supplement was formulated to contain 25% CP. The supplement was offered in an amount of 6 g / kg body weight, and the mineral blend was offered ad libitum. Thirty days postpartum were performed on progeny biopsy of skeletal muscle tissue to determine number, size of muscle fibers and expression of genetic markers. During the reproduction phase, the weight gain, the thickness of the subcutaneous fat and the loin eye area were evaluated in the progeny. A lower concentration of NEFA and BHBA ($P < 0.01$) at 180 and 281

days of gestation was observed for MID and LATE matrices respectively. Higher glucose concentration (P <0.1) during pregnancy was observed for MID matrices at 180 days and LATE at 281 days of gestation. Involution of the uterus was observed at 30 days postpartum, with a size of 14.40 ± 1.31, 15.70 ± 1.20 and 14.70 ± 1.25 mm, respectively for the treatments UNS, MID and LATE. Progeny performance (P = 0.953) was not influenced by supplementation of the pregnant matrices; greater subcutaneous fat thickness (P = 0.006) was observed in the progeny of LATE matrices; there was larger loin eye area (P = 0.077) for progenies of MID and LATE matrices when compared to the UNS. A higher number of muscle fibers (P = 0.093) was observed for the progeny of the MID matrices when compared to the UNS, but no differences were observed in the LATE matrices and in the UNS matrices. No influence of supplementation of pregnant matrices on the size of the progeny muscle fibers (P = 0.208) was detected. A higher expression of FGF2 (P = 0.003) was observed for the progeny of the MID treatment. Higher PPARα expression (P = 0.073) was also observed for progenies of MID matrices when compared to LATE. Prepartum supplementation improves energy balance by increasing glucose concentration and decreasing the concentration of NEFA and BHBA, without altering uterine involution and postpartum fertility. Strategic supplementation of pregnant grazing matrices does not alter progeny performance during the breeding phase, but improves the expression of genetic markers (FGF2 e PPARα) capable of positively influencing fetal myogenesis and adipogenesis.

RESUMO

MARQUEZ, David Contreras, D.Sc., Universidade Federal de Viçosa, fevereiro de 2017. **Suplementação estratégica de vacas pastando durante a gestação: Efeitos sobre as características reprodutivas pós-parto das matrizes e desenvolvimento muscular esquelético da progênie**. Orientador: Mário Fonseca Paulino. Coorientadores: Marcio de Sousa Duarte e Luciana Navajas Rennó.

O presente estudo foi realizado com objetivo de determinar o efeito da suplementação de matrizes Nelore mantidas em pastejo nos diferentes períodos da gestação sobre fertilidade pós-parto e desempenho da progênie durante a fase de cria. Assim, foram utilizadas 27 matrizes Nelore, divididas em três grupos (n=9 para cada grupo) como segue: matrizes não suplementadas durante a gestação (UNS), matrizes suplementadas de 30 a 180 dias de gestação (MID) e matrizes suplementadas de 181 a 281 dias de gestação (LATE). A composição percentual dos suplementos fornecido as matrizes foi: grãos de milho moído (26,25%), farelo de trigo (26,25%) e farelo de soja (47,5%). O suplemento foi formulado para conter 30% PB. Foram fornecidos 150 kg de suplemento por animal durante cada período de suplementação (1 e 1,5 kg / dia para vacas nos grupos MID e LATE, respectivamente), acompanhados de mistura mineral oferecida ad libitum. As matrizes UNS, receberam apenas mistura mineral ad libitum durante a gestação. Coletas de sangue para determinar as concentrações de NEFA (ácidos graxos não esterificados), BHBA (β-hidroxibutirato) e glicose foram realizadas nos dias 30, 105, 180, 230 e 281 da gestação das matrizes. Após o parto, nos dias 10, 18 e 30, foram feitas coletas de sangue para determinar a concentração de progesterona (P4), assim como avaliações ultrassonográficas para determinar o tamanho, ausência de líquidos nos cornos uterinos e presença de folículos e corpos lúteos ováricos. Inseminação artificial a tempo fixo (IATF) foi realizada em todas as matrizes aos quarenta e cinco dias pós-parto, e vinte oito dias após inseminação foi feito diagnostico para determinar o porcentual de gestação. Após o nascimento, todas as progênies foram suplementadas por oito meses até o desmame, com o mesmo suplemento em quantidades iguais. A composição percentual do suplemento foi: grão de milho moído (30%), farelo de trigo (30%), farelo de soja (35%) e melaço (5%). O suplemento foi formulado para conter 25% PB. O suplemento foi oferecido numa quantidade de 6 g / kg do peso vivo e a mistura mineral foi oferecida ad libitum. Trinta dias após o parto foi realizado na progênie biópsia de tecido muscular esquelético para determinação do número, tamanho das fibras musculares e expressão de marcadores genéticos. Durante a fase de cria foi avaliado na progênie o ganho de peso, espessura de gordura subcutânea

e área de olho de lombo. Menor concentração de NEFA e BHBA (P <0,01) aos 180 e 281 dias de gestação foi observada para matrizes MID e LATE respectivamente. A maior concentração de glicose (P <0,1) durante a gravidez foi observada para matrizes MID a 180 dias e LATE a 281 dias de gestação. A involução do útero foi observada aos 30 dias pós-parto, com tamanho de 14.40±1.31, 15.70±1.20 e 14.70±1.25 mm, respectivamente para os tratamentos UNS, MID e LATE. O desempenho da progênie (*P*=0,953) não foi influenciado pela suplementação das matrizes gestantes; maior espessura de gordura subcutânea (*P*=0,006) foi observada na progênie de matrizes LATE; houve maior área de olho de lombo (*P*=0,077) para progênie de matrizes MID e LATE quando comparadas com UNS. Maior número de fibras musculares (*P*=0.093) foi observado para progênie de matrizes MID quando comparadas com UNS, mais não foi observado diferencia com matrizes LATE assim como destas com matrizes UNS. Não foi detectado influência da suplementação de matrizes gestantes sobre o tamanho das fibras musculares da progênie (*P*=0.208). Maior expressão de *FGF2* (*P*=0.003) foi observada para progênie do tratamento MID, igualmente foi observado maior expressão de *PPARα* (*P*=0.073) para progênie de matrizes MID quando comparadas com LATE mais não houve diferencia destes dois tratamentos com UNS. A suplementação pré-parto, melhora o balanço energético pelo aumento na concentração de glicose e diminuição na concentração de NEFA e BHBA, sem alterar a involução do útero e a fertilidade pós-parto. A suplementação estratégica de matrizes gestantes em pastejo não altera o desempenho da progênie durante a fase de cria, porém, melhora a expressão de marcadores genéticos (FGF2 e PPARα) com capacidade de influenciar positivamente a myogenesis e adipogenesis fetal.

INTRODUTION

Bovine gestation is represented by a period of approximately 285 days according to the animal's racial type (Cavalcante *et al.*, 2001), during this period several physiological processes occur that can positively or negatively influence the productive future not only of the matrix itself As well as the fetus and calf that are probably breastfeeding. These events are dependent on the nutritional state in which the matrix is found, as well as on the diet plan provided during gestation, favoring the supply of nutrients necessary to maintain the cow's body condition score, fetal tissue development as well as milk production for Lactation and calf rearing.

In the case of the matrix, the maintenance of the body condition score is of vital importance, since in the presence of catabolic processes mainly of adipose tissue, in states of nutritional restriction, metabolic products such as NEFA, BHBA, triglycerides, leptin, etc. provide intermediate signals that may have an effect on energy balance (EB), thus on health and especially on reproduction at the beginning of lactation (Van Knegsel *et al.*, 2007).

According to Ayres *et al.* (2014), therefore, BE becomes a determinant condition for the resumption (Ferreira *et al.*, 2013; Torres *et al.*, 2015), therefore, BE becomes a determinant condition for the resumption of ovulation and involution of the uterus.

During the postpartum period, the rapid involution of the uterus, together with the resumption of ovulation, become essential to achieve intervals between ideal deliveries, so the earlier the matrix initiates the postpartum uterine cyclicity and involution, the greater will be The fertility achieved during the breeding period (Giuliodori *et al.*, 2011).

In the case of the fetus, calf growth, health and performance are important prerequisites for a successful cattle breeding program. Therefore, several attempts have been made to improve these parameters through nutritional programming (Bollwein *et*

al., 2016). This nutritional management begins during the fetal life, because the prenatal development is extremely sensitive to environmental and maternal challenges (Zhu *et al.*, 2006; Van Eetvelde *et al.*, 2016). Thus, the pregnant matrix is supplemented during the gestational period in order to meet the nutritional requirements for the optimal development of fetal tissues, even more so for those who participate in a marked way in animal performance (Du *et al.*, 2015) or of greater commercial importance such as muscle tissue.

In the case of the lactating calf, the nutritional status of the matrix during pre-calving exerts a direct effect on the production and quality of colostrum and milk, therefore, on the health and productive performance of the infant (Climeni *et al.*, 2008).

Since the matrix is the gateway to the nutritional support necessary for the maintenance and development of the mother, fetus and infant, the gestation period in which a given nutritional supply exerts greater influence on the trinomium is still unknown.

As shown previously, these situations are interconnected, where the benefit of one individual may be affecting the other, so this experiment with Nellore matrices supplemented at different periods of gestation was performed with the objective of determining if there is a gestation period where Food supplementation exerts positive influences on the trinomial, mother, fetus and infant.

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CHAPTER 1

Supplementation of grazing beef cows during gestation as a strategy to improve skeletal muscle development of the offspring

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Short title: Gene expression and performance of progeny

Abstract

The appropriate supply of nutrients in pregnant cows has been associated with the optimal development of foetal tissues, performance of their progeny and their meat quality. The aim of this study was to evaluate supplementation effects of grazing cows in different stages of gestation on skeletal muscle development and performance of the progeny. Thereby, 27 Nellore cows were divided into three groups (n=9 for each group) and their progeny as follows: UNS, unsupplemented during gestation; MID, supplemented from the 30 to 180-day of gestation; LATE, supplemented from the 181 to 281-day of gestation. The percentage composition of the supplement provided for the matrices was: ground corn (26.25%), wheat bran (26.25%) and soybean meal (47.5%). The supplement was formulated to contain 30% CP. Supplemented matrices received 150 kg of supplement (1 and 1.5 kg / day for cows MID and LATE groups,

respectively). After birth, a biopsy was performed to obtain samples of skeletal muscle tissue from calves to determine number and size of muscle fibres and for mRNA expression analysis. The percentage composition of the supplement provided for the progeny was: ground corn grain (30%), wheat bran (30%), soybean meal (35%) and molasses (5%). The supplement was formulated to contain 25% CP and offered in an amount of 6 g / kg body weight. Performance of the progeny was monitored throughout the suckling period. Means were submitted to analysis of variance and regression, and UNS, MID and LATE periods of supplementation were compared. Differences were considered at $P<0.10$. Birth weight, average daily gain, and weaning weight of the offspring did not differ among treatments ($P>0.10$). Similarly, no differences were observed between calves for nutrient intake ($P>0.10$). However, greater subcutaneous fat thickness ($P=0.006$) was observed in the calves of LATE group. The ribeye area ($P=0.077$) was greater in calves born from supplemented compared to UNS cows. The supplementation of pregnant cows did not affect the muscle fibre size of their progeny ($P=0.208$). On the other hand, calves born from dams supplemented at mid-gestation had greater muscle fibre number ($P=0.093$) compared to calves from UNS group. Greater mRNA expression of *PPARα* ($P=0.073$) and *FGF2* ($P=0.003$) was observed in the calves born from MID cows. Although strategic supplementation did not affect the body weight of offspring, it did cause changes in carcass traits, number of myofibres, and mRNA expression of a muscle hypertrophy and lipid oxidation markers in skeletal muscle of the offspring.

Keywords: Adipogenesis, hyperplasia, foetal programming, myogenesis, pregnancy.

Implications

Many studies are reported the importance of providing the adequate nutrients to pregnancy cows, for the better progeny performance. However, studies evaluating the

capacity of tropical grass to supply nutrients necessary for fetal development, are scarce. These studies are essential due to the grass have many qualitative and quantitative variations during the gestation period. This study aimed establishes if the grass can optimize the fetal development and evaluated the effects of supplementary on pregnancy cows and their progeny. Our results showed that even the grass present a good nutritional status, the supplementation improve the muscle development and adipogenesis in the progeny.

Introduction

Development and growth of foetal skeletal muscle in cattle are processes regulated by interactions between undifferentiated mesenchymal cells in the embryo phase, and also nutrients supplied from the mother, which may be supplied from diet or by mobilization from body reserves (energy and protein source) (Jennings *et al.,* 2016). Undifferentiated mesenchymal cells have the ability to create myocytes, adipocytes and fibroblasts (Du *et al.*, 2013). These cells can present hypertrophy or hyperplasia, processes that are regulated by nutrition during gestation. For example, hyperplasia in myocytes is stimulated by nutrient supply in the early-mid gestation. On the other hand, myocyte and adipocyte hypertrophy, adipogenesis and fibrogenesis, are stimulated by nutrient supply during late gestation (Picard *et al.*, 2002, Bonnet *et al.*, 2010, Du *et al.*, 2010).

Consequently, the nutritional manipulation of the feed of pregnant cows has been used to improve the foetal development and performance of their progeny, a process known as 'foetal programming' (Du *et al.*, 2011). Considering the fact that there is no increase the number of muscle fibres in cattle after birth (Russell and Oteruelo, 1981, Picard *et al.*, 2002), muscle tissue development programming during the foetal phase is a priority, since it can lead to an increase in the number of muscle fibres and muscle

mass, improving the carcass yield (Zhu *et al.*, 2004). Moreover, maternal nutrition may also change energy metabolism of skeletal muscle, where fatty acids may become the major energy source instead of carbohydrate (Ozanne *et al.*, 2003, Selak *et al.*, 2003, Aragão *et al.*, 2014).

In production systems for growing beef cattle, qualitative and quantitative pasture variations are one of the major concerns for a short cycle of animal production. In this case supplementation system becomes a viable tool to enhance animal performance (Paulino *et al.*, 2008). Nevertheless, the ability of pastures to provide required nutrients and the existence of additional gains in foetal muscle development when grazing cows are supplement during gestation is not completely understood. In this sense, the present work aimed to determine the gestation period at which supplementation of grazing cows exerts higher influence on performance and carcass composition as well as on the mRNA expression of skeletal muscle development and energy metabolism markers of the progeny during the suckling phase.

Materials and methods

Location and weather conditions

This study was approved by the Brazilian Ethics Committee on Animal Use (CEUAP/UFV – Process nº 26/2014), according to ethical principles of animal experimentation established by the National Council of Animal Experimentation Control (CONCEA). The experiment was performed at the Beef Cattle Farm of Animal Science Department of Universidade Federal de Viçosa, Viçosa-MG, Brazil. Weather conditions during the experiment were: 284 mm of rain and an average temperature of 22.6 °C (first trimester of pregnancy); 94 mm of rain and average temperature 18.6 °C (second trimester of pregnancy); 61 mm of rain and average temperature 18.7 °C (last

trimester of pregnancy); and 983 mm of rain and average temperature 22.6 °C (rearing phase of progeny).

Animals, experimental design and supplements

In the present study, twenty-seven pregnant Nellore cows were used with a 490 ± 12.82 kg average initial weight. Cows were artificially inseminated at a fixed time using semen from the same bull. At sixty days of gestation, foetal sexing was performed to obtain homogeneous treatment. Animals were allocated to six-hectare paddocks per treatment, evenly covered with Urochloa decumbens grass, equipped with a drinker and feeders.

The study was performed as a completely randomized design, with three treatments and nine replications as following: UNS, unsupplemented during gestation; MID, supplemented from the 30 to 180-day of gestation; LATE, supplemented from the 181 to 281-day of gestation. The supplementation periods were chosen based on the theoretical development of skeletal muscle during gestation as suggested by Du *et al.* (2010). We hypothesized that the supplementation periods tested would be the most effective period to modulate the skeletal muscle development, once the mid-gestation is when most of myogenesis process occurs (Bonnet *et al.*, 2010, Du *et al.*, 2010) while late gestation is when intramuscular adipogenesis/fibrogenesis would initiate (Du *et al.*, 2010). We also hypothesized that maternal supplementation in different stages of gestation would also change the transcriptional metabolic switch from carbohydrate to lipid oxidation, which would contribute for less intramuscular fat deposition in the offspring at slaughter.

Composition of supplements and pastures are shown in Table 1. Percentage composition of supplements was the: ground corn grain (26.25%), wheat meal (26.25%)

and soybean meal (47.5%) for cows. A total of 150 kg of supplement per animal were provided during the total experiment period for each treatment (1 and 1.5 kg/day to cows in the MID and LATE groups respectively), accompanied by a mineral mixture offered ad libitum (Table 1). The control group, the UNS cows, received only a mineral mixture ad libitum during gestation.

After birth, all progeny were kept at the same conditions and supplemented for 8 months until weaning, with the same supplement in equal amounts. Percentage composition of supplements was the: ground corn grain (30%), wheat meal (30%), soybean meal (35%) and molasses (5%). The supplement was offered in an amount of 6 g/kg of the live weight, and the mineral mixture was offered ad libitum. The experiment was carried out for sixteen months, corresponding to the last eight months of gestation (after pregnancy diagnosis, thirty days after insemination) and eight months of growth of the progeny.

Experimental procedures and sampling

Cows were weighed after 14 hours of fasting at the beginning (30 days of gestation) and at the end of the experiment (immediately after birth of calves) to determine their average daily gain (ADG). Calves were weighed immediately after birth and at the end of the suckling period (after 14 hours of fasting) and every 30 days calves were weighed (without fasting) for performance monitoring and adjustment of the supplement offered.

In order to minimize possible paddock effects, all animals were rotated every seven days; all groups stayed the same period in each paddock. Pastures samples were collected via manual grazing simulation, every 15 days, dried in an forced-air circulation oven (SOLAB®, Ar SL - 102) at 55–60ºC for 72 hours and then grounded with 1 and 2 mm knife mill (Willye® TE-680).

To evaluate the nutritional characteristics of diet, a digestibility trial was performed for nine days at both 100 and 230 days of gestation as well as 120 days post calving. The three-marker method was used as it follows. Chromic oxide (Cr_2O_3) was used estimate the faecal excretion of animals, which was wrapped in paper cartridges in an amount of 20 g per animal/day for cows and 10 g per animal/day for calves and administered with a metal probe via the oesophagus at 10 hours (Detmann *et al.*, 2001b). Titanium dioxide (TiO2) was used to estimate the individual supplement intake, provided via supplement at the proportion of 10 g/kg of supplement (Titgemeyer *et al.*, 2001). Indigestible neutral detergent fibre (iNDF) was used to estimate the pasture dry matter intake (Detmann *et al.*, 2001a). The first five days of each trial were used for animal adaptation to TiO_2 and Cr_2O_3. Faecal samples were collected immediately after of defecation or directly from the rectum of the animals in the last 4 days of the digestibility period (one sample for day) at the times 18, 14, 10 and 06 hours. After collection, faeces were dried in a forced-air circulation oven (SOLAB®, Ar SL - 102) at 60 °C for 72 hours and were grounded with 1 and 2 mm knife mill (Willye® TE-680). A composite sample was prepared for each animal and stored for subsequent analysis.

Chemical analysis

Dry matter content (DM), crude protein (CP), ether extract (EE), neutral detergent fibre corrected to ash and protein (NDFap) and iNDF were quantified in supplement, pasture and faecal samples processed as mentioned above. Measurements of DM, CP and EE were performed according to AOAC (Association of Official Analytical and Helrich, 1990); NDFap, according to (Mertens, 2002), using thermostable a-amylase, without using sodium sulphite; insoluble NDF content (NDIN) was quantified by following the recommendations of Van Soest and Robertson (1985), with ash corrections; iNDF, according to (Valente *et al.*, 2011), obtained after in situ incubation

in F57 Ankom® bags (Macedon, NY, USA) for 288 h. In faecal samples, Cr_2O_3 was determined by atomic absorption spectrophotometry (Williams *et al.*, 1962) and TiO_2 by colorimetric determination (Titgemeyer *et al.*, 2001).

Faecal dry matter excretion was estimated based on the amount of indicator ingested and its concentration in the faeces (Lopes *et al.*, 2014):

$$\text{Faecal DM (kg/day)} = \text{AOI} / \text{ICF},$$

where AOI is the amount of indicator ingested (g), and ICF is the indicator concentration in faeces (g/kg of faeces).

Estimates of individual supplement intake were obtained as follows:

$$\text{ISI} = [(\text{FE} \times \text{ICaF}) / \text{IOG}] \times \text{SOG},$$

where ISI is the individual supplement intake (kg/day); FE is faecal excretion (kg/day); ICaF is the indicator concentration in animal faeces (kg/kg); IOG is the indicator present in the supplement offered to each group (kg/day); and SOG is the supplement amount offered to the group of animals or treatment (kg/day).

Estimation of voluntary DM intake of forage (DMIF) was performed using iNDF as the internal marker, as follows:

$$\text{DMIF (Kg/day)} = (\text{FE} \times \text{FIC}) / \text{FOIC},$$

where FIC is the indicator concentration in the faeces (kg/kg); FOIC is the indicator concentration in forage (kg/kg); and FE is faecal extraction (Kg/day).

Total dry matter intake was obtained by the sum of cows concentrate and forage consumption.

To estimate the dry matter, CP and EE of milk consumed by calves, three milk collections were made with a mechanical milking machine (Milkban MB 300-1) at 30, 75 and 135 days of the suckling phase. Before milking, oxytocin (Forte UCB, 2.5 U.I, 0.5 ml/animal) was applied to facilitate the milk descent. For calves, the milk dry matter intake was also considered in addition to forage and concentrate to quantify total dry matter intake.

Histological analyses

At 30 days after birth, biopsies of skeletal muscle tissue from calves were collected to obtain samples of longissimus lumborum (LL) muscle (Arrigoni *et al.*, 2004). A 2 cm skeletal muscle samples were immediately fixed in 4% paraformaldehyde (pH = 7.03) and incubated at room temperature for 24 hours. After incubation, samples were dehydrated in an increasing concentration series of ethanol solutions (70, 80, 90 and 100%) for 2 hours, diaphonized in xylene for 1 h and embedded in paraffin. Sections of 4 µm were stained with haematoxylin-eosin (H&E) (Timm, 2005). A total of 30 images (magnification = x10, scale bar = 20 µm, image's resolution = 1 600 x 1 200 pixels) were obtained from each animal using an Olympus BX50 microscope with attached camera CMOS 1.3MP BioCAM (Japan). Muscle cell number and size were analysed with ImageJ software (Schindelin *et al.*, 2015).

Gene expression

Total RNA was extracted from 500 mg of muscle tissue biopsies (n=9) with Trizol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer instructions. To remove contamination by genomic DNA, RNA samples were treated with DNase-I Amplification Grade (Invitrogen). cDNA synthesis was performed from 1 µg of total RNA using the GoScript Reverse Transcription System (Promega, Madison, WI). The mRNA expression levels of markers for fibrogenesis (*TGF*-β, *COL1A1*, *COL3A3*), adipogenesis (*PPARγ*, *Zfp423*, *C/EBPα*), muscle hypertrophy (*FGF2R1*, *FGF2*), and energy metabolism (*PPARα, MCAD, UCP3*, *PRKAA2*, *HADH*, *MYH7*, *PDK4*, *PGC1α*, *CPT1*), were determined by the SYBR Green RT-PCR kit from Bio-Rad using a 7 300 Real-Time PCR System (Applied Biosystems, Foster City, CA). We have not evaluated the mRNA expression for myogenesis markers because formation of skeletal muscle fibres in bovine occurs prenatally (Bonnet *et al.*, 2010, Du *et al.*, 2010). Thus, we have only evaluated the effects of maternal nutrition on myogenesis through histological analysis. The endogenous gene used was 18S and the sequence primers are shown in Table 2. PCR reactions were performed at 95 °C for 3 min and subjected to 40 cycles of 95 °C for 10 s, and 60 °C for 30 s, followed by melting curve (0.01 °C/s). Results are expressed by ΔΔCt method (Bustin, 2002, Nolan *et al.*, 2006, Duarte *et al.*, 2013, Martins *et al.*, 2015).

Evaluation of carcass traits by ultrasonography

In the final measurement in the suckling phase, carcass traits of the calves were evaluated by ultrasound (Aloka, model: SSD 500 v, with linear probe of 18 cm). Carcass images were obtained from the right side of the animal where images for ribeye area measurement was taken from transversal section of the Longissimus lumborum muscle (between the T12 and T13 thoracic vertebrae); subcutaneous fat thickness, from

an average of two measurements (the first from the same place where the image for ribeye area was taken and the second at the pelvic region, between the ischium and pubis. Images were analysed in the BioSoft Toolbox® II for Beef software (Biotronics Inc., Ames, Iowa, USA).

Statistical analysis

The PROC MIXED procedure of SAS software (version 9.0) was used for all statistical analyses. For all statistical procedures we adopted α=0.10 of probability for type-I error, due the high incidence of type-II error in this kind of study. Means were submitted to analysis of variance and regression, and UNS, MID and LATE periods of supplementation were compared. Sex of the foetus was tested as fixed effect and removed from the model as it was not significant. Initial BW was used as a co-variable when significant and removed from the model when not significant. The variables on the effect of different supplementation (treatments) on the measured parameters were analysed in a completely randomized design.

Results

Performance

A greater intake of total digestible nutrients (TDN) and protein (CP) was observed in cows from MID group (P=0.002) in the 30–180-day period of gestation, when compared with those from LATE and UNS treatments (Table 3). Cows from LATE group presented greater consumption of DM, pasture dry matter, TDN and CP (P<0.1), at the 181–281-day period of gestation (Table 3). Cow supplementation did not affect the consumption (P>0.1) in calves during the suckling phase (Table 3).

In cows, no difference were observed in the daily weight gain (P=0.316) among MID, LATE and UNS, during the 30–180-day period of gestation. The average daily

gain was lower in the UNS group, at 181–281-day period of gestation (P=0.009). Supplementation during gestation did not influence the birth weight (P=0.883), final weight (P=0.953), average daily gain (P=0.986) and muscle fibre size (P=0.208) in calves. Higher values of subcutaneous fat thickness were observed in the progeny of LATE, also, higher values of ribeye area were observed in calves born from cows of LATE and MID groups, respectively (Table 4 and Fig. 1A e C). Muscle fibre number was greater in calves born from cows of MID compared with those born from cows of UNS group but was not different compared with LATE group (Fig 1B).

mRNA expression

The progeny of MID group presented greater mRNA expression of *FGF2* (P=0.003) and *PPARα* (P=0.073) genes. The mRNA expression of *PPARα* was decreased in skeletal muscle of calves from LATE group (Figure 2). Biomarkers associated with fibrogenic (*TGFβ*, *FGF2R1*, *COL1A1*, *COL3A3*), adipogenic (*ZFP423*, *PPARɣ*, C/*EBPα*) and beta oxidation (*MCAD*, *UCP3*, *PRKAA2*, *HADH*, *MYH7*, *PDK4*, *PGC1α*, *CPT1*) processes did not differ among the treatments (P>0.10) (Figure 2).

Discussion

It is well known that maternal nutrition during gestation affects the foetuses skeletal muscle development and thus impacts the postnatal development of the progeny (Du *et al.*, 2010). Among several ways that maternal nutrition may affects foetuses skeletal muscle development is by changing the mesenchymal stem cell commitment into different cell types that composes skeletal muscle (Du *et al.*, 2013, Duarte *et al.*, 2014). Among these types of cells are myocytes, adipocytes and fibroblasts, which are all derived from the same pool of mesenchymal stem cells and are the cells that contribute not only on muscle mass but also on marbling and collagen deposition. Thus, in the

present study we investigated not only if maternal supplementation would affect the animal performance but also if it would change the number of myocytes and the transcription profile of adipogenesis, fibrogenesis and energy metabolism markers in skeletal muscle of the offspring.

With regard to myogenesis, our data revealed that maternal supplementation during mid-gestation increased the number of myofibres in skeletal muscle of the offspring when compared to calves born from dams that were not supplemented during gestation. However, the number of myofibres in skeletal muscle of calves born from dams supplemented at late gestation did not differ from those born from non-supplemented cows. As reported by Bonnet *et al.* (2010) the total number of muscle fibres is set by the end of the second trimester of gestation. Therefore, a greater availability of nutrients due to maternal supplementation at mid-gestation may led to a greater number of myofibres in skeletal muscle of calves. On the other hand, it has been suggested that during the last trimester of gestation is when foetal skeletal muscle mass increase mainly due to muscle fibres hypertrophy (Du *et al.*, 2010). Thus, our results indicate that supplementation at late gestation may not substantially contributed to increase myogenesis in foetal skeletal muscle, which may explain the similarity of number of myofibers in skeletal muscle of calves from LATE and UNS groups. Collectively, these data indicates that supplementation at mid gestation may be more effective to increase the commitment of mesenchymal stem cells into myogenesis leading to a greater number of myofibres at birth.

Our data revealed that supplemental feed for grazing beef cows during gestation did not influence the progeny birth weight, weaning weight and the average daily gain. However, despite the lack of differences in BW gain, maternal supplementation during gestation did change the carcass composition of the calves. A greater rib eye area was

observed in calves from cows that were supplemented at both mid and late gestation compared to those born from non-supplemented cows. This difference in rib eye area may be a consequence of an increase in both hyperplasia of skeletal muscle myofibers of calves born from supplemented dams. It should be mentioned that no differences were observed in nutrient intake by calves between treatments, which strongly suggest that differences observed in calves were likely due to changes occurred in skeletal muscle development during gestation.

As hyperplasia of skeletal muscle cells does not occur after birth, we evaluated the mRNA expression of *FGF2* as a marker for skeletal muscle hypertrophy. It has been shown that *FGF2* is up-regulated during skeletal muscle hypertrophy being predominantly localized to the myofibers during skeletal muscle hypertrophy (Mitchell *et al.*, 1999). The main role of *FGF2* during postnatal skeletal muscle cell development is to stimulate myoblast (satellite cells) proliferation while inhibits its differentiation (Allen and Rankin, 1990, Mitchell *et al.*, 1999). We observed a greater mRNA expression of *FGF2* in skeletal muscle of calves born from dams that were supplemented at mid-gestation compared to the other treatments. Previously study has demonstrated that proliferation capacity of skeletal muscle satellite cells in lambs` offspring is affected by maternal nutrition during gestation (Raja *et al.*, 2016). Collectively, our results suggest that maternal supplementation during mid-gestation leads not only to an increased number of myofibres but also stimulate the proliferation capacity of satellite cells by increasing *FGF2* expression, which may contribute to an increase in skeletal muscle deposition later in life.

It has been shown that maternal over-nutrition increases the mRNA expression of adipogenesis markers in foetal skeletal muscle of beef cattle (Duarte *et al.*, 2014) and fibrogenesis in skeletal muscle of ovine offspring (Huang *et al.*, 2012). Therefore, we

hypothesized that maternal supplementation at late gestation would shift the commitment the mesenchymal stem cells towards to adipogenesis and fibrogenesis since both adipocytes and fibroblasts shear a common immediate common progenitor cells (Uezumi *et al.*, 2011). However, no differences were observed for mRNA expression of any of adipogenic and fibrogenic markers evaluated in this study. Although the number of intramuscular adipocytes and the amount of connective tissue was not evaluated, the mRNA expression data indicates that maternal supplementation did not affect the transcriptional level of the main markers of adipogenesis and fibrogenesis. It is noteworthy that although cows were supplemented, it does not characterize overnutrition of the dams as was described by previous studies. Thus, our data suggests that to enhance intramuscular fat deposition in skeletal muscle of the offspring through maternal nutrition during gestation it would be necessary a higher energy or global nutrient intake by the dams during pregnancy, which warranty further studies using grazing beef cows as a model.

Energy metabolism of skeletal muscle of the offspring is also affected by maternal nutrition during gestation (Zhu *et al.*, 2006). These adaptive changes that occurs in skeletal muscle of the offspring due maternal nutrition includes decreased glucose transport and ATP production (Selak *et al.*, 2003) and impairment of transcriptional metabolic flexibility of skeletal muscle, which means the decrease of skeletal muscle capacity to change the source for ATP synthesis. Thus, in certain cases maternal nutrition may program skeletal muscle of the offspring for a preferable use of fatty acid instead the use of carbohydrates as a source to produce ATP, even when high levels of glucose are available for the skeletal muscle (Aragão *et al.*, 2014). Consequently, this scenario would lead to a lower marbling deposition in beef animals even when they are fed high-energy diets.

In the present study we did not observed differences in mRNA expression of energy metabolism markers in the skeletal muscle of the offspring, with exception of *PPARα*.

The *PPARα* gene regulates the mitochondrial biosynthesis of muscle through adipose triglyceride lipase (AGTL) expression. This enzyme is responsible for fatty acid oxidation at the mitochondrial level, providing energy primarily (in the ATP form) for muscle metabolism (Biswas *et al.*, 2016). Biosynthesis of muscle fibres requires a major input of energy for metabolism and hyperplasic growing of muscle. In this context, the *PPARα* expression observed in this study can be associated to increase of muscle fibre number. Thus, the greater expression of *PPARα* in skeletal muscle of calves born from damns supplemented at mid-gestation may be due to the greater number of myofibres presented by these animals. Since a biological role of intramuscular fat is to provide readily carbon source for oxidation by muscle cells (Duarte *et al.*, 2017), the greater number of myofibres would decrease the lipid storage by intramuscular adipocytes in these animals, leading to a decrease in intramuscular fat deposition. The lower mRNA expression of *PPARα* may also explain the greater subcutaneous fat deposition in calves from LATE group.

This may represent a lower energy demand by the skeletal muscle, likely allowing the energy partitioning towards other fat depots. Additionally, it must be noted that the main focus of the present study was the skeletal muscle development and the cellularity of other fat depots was not investigated. Thus, as hypothesized for intramuscular fat depot, maternal supplementation at late gestation may also increase adipogenesis in subcutaneous fat depot, leading to a greater carcass back fat in the offspring, which need further investigation.

Conclusion

Supplementation of grazing beef cows during gestation promotes additional gain of skeletal muscle fibres number and ribeye area of the progeny but does not seem to improve intramuscular adipogenesis and/or fibrogenesis, which might require a greater energy or global nutrient intake. Calves born from damns supplemented during mid-gestation had increased mRNA expression of *FGF2*, which may indicate a higher hypertrophy ability of skeletal muscle of the offspring. On the other hand, calves born from dams supplemented at late gestation had greater subcutaneous fat deposition. Further studies are needed to investigate not only when but also the level of maternal supplementation at different stages of gestation to develop a precise maternal plane of nutrition to improve performance of the offspring.

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Table 1 Nutrient content of dietary supplements and pastures at times A, B, C offered to cows during gestation and your progeny.

Nutrient measure	Supplements		Pastures		
	Pregnant cows	Progeny	A	B	C
DM, g/kg fresh matter[1]	879.8	882.5	877.2	886.7	877.8
MO, g/kg DM[2]	958.7	890.9	907.4	906.9	910.8
CP, g/kg DM[3]	282.7	246.1	92.5	74.8	82.9
EE, g/kg DM[4]	34.6	27.2	49.5	41.7	16.2
NDFap, g/kg DM[5]	196.2	144.7	565.7	587	565.7
NFC, g/kg DM[6]	445.1	472.9	199.7	203.3	245.9
iNDF, g/kg DM[7]	35.5	16.2	207.5	191.8	193.3

A - Manual grazing simulation, early-mid gestation; B - Manual grazing simulation, late gestation; C - Manual grazing simulation, growing period.

Mineral mix - $CaHPO_4$= 50.00%; NaCl= 47.15%; $ZnSO_4$= 1.50%; Cu_2SO_4= 0.75%; $CoSO_4$= 0.05%; KIO_3= 0.05% and $MnSO_4$= 0.05%.

[1]DM dry matter.

[2]MO matter organic.

[3]CP crude protein.

[4]EE ether extract.

[5]NDFap neutral detergent fibre corrected to ash and protein.

[6]NFC non-fibrous carbohydrates.

[7]iNDF neutral detergent fibre indigestible

Table 2 Primer sequence for amplifying mRNA transcripts of genes of interest in longissimus muscle tissue of the progeny of cows supplemented during pregnancy

Gene	Forward sequence 5'–3'	Reverse sequence 5'–3'
TGFβ	AGCCAGGGGGATGTGCCA	TAGCACGCGGGTGACCTCCT
COL1A1	CCACCCCAGCCGCAAAGAGT	ACGCAGGTGACTGGTGGGATGTC
FGF2R1	AGGAGGATCGAGCCCACGGC	CTTGCTCCGGCAAGGTCGGGG
COL3A3	GGCCCCCTGGAAAGGACGGA	CCCCGCCAGCACCACAACAT
FGF2	GGAGCATCACCACGCTGCCA	GTGGGTCGCTCTTCTCGCGG
PPARα	GCCAGAGGAGGAGAAAGA	CTGGGAGAGGTCTGTGTAG
PPARγ	TGGAGACCGCCCAGGTTTGC	AGCTGGGAGGACTCGGGGTG
MCAD	CGAGTACCCTGTCCCATTA	CCAAGACCTCCACAACTTTC
UCP3	GCCAGAGGAGGAGAAAGA	CTGGGAGAGGTCTGTGTAG
HADH	CCTGCCTCTTGCCTTTAC	CCAGCAGCAACATCTCAA
MYH7	GGATGCACTCGTTTCTCAG	GGCACTCTTGGCCTTTATC
PDK4	GGATGGGTGCTCTCATTTC	CTGTCCACCTGCACATTC
PGC1α	GAAGCGGGAATCCGAAAG	CTCAGTTCTGTCCGTGTTG
CPT1	GTCCCTTCCCTTGCTCTA	GGACAGCAGAGACCCATA
Zfp423	GGATTCCTCCGTGACAGCA	TCGTCCTCATTCCTCTCCTCT
C/EBPα	TGCGCAAGAGCCGGGACAAG	ACCAGGGAGCTCTCGGGCAG
18S	CCTGCGGCTTAATTTGACTC	AACTAAGAACGGCCATGCAC

TGFβ: Transforming growth factor, beta; *COL1A1*: Collagen type I, alpha 1; *FGF2R1*: Fibroblast growth factor 2, receptor 1; *COL3A3*; Collagen type III, alpha 3; *FGF2*: Fibroblast growth factor 2; *PPARα*, *PPARγ*: Peroxysome proliferator activated-receptor, alpha/gamma; *MCAD*: Medium-chain acyl-CoA dehydrogenase; *UCP3*: Uncoupling protein 3; *HADH*: Hydroxyacyl-CoA dehydrogenase; *MYH7*: Myosin heavy chain 7; *PDK4*: Pyruvate dehydrogenase kinase 4; *PGC1α*: Peroxisome proliferator activated receptor gamma coactivator 1 alpha; *CPT1*: Carnitine palmitoyltransferase 1; *Zfp423*: Zinc finger protein 423; *C/EBPα*: CCAAT enhancer binding protein, alpha.

Table 3 Dry matter and nutrient intake of dietary treatments offered to cows during gestation and their progeny

Item	Treatments			SEM	P value
	UNS	MID	LATE		
Dry matter 30-180 day, kg	14.1	14.9	14.1	0.31	0.182
Dry matter 181-281 day, kg	13.5[b]	13.3[b]	15.5[a]	0.25	0.001
Pasture dry matter 30-180 day, kg	14.1	13.9	14.1	0.29	0.697
Pasture dry matter 181-281 day, kg	13.5[b]	13.3[b]	14.2[a]	0.26	0.041
Total digestible nutrients 30-180 day, kg	6.8[b]	7.9[a]	6.8[b]	0.18	0.002
Total digestible nutrients 181-281 day, kg	7.3[b]	7.4[b]	9.5[a]	0.20	0.001
Crude protein 30-180 day, kg	1.3[b]	1.6[a]	1.3[b]	0.03	0.001
Crude protein 181-281 day, kg	1.0[b]	1.0[b]	1.4[a]	0.01	0.001
Progeny					
Dry matter, kg	3.5	4.2	3.9	0.52	0.690
Dry matter supplement, kg	1.0	1.1	1.2	0.19	0.719
Dry matter milk, kg	0.7	0.7	0.8	0.05	0.574
Matter organic, kg	3.3	3.8	3.6	0.48	0.730
Crude protein, kg	0.4	0.5	0.5	0.07	0.786

UNS = cow unsupplemented during the gestation; MID = cows supplemented during 30 to 180-day of gestation; LATE = cows supplemented during 181 to 281-day of gestation.

[a,b] Values within a row with different superscripts differ significantly at $P<0.1$.

Table 4 Performance and body condition of cows during gestation and their progeny.

Item	Treatments			SEM	P value
	UNS	MID	LATE		
Final weight 180 day, kg	490.3	476.4	490.3	10.41	0.433
Final weight 281 day, kg	476.7	489.4	510.4	14.00	0.245
Average daily gain 180 day, g	-22.9	-57.3	-22.9	20.15	0.316
Average daily gain 281 day, g	-99.4[b]	156.8[a]	220.9[a]	70.05	0.009
Fat thickness 180 day, mm	4.6	5.4	4.6	0.60	0.441
Fat thickness 281 day, mm	3.4	4.1	3.5	0.51	0.568
		Progeny			
Birth weight, kg	33.0	34.2	33.9	1.84	0.883
Final weight, kg	238.0	240.2	240.5	6.22	0.953
Average daily gain, g	855.0	861.0	858.0	26.23	0.986
Fat thickness, mm	2.2[b]	2.2[b]	3.1[a]	0.21	0.006

UNS = cow unsupplemented during the gestation; MID = cows supplemented during 30 to 180-day of gestation; LATE = cows supplemented during 181 to 281-day of gestation.

[a,b] Values within a row with different superscripts differ significantly at P<0.1.

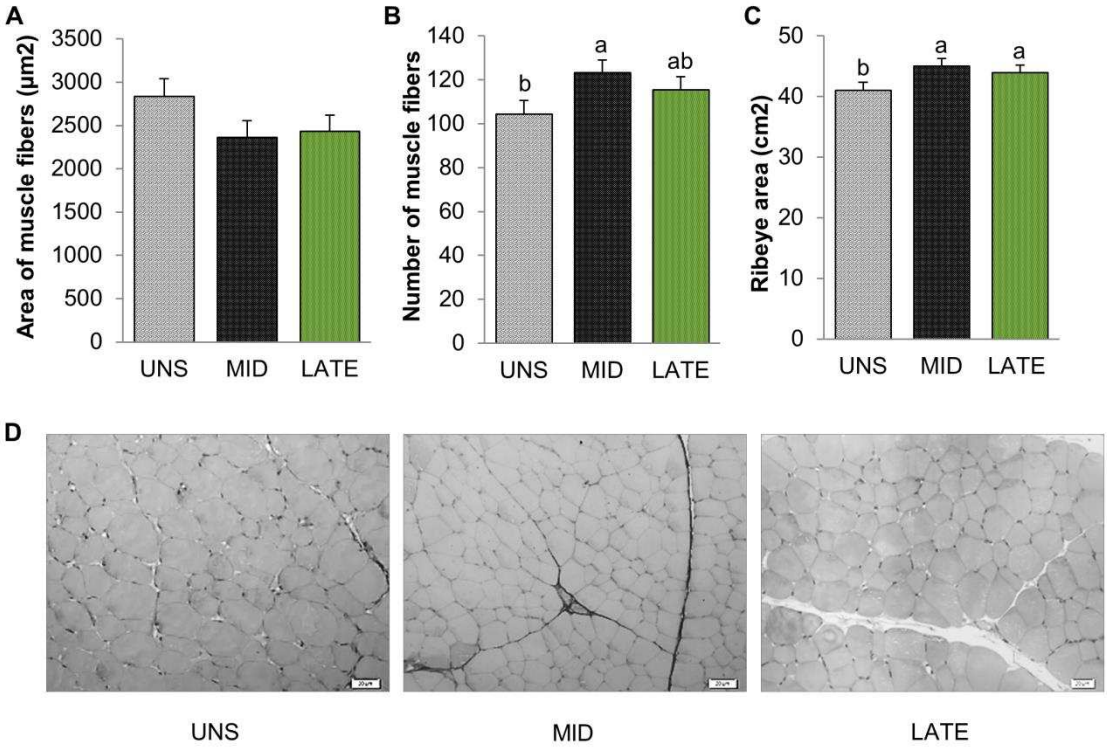

Figure 1 Effect of maternal nutrition during gestation on characteristics of histological muscle of progeny of UNS cow unsupplemented during the gestation; MID cows supplemented during 30 to 180-day of gestation; LATE cows supplemented during 181 to 281-day of gestation. Samples were collected per biopsies of Longissimus lumborum (LL) muscle. Bars represent means ± SEM (n = 9). Different letters indicate significant differences between the groups ($P<0.1$). There was no significant variation by sex effect. Cell number per reading area (10x).

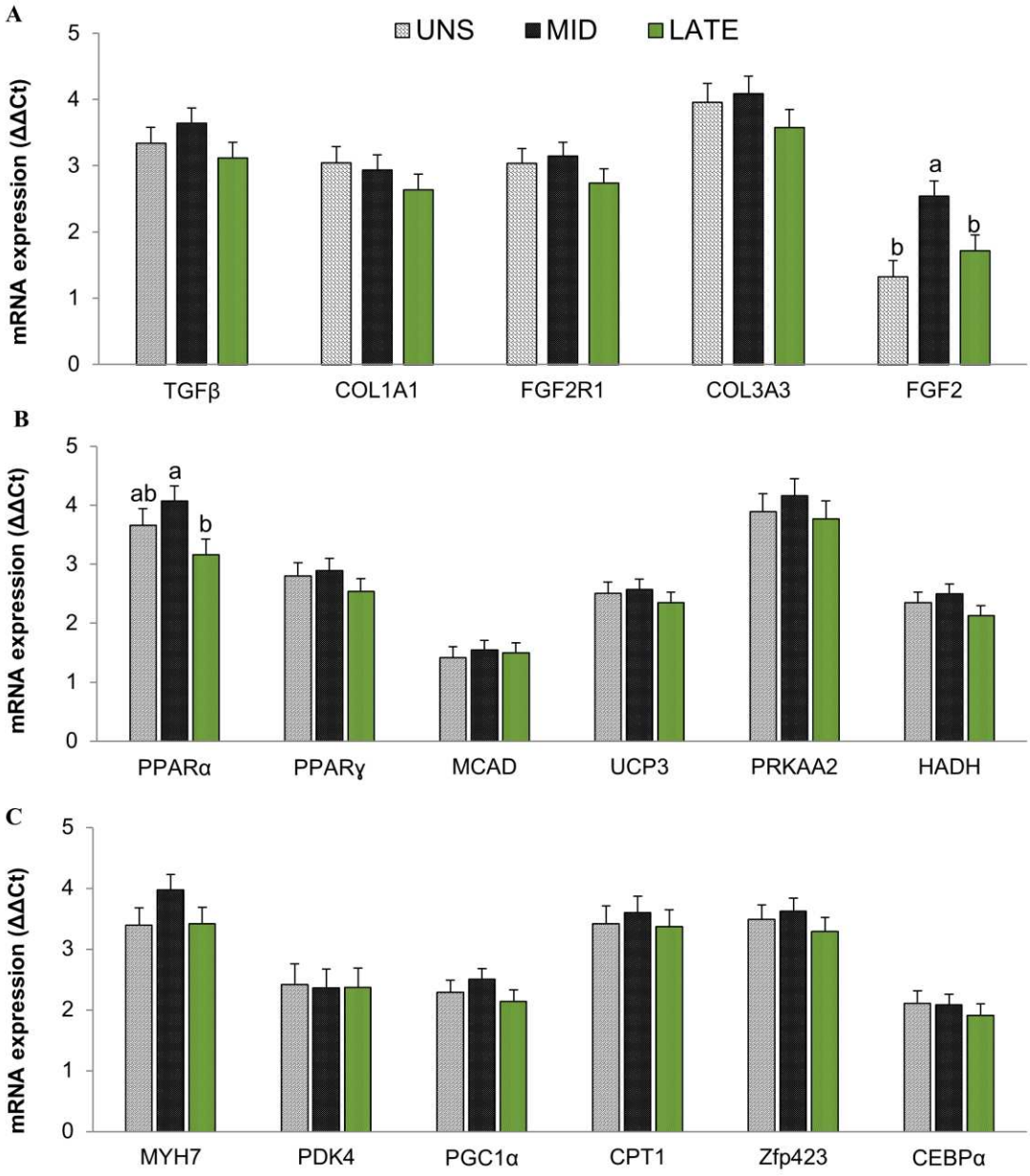

Figure 2 Real-time PCR analysis of the influence of maternal nutrition during gestation on characteristics muscle of progeny of UNS cows (unsupplemented during gestation), MID cows (supplemented 30 to 180-day of gestation) and LATE cows (supplemented 181 to 281-day of gestation). Samples were collected per biopsies of longissimus lumborum (LL) muscle. Bars represent means ± SEM (n=9). Different letters indicate significant differences between the groups ($P<0.1$). There was no significant variation by sex effect.

CHAPTER 2

Supplementation of grazing beef cows during gestation as a strategy to improve the prepartum energy balance and postpartum fertility

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Abstract

The objective of this study was to determine the effect of cow's grazing supplementation during pregnancy on preterm concentration of metabolites in blood and its relation with uterine involution and postpartum fertility. Thereby, 27 Nellore cows were divided into three groups (n=9 for each group) as follows: UNS, unsupplemented during gestation; MID, supplemented from the 30 to 180-day of gestation; LATE, supplemented from the 181 to 281-day of gestation. Blood samples to determine the concentrations of NEFA, BHBA (β-hydroxybutyrate) and glucose were performed on days 30, 105, 180, 230 and 281 of gestation. After delivery, on days 10, 18 and 30, ultrasound measurements were taken to determine the size, position, and absence of fluids in the uterine horns, and thus to establish the uterine involution time. Fixed-time artificial insemination (FTAI) was performed in all matrices at forty-five days postpartum. Lower NEFA and BHBA concentrations were observed in the MID and

LATE group at 180 and 281 days of gestation respectively for each treatment. Higher glucose concentration was observed at 180 and 281 days of gestation for MID and LATE treatment respectively. There was no difference in uterine involution and percentage of gestation between treatments. Involution of the uterus was observed at 30 days postpartum, with a size of 14.40 ± 1.31, 15.70 ± 1.20 and 14.70 ± 1.25 mm, respectively for the UNS, MID and LATE treatments. Pre-birth supplementation improves energy balance, increased glucose concentration, and decreased NEFA and BHBA concentration, without altering uterine involution and postpartum fertility.

Keywords: Reproduction, metabolites, gestation, energetic status.

Introduction

The onset of lactation represents a transition period with physiological and metabolic changes that disturb homeostatic balance and reproduction. This process begins in the final stage of gestation and continues during the initial period of lactation, where the growth of the pregnant uterus negatively influences rumem size and dry matter intake. This change continues after calving, coinciding with an increase in nutritional requirements for milk synthesis, leading to a negative energy balance (SB) of the matrices.

As reproduction is a characteristic of low heritability, reproductive performance is influenced by non-genetic factors such as environment and nutrition (Vasconcelos et al., 1999), the latter being an important relation with the prepartum metabolic state and post-fertility -parto (Ayres et al., 2014). Thus, EB becomes a determinant condition for the resumption of ovulation and involution of the uterus. BE may be estimated by the plasma concentration of blood metabolites such as NEFA, βHBA and glucose and even by the body condition score. NEFA are free fatty acids products of the degradation of

lipids stored as fat, βHBA are ketone bodies produced mainly in the liver, from the free volatile fatty acids (aceto acetate), butyrate and aceto acetate present in the portal circulation. Ruminal epithelium also produces small amounts of βHBA from acetate and butyrate. Thus, these metabolites represent negative states of energy balance. Glucose, on the other hand, is a metabolite of synthesis, mainly produced in the liver from propionate, but also produced by the intestinal epithelium normally when animals are fed grain without heat treatment, where part of the starch escapes ruminal degradation.

Hypothalamic nuclei are responsible for the return of ovulation, responding with release of GnRH pulses when the animal is in positive energy balance (BE), because GnRH stimulates the release of FSH and LH by the pituitary hormones responsible for growth And ovulation of the follicle.

During the postpartum period, the rapid involution of the uterus, together with the resumption of ovulation, become essential to achieve intervals between ideal deliveries, so the earlier the matrix initiates the postpartum uterine cyclicity and involution, the greater will be the fertility achieved during the breeding period (Giuliodori *et al.*, 2011).

Pregnant matings, which graze in the dry season during the last gestation period, have been related to weight loss, loss of body condition score and high concentration of NEFA and βHBA, leading to long periods of negative energy balance, thus , Adequate prepartum nutrition through supplementation, improved nutrient intake, and positive energy balance return. Feeding and other care with the matrices in the three weeks before and three weeks after childbirth become the success of the reproductive process; however, nutrition in the early stages of gestation is of great importance for fetal development and offspring performance (Zhu *et al.*, 2006, Du *et al.*, 2010, Du *et al.*, 2011, Du *et al.*, 2015).

Therefore, the present study aims to determine the effect of supplementation in the early gestation periods, on the prepartum energy balance and its relation to the return of ovulation, uterine involution and fertility.

Materials and methods

Location and weather conditions

This study was approved by the Brazilian Ethics Committee on Animal Use (CEUAP/UFV – Process nº 26/2014), according to ethical principles of animal experimentation established by the National Council of Animal Experimentation Control (CONCEA). The experiment was performed at the Beef Cattle Farm of Animal Science Department of University Federal de Viçosa, Viçosa-MG, Brazil. Weather conditions during the experiment were: 284 mm of rain and an average temperature of 22.6 °C (first trimester of pregnancy); 94 mm of rain and average temperature 18.6 °C (second trimester of pregnancy); 61 mm of rain and average temperature 18.7 °C (last trimester of pregnancy).

Animals, experimental design and supplements

In the present study, twenty-seven pregnant Nellore cows were used with an 490 ± 12.82 kg average initial weight. Cows were artificially inseminated at a fixed time using semen from the same bull. At sixty days of gestation, foetal sexing was performed to obtain homogeneous treatment. Animals were allocated to six-hectare paddocks per treatment, evenly covered with *Urochloa decumbens* grass, equipped with a drinker and feeders. Experimental design was a completely randomized design, with three treatments and nine replications as following: UNS, unsupplemented during gestation; MID, supplemented from the 30 to 180-day of gestation; LATE, supplemented from the 181 to 281-day of gestation. Composition of supplements and pastures are shown in

Table 1. Percentage composition of supplements was the: ground corn grain (26.25%), wheat meal (26.25%) and soybean meal (47.5%) for cows. A total of 150 kg of supplement per animal were provided during the total experiment period for each treatment (1 and 1.5 kg/day to cows in the MID and LATE groups respectively), accompanied by a mineral mixture offered *ad libitum* (Table 1). The control group, the UNS cows, received only a mineral mixture *ad libitum* during gestation.

Experimental procedures and sampling

Cows were weighed after 14 hours of fasting at the beginning (30 days of gestation) and end of their portion of the experiment. In order to minimize possible paddock effects on the treatments, all animals were rotated every seven days; all groups stayed the same period in each paddock. Pastures samples were collected via manual grazing simulation, every 15 days. Samples were dried in an forced-air circulation oven (SOLAB®, Ar SL - 102) at 55–60ºC for 72 hours and were crushed in an 1 and 2 mm knife mill (Willye® TE-680).

To evaluate the nutritional characteristics of diet, a digestibility trial was performed for nine days at both 100 and 230 days of gestation. The three-marker method was used as follows. Chromic oxide (Cr_2O_3) was used estimate the faecal excretion of animals, which was wrapped in paper cartridges in an amount of 20 g per animal/day and administered with a metal probe via the oesophagus at 10 hours (Detmann *et al.*, 2001b). Titanium dioxide (TiO2) was used to estimate the individual supplement intake, provided via supplement at the proportion of 10 g/kg of supplement (Titgemeyer *et al.*, 2001). Indigestible neutral detergent fibre (iNDF) was used to estimate the pasture dry matter intake (Detmann *et al.*, 2001b). The first five days of each trial were used for animal adaptation to TiO_2 and Cr_2O_3. Faecal samples were collected immediately after of defecation or directly from the rectum of animals, in the last 4 days of experiment

(one sample for day) at the times 18, 14, 10 and 06 hours. After collection, faeces were dried in a forced-air circulation oven (SOLAB®, Ar SL - 102) at 60 °C for 72 hours and were crushed in an 1 and 2 mm knife mill (Willye® TE-680). A composite sample was prepared for each animal and stored in glass pots for subsequent analysis.

Spot urine samples were obtained on the last day of the digestibility trial by spontaneous urination and blood samples collected via jugular venipuncture, both ~4 h after supplementation. Blood samples were collected using vacuum tubes containing a clot activator and separator gel (BD Vacuntainer®, SST II Advance, Cidade Industrial, Curitiba – Paraná, Brazil). After collection, the samples of urine were diluted in H2SO4 (0.036 N) and frozen at -20°C (Valadares *et al.*, 1999) for later evaluation of the contents of creatinine, urea and purine derivatives. The blood was immediately centrifuged at 2700g for 15 min at room temperature, and the serum was stored at -20°C, for later evaluation of the contents of urea.

Chemical analysis

The dry matter content (DM), crude protein (CP), ether extract (EE), neutral detergent fibre corrected to ash and protein (NDFap) and iNDF were quantified in supplement, pasture and faecal samples processed as mentioned above. DM, CP and EE were processed according to AOAC (Association of Official Analytical and Helrich, 1990); NDFap, according to (Mertens, 2002), using thermostable a-amylase, without using sodium sulphite; insoluble NDF content (NDIN) was quantified by following the recommendations of (Van Soest and Robertson, 1985), with ash corrections; iNDF, according to (Valente *et al.*, 2011), obtained after *in situ* incubation in F57 Ankom® bags (Macedon, NY, USA) for 288 h. In the faecal samples, chrome was evaluated by atomic absorption spectrophotometry (Williams *et al.*, 1962), and titanium by colorimetric determination (Titgemeyer *et al.*, 2001).

Fecal dry matter excretion was estimated based on the amount of indicator and its concentration in the faeces (Lopes *et al.*, 2014):

$$\text{Fecal DM (kg/day)} = \text{AOI} / \text{ICF},$$

where AOI is the amount of indicator (g), and ICF is the indicator concentration in faeces (g/kg of faeces).

Estimates of individual supplement intake were obtained as follows:

$$\text{ISI} = [(\text{FE} \times \text{ICaF}) / \text{IOG}] \times \text{SOG},$$

where ISI is the individual supplement intake (kg/day); FE is faecal excretion (kg/day); ICaF is the indicator concentration in animal faeces (kg/kg); IOG is the indicator present in the supplement offered to each group (kg/day); and SOG is the supplement amount offered to the group of animals or treatment (kg/day).

Estimation of voluntary DM intake of forage (DMIF) was performed using iNDF as the internal marker (Detmann *et al.*, 2001a), as follows:

$$\text{DMIF (Kg/day)} = (\text{FE} \times \text{FIC}) / \text{FOIC},$$

where FIC is the indicator concentration in the faeces (kg/kg); FOIC is the indicator concentration in forage (kg/kg); and FE is faecal extraction (Kg/day).

Total dry matter intake was obtained by the sum of cows concentrate and forage consumption.

Creatinine, uric acid and urea were quantified by the kinetic colorimetric, enzymatic colorimetric and fixed-time kinetic methods, respectively, using an automatic device for biochemistry (Mindray® model BS200E) and kits. The daily urinary volume was

calculated by employing the ratio between the day excretion of creatinine (ECU), adopting the equation proposed by (Silva *et al.*, 2012) and its concentration in the spot samples as reference:

$$ECU\ (g/day) = 0.0345 \times BW^{0.9491}$$

where: BW= body weight.

Allantoin in the urine was analyzed as described by (Chen and Gomes, 1992). The total excretion of purine derivatives was calculated as the sum of the amounts of allantoin and uric acid excreted in the urine. Absorbed purines (Y, mmol/day) were calculated from the excretion of purine derivatives (X, mol/day), by the following equation:

$$Y = (X - 0.301 \times BW^{0.75}) / 0.80$$

where: 0.80 is the recovery of absorbed purines as purine derivatives and $0.301 \times BW^{0.75}$ is the endogenous contribution to the excretion of purines (Barbosa *et al.*, 2011).

The ruminal synthesis of nitrogen compounds (Y, g micN/day) was calculated as a function of the absorbed purines (X, mmol/day), using the equation described by (Barbosa *et al.*, 2011).

$$Y = 70X / 0.93 \times 0.137 \times 1000$$

where 70 is the N content in the purines (mgN/mol); 0.137 is the purine N:total bacterial N ratio; and 0.93 is the digestibility of the bacterial purines.

Microbial efficiency was expressed as g microbial CP/kg of total digested organic matter (g micCP/kg DOM).

Metabolites of energy status

Blood samples were obtained via puncture of the jugular vein, for analysis of glucose, NEFA and βHBA on days 30, 105, 180, 230 and 280 of gestation. After collection, the blood was immediately centrifuged at 3600 rpm × 20 minutes, the serum stored (-20 ° C) for further analysis.

The method for quantification of NEFA and βHBA was the colorimetric methods, using a Bioclin® kit and Randox® kits respectively, using the automatic equipment for biochemistry of Mindray brand, model-BS200E. Glucose levels were quantified by enzymatic colorimetric method using commercial kits of the brand Bioclin®. In these analyzes we used the automatic equipment for biochemistry of the brand Mindray, model-BS200E.

The different procedures were carried out according to the protocols recommended by the companies supplying the respective kits.

Postpartum reproductive assessments

Using transrectal ultrasonography (Aloka 500 with a 5 MHz linear probe, Aloka, Wallingford, CT) were performed on days 10, 18 and 30 postpartum evaluations to determine uterine involution and return of cyclic luteal activity. After delivery, the non-pregnant horn was measured and considered uterine involution when the horn that supports the gestation is measured close to that of the non-pregnant horn. The measurements were longitudinally and vertically over the curvature of the horn.

Presence of fluid and position of the uterus (abdominal or pelvic) were evaluated to determine complete uterine involution.

Forty-five days postpartum, the matrices were protocolized and inseminated by IATF, the protocol used was as follows: Day 0: application of intra-vaginal slow-release progesterone device plus 2 ml of estrogen (estradiol benzoate); Day 7: withdrawal of the intra-vaginal slow-release progesterone device plus application of 2 ml prostaglandin F2α (Cloprostenol); Days 8: application of 0.5 ml of estrogen (estradiol cypionate); Day 10: 48 hours after application of estrogen artificial insemination was performed in all matrices; Day 31: the intra-vaginal slow-release progesterone device was reapplied in all matrices; Day 38: withdrawal of intra-vaginal device from slow release of progesterone in all matrices. Pregnant cows were sent to the batch of pregnant women and counted as a positive result of the first IATF, empty cows continued the protocol with application of 2 ml of prostaglandin F2α (cloprostenol); Day 39: application of 0.5 ml of estrogen (estradiol cypionate) in the empty matrices; Day 41: forty-eight hours after application of estrogen artificial insemination was performed in all matrices. Twenty eight days after the second insemination the diagnosis of gestation was made by ultrasonography and the percentage of total gestation was determined.

Statistical analyzes

The PROC MIXED procedure of the SAS (Statistical Analysis System, version 9.0) software was used for all statistical analyses. For all statistical procedures we adopted α=0.10 of probability for type I error, due the high incidence of type II error in this kind of study. Means were submitted to analysis of variance and regression, comparison different periods of supplementation, being UNS, MID, LATE = unsupplemented during gestation, supplemented from the 30 to 180-day of gestation and supplemented

from the 181 to 281-day of gestation. Sex of the fetus was tested as fixed effect and removed from the model while not significant. Initial BW was used as a co-variable when significant and removed from the model while not significant. The variables on the effect of different supplementation (treatments) on the measured parameters were analyzed in a completely randomized design. Posture of the uterus and presence of fluid in the uterus were evaluated by generalized models.

Result

Intake and Performance

During the period between 30-180 days of gestation, MID matrices presented higher consumption ($P < 0.01$) of NDT and PB compared to UNS and LATE. Greater consumption ($P < 0.05$) of MS, NDT and PB were observed for LATE matrices during 181-281 in comparison with UNS and MID (Table 2). As observed in Table 3, there was no difference in live weight and subcutaneous fat thickness of the matrices during gestation ($P > 0.1$).

Energy status

A lower concentration of NEFA and BHBA ($P < 0.01$) at 180 and 281 days of gestation was observed for MID and LATE matrices respectively (Figure 1A-B). Higher glucose concentration ($P < 0.1$) during pregnancy was observed for MID matrices at 180 days and LATE at 281 days of gestation (Figure 1C).

As shown in Figure 2, higher microbial efficiency was detected for UNS and MID matrices during 30-180 days of gestation. During this same period, MID matrices presented higher concentration of SUN and UUN compared to UNS and LATE. Greater MICN, SUN and UUN for LATE matrices were observed between 181-281 days of

gestation when compared to UNS and MID, however, there was a higher concentration of UUN for LATE compared to MID.

Postpartum Reproductive Parameters

There was no significant difference between treatments for uterine involution at 10, 18 and 30 days postpartum (Figure 3A). During the postpartum evaluation period, there was no presence of ovarian corpora lutea. Uterine involution was observed at 30 days of gestation with a size of 14.40 ± 1.31, 15.70 ± 1.20 and 14.70 ± 1.25 mm, respectively for the UNS, MID and LATE treatments.

UNS, MID and LATE treatments presented 4/9, 6/9 and 7/9 pregnant matrices respectively during the first artificial insemination (Figure 3B).

Discussion

Pre-partum - metabolic markers

Increased lipomobilization during the last four weeks of gestation was not associated with delay in first ovulation and altered fertility in multiparous cows. Similar data were observed in the present study, where, although no difference was observed between treatments in relation to Fat thickness, higher concentration of NEFA and BHBA at 281 days for MID did not affect uterine involution and fertility. Increased concentrations of NEFA in MID matrices at 281 days of gestation can be explained by the fact that pregnant cows metabolize energy reserves and alter their metabolism to meet the energetic demands of the growing fetus during the last gestation period (Wood *et al.*, 2013).

The best energetic status presented by LATE matrices at 281 days of gestation, due to the higher concentration of glucose and lower concentration of NEFA can be

explained by the effect of supplementation, generating greater consumption of DM, TDN and CP between 181 and 281 days of gestation.

Percentage of gestation

Increased thickness of subcutaneous fat at delivery has been associated with a high probability of postpartum gestation (Ayres *et al.*, 2014). Although supplemented animals presented a balanced energy balance when compared to non-supplemented, no difference in subcutaneous fat thickness and body condition score was observed at 180 and 281 days of gestation, and may explain, in a way, the equality in gestation percentage between the treatments.

Even if no statistical difference was observed in relation to the percentage of gestation, I should note that in order to reach the same percentage of total gestation, matrices not supplemented during gestation would spend higher numbers of semen doses when compared with supplemented matrices.

Conclusion

Supplementation during gestation improves energy balance with decreased levels of NEFA, BHBA and increased glucose levels, with no change in uterine involution and postpartum fertility. Supplementation during pregnancy does not alter pre-delivery subcutaneous fat thickness or postpartum fertility. Feeding strategies can be applied when looking for a joint effect between fetal muscle hyperplasia and postpartum fertility of the matrices.

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Table 1

Nutrient content of dietary supplements and pastures offered to cows during gestation

Item	**Supplement**	**[1]Pasture**	**[2]Pasture**
Dry matter, g/kg fresh matter	879.85	877.20	886.74
Matter organic, g/kg dry matter	958.72	907.36	906.95
Crude protein, g/kg dry matter	282.70	92.46	74.84
Ether extract, g/kg dry matter	34.64	49.51	41.75
NDFap, g/kg dry matter	196.26	565.70	587.00
Non-fibrous carbohydrates, g/kg dry matter	445.10	199.68	203.35
i-NDF, g/kg dry matter	35.50	207.48	191.81

[1]Manual grazing simulation, early-mid gestation; [2]Manual grazing simulation, late gestation.

Mineral mix - $CaHPO_4$= 50.00%; NaCl= 47.15%; $ZnSO_4$= 1.50%; Cu_2SO_4= 0.75%; $CoSO_4$= 0.05%; KIO_3= 0.05% and $MnSO_4$= 0.05%.

Table 2

Dry matter and nutrient intake of dietary treatments offered to cows during gestation.

Item	Treatments			P-value
	UNS	MID	LATE	
Dry matter 30-180 day, kg	14.1 ± 0.31	14.9 ± 0.41	14.1 ± 0.31	0.182
Dry matter 181-281 day, kg	13.5 ± 0.25[b]	13.3 ± 0.23[b]	15.5 ± 0.24[a]	0.001
PDM 30-180 day, kg[1]	14.1 ± 0.29	13.9 ± 0.28	14.1 ± 0.29	0.697
PDM 181-281 day, kg[1]	13.5 ± 0.26[b]	13.3 ± 0.25[b]	14.2 ± 0.26[a]	0.041
TDN 30-180 day, kg[2]	6.8 ± 0.18[b]	7.9 ± 0.25[a]	6.8 ± 0.18[b]	0.002
TDN 181-281 day, kg[2]	7.3 ± 0.20[b]	7.4 ± 0.27[b]	9.5 ± 0.20[a]	0.001
Crude protein 30-180 day, kg	1.3 ± 0.03[b]	1.6 ± 0.04[a]	1.3 ± 0.03[b]	0.001
Crude protein 181-281 day, kg	1.0 ± 0.01[b]	1.0 ± 0.01[b]	1.4 ± 0.01[a]	0.001

UNS = cow unsupplemented during the gestation; MID = cows supplemented during 30 to 180-day of gestation; LATE = cows supplemented during 181 to 281-day of gestation.

[1]Pasture dry matter; [2]Total digestible nutrients.

[a,b] Values within a row with different superscripts differ significantly at P<0.1.

Table 3

Performance of cows during gestation.

Item	Treatments			P-value
	UNS	MID	LATE	
Final weight 180 day, kg	490.3 ± 10.41	476.4 ± 13.96	490.3 ± 10.41	0.433
Final weight 281 day, kg	476.7 ± 14.00	489.4 ± 13.28	510.4 ± 14.00	0.245
Fat thickness 180 day, mm	4.6 ± 0.60	5.4 ± 0.81	4.59 ± 0.60	0.441
Fat thickness 281 day, mm	3.4 ± 0.51	4.1 ± 0.49	3.5 ± 0.52	0.568

UNS = cow unsupplemented during the gestation; MID = cows supplemented during 30 to 180-day of gestation; LATE = cows supplemented during 181 to 281-day of gestation.

[a,b] Values within a row with different superscripts differ significantly at P<0.1.

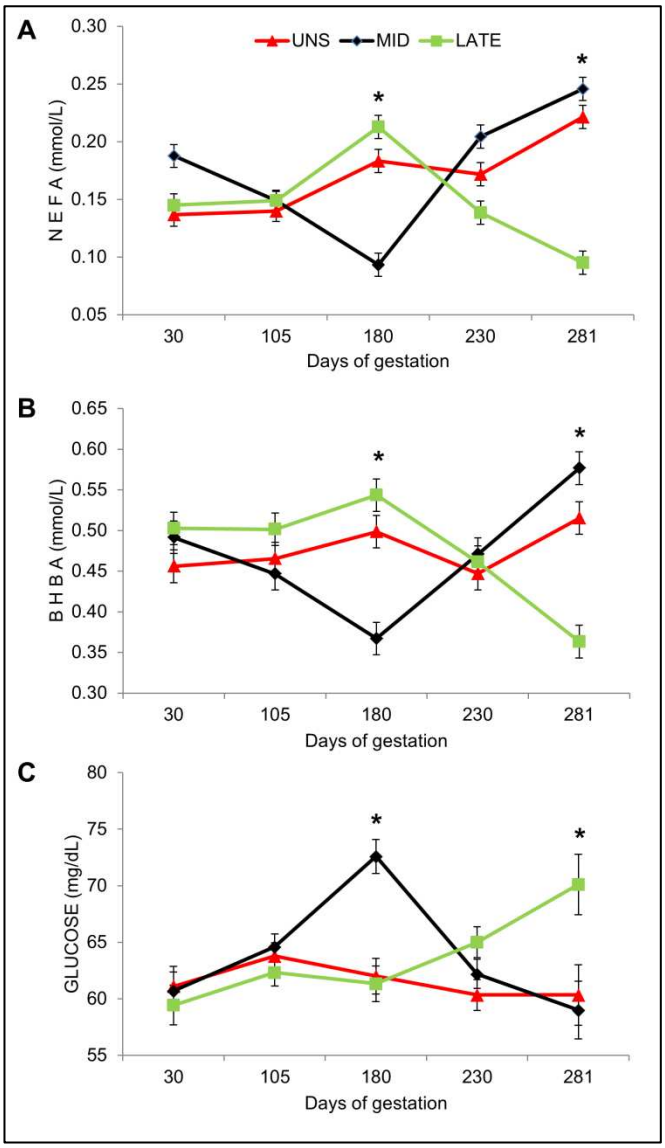

Figure 1. Pre-partum, energetic status of females during gestation. UNS, unsupplemented during gestation; MID, supplemented from the 30 to 180 day of gestation and LATE, supplemented from the 181 to 281 day of gestation. Bars represent means ± s.e (n = 9). Asterisks indicate significant differences between the groups (*P<0.1). (A) NEFA: fatty acids not esterified, (B) BHBA: beta hydroxybutyrate and (C) Glucose during gestation.

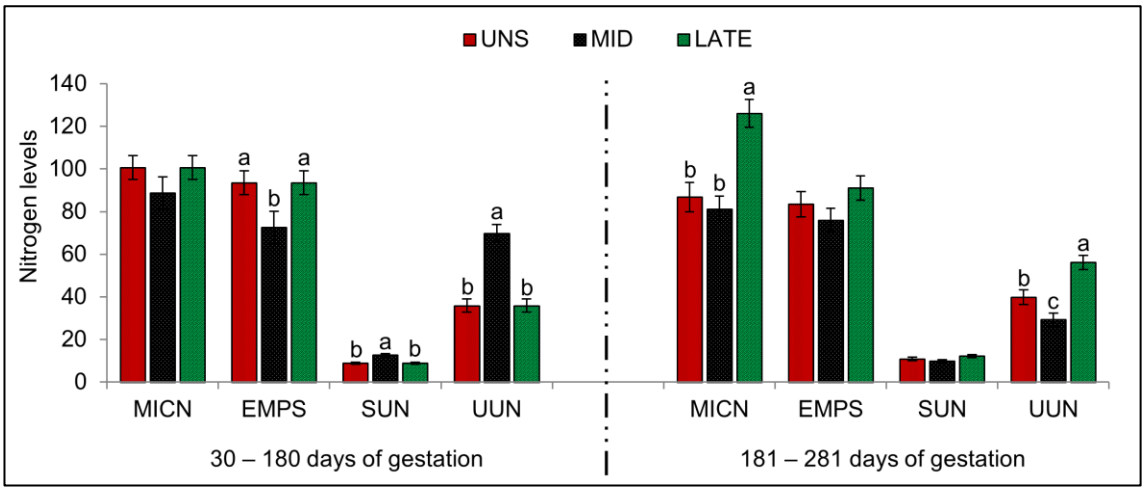

Figure 2. Microbial efficiency and nitrogen balance.

UNS, unsupplemented during gestation; MID, supplemented from the 30 to 180-day of gestation; LATE, supplemented from the 181 to 281-day of gestation. Bars represent means ± s.e (n = 9). Letters indicate significant differences between the groups (*P<0.1).

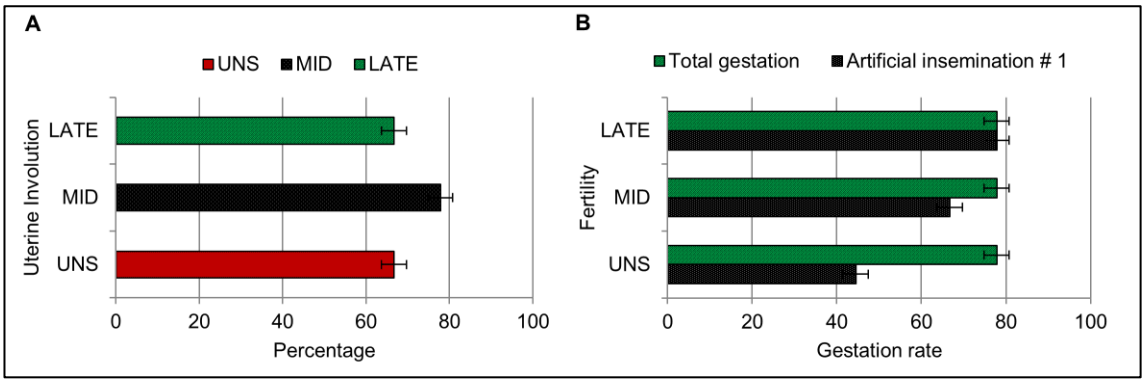

Figure 3. Postpartum reproductive parameters of cows supplemented during pregnancy.

UNS, unsupplemented during gestation; MID, supplemented from the 30 to 180-day of gestation; LATE, supplemented from the 181 to 281-day of gestation. Bars represent means ± s.e (n = 9). Asterisks indicate significant differences between the groups (*P<0.1).