MARTA MARIA DOS SANTOS

IMPLICATIONS OF NUTRITIONAL STRATEGIES DURING GESTATION AND PRE- WEANING FOR SKELETAL MUSCLE COMPOSITION IN BOVINES

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: Marcio de Souza Duarte

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“Estou entre aqueles que pensam que a ciência tem uma grande beleza”.

(Marie Curie)

BIOGRAPHY

MARTA MARIA DOS SANTOS, filha de Francisco Paulo dos Santos e Maria da Conceição dos Santos, nasceu em Viçosa – MG, em 13 de Agosto de 1990.

Em Março de 2013, ingressou no curso de Zootecnia na Universidade Federal de Viçosa, onde graduou-se em 26 de Janeiro de 2018.

Em Fevereiro de 2018, iniciou o Mestrado em Zootecnia, na Universidade Federal de Viçosa, sob orientação do Prof. Dr. Marcio de Souza Duarte, obtendo seu título de mestre em Fevereiro de 2020.

Em Março de 2020 iniciou o Doutorado em Zootecnia, na Universidade Federal de Viçosa, sob orientação do Prof. Dr. Marcio de Souza Duarte, concentrando seus estudos nas linhas de pesquisa de nutrição gestacional e programação fetal em ruminantes.

ABSTRACT

SANTOS, Marta Maria dos, D.Sc., Universidade Federal de Viçosa, February, 2024. **Implications of nutritional strategies during gestation and pre- weaning for skeletal muscle composition in bovines**. Adviser: Marcio de Souza Duarte.

The productive traits of beef cattle can be defined by pre and post-natal environmental conditions. Different strategies have been used in beef cattle during gestation and pre- weaning to improve animal performance and meat quality. The intrauterine conditions modulate the skeletal muscle development and establish, at least partially, the postnatal body composition, influencing the final meat quality. Therefore, supplementation of nutrients during the fetal stages can improve the maternal metabolism to regulate the nutrient partitioning needed to supply the fetus and, consequently improve pos natal performance. In addition, supplementation of nutrients during the early weaning stage to about 250 d of age can be a strategy to specifically enhance intramuscular adipogenesis, resulting in adipocyte hypertrophy and high marbling. Thus, the current study was developed based on two experiments. The aim of the first study was to evaluate the impact of shifting urea release from rumen to the intestinal tract at late gestation on liver metabolism, and nutritional status of pregnant cows at late gestation. Twenty-four Brahman dams, pregnant from a single sire, and weighing 545 kg ± 23 kg were confined into individual pens at 174 ± 23 d of gestation, and randomly assigned into one of two dietary treatments up to 270 d of gestation: Control (CON, n = 12), consisting of a basal diet supplemented with conventional urea, where the cows were fed with diets containing 13.5 g conventional urea per kg dry matter; and PRU (PRU, n = 12), consisting of a basal diet supplemented with a urea coated to extensively prevent ruminal degradation while being intestinally digestible, where the cows were fed with diets containing 14,8 g urea protected from ruminal degradation per kg dry matter. Post-ruminal supply of urea reduced the urine levels of 3-methylhistidine (P = 0.02). There were no differences between treatments for dry matter intake (DMI; P = 0.76), total digestible nutrient (TDN) intake (P = 0.30), and in the body composition variables, such as, subcutaneous fat thickness (SFT; P = 0.72), and rib eye area (REA; P = 0.85). In addition, there were no differences between treatments for serum levels of glucose (P = 0.87), and serum levels of glucogenic (P = 0.28), ketogenic (P = 0.72), glucogenic, and ketogenic (P = 0.45) amino acids, neither for urea in urine (P = 0.51) as well as urea serum (P = 0.30). One the other hand, enriched pathways were differentiated related to carbohydrate digestion, and absorption, glycolysis, pyruvate metabolism, oxidative phosphorylation, pentose phosphate pathway, and biosynthesis of amino acids of the exclusively expressed proteins in

PRU cows. Shifting urea supply from the rumen to post-ruminal compartments decreases muscle catabolism in cows during late gestation. Our findings indicate that postruminal urea supplementation for beef cows at late gestation may improve the energy metabolism to support maternal demands. In addition, the post-ruminal urea release seems to be able to trigger pathways to counterbalance the oxidative stress associated to the increase liver metabolic rate. The second study were developed to elucidate the impact of nutrient supplementation of beef female calves at pre-weaning (100–250 days of age) on intramuscular adipogenic determination. Thirty-four female calves were assigned to two experimental treatments: Control (CON, n = 17), where animals were supplemented only with mineral mixture; Supplemented (SUP, n = 17), where animals received energy-protein supplement containing minerals (5 g/kg of BW per day) of their body weight. Animals were supplemented from 100 to 250 days of age, and muscle samples were biopsied at the end of the supplementation period. Regarding the performance variables, there were no differences between treatments for initial body weight (P = 0.75). The final body weight (P = 0.07), average daily gain (P = 0.07), rib eye area (P = 0.03), and rib fat thickness (P = 0.08) were greater in SUP female calves compared with CON treatment. The number of fibro-adipogenic progenitor cells (P = 0.69) did not differ between treatments, while a greater number of intramuscular pre-adipocytes were observed in SUP than CON female calves (P = 0.01). The expression of miRNA-4429 (P = 0.20) did not differ between treatments, while the expression of miRNA-129-5p (P = 0.09) and miRNA-129-2-3p (P = 0.05) was greater in CON than SUP female calves. Our results suggest that nutrient supplementation at early postnatal stages of development enhances the commitment of fibro-adipogenic progenitor cells into the adipogenic lineages allowing to an increase in intramuscular fat deposition potential of the animals later in life. Altogether the results suggest that nutritional strategies during the fetal and pre-weaning stages improve maternal metabolism to maintain an adequate fetus' development and enhance the number of pre-adipocytes in the skeletal muscle preparing intramuscular adipose tissue for fat deposition during the postweaning period.

Keywords: Beef cattle. Creep feeding. Female calves. Marbling. Preadipocytes. Proteome. Skeletal Muscle.

RESUMO

SANTOS, Marta Maria dos, D.Sc., Universidade Federal de Viçosa, fevereiro de 2024. **Implicações de estratégias nutricionais durante a gestação e pré-desmame na composição muscular esquelética em bovinos**. Orientador: Marcio de Souza Duarte.

As características produtivas na pecuária de corte podem ser definidas pelas condições ambientais pré e pós-natais. Diferentes estratégias têm sido utilizadas em bovinos de corte durante a gestação e pré-desmame para melhorar o desempenho do animal e a qualidade da carne. As condições intrauterinas modulam o desenvolvimento muscular esquelético e estabelecem, pelo menos parcialmente, a composição corporal pós-natal, influenciando a qualidade final da carne. Portanto, a suplementação de nutrientes durante a fase fetal pode melhorar o metabolismo materno para regular a partição de nutrientes necessária para suprir o feto e, consequentemente, melhorar o desempenho pós-natal. Além disso, a suplementação de nutrientes durante a fase de desmame até cerca de 250 dias de idade pode ser uma estratégia para aumentar especificamente a adipogênese intramuscular, resultando em hipertrofia de adipócitos e alto marmoreio. Assim, o presente estudo foi desenvolvido com base em dois experimentos. O objetivo do primeiro estudo foi avaliar o impacto do deslocamento da liberação de uréia do rúmen para o trato intestinal no final da gestação no metabolismo hepático e no estado nutricional de vacas prenhas no final da gestação. Vinte e quatro matrizes Brahman, prenhas de um único touro e pesando 545 kg ± 23 kg foram confinadas em baias individuais aos 174 ± 23 dias de gestação e distribuídas aleatoriamente em um dos dois tratamentos dietéticos até 270 dias de gestação: Controle (CON, n = 12), composta por dieta basal suplementada com uréia convencional, onde as vacas foram alimentadas com dietas contendo 13,5 g de uréia convencional por kg de matéria seca; e PRU (PRU, n = 12), consistindo de uma dieta basal suplementada com uréia revestida para prevenir extensivamente a degradação ruminal e ao mesmo tempo digestível intestinalmente, onde as vacas foram alimentadas com dietas contendo 14,8 g de uréia protegida da degradação ruminal por kg matéria seca. O fornecimento pós-ruminal de uréia reduziu os níveis urinários de 3-metilhistidina (P = 0,02). Não houve diferenças entre os tratamentos para consumo de matéria seca (CMS; P = 0,76), consumo de nutrientes digestíveis totais (NDT) (P = 0,30) e nas variáveis de composição corporal, como espessura de gordura subcutânea (EGS; P = 0,72).) e área de olho de lombo (REA; P = 0,85). Além disso, não houve diferenças entre os tratamentos para níveis séricos de glicose (P = 0,87), níveis séricos de aminoácidos glicogênicos (P = 0,28), cetogênicos (P = 0,72), glicogênicos e cetogênicos (P = 0,45), ureia na urina (P = 0,51), e ureia sérica (P = 0,30).

Por outro lado, houve enriquecimento de vias relacionadas à digestão e absorção de carboidratos, glicólise, metabolismo do piruvato, fosforilação oxidativa, via da pentose fosfato e biossíntese de aminoácidos das proteínas exclusivamente expressas em vacas PRU. A mudança do fornecimento de uréia do rúmen para os compartimentos pós-ruminais diminui o catabolismo muscular em vacas durante o final da gestação. Nossos resultados indicam que a suplementação de uréia pós-ruminal para vacas de corte no final da gestação pode melhorar o metabolismo energético para suportar as demandas maternas. Além disso, a liberação pós-ruminal de uréia parece ser capaz de desencadear vias para contrabalançar o estresse oxidativo associado ao aumento da taxa metabólica hepática. O segundo estudo foi desenvolvido para elucidar o impacto da suplementação nutricional de bezerras de corte no pré-desmame (100-250 dias de idade) na determinação adipogênica intramuscular. Trinta e quatro bezerras foram distribuídas em dois tratamentos experimentais: Controle (CON, n = 17), onde os animais foram suplementados apenas com mistura mineral; Suplementado (SUP, n = 17), onde os animais receberam suplemento energético-protéico contendo minerais (5 g/kg de PC por dia). Os animais foram suplementados dos 100 aos 250 dias de idade e amostras musculares foram coletadas ao final do período de suplementação. Em relação às variáveis de desempenho, não houve diferenças entre os tratamentos para peso corporal inicial (P = 0,75). O peso corporal final (P = 0,07), ganho médio diário (P = 0,07), área de olho de lombo (P = 0,03) e espessura de gordura subcutânea (P = 0,08) foram maiores nas bezerras SUP em comparação com o tratamento CON. O número de células progenitoras fibro-adipogênicas (P = 0,69) não diferiu entre os tratamentos, enquanto um maior número de pré-adipócitos intramusculares foi observado em bezerras SUP do que CON (P = 0,01). A expressão do miRNA-4429 (P = 0,20) não diferiu entre os tratamentos, enquanto a expressão do miRNA-129-5p (P = 0,09) e do miRNA-129-2-3p (P = 0,05) foi maior no CON do que no SUP. Nossos resultados sugerem que a suplementação de nutrientes nos estágios iniciais de desenvolvimento pós-natal aumenta o comprometimento das células progenitoras fibro-adipogênicas nas linhagens adipogênicas, permitindo aumento no potencial de deposição de gordura intramuscular dos animais mais tarde na vida. Em conjunto, os resultados sugerem que as estratégias nutricionais durante as fases fetal e pré-desmame melhoram o metabolismo materno para manter um desenvolvimento adequado do feto e aumentam o número de pré-adipócitos no músculo esquelético, preparando o tecido adiposo intramuscular para a deposição de gordura durante o período pós-desmame.

Palavras-chave: Bezerras. Bovinos de corte. *Creep feeding*. Marmoreio. Musculo Esquelético. Preadipócitos. Proteoma.

SUMMARY

1. CHAPTER 1

1.1. General Introduction

Cow-calf phase is the foundation of the entire beef cattle production cycle (López-González et al., 2020). The productivity of cow-calf systems is associated with many factors, such as nutrition, genetics and, environmental conditions (Warner et al., 2010). The understanding the interaction between these factors is crucial to shorten the cycle of animal production and produce good quality meat, which meets the global demand and causes less environmental impact as possible. It is well established that growth and carcass characteristics of beef cattle are dependent on genetics and all environmental conditions that the animals are exposed to in their postnatal lives (Warner, et al., 2010). However, the skeletal muscle, which provides meat, begins to develop during the intrauterine stage (Costa et al., 2021). Therefore, the prenatal nutrition, can also reshape the animal development trajectory and cause persistent long-term consequences-a concept known as fetal programming.

Maternal nutrition affects the skeletal muscle development of the fetus, exerting long-term effects on offspring performance and growth (Santos et al., 2022). Overall, these studies have shown that inadequate maternal diets in beef cattle have negative consequences such as a lower population of muscle fibers (Marquez et al., 2017; Costa et al., 2021a), due to changes in the mRNA abundance of myogenic regulatory factors (Jennings et al., 2016) involved with cell determination, proliferation, and differentiation. In addition, during pregnancy the maternal physiology and metabolism are altered to maintain an adequate fetus' development and health (Costa et al., 2022). According to Bell et al. (2005) more than 50% of placental energy demand during the last third of gestation is supplied by amino acids in bovines. It has been demonstrated that urea supplementation can improve the efficiency of the utilization of amino acids in body anabolism (Batista et al., 2016). Recently, it has been shown that supplementation with post-ruminal urea release provides greater efficiency in nitrogen usage in rumen, and animal body due to greater, and more stable ruminal N recycling, greater uptake of recycled N by rumen microorganisms, decreased urinary-N loss, and improvement in fiber digestion when compared to the classical releasing of urea as pulse dose in the rumen (Carvalho et al., 2020; Oliveira et al., 2020). Thus, moving the release of dietary urea from the rumen to the intestinal tract may be strategically used to overcome the challenging nutritional conditions of pregnant cows at late gestation.

In addition to strategies during gestation, the pre-weaning phase is reported to be time window period in which marbling is enhanced without the increase in other fat depots (Du et al., 2013), reflecting on the quality of the meat. Despite formation of adipose tissue begins before mid-gestation in beef cattle most adipocytes are formed during the fetal and early postnatal stages (Bonnet et al.,2010). As such, supplementation of nutrients or other bioactive compounds to enhance adipogenesis during the early weaning stage to about 250 d of age can be a strategy to specifically enhance intramuscular adipogenesis, which provide sites for lipid accumulation during the "fattening" stage, resulting in adipocyte hypertrophy and high marbling (Du et al., 2013). Moreover, nutritional strategies during the fetal stages can improve the maternal metabolism to regulate the nutrient partitioning needed to supply the fetus and, consequently improve pos natal performance.

1.2. Thesis Objectives

This study was developed aiming to:

1 – Summarize through a review article reporting the impacts of fetal programming on the skeletal muscle development and final body composition in ruminants.

2 –Evaluate the impact of shifting urea release from rumen to the intestinal tract at late gestation on liver metabolism, and nutritional status of pregnant cows at late gestation.

3 –Elucidate the impact of nutrient supplementation of beef female calves at pre-weaning (100–250 days of age) on intramuscular adipogenic determination.

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2. CHAPTER 2

Prenatal origins of productivity and quality of the beef[1]

Marta Maria dos Santos [1,2], Thaís Correia Costa [1,2], Germán Darío Ramírez-Zamudio [3], Karolina Batista Nascimento [3], Mateus Pies Gionbelli [3] , Marcio de Souza Duarte [4]

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Review Article

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2.1. Abstract

The productive traits of beef cattle are orchestrated by their genetics, postnatal environmental conditions, and by the intrauterine background. Both under- or overnutrition, as specific dietary components, are able to promote persistent effects on the offspring. This occurs because dietary factors act not only affecting the availability of substrates for fetal anabolism and oxidative metabolism, but also as signals that regulate several events toward fetal development. Therefore, this study aimed to summarize the gestational nutrition effects on the offspring performance and meat quality in a long term. Overall, studies have shown that many of these alterations are under the control of epigenetic mechanisms, as DNA methylation, histones modification, and non-coding RNA. The current knowledge has indicated that the fetal programming responses are dependent on the window of fetal development in which the dietary treatment is applied, the intensity of maternal nutritional stimuli, and the treatment application length. Collectively, studies demonstrated that muscle cell hyperplasia is impaired when maternal requirements were not achieved in the second third of gestation, which limits the formation of a greater number of muscle fibers and the offspring growth potential in a long term. Changes in muscle fibers metabolism and in collagen content were also reported as consequence of a dietary perturbation during pregnancy. In contrast, a maternal overnutrition during the late pregnancy has been associated with beneficial responses on meat quality. In summary, ensuring an adequate maternal environment during the fetal development is crucial to enhance the productive responses in beef cattle operations.

Keywords: adipogenesis, bovine, fibrogenesis, maternal nutrition, myogenesis, progenitor cells

2.2. Introduction

Fetal programming is the response of an organism to an environmental challenge during a critical period of intrauterine development, which leads to persistent changes (Nathanielsz et al., 2007). Both maternal under- and overnutrition can trigger changes in the development, metabolism, and physiology of the offspring (Nissen et al., 2003; Greenwood and Cafe, 2007; Duarte et al., 2014; Gionbelli et al., 2018; Costa et al., 2021a). Such modifications are under the control of epigenetics, which act as a memory of the environment exposure (Wu et al., 2006; Sinclair et al., 2016; Paradis et al., 2017; Batistel et al., 2019).

Dietetic manipulations over pregnancy are an opportunity to improve the offspring performance and meat quality, but at the same time, is a way to compromise these

characteristics irreversibly. The basic structure of skeletal muscle tissue is composed by muscle fibers, adipocytes, and connective tissue, all derived from the mesenchymal stem cells of mesoderm (Du et al., 2013) (Figure 1). In this sense, maternal nutrition acts to control the fate steam cell in the different lineages, regulating the balance between myogenesis, adipogenesis, and fibrogenesis (Du et al., 2013; Blair et al., 2021).

Overall, studies have shown that inadequate maternal diets in beef cattle have negative consequences such as a lower population of muscle fibers (Marquez et al., 2017; Costa et al., 2021a), due to changes in the mRNA abundance of myogenic regulatory factors (Jennings et al., 2016) involved with cell determination, proliferation, and differentiation. Those changes lead to a lower muscle growth potential (Costa et al., 2021b), which affects the whole-body energy expenditure in the post-natal life, once skeletal muscle is the major glucose utilization site (Mohammadabadi et al., 2021). Less muscle fiber hyperplasia can be replaced by intramuscular collagen deposits (Costa et al., 2021a), which may contribute to increase meat toughness (Fontes et al., 2021). Moreover, prenatal nutritional insults can also cause changes in muscle fiber metabolism regulated by transcription factors (Ramírez-Zamudio et al., 2022) known as skeletal muscle metabolic plasticity (Aragão et al., 2014), which can negatively affect marbling deposition (Marquez et al., 2017). Therefore, monitoring the gestational environment is crucial to enhance the efficiency of meat production.

This comprehensive review aimed to highlight the effects of maternal nutrition on the offspring performance and meat quality, once the identification of these responses plays a central role in the global beef satisfying demand. Here, we first discuss the maternal and placental metabolism changes in response to the availability of nutrients over gestation, as well as the underlying mechanisms involved with phenotypic alterations observed in a long term. Then, we summarized the main effects of maternal nutrition on productive traits of beef cattle.

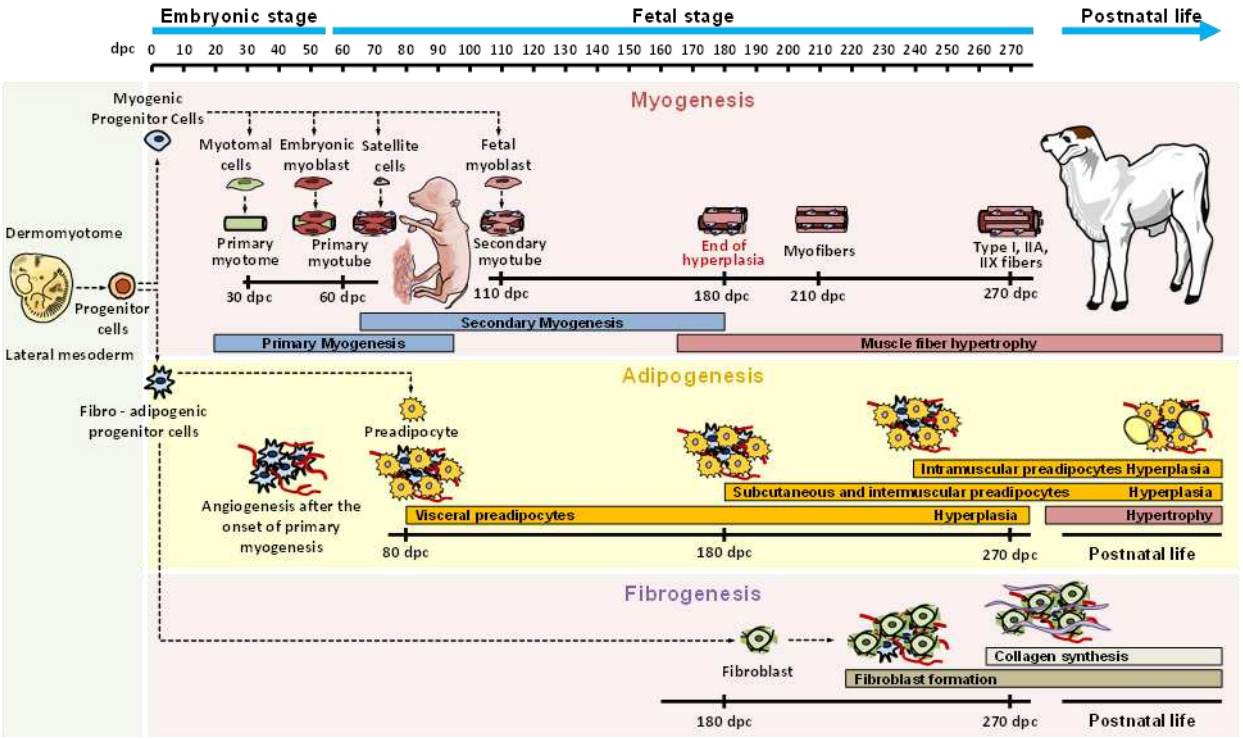

Figure 1: Mesenchymal progenitor cells differentiate into myogenic and fibroadipogenic cells during fetal muscle development in beef cattle. Scheme based on studies from: Picard et al., (2002); Bonnet et al., (2010); Du et al., (2015); Wang et., (2016). Summary of the main processes involved in skeletal muscle cell differentiation during the embryonic and fetal stage, and postnatal life according to the days post conception (dpc). Mesenchymal progenitor cells differentiate into myogenic and fibro-adipogenic cells during the embryonic stage in beef cattle. Primary myogenesis occurs in the embryonic stage, followed by secondary myogenesis that occurs in the fetal stage up to 180 dpc, approximately, occurring subsequent secondary myofibers mature into type I, IIA, and IIX. Hypertrophy of muscle fibers begins around 170 dpc and extends to postnatal life. The determination of fibroadipogenic cells begins in the fetal phase, with the formation of preadipocytes in different fat deposits, including visceral, subcutaneous, intermuscular, and intramuscular. Fibrogenesis begins around 180 dpc, with the formation of fibroblasts followed by collagen synthesis that begins around 260 dpc and extends to postnatal life.

2.3. Changes in maternal and placental metabolism in response to nutritional challenges during gestation

During gestation, females from all species undergo homeorhesis, in which several physiological changes occur to ensure the continuous supply of essential metabolites to support fetal growth and development (Redmer et al., 2004). Thus, when fetal development is critical, due to nutrient deficiency, the mother tends to favor the fetal system, with coordinated changes in her own tissue metabolism that regulate the nutrient partitioning needed to supply the fetus (Bauman and Currie, 1980).

In addition to the utilization of propionate for glucose production during periods of low availability or high demand for glucose, energy reserves may also be mobilized and used as a gluconeogenic precursor (Funston et al., 2010a). Under conditions of nutrient deficiency, the amino acids provided by the mobilization of maternal skeletal muscle are used to improve fetal access to amino acids (Bell et al., 2005) or may also be used in maternal gluconeogenesis. The utilization of long-chain fatty acids, non-esterified fatty acids (NEFA), or ketoacids by the fetuses is limited due to the low placental ability to transport these substrates (Bell et al., 2005). However, although NEFA seem to be not utilized by the fetus as a carbon source for energy production, this substrate supplies the pregnant dam with substrates for their own maintenance, and thus it indirectly contributes to spare glucose and amino acids to supply fetal requirements (Bell and Ehrhardt, 2000).

Maternal tissue mobilization or deposition occurs as a function of dietary substrate supplies (McNeill et al., 1997). Thus, nutritional adjustments for pregnant cows undergoing nutritional restrictions have been the subject of studies (Lopes et al., 2020), which, in general, aimed to establish nutritional management that minimizes lean tissue catabolism and the negative effects on the fetus. For instance, Lopes et al. (2020) showed the importance of supplementation for undernourished beef cows and reported a tendency toward greater mRNA expression of skeletal muscle synthesis markers in cows that received protein supplementation during late gestation. Such results likely demonstrate that a consequence of protein supplementation during gestation is a reduction in the intensity of lean tissue mobilization.

Under conditions of low nutrient availability causing intrauterine growth restriction, an additional compensatory mechanism involving the placenta may occur (Redmer et al., 2004). Borowicz et al. (2007) reported that when metabolizable protein is reduced to 60% of requirements in sheep, uterine blood flow increased, indicating an adaptation of placental vasculature. Therefore, it is possible that nutrient deprivation due to inadequate placenta size and function affects fetuses from well-nourished dams. Additionally, fetuses from undernourished dams may not have difficulty meeting their nutrient requirements due to compensatory mechanisms in the placental system (Redmer et al., 2004). For instance, Vonnahme et al. (2007) showed that nutrient restriction from 30 to 125 days of gestation in bovine increased placental mRNA concentrations of placental growth factor, improving fetal weight due to a greater transfer of nutrients through the placenta. Under a moderate nutritional restriction, the placenta may contribute to an increase in the abundance of Glucose transporter 3 (GLUT-3) as an attempt to increase its ability to glucose transfer (Bell and Ehrhardt, 2000). However, under severe and prolonged nutrient restriction, the placenta may reduce glucose

uptake and use glucose for its own demands (Bell and Ehrhardt, 2000). McCrabb et al. (1992) showed that pregnant sheep subjected to nutrient restriction in mid-gestation presented a decrease in placenta size without changing the number of individual placentomes or the fetal weight and dimensions. In contrast, Zhang et al. (2016) observed that undernourished animals presented lower concentrations of serum total polyamines in the uterine artery, fetal umbilical vein, and amniotic and allantoic fluids, which are crucial mediators of placental growth and angiogenesis, of fetal cellular function and synthesis of DNA and protein (Zhang et al., 2016). Therefore, the compensatory mechanisms related to placental functioning may occur under conditions of nutritional restriction by the pregnant dam during gestation in attempt to mitigate the effects on fetal development, which may buffer the negative effects on the development of fetuses.

2.4. Maternal nutrition effects on epigenetic mechanisms underlying the skeletal muscle development

It is well established that among omics extracts (transcripts, proteins, and metabolites), a set of regulations and interactions generates a specific response according to the environment. These modulations may be explored through epigenetic analysis and systems biology approaches. Epigenetics explains how gene expression might be altered without affecting the nucleotide sequence (Feil, 2006). Moreover, this set of mechanisms is transferred between cell generations, constituting epigenetic memory. Of the epigenetic modifications, DNA methylation, chromatin remodeling, and noncoding RNA are relevant mechanisms for maternal nutrition and fetal programming.

DNA methylation is related to gene silencing, since the inclusion of a methyl group at the 5’ position of the cytosine residues located in the CpG islands in the promoter region of a gene inhibits the interaction between the transcriptional machinery complex and the target gene (Osorio et al., 2017). This process is widely influenced by dietary precursors, which are responsible for donating chemical groups to positively or negatively regulate DNA methylation (Osorio et al., 2017). The methyl donor S-adenosylmethionine (SAM), synthesized in the methionine cycle, is transferred to DNA through DNA methyltransferases (DNMT) (Triantaphyllopoulos et al., 2016). Demethylation and, consequently, the reversion of gene silencing are catalyzed by the α-ketoglutarate (α-KG)-dependent ten-eleven translocation (TET) family of proteins (Ito et al., 2010). At the transcriptional level, energy restriction during late gestation has been found to alter the skeletal muscle and blood transcriptome of calves; specifically, genes related to energy metabolism and muscle development are downregulated in

muscle cells, accompanied by a decrease in the expression of genes associated with the immune response (Sanglard et al., 2018). While evaluating the DNA methylation level of some important gene inducers of cell differentiation, Paradis et al. (2017) observed hypermethylation in the promoter region of IGF2 in fetal skeletal muscle of offspring born from cows that were nutrient-restricted during mid- to late gestation, emphasizing the interaction between the nutritional plan and changes in gene expression.

Chromatin remodeling is mediated by histone post-translational modification (PTM), which involves the inclusion of a set of chemical or protein groups (e.g., methyl, acetyl, phosphate, and ubiquitin) to the histone tails (Triantaphyllopoulos et al., 2016). The combination of different PTM in a specific histone is called the histone code (Jenuwein and Allis, 2001). Depending on the histone code, chromatin may assume the structure of heterochromatin (compacted) or euchromatin (relaxed), which are associated with the repression or activation of gene expression, respectively (Jenuwein and Allis, 2001). As an example, a decrease in the histone code H3K27me3 (histone 3 lysine 27 trimethylation) marker of gene silencing promoted an increase in overall adipogenesis in fetal mice from obese mothers (Yang et al., 2013). In contrast, the increase in the histone codes H3K9Ac (histone 3 lysine 9 acetylation) and H3K4me3 (histone 3 lysine 4 trimethylation) markers of gene activation, in the promoter region of myostatin, resulted in the reduction in muscle mass of piglets born from sows fed low-protein diets during pregnancy and lactation (Jia et al., 2016).

When the effects of maternal feed restriction during different stages of gestation on the newborn goat skeletal muscle transcriptome (Costa et al., 2021c) and proteome (Costa et al., 2022) were evaluated, it was observed that proteins exclusively expressed in each treatment (feed restriction in the first vs. last half of gestation) were present in both treatments at the transcriptional level. This suggested possible posttranscriptional regulation that repressed a set of genes in one of the treatments. The mechanism of post-transcriptional regulation may be mediated by noncoding RNA, called microRNA (miRNA). The inhibitory role of miRNA involves base-pairing with the target mRNA, which promotes repression (Wang et al., 2013). Imperfect base-pairing with the target mRNA inhibits translations and, consequently, protein synthesis, while perfect complementation causes the degradation of the target mRNA (Wang et al., 2013).

Therefore, maternal nutrition directly affects fetal metabolism through the pool of available nutrients, which mediate epigenetic mechanisms. The integration of omics data using a systems biology approach, combined with epigenetic analysis, may contribute valuable information on the effects of maternal nutrition on offspring skeletal muscle development and

metabolism at the cellular level, which is reflected in the skeletal muscle growth and development and may cause changes in the quality traits of meat.

2.5. The impact of maternal nutrition on the performance, carcass, and meat quality traits of the offspring

In tropical and subtropical regions, forages are the main components of the diet in most cow-calf herds (Bell and Greenwood, 2013). Such a scenario promotes variation in pasture availability and quality throughout the year, which is insufficient to meet the nutritional requirements of pregnant cows, mainly during mid- to late gestation (Lemos et al., 2012). Therefore, maternal restriction during critical periods of fetal skeletal muscle and adipose tissue development may compromise the performance and meat quality of the offspring (Figure 2).

During the dry season, pastures are deficient in proteins; thus, the restriction of energy and other nutrients in pregnant cows is also observed. In fact, the reduction of protein intake affects ruminal microorganism growth, which is responsible for the degradation of dietary fibers, causing a limitation of energy and dry matter intake (DMI) by cows (Sampaio et al., 2010). Therefore, the use of nutritional strategies that increase the protein intake of pregnant cows improves the digestibility of low-quality fibers and, consequently, enhances maternal-fetal nutrient flow (Marquez et al., 2017). However, studies examining the effects of maternal nutritional strategies on offspring performance and carcass characteristics have had variable results (Tables 1a and 1b)

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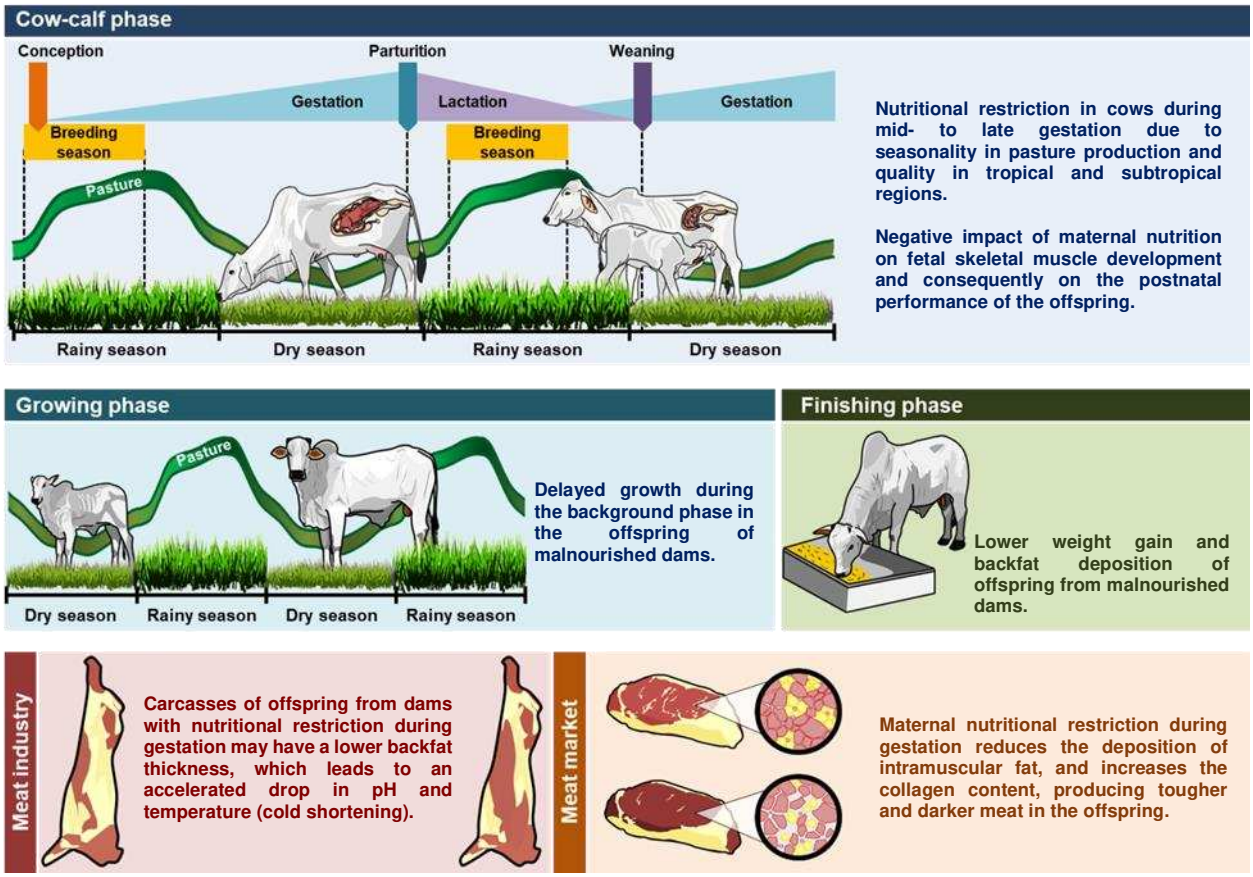

Figure 2: The impact of maternal nutrition on the performance and carcass characteristics of the offspri

Table 1a. Effects of the nutritional management of pregnant cows on the performance and carcass characteristics of the offspring

Item	Underwood et al., 2010 [A,1]		Stalker et al., 2007 [B,2]		Stalker et al., 2006 [C,3]		Larson et al., 2009 [D,3]	
	REST*	NR**	REST	NR	REST	NR	REST	NR
Birth weight, kg	38.7 ± 2.0	36.6 ± 1.9	38.4 ± 0.4 [a]	39.8 ± 0.4 [b]	36.1 ± 0.5	36.6 ± 0.5	36.9 ± 1.1 [a]	38.4 ± 1.1 [b]
Weaning weight, kg	242.1 ± 3.7 [a]	256.2 ± 3.5 [b]	199.8 ± 2.3 [a]	210.1 ± 2.3 [b]	211.0 ± 2 [a]	217.5 ± 2 [b]	235 ± 8	241 ± 8
Feedlot performance								
Slaughter weight, kg	520.6 ± 7.7 [a]	543.9 ± 7.1 [b]	557.5 ± 8 [a]	586.5 ± 8 [b]	-	-	602.5 ± 13	615.5 ± 13
Dry matter intake, kg/d	-	-	11.2 ± 0.2 [a]	12.1 ± 0.2 [b]	8.48 ± 0.17	8.53 ± 0.17	8.94 ± 0.21 [a]	9.19 ± 0.24 [b]
Average daily gain, kg/d	1.49 ± 0.07 [a]	1.66 ± 0.06 [b]	1.60 ± 0.04 [a]	1.68 ± 0.04 [b]	1.56 ± 0.02	1.56 ± 0.02	1.66 ± 0.05 [a]	1.70 ± 0.05 [b]
Feed efficiency, kg: kg	-	-	0.144 ± 0.004	0.139 ± 0.004	0.185 ± 0.002	0.183 ± 0.002	0.186 ± 0.07	0.186 ± 0.07
Carcass characteristics								
Carcass weight, kg	329.5 ± 4.8 [a]	348.2 ± 4.5 [b]	346.5 ± 5 [a]	365.0 ± 5 [b]	363 ± 4	369 ± 4	364 ± 8	372 ± 8
Yield, %	-	-	-	-	64.6 ± 2	64.9 ± 2	-	-
Rib eye area, cm^2	-	-	87.2 ± 1.8	87.7 ± 1.8	86.9 ± 1.0	88.1 ± 1.0	89.1 ± 1.9	89.4 ± 1.9
Backfat thickness, cm	1.11 ± 0.15 [a]	1.51 ± 0.14 [b]	1.24 ± 0.06	1.21 ± 0.06	1.31 ± 0.06	1.34 ± 0.06	1.17 ± 0.08	1.24 ± 0.08
Marbling score	420 ± 16	455 ± 15	548 ± 20	561 ± 20	467 ± 9	479 ± 9	443 ± 20 [a]	492 ± 20 [b]

*** REST** = nutritional restriction during gestation; ****NR** = no nutritional restriction during gestation.

[A] REST = native pasture with ~6% CP; **NR** = improved pasture with ~11% CP; treatment for 30 days during the second half of gestation. Twenty-six crossbred dams carrying male offspring from Angus sires (REST, n = 12; NR, n = 14). Offspring slaughtered at 430 ± 2 days of age.

[B] REST = native pasture with ~7,5 CP; **NR** = native pasture with ~7,5% CP + 0,45 kg supplement/day/3 times a week (42% CP) during the last third of gestation. One hundred thirty-six dams (¼ Angus, ¼ Gelbvieh, ¼ Hereford, and ¼ Simmental) from 3 to 5 years of age pregnant with male offspring. Offspring slaughtered at ~314 days of age.

[C] REST = native pasture with ~7,5 CP; **NR** = native pasture with ~7,5% CP + 0,45 kg supplement/day/3 times a week (42% CP) during the last third of gestation. One hundred thirty-six dams (¼ Angus, ¼ Gelbvieh, ¼ Hereford, and ¼ Simmental) from 3 to 5 years of age pregnant with male offspring. Offspring slaughtered at ~372 days of age.

[D] REST = native pasture with ~6,8% CP or corn residues with ~5,2% CP; **NR** = native pasture with ~6,8% CP or corn residues with ~5,2% CP + 0,45 kg supplement (28% CP) during the last third of gestation. One hundred and nine Red Angus × Simmental cows between 3 and 5 years of age pregnant with male offspring. The offspring was slaughtered when they reached 1.3 cm of backfat thickness.

[1] Marbling score: trace = 200; slight = 300; small = 400.

[2] Marbling score: slight = 400; small = 500.

[3] Marbling score: small = 400; modest = 500. [a,b] Significant differences between the groups ($P < 0.05$).

Table 1b. Effects of the nutritional management of pregnant cows on the performance and carcass characteristics of the offspring

Item	Mulliniks et al., 2012 [E,2]		McLean et al., 2018 [F,4]		Maresca et al., 2019 [G,5]	
	REST*	NR**	REST	NR	REST	NR
Birth weight, kg	-	-	37.6 ± 0.5	37.7 ± 0.5	-	-
Weaning weight, kg	253 ± 5	253 ± 5	176.2 ± 4.2	180.8 ± 4.2	-	-
Feedlot performance						
Slaughter weight, kg	511 ± 8	512 ± 8	647.9 ± 7.7 [a]	631.2 ± 7.7 [b]	493.6 ± 12.5	480.5 ± 16.0
Dry matter intake, kg/d	-	-	-	-	11.6 ± 0.66	10.4 ± 0.68
Average daily gain, kg/d	1.38 ± 0.04	1.46 ± 0.04	1.86 ± 0.03 [a]	1.78 ± 0.03 [b]	1.45 ± 0.10	1.29 ± 0.23
Feed efficiency, kg: kg	-	-	-	-	-	-
Carcass characteristics						
Carcass weight, kg	322 ± 6	323 ± 6	386.6 ± 5.5	377.8 ± 5.5	284.3 ± 7.7	289.5 ± 9.9
Yield, %	63.1 ± 0.19	62.9 ± 0.19	63.6 ± 0.4	63.8 ± 0.4	57.6 ± 0.6 [a]	60.2 ± 0.8 [b]
Rib eye area, cm^2	80.5 ± 1.16	82.3 ± 1.16	87.0 ± 1.4	87.8 ± 1.4	63.7 ± 1.65 [a]	69.4 ± 2.22 [b]
Backfat thickness, cm	1.36 ± 0.07	1.42 ± 0.07	1.55 ± 0.09	1.45 ± 0.09	0.67 ± 0.07	0.57 ± 0.08
Marbling score	487 ± 13	487 ± 13	364 ± 16	366 ± 16	455 ± 15	435 ± 20

***REST** = nutritional restriction during gestation; ****NR** = no nutritional restriction during gestation. [E] **REST** = forage *Bouteloua gracilis, Aristida* spp*., Lycurus phleoides* + supplement (36% CP) in high stress phases; **NR** = forage *Bouteloua gracilis, Aristida* spp*., Lycurus phleoides* + 202 g/cow/day supplement with 50% NDCP (blood and feather meal) during the last third of gestation. Three hundred thirty-three Angus dams or crossbreds with Angus from 3 to 9 years of age pregnant with male. The offspring was slaughtered at ~340 days of age. [F] **REST** = native pasture (≤ 6% CP) + 0,2 kg/day/4 days a week supplement (8% CP); **NR** = native pasture (≤ 6% CP) + 1,82 kg/day/ 4 days a week supplement (38% CP). Supplementation of the treatments REST and NR during the breeding season and the initial third of gestation. One hundred eighty-nine multiparous Angus cows with male pregnancy. Slaughter of offspring at ~699 days of age.

[G] **REST** = feedlot diet with 6% CP; **NR** = feedlot diet with 12% CP. Both diets were isoenergetic and were provided from 134 ± 14 days of gestation to term. Sixty-eight multiparous Angus cows pregnant with an Angus sire. Twenty-four male offspring was selected and subsequently slaughtered at 687 ± 13 days of age.

[2] Marbling score: slight = 400; small = 500. [4] Marbling score: slight = 200-299; small = 300-399. [5] Marbling score: trace = 200; slight = 300; small = 400; modest = 500

[a,b] Significant differences between the groups ($P < 0.05$).

Underwood et al. (2010) showed that cows fed improved pasture for 30 days at mid-gestation exhibited an increase of 10% in the offspring weaning and feedlot weight compared with the offspring resulting from cows fed native range pasture (~6% crude protein). In addition, an increase of approximately 19 kg carcass and 13.6% subcutaneous fat was observed in the resulting offspring (Underwood et al., 2010). These results may suggest that outcomes of fetal programming through maternal nutrition may have an indirect effect on meat quality traits. The increased subcutaneous fat thickness in the carcass of offspring born from dams in better nutritional conditions likely help to prevent the rapid decline in temperature during the transformation process of muscle to meat, avoiding cold-shortening, which contributes to meat toughness (Ockerman and Basu, 2014). Moreover, the heaviest carcasses in progeny born from dams that received adequate nutrition during gestation may result from greater muscle fiber development during the fetal stage. Costa et al. (2021a) showed that maternal protein restriction during mid-gestation reduces the number of muscle fibers in offspring.

In general, progeny from dams that receive an adequate nutritional plan shows better performance during the initial stages of life (Stalker et al., 2006; Stalker et al., 2007; Funston et al., 2010a; Funston et al., 2010b; Rodrigues et al., 2020), while few studies have reported significant gains throughout the production cycle (Stalker et al., 2007; Underwood et al., 2010). In contrast, other studies have failed to find an effect of adequate nutrition during mid- to late gestation on offspring performance throughout the production cycle or on carcass characteristics (Larson et al., 2009; Mulliniks et al., 2012; Mulliniks et al., 2013). Such variations in the phenotypic responses of progeny affected by maternal nutrition depend on multiple factors, such as herd management during the production phases, genetic composition, maternal nutritional history, and adaptability to the environment (Broadhead et al., 2019).

Maternal nutritional status during gestation may impact the qualitative properties of the meat from offspring (Alvarenga et al., 2016; Maresca et al., 2019; Webb et al., 2019). For instance, the meat of steers born from dams raised under improved pastures for 30 days at mid-gestation was more tender than that of steers born from dams fed native pasture (~6% crude protein) (Underwood et al., 2010). In addition to tenderness, the pH, color, water-holding capacity, and marbling of the meat may be affected by maternal nutrition due to alterations in the metabolic characteristics of muscle fibers (Fahey et al., 2005; Picard and Gagaoua, 2020), as well as the proportion of muscle, adipose, and connective tissue formed during the prenatal phase (Duarte et al., 2014; Du et al., 2015). For example, a 50% nutrient restriction in sheep during the first 30 days of gestation enhanced the proportion of muscle fibers with the characteristics of slow contraction and oxidative metabolism in offspring (Fahey et al., 2005).

Muscles with a greater proportion of slow-twitch fibers and oxidative metabolism show a low rate of postmortem pH decline due to low glycogen storage, resulting in an elevated final pH of the meat (Kim et al., 2016). When the pH is higher than 5.6, there is a change in the negative charge and structures of the muscular matrix, which results in greater intracellular water retention, negatively affecting meat color (Ramanathan et al., 2020). Moreover, changes in the final pH interfere with the activity of proteolytic enzymes, which are responsible for tenderness (Matarneh et al., 2017).

However, some changes may occur at the molecular level (Table 2) without resulting in phenotypic changes. Jennings et al. (2016), evaluated the effects of energy levels [72, 87, or 146% of net energy for maintenance (NEm) requirements] during early to mid-gestation and did not find effects of maternal nutrition on muscle histology characteristics (fiber area, diameter, and number), despite the effects on mRNA expression in skeletal muscle. In this study, myogenin was upregulated in the skeletal muscle of fetuses from cows fed at 72% NEm compared with those from cows fed at 87% NEm, indicating a potential reduction in myoblast differentiation, followed by an earlier fusion of these cells in fetuses exposed to undernutrition.

Rodrigues et al. (2020) investigated the effects of protein supplementation during mid- to late gestation in grazing beef cows with moderate nutritional restriction on performance and molecular markers in offspring (Table 2). Protein supplementation of the dams did not affect the expression of myogenic genes. However, a downregulation of C/EBPA and FABP4 was observed in 11-day-old calves from supplemented dams. These findings indicate that offspring from non-supplemented cows showed early adipogenic differentiation, which may impair the proliferation of intramuscular adipocytes. In summary, maternal restriction during critical periods of fetal skeletal muscle and adipose tissue development may compromise the performance and meat quality of the offspring; however, the use of maternal nutritional strategies shows better performance and carcass characteristics on offspring.

Table 2. Effects of prenatal nutrition on gene expression and characteristics of skeletal muscle in cattle

Reference	Period of gestation	Altered genes expressions	Unaltered gene expression	Phenotypic trail
Jennings et al. (2016)[A]	85 to 180 d	upregulated in **OVER**: *PREF-1***; *μ-Calpain***; *IGF-II**; upregulated in **RES**: *CEBPB***; *FAS**; *MyoG***; *IGF-II**	*PPARG*; *C/EBPA*; *SCD*; *MyoD; MyF5*; *MRF4*; *Myostatin*; *m-Calpain*; *Calpastatin*; *IGF-I*	No effects on fetal weight; fiber area (μm²); fiber diameter (μm) or fiber number in *Longissimus dorsi* and *Semitendinosus*
Paradis et al. (2017)[B]	147 to 247 d	upregulated in **RES** *Longissimus dorsi*: *MyoD***; *MyoG***; *PPARG***; *IGFI***; *IGF1R***; *IGF2R***; *INSR***	*IGF2*; *MEF2A*; *SRF*	No effects on fetal weight or crown-rump length
Marquez et al. (2017)[C]	**MID** = 30 to 180 d **LATE** = 181 to 281 d	*PPARA*** = greater for **MID** and lower for **LATE**; *FGF2*** = greater for **MID**, lower for **CON** and **LATE**	*TGFβ1*; *COL1A1*; *FGF2R1*; *COL3A3*; *PPARG*; *MCDA*; *UCP3*; *PPKAA2*; *HADH*; *MYH7*; *PDK4*; *PGC1α*; *CPT1*; *ZFP423*; *C/EBPA*	No effects on fiber area (μm²) or BW at birth and weaning. Greater number of muscle fibers for MID and lower for **CON****. Greater ribeye area (cm²) for **MID** and **LATE****.
Gionbelli et al. (2018)[D]	60 to 139, 199, 241 or 268 d	*CTNNB***; *ZFP423*** and *PPARG*** = greater expression at 139 d for **OVER**; *FN1*** = greater for **OVER**	*MyoD*; *MyoG*; *C/EBPA*; *COL1A1*; *COL3A1*; *TGFβ1*	Crude protein content in skeletal muscle (g/kg) greater for **OVER****. No effects on fat content of skeletal muscle; intramuscular collagen deposition (percentage) or the number of myocytes
Rodrigues et al. (2020)[E]	124 to 270 d	*Birth*: *PPARG***, *WNT10B***, *CD36***, *TGFβ1*** = greater for **SUP**; *11 days of age*: *C/EBPA***; *FABP4* **= lower for **SUP**; *Weaning*: *PPARG**, *ZFP423***; *TGFβ1*** = greater for **SUP**	*β-catenin*; *COL3A1*; *FN1*; *MyoD*; *MyoG*; *IGFR1*; *mTOR*; *MyHC1*; *MyHC2a*; *MyHC2x*	Greater BW at birth for **SUP****. No effects on BW at 120 and weaning.
Costa et al. (2021a)[F]	100 to 200 d	*30 d: PAX7** and *MHC2X*** = greater for **CON**. 450d: no effects. Maternal nutrition × offspring sex interactions: 30d: *ZFP423*; FN*; PDGFRα*; MHC1*; MHC2X*; LOX.* 450 d: *FN**; *TGFβ**; *MMP2**; *MHC1**; *COL1***; *COL3***; *MHC2X***	30 d: *C/EBPA*; *PPARG*; *TGFβ*; *COL1A1*; *COL3*; *P4Ha1*; *TIMP1*; *TIMP2*; *MHC1*; *MHC2A*; 450 d: *ZFP423*; *C/EBPA*; *PPARG*; *LOX*; *P4Ha1*; *TIMP1*; *TIMP2*; *PAX7*; *PDGFFRα*; *MHC1*; *MHC2A*; *MHC2X*	Lower number of muscle fibers for **CON****; Increase skeletal muscle collagen content for **CON****.

ABREVIATIONS: ***18S*** *= 18 S ribosomal;* ***CD36*** *= Cluster of differentiation 36;* ***C/EBPA*** *= Enhancer-binding protein α;* ***C/EBPB*** *= Enhancer-binding protein β;* ***COL1A1*** *=collagen type I, α 1; receptor 1;* ***COL3A3*** *= collagen type III, α 3;* ***CPT1*** *= carnitine palmitoyltransferase 1;* ***CTNNB1*** *= Cadherin-associated protein, beta-1;* ***FABP4*** *= Adipocyte-type fatty acid-binding protein;* ***FAS*** *= fatty acid synthase;* ***FGF2*** *=fibroblast growth factor 2;* ***FGF2R1*** *=fibroblast growth factor 2,* ***FN1*** *= Fibronectin 1;* ***HADH*** *=hydroxyacyl-CoA dehydrogenase;* ***IGF1 =*** *Insulin Like Growth Factor 1;* ***IGF1R*** *= Insulin Like Growth Factor 1 Receptor;* ***IGF2 =*** *Insulin Like Growth Factor 2;* ***IGF2R =*** *Insulin Like Growth Factor 2 Receptor;* ***INSR=*** *Insulin Receptor;* ***LOX*** *= Lysyl oxidase;* ***MCAD*** *= medium-chain acyl-CoA dehydrogenase;* ***MEF2A =*** *Myocyte Enhancer Factor 2A;* ***MMP2*** *= Matrix metalloproteinase-2;* ***MRF4*** *= myogenic regulatory factor-4;* ***mTOR*** *= Mammalian target of rapamycin;* ***MyoD*** *= Myogenic Differentiation 1;* ***MyoG*** *= Myogenin;* ***MYH7*** *= myosin heavy chain 7;* ***MyHC1*** *= Myosin heavy chain type I;* ***MyHC2a*** *= Myosin heavy chain type IIa;* ***MyHC2x*** *= Myosin heavy chain type IIx;* ***P4Ha1*** *= Prolyl 4-Hydroxylase Subunit Alpha 1;* ***PDK4*** *=pyruvate dehydrogenase kinase 4;* ***Pax7*** *= Paired box 7;* ***PDGFRa*** *= Platelet-derived growth factor receptor A;* ***PGC1α*** *=peroxisome proliferator-activated receptor γ coactivator 1 α;* ***PPARα*** *= Peroxysome proliferator-activated receptor α;* ***PPARG*** *= Peroxysome proliferator-activated receptor γ;* ***PRKAA2*** *= protein kinase AMP - activated catalytic subunit α 2;* ***PREF-1*** *= preadipocyte factor-1;* ***SCD*** *= stearoyl-CoA desaturase;* ***SRF =*** *Serum Response Factor;* ***TGFβ1*** *= Transforming growth factor- β1;* ***UCP3*** *= uncoupling protein 3;* ***TIMP1/2*** *= TIMP metallopeptidase inhibitor 1 or 2;* ***ZFP423*** *= Zinc finger protein 423;* ***WNT10B*** *= wingless-type MMTV integration site family member 10B.*

[A] **LOW** = Fed 72% of NEm of NRC requirements; **CON** = Fed 87% of NEm of NRC requirements; **HIGH** = Fed 146% of NEm of NRC requirements

[B] **LOW** = 85% of ME requirements; **HIGH** = 140% of ME requirements
[C] **CON**= Unsupplemented; **MID** = Fed with a 30% CP supplement from 30 to 180 days of pregnancy; **LATE** = Fed with a 30% CP supplement from from 181 to 281 days of pregnancy
[D] **CON** = Fed 100% of NRC requirements; **ON** = Fed 190% of NRC requirements
[E] **CON** = Unsupplemented; **SUP** = Fed pasture plus a mineral-protein supplement (36% CP) from 124 days of pregnancy to parturition
[F] **CON** = Unsupplemented; **SUP** = Fed pasture plus a protein supplement (40% CP) from 100 to 200 days of pregnancy
* $P < 0.10$.; **P < 0.05.

2.6. Summary and future perspectives

Maternal nutrition affects the skeletal muscle development of the fetus, exerting long-term effects on offspring performance and growth. Maternal undernutrition during fetal development reduces the number of muscle fibers, alters muscle fiber composition, and impacts fetal adipogenesis. However, adequate supplementation with nutrients improves skeletal muscle development and adipogenesis, increasing marbling in offspring. Thus, understanding the effects of maternal supplementation during different gestational periods on the performance and final carcass composition of the progeny may help improve meat production and carcass and meat quality traits.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

Conceptualization: M.M. Santos, T.C. Costa, G.D. Ramírez-Zamudio, K.B. Nascimento, M.P. Gionbelli and M.S. Duarte. Funding acquisition: M.P. Gionbelli and M.S. Duarte. Investigation: M.M. Santos, M.P. Gionbelli and M.S. Duarte. Methodology: M.M. Santos. Project administration: M.P. Gionbelli and M.S. Duarte. Resources: M.P. Gionbelli and M.S. Duarte. Supervision: M.P. Gionbelli and M.S. Duarte. Writing – original draft: M.M. Santos, T.C. Costa, G.D. Ramírez-Zamudio, K.B. Nascimento, M.P. Gionbelli and M.S. Duarte. Writing – review & editing: M.M. Santos, T.C. Costa, G.D. Ramírez-Zamudio, K.B. Nascimento, M.P. Gionbelli and M.S. Duarte.

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3. CHAPTER 3

Can the post-ruminal urea release impact liver metabolism, and nutritional status of beef cows at late gestation?[2]

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3.1. Abstract

We aimed to evaluate the effects of post-ruminal supply of urea (PRU) on nutritional status, and liver metabolism of pregnant beef cows during late gestation. Twenty-four Brahman dams, pregnant from a single sire, and weighing 545 kg ± 23 kg were confined into individual pens at 174 ± 23 d of gestation, and randomly assigned into one of two dietary treatments up to 270 d of gestation: Control (CON, n = 12), consisting of a basal diet supplemented with conventional urea, where the cows were fed with diets containing 13.5 g conventional urea per kg dry matter; and PRU (PRU, n = 12), consisting of a basal diet supplemented with a urea coated to extensively prevent ruminal degradation while being intestinally digestible, where the cows were fed with diets containing 14,8 g urea protected from ruminal degradation per kg dry matter. Post-ruminal supply of urea reduced the urine levels of 3-methylhistidine (P = 0.02). There were no differences between treatments for dry matter intake (DMI; P = 0.76), total digestible nutrient (TDN) intake (P = 0.30), and in the body composition variables, such as, subcutaneous fat thickness (SFT; P = 0.72), and rib eye area (REA; P = 0.85). In addition, there were no differences between treatments for serum levels of glucose (P = 0.87), and serum levels of glucogenic (P = 0.28), ketogenic (P = 0.72), glucogenic, and ketogenic (P = 0.45) amino acids, neither for urea in urine (P = 0.51) as well as urea serum (P = 0.30). One the other hand, enriched pathways were differentiated related to carbohydrate digestion, and absorption, glycolysis, pyruvate metabolism, oxidative phosphorylation, pentose phosphate pathway, and biosynthesis of amino acids of the exclusively expressed proteins in PRU cows. Shifting urea supply from the rumen to post-ruminal compartments decreases muscle catabolism in cows during late gestation. Our findings indicate that postruminal urea supplementation for beef cows at late gestation may improve the energy metabolism to support maternal demands. In addition, the post-ruminal urea release seems to be able to trigger pathways to counterbalance the oxidative stress associated to the increase liver metabolic rate.

3.2. Introduction

In bovines, more than 50% of placental energy demand during the last third of gestation is supplied by amino acids [1]. The potential of fetal growth based on maternal fat reserves is low [2] due to the limited transport of fatty acids, and ketone bodies through the placenta [3]. Studies have shown changes in plasmatic regulators of protein catabolism, and nitrogen losses of ewes during the last third of gestation, suggesting nitrogen mobilization from maternal lean tissue [4,5] as an attempt to meet fetal requirements.

However, nitrogen (N) metabolism in ruminants also involve other high-priority functions, such as nitrogen recycling to the gastrointestinal tract [6]. Normally, urea synthesis is performed from cytoplasmatic, and mitochondrial N pools, where N atoms can be absorbed from both ammonia, and amino acids [7]. It has been demonstrated that urea supplementation can improve the efficiency of the utilization of amino acids in body anabolism [8]. Recently, it has been shown that supplementation with post-ruminal urea (PRU) release provides greater efficiency in nitrogen usage in rumen, and animal body due to greater, and more stable ruminal N recycling, greater uptake of recycled N by rumen microorganisms, decreased urinary-N loss, and improvement in fiber digestion when compared to the classical releasing of urea as pulse dose in the rumen [9,10]. In addition, the absorption of urea in the small intestine can avoid an overload of ammonia in the liver, which occurs after ruminal degradation of dietary urea [10].

Late gestation is a critical period for a pregnant dam [11] as the nutritional requirements are enhanced due to an increase in growth rate of the fetus, while the dry matter intake capacity by the cows is limited [12]. Thus, moving the release of dietary urea from the rumen to the intestinal tract may be strategically used to overcome the challenging nutritional conditions of pregnant cows at late gestation. Therefore, we aimed to evaluate the impact of shifting urea release from rumen to the intestinal tract at late gestation on liver metabolism, and nutritional status of pregnant cows at late gestation.

3.3. Materials and Methods

3.3.1. Animal ethics

All experimental procedures were approved by the Ethical Committee on Animal Use of the Department of Animal Science at Universidade Federal de Viçosa, Minas Gerais, Brazil (protocol 33/2020).

3.3.2 Animals and experimental diets

Twenty-four Brahman dams, pregnant from a single sire, weighing 545 kg ± 23 kg, and 174 ± 23 d of gestation were confined at into individual pens with an area of 12 m^2, and provided with bunkers, and water bowls. The twenty-four cows used in the trial were pulled from a herd that was submitted to a reproductive protocol using three attempts of fixed-time artificial insemination. Consequently, a total of three groups of cows according to days of gestation was obtained. Thus, at the beginning of the trial the cows within the gestational time groups were evenly allocated to the two experimental treatments. However, the endpoint of the trial was 270 days of gestation to all cows and thus, the gestation group (1 to 3) was considered as a fixed effect in the statistical model. All cows were initially submitted to an adaptation period of 10 d receiving water ad libitum, and diet containing corn silage, ground corn, soybean meal, and mineral mixture. After the adaptation period, the fetal sex was determined via ultrasound on every cow, and within each pregnancy group, and fetal sex combination, cows were randomly assigned into two experimental treatments up to 270 d of gestation: Control (CON, n = 12), where cows were fed a basal diet containing conventional urea; and PRU (PRU, n = 12), where cows were fed a basal diet containing urea protected from ruminal degradation to partially be released in the small intestine (Trouw Nutrition, Netherlands) (Table 1). The post-ruminal urea (PRU) product was subjected to an in vitro evaluation to estimate ruminal protection rate [13]. Approximately 35% of this urea is released into the rumen, and the remainder (65%) is released into the post-ruminal compartments. The dry matter intake was adjusted weekly to meet 100% maintenance requirements [14] based on the body weight, and gestational age.

Table 1. Ingredients, and chemical composition of the experimental diets

Ingredients	CON (g/kg)	PRU (g/kg)
Corn silage[a]	700.0	700.0
Ground Corn [a]	227.5	226.2
Soybean meal[a]	45.0	45.0
Feed-grade urea[a]	13.5	-
Rumen protected urea[a]	-	14.8
Mineral mixture[a,b]	14.0	14.0
Chemical composition	g/kg	
Dry matter[c]	509	509
Crude protein[a]	116	119
Ether extract[a]	31	31
Neutral detergent fiber[a]	364	364
Non-fibrous carbohydrates[a]	430	427
Crude ash[a]	59	59

CON= Control treatment; PRU= post-ruminal releasing of urea
[a]g/kg dry matter
[b]Composition per kg: 76 g of calcium, 65.6 g of phosphorus, 34 g of sulfur, 14.4 g of magnesium, 61 g of sodium, 890 mg of copper, 48.4 mg of cobalt, 61 mg of iodine, 740 mg of manganese, 3340 mg of zinc, 16.5 mg of selenium.
[c]g/kg as fed

3.3.3 Animal performance data, and sample collection

To evaluate the nutritional characteristics of the diet, samples of roughage, and concentrate were collected weekly, and stored at -20˚C for further chemical analysis. To evaluate the total digestibility, the experiment was divided into three periods of 28 d each, and approximately 200 g of feces were sampled, via manual collection directly from the rectum, on the 17 d at 6 am, 18 d at 12 pm, and 19 d (at 6 pm, three hours after second feeding) of each experimental period. Simultaneously to fecal sample collection, approximately 50 ml of urine was sampled by stimulated urination. Immediately after sampling, 5 ml of urine was filtered, diluted in 20 ml of sulfuric acid (0.036 N), and stored at -20˚C. Upon the third urine collection (collected at 19 d of the experimental period), the first two samples (previously collected at the 17 d, and 18 d of the experimental period)

were thawed, and the three samples were mixed, and stored -20˚C for further analyses for urea, and 3 methyl-histidine.

On the 28 d of each experimental period, cows' blood samples were collected in the morning via jugular venipuncture into vacuum tubes (BD Vacutainer, NJ, USA) containing heparin to evaluate the amino acid profile, tubes containing sodium fluoride to evaluate the concentration of glucose, and tubes containing gel, and clot activator to evaluate the concentration of urea. Immediately after collection, the samples were sent to a commercial laboratory (Viçosa Lab, Vic¸osa, Brazil). Simultaneously with the blood collection, carcass ultrasound images were collected by using a Aloka SSD500 with a 3.5 Mhz 18 cm linear probe to monitor the body condition score of the cows. Images were captured and analyzed by using the Biosoft ToolBox for Bovine (Biotronics, Ames, Iowa, USA). On the 54 d of the feeding trial, liver biopsies were performed using specific needles (TruCut biopsy needle, Care Fusion Corporation, San Diego, California, USA), according to the procedures previously described [15]. The area between the 11th -12th rib was initially cleaned with 70% ethanol, and the incision was performed 10 min after local anesthesia treatment (Lidocaine 2%). Liver samples (~ 30 mg) from the right lobe were collected. Immediately after collection, the samples were rinsed with phosphate saline buffer (pH = 7.4) and immediately snap-frozen in liquid nitrogen and kept at -80˚C for further analysis.

3.3.4. Feed, and fecal samples chemical analysis

Samples of feeds, and feces were oven-dried (60˚C), and ground in a Wiley mill model 3 (Thomas Scientific, NJ, USA) to pass through a 2- mm screen. After that, half of each ground sample was ground again to pass through a 1- mm screen. The fecal samples were pooled on an air-dry weight basis per day, and animal according to the periods of collection (equal weight for each collection time).

The samples ground to pass through a 1- mm screen sieve were used to perform the analyses of dry matter [16] (DM; method 934.01), crude protein [16] (CP; method 990.13), ether extract [16] (EE; method 920.39), ash [16] (method 942.05), neutral detergent fiber corrected for ash, and protein (NDFap; using a heat stable α-amylase omitting sodium sulfite; [17]; methods INCT-CA F-002/1; N-004/1, and M-002/1). The non-fibrous carbohydrates (NFC) were calculated as previously proposed [18]. In addition, the samples ground to pass a 2- mm screen sieve were incubated in quadruplicate in the rumen of two cows for 240 h using nonwoven textile bags (100 g/m2) to quantify the indigestible NDF (iNDF) [19]. The fecal excretion of DM was estimated by

using iNDF as an internal marker. We calculated the digestibility coefficients for the different feed components to assess the digestible energy intake, which was expressed in terms of total digestible nutrients (TDN).

3.3.5. Analysis of blood, and urinary parameters

In urine samples the concentrations of urea (Atellica Solution™, Siemens Healthineers, Erlangen, Forchheim, Germany), and 3 methyl-histidine (HPLC, Hermes Pardini, Belo Horizonte, Minas Gerais, Brazil) were determined. In blood samples, analyses of glucose (Cobas C311 analyzer, Roche Diagnostics, Rotkreuz, Switzerland), urea (Cobas C311 analyzer, Roche Diagnostics, Rotkreuz, Switzerland), and the amino acid profile were performed, and the following serum amino acids were quantified: glutamic acid, alanine, glycine, methionine, valine, citrulline, ornithine, leucine, phenylalanine, tyrosine, and isoleucine (LC-MS/MS, Xevo™ TQD, Waters Corporation, Milford, Massachusetts, USA).

3.3.6. Liver tissue protein extraction

Total proteins were extracted from 1 mg of tissue in a 1 mL of lysis buffer (7 M urea, 2 M thiourea, 4% 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS) detergent, 1% dithiothreitol (DTT), and 10 ul protease inhibitor cocktail (Sigma-Aldrich1, St. Louis, Missouri, USA), homogenized using a shaft-type homogenizer (LabGEN 125, Cole-Parmer, Bunker Hill, IL, USA), and then centrifuged at 10000 × g for 30 min at 4˚C. The supernatant was collected, aliquoted, and stored at -80˚C. Protein content was estimated by the Bradford Protein Assay (Bio-Rad, Hercules, CA, USA). After protein quantification, 50 µg of sample was transferred to 2.5 µL tuber containing 100 mM of DTT (1.4-dithiothreitol). The solution was then stirred, and placed in a thermal block at 60˚C for 30 min. After reaching the room temperature, 2.5 µL of 300 mM iodoacetamide was added, for cysteine alkylation, and transferred to the dark at room temperature for 30 min. 10µL of trypsin (Promega Corporation, Madison, WI, USA) was added to an ammonium bicarbonate (Ambic) solution, vortexed, and digested overnight at 37˚C. The samples were then dried using a SpeedVacTM centrifuge (Thermo Fisher Scientific, Waltham, MA, USA), resuspended in 50 µL of 0.1% trifluoroacetic acid (TFA) solution prepared in H2O milliQ, and desalted using ZipTip1C18 (Merck Millipore, Billerica, MA, USA).

3.3.7. Protein identification, and data processing

Protein identification, and quantification was performed in a NanoAquity high-performance liquid chromatographer (HPLC) coupled with a maXis 3G high-resolution Q-TOF mass spectrometer (Bruker Daltonics, Billerica, Massachusetts, USA). The raw data were processed with MaxQuant software (v. 1.6.3.3), with the parameters set to default values, considering the protein amino terminal acetylation, methionine oxidation as variable modification, and the fixed modification as carbamidomethylation of cysteine. The trypsin specificity was kept as the digestion mode, and the instrument selected was Bruker-QTOF, set to default, including the parameters of first (20 ppm), and main (10 ppm) search peptide tolerance. The label-free quantification (LFQ) mode was added, and at least two unique peptide ratios (min LFQ ratio count = 2) were considered. The bovine reference proteome was obtained from UniProt (ID: UP000009136) available in (www.uniprot.org, accessed on 23 March 2021). A total of 807 proteins were identified in the liver of cows.

Prior to normalization of the data, the processed proteomic abundance data were subjected to quality control. For this, samples (i.e., animals), and proteins identified as potential contaminants, only identified by site, and reverse sequence were removed from the dataset to avoid issues with the statistical analyses, and inferences. First, samples with less than 1% of the proteins identified were removed from the dataset. In this step, two samples (both from CON) were removed, resulting in a final dataset with 22 animals (10 from CON, and 12 from PRU) with proteomic data. Afterwards, PTNs represented in less than 10% of samples (i.e., in 2 or fewer samples) were removed. The final dataset included 382 proteins.

After quality control, the data were subjected to normalization of the library size. Normalizing factors were obtained for each protein using the Trimmed Mean of M-values method, via the TMM package [20] implemented in R (R Core Team, Vienna, Austria).

3.3.8. Statistical analyses

3.3.8.1 Analyses of data of animal performance, nutritional, and physiological blood characteristics

Data from animal nutritional, and physiological blood parameters were analyzed according to the following model below:

$$y_{ijkl} = \mu + T_i + GG_j + FS_k + b_1 IW_{ijkl} + e_{ijkl}$$

[Eq. 1]

where y_{ijkl} is the observed data for the l^{th} cow; μ is the intercept; T_i is the fixed-effect of the i^{th} Treatment (i = 1, 2); GG_j is the fixed-effect of the j^{th} Gestation Group (i = 1 to 3); and FS_k is the fixed-effect of the k^{th} Sex of the Fetus (k = 1, 2); b_1 is the partial regression coefficient for the fixed-effect covariate of initial body weight; IW_{ijkl} is the initial body weight of the cow at the beginning of the feeding period (in kg) of the l^{th} cow; and e_{ijkl} is the random error for the l^{th} cow, assuming $e_{ijkl} \sim N(0, \boldsymbol{I}\sigma_e^2)$, where $\boldsymbol{I}$ is the identity matrix, and σ_e^2 the residual variance. The covariate of initial body weight of the cow at the beginning of the feeding period in Eq. 1 was kept in the model for traits when highly significant ($P < 10^{-5}$).

Prior to analyses, residuals were evaluated for distributional assumptions. Studentized residuals outside ± 3 SD were removed, one at a time, while simultaneously assessing their homogeneity across fixed-effects with visual inspection, and their normality with Shapiro-Wilk's test ($P > 0.05$) [21]. After removal of outliers, all the data showed normality, and homogeneity of the residual, and were subjected to subsequent final analyses. All analyses were performed using the GLIMMIX, and UNIVARIATE procedures of SAS 9.4 (Statistical Analysis System Institute, Inc., Cary, NC, USA). Significances were declared at $P < 0.05$, and trends were discussed when $0.10 < P \leq 0.05$.

3.3.8.2 Models evaluated for proteomic analysis

Multiple distributions of the normalized data, and models were evaluated for each of the 382 proteins analyzed. In this step we evaluated Negative Binomial (NB) distributions assuming or not zero-inflated (ZI) data using a log-link function [22]. Therefore, two combinations were evaluated, one not assuming zero-inflation of the data (i.e., many samples without abundance for the protein analyzed), and referred to just as NB, and one assuming zero-inflation (ZINB).

For the NB model, the data were analyzed as:

$$ln\left(\frac{y_{ijkl}}{LS_{ijkl}}\right) = \mu + T_i + GG_j + FS_k$$

[Eq. 2]

where $ln\left(\frac{y_{ijkl}}{LS_{ijkl}}\right)$ represents the natural logarithm of the normalized protein abundance analyzed adjusted for the normalized library size for the l^{th} animal, and all other terms have been defined in Eq. 1.

When assuming ZI data, we used three models where the count (i.e., non-ZI) part of the model was the same as in Eq. 2, and the ZI part was different. The first ZI model ($ZINB_{basic}$) assumed only the intercept, as:

$$ln\left(\frac{y_{ijkl}}{LS_{ijkl}}\right) = \begin{cases} \mu + T_i + GG_j + FS_k & if\ y_{ijkl} > 0 \\ \mu_{ZI} & if\ y_{ijkl} = 0 \end{cases}$$

[Eq. 3]

where μ_{ZI} represents the intercept of the ZI part of the model, and all other terms have been previously described in Eq. 2. In $ZINB_{basic}$, we assumed that over the presence of many zero in the data is only a function of the intercept.

In the other two models, Eq. 3 was expanded to include the effects in Eq. 1. First, only the effect of treatment was used ($ZINB_{TRT}$):

$$ln\left(\frac{y_{ijkl}}{LS_{ijkl}}\right) = \begin{cases} \mu + T_i + GG_j + FS_k & if\ y_{ijkl} > 0 \\ \mu_{ZI} + T_{ZI_i} & if\ y_{ijkl} = 0 \end{cases}$$

[Eq. 4]

where T_{ZI_i} represents the i^{th} fixed effect of Treatment (i=1,2) in the ZI part of the model; and all other terms have been previously described in Eq. 1, and 2. The ZI_{TRT} model was used to evaluate whether the presence of many zero in the data could also be explained by the maternal treatment. Finally, the last model assumed the same effects in both the non-ZI, and ZI parts of the model ($ZINB_{full}$):

$$ln\left(\frac{y_{ijkl}}{LS_{ijkl}}\right) = \begin{cases} \mu + T_i + GG_j + FS_k & if\ y_{ijkl} > 0 \\ \mu_{ZI} + T_{ZI_i} + GG_{ZI_j} + FS_{ZI_k} & if\ y_{ijkl} = 0 \end{cases}$$

[Eq. 5]

where GG_{ZI_j} represents the j^{th} fixed-effect of Gestation Group (j=1 to 3) in the ZI part of the model; FS_{ZI_k} represents the k^{th} fixed-effect of Fetus Status (k=1 to 3) in the ZI part of the model; and all other terms have been previously described in Eq. 2 to 4. With this, four models were

evaluated for each protein. Analyses of all models were performed in SAS 9.4 (Statistical Analysis System Institute, Inc., Cary, NC, USA) with the GLIMMIX procedure.

3.3.8.3 Model selection and identification of differentially abundance proteins (DAPs)

The NB, $ZINB_{basic}$, $ZINB_{TRT}$, and $ZINB_{full}$ models were compared within each protein analyzed. Only models that converged were evaluated. Model comparisons followed a nested strategy, from the simplest (NB) to the most complex ($ZINB_{full}$) model using a likelihood ration test (LRT) to evaluate the inclusion of additional parameters into the model. That is, first, NB was compared to $ZINB_{basic}$, and if the LRT was not significant ($P > 0.05$), then the NB model was used for the protein. However, if the LRT between NB, and $ZINB_{basic}$ was significant ($P > 0.05$), results from the $ZINB_{basic}$ were compared with the $ZINB_{TRT}$. Likewise, if the LRT test between these were significant ($P < 0.05$), then the $ZINB_{TRT}$ model was chosen, otherwise, $ZINB_{basic}$ was used for the protein. Finally, if $ZINB_{TRT}$ was selected in place of $ZINB_{basic}$, it was then compared with $ZINB_{full}$ to evaluate the best model for the protein analyzed. This process was used for each of the 382 proteins analyzed, and hence, the final results include different models, according to the protein.

After models were selected for each protein, effects in the model were deemed significant after adjusting *P*-values for multiple comparisons using False-Discovery Rate (FDR) [23] as *q*-values. Differentially abundant proteins (DAP) were identified at *q*-value < 0.05. The calculation of *q*-values was obtained with the *qvalue* package [24] in *R* version 3.6.3 [25] in *RStudio* version 3.0.386 [26].

3.3.8.4 Gene ontology (GO) and signaling pathways (KEGG) analyses

The protein-protein interaction network, Kyoto Encyclopedia of Genes, Genomes (KEGG), and Gene Ontology (GO) enrichment analyses were performed by String 11.0 (string-db.org). The interaction network of the exclusive proteins from the treatments CON, PRU, and the DAPs were obtained using the available interaction map from *Bos taurus* with the default option (medium confidence given by score of 0.4) [27]. The functional classification of the GOs and KEGG signaling pathways were deemed significant at an FDR-adjusted *P*-value (P_{FDR}) < 0.05 based on Benjamini-Hochberg's method [28].

3.4. Results

3.4.1. Animal performance, digestibility, and blood parameters

There were no differences between treatments for dry matter intake (DMI; P = 0.99), total digestible nutrient (TDN) intake ($P = 0.99$). The digestibility variables, dry matter ($P = 0.58$), crude protein ($P = 0.51$), ether extract ($P = 0.73$), non-fiber carbohydrates ($P = 0.68$), neutral detergent fibre corrected for ash, and protein ($P = 0.95$) did not differ between treatments. In addition, there were no differences in body composition variables, such as subcutaneous fat thickness (SFT; $P = 0.72$), and rib eye area (REA; $P = 0.85$). The concentrations of urea in urine ($P = 0.51$), and serum ($P = 0.30$) were also not different among treatments. Similarly, no changes in serum levels of glucose ($P = 0.87$), and in glucogenic ($P = 0.28$), ketogenic ($P = 0.72$), glucogenic, and ketogenic ($P = 0.45$) amino acids were observed among treatments. Similarly, the maternal treatment did not influence ($P = 0.59$) the birth weight of the progeny. On the other hand, the levels of 3 methyl-histidine, in mmol/mol creatine, were higher ($P = 0.02$) in CON. Results are presented in Table 2.

Table 2. Intake, digestibility, performance, urine, and serum parameters from treatment PRU, and CON.

Item	CON	PRU	*P*-value
Intake			
Dry matter intake (kg/day)	5.9 ± 0.28	5.9 ± 0.27	0.99
Total digestible nutrient intake (kg/day)	4.0 ± 0.19	4.0 ± 0.18	0.95
In vivo digestibility (%)			
Dry matter	65.7 ± 0.48	66.1 ± 0.44	0.58
Crude protein	62.5 ± 0.97	61.6 ± 0.92	0.51
Ether extract	81.6 ± 0.73	81.3 ± 0.69	0.73
Non-fiber carbohydrates	78.3 ± 1.25	77.6 ± 1.18	0.68
NDFap[a]	59.8 ± 0.56	59.9 ± 0.53	0.95
Performance			
Rib eye area (cm^2)	62.3 ± 3.79	63.2 ± 3.60	0.85
Rib fat thickness (mm)	3.5 ± 0.63	3.8 ± 0.59	0.72
Average daily gain (kg/day)	0.38 ± 0.1	0.32 ± 0.09	0.66
Final body weight (kg)	589 ± 6.86	583 ± 6.44	0.47
Serum parameters			
Glucogenic amino acids[b] (µmol/L)	163.0 ± 5.59	170.9 ± 5.31	0.28
Ketogenic amino acids[c](µmol/L)	19.7 ± 1.02	20.1 ± 0.98	0.72
Glucogenic, and ketogenic amino acids[d] (µmol/L)	39.7 ± 3.19	42.8 ± 3.03	0.45
Glucose (mg/dL)	60.4 ± 2.42	60.9 ± 2.29	0.87
Urea (mg/dL)	28.1 ± 1.44	26.2 ± 1.36	0.30
Urine parameters			
Urea (mg/dL)	1157.5 ± 143.3	1036.4 ± 135.8	0.51
3 methylhistidine (mmol/mol creatinine)	8.76 ± 0.50	7.19 ± 0.48	0.02
Offspring's performance (kg) Birth weight	33.1 ± 1.64	34.2 ± 1.60	0.59

CON= Control treatment; PRU= Post-ruminal releasing of urea

[a]Neutral detergent fibre corrected for ash, and protein

[b]Glutamic acid, alanine, glycine, methionine, valine, citrulline, and ornithine

[c]Leucine;

[d]Phenylalanine, tyrosine, and isoleucine

3.4.2. Proteomic profile of hepatic tissue

To identify the differentially abundant proteins (DAPs), the 382 proteins were tested. From these, 19 DAPs were identified (q-value < 0.05) between treatments, seven, and 12 proteins showing greater, and lower abundance in the PRU group compared to CON (Table 3). Among them, S-adenosylmethionine synthase isoform type 1 (MAT1A), methyl malonyl-CoA isomerase

(MMUT), and persulfide dioxygenase (ETHE1) were more abundant in PRU compared to CON cows. On the other hand, the proteins apoptosis inducing factor mitochondrial associated (AIFM1), galectin-1 (LGALS1), and e lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex mitochondrial (DBT) were less abundant in PRU compared to CON cows.

3.4.3. Functional analysis of the differentially abundant proteins

The GO can be classified as biological processes, molecular function, and cellular components. The DAPs network showed biological processes related to sulfur amino acid metabolism (GO:0000096; PFDR = 0.02), and nitrate metabolic process (GO:0042126; PFDR = 0.02) upregulated in PRU compared to CON cows. The enriched molecular function related to catalytic activity (GO:0003824; PFDR < 0.03), and binding proteins (GO:0005488; PFDR < 0.03), showed up, and down-regulation in PRU compared to CON cows, respectively. Regarding the cellular components, mitochondrion term (GO:0005739; PFDR < 0.01) was upregulated in PRU compared to CON cows. The KEGG signaling pathways analysis identified the upregulation of fatty acids biosynthesis (WP1020; PFDR = 0.03), and metabolism of vitamin B12 (WP3193; PFDR = 0.04) in PRU compared to CON cows. Conversely, the apoptosis (bta04210; PFDR < 0.01), and FAS (WP1019; PFDR < 0.01) pathways were downregulated in PRU compared to CON cows.

3.4.3. Functional analysis of the exclusive protein

We found 280 and 83 exclusive proteins in the treatments PRU and CON, respectively. The protein-protein interaction network of proteins found to be exclusive in each treatment were significant (PRU: PFDR < 1.0e-16; CON: PFDR = 2.1e-7), indicating that the proteins are biologically connected.Due to the greater number of proteins found to be exclusively present in each treatment, we identified a variety of proteins which participate in pathways involved in nitrogen and energy metabolism that will be further emphasized in discussion section. The analysis of KEGG pathways from the exclusive proteins in treatment PRU indicate pathways of interest related to pentose phosphate pathway (PGD), pyruvate metabolism (DLAT, LDHA), glycolysis (ALDOA, PGAM1, PKLR), carbohydrate digestion, and absorption (ATP1A1, ATP1A4, ATP1A3, ATP1A2), cysteine, and methionine metabolism (MTAP, ADI1), and oxidative

phosphorylation (NDUFA8, NDUFAB1, NDUFS1, NDUFV2, UQCRB, UQCRH, UQCRC1, COX72A, COX6B1, COX5B, MGC148714, ATP5D, ATP5F1, ATP5J, ATP5J2) (Table 4).

Table 3. Differentially abundant proteins (DAPs) in the hepatic tissue of the cow.

Accession	Protein name	Gene name	*q*-value	ln (FC)[a] [95%CI][b]
Q1LZH1:G5E6I5	Mitochondrial amidoxime reducing component	*MTARC*	1.65E-271	0.49
A0A3Q1LUG9;F1MKS3	Thioredoxin domain-containing protein 5	*TXNDC5*	1.65E-271	0.58
F1N5J8;A0A3Q1LNW7	2,4-dienoyl-CoA reductase 1	*DECR1*	1.65E-271	1.03
Q3T094	Persulfide dioxygenase	*ETHE1*	1.92E-139	0.26
Q9GK13;A0A3S5ZPN0	Methylmalonyl-CoA isomerase	*MMUT*	2.98E-134	0.24
A0A3Q1M909;P54149;A0A3Q1N5G3	Mitochondrial peptide methionine sulfoxide reductase	*MSRA*	3.51E-70	0.17
A0A3Q1NCY0;G5E5U7;Q2KJC6;A7E3T7;A0A3Q1MZ37	S-adenosylmethionine synthase isoform type-1	*MAT1A*	2.57E-44	0.16
F1MYG5;E1B8N6;A7YY47;A0A3Q1LNG7; F1MJI7	Lamin	*LMN*	1.65E-271	-1.53
A0A3Q1MTB8;Q28034;A0A3Q1MKJ7	Glucosidase 2 subunit beta	*PRKCSH*	1.65E-271	-1.48
F6PRB5;A0A3Q1LS67	Enoyl-CoA hydratase 1	*ECH1*	1.65E-271	-1.15
Q5I597;A0A452DHV7;A0A3Q1NDS5	Betaine--homocysteine S-methyltransferase	*BHMT*	1.65E-271	-0.35
P11116	Galectin-1	*LGALS1*	2.06E-264	-0.36
Q0VCU1;F1MS05;A0A3Q1MHC4	Cytoplasmic aconitate hydratase	*ACO1*	3.92E-155	-0.18
F1N5Q0;E1BJ78;A0A3Q1MP36;A0A3Q1M941; E1BIS9	Sulfotransferase	*SULT*	8.00E-148	-0.24
E1BJA2	Apoptosis inducing factor mitochondria associated	*AIFM1*	1.19E-43	-0.11
A7MBI6	Glyoxalase domain-containing protein 4	*GLOD4*	1.09E-33	-0.11
P68530	Cytochrome c oxidase subunit 2	*MT-CO2*	3.06E-18	-0.08
A0A3Q1MC79;F1MYV0;A0A3Q1LW09	Solute carrier organic anion transporter family member 1B3	*SLCO1B3*	1.17E-15	-0.07
A0A3Q1MED4;P11181	Lipoamide acyltransferase	*DBT*	4.42E-09	-0.08

CON= Control treatment; PRU= Post-ruminal supplementation with urea; [a]Negative, and positive natural log fold changes [ln(FC)] indicate, respectively, lower, and greater protein abundance in the treatment PRU compared to CON; [b]95% confidence interval.

Table 4. Enriched metabolic pathways of the exclusive proteins from treatment PRU, and CON.

KEGG ID	Description	P_{FDR}[a]	Protein symbols[b]
Treatment PRU			
bta00190	Oxidate phosphorylation	2.15E-09	ATP5D, UQCRB, NDUFA8, COX7A2, COX6B1, NDUFAB1, ATP5F1, PPA1, UQCRH, CYC, MGC148714, COX5B, UQCRC1, NDUFS1, ATP5J, ATP5J2, NDUFV2
bta00030	Pentose phosphate pathway	0.0021	DERA, TALDO1, ALDOA, PGD, GLYCTK
bta00280	Valine, leucine, and isoleucine degradation	0.0424	AOX1, HMGCS1, BCKDHA, ALDH1B1
bta04975	Cholesterol metabolism	0.0021	LIPA, CYP27A1, VAPA, VAPB, NPC2, ABCB11
bta00010	Glycolysis / gluconeogenesis	0.0045	LDHA, DLAT, ALDOA, ALDH1B1, PGAM1, PKLR
bta04973	Carbohydrate digestion, and absorption	0.0302	ATP1A1, ATP1A4, ATP1A3, ATP1A2
bta00270	Cysteine, and methionine metabolism	0.0424	ADI1, LDHA, MTAP, GCLC
bta01230	Biosynthesis of amino acids	0.0332	ADI1, LDHA, MTAP, GCLC
bta00620	Pyruvate metabolism	0.0220	LDHA, DLAT, ALDH1B1, PKLR
bta04141	Protein processing in endoplasmic reticulum	0.0077	CRYAB, SSR1, LMAN2, HYOU1, SAR1A, BCAP31, SAR1B, HSPA6, RPN2
bta03050	Proteasome	0.0021	PSMB2, PSMA7, PSMB1, PSMA4, PSMA8, PSME1
bta03010	Ribosome	0.000022	RPS17, RPS28, RPL23, RPS9, RPL12, RPS2, RPS19, RPS5, RPS14, RPS24
bta04145	Phagosome	0.0021	CTSL1, LOC100141266, TUBA4A, ACTG1, MBL2, SEC22B, RAB7A, CGN1, CTSV, LAMP1
bta00480	Glutathione metabolism	0.0157	GSR, MGST3, PGD, GSTA5, GCLC
bta04142	Lysosome	1.22E-9	GUSB, CTSL1, AP1B1, MAN2B1, NAGA, SCARB2, ASAH1, LIPA, HEXA, GBA, TPP1, CTSV, CTSZ, NPC2, LAMP1, HEXB, LBMN
Treatment CON			
bta00380	Tryptophan metabolism	0.0209	CYP1A2, DLD, GCDH
bta00830	Retinol metabolism	0.0036	CYP1A2, CYP3A24, LOC100138004, UGT1A1

CON= Control treatment; PRU= post-ruminal supplementation with urea; [a]False Discovery Rate (FDR)-adjusted *P*-value; [b]Proteins corresponding to the connected proteins in the protein-protein interaction network

For the proteins exclusively found in CON cows, KEGG analysis identified enriched pathways related to retinol (CYP1A2, CYP3A24, LOC100138004, UGT1A1), and tryptophan metabolism (CYP1A2, DLD, GCDH) (Table 4).

3.5. Discussion

Studies evaluating the post-ruminal urea supplementation have recently emerged [9,10,13], however, the effects of the post-ruminal supply of urea in cows during late gestation are not fully understood. Therefore, in the present study we evaluated the impact of shifting urea release from rumen to the intestinal tract at late gestation on liver metabolism, and nutritional status of pregnant cows at late gestation. To further explore the consequences of PRU supplementation in the metabolism, we employed a shot-gun proteomic approach, and a pathway analysis to identify the possible biological changes in the liver. We were able to identify the enhancement of S-adenosylmethionine synthase isoform type 1 (MAT1A) in PRU compared to CON cows. The protein MAT1A is involved in methionine metabolism, catalyzing the conversion of methionine into S-adenosylmethionine (SAM), which is demanded for the biosynthesis of polyamines, and glutathione (GSH) [29] (Fig 1). The proteins methylthioadenosine phosphorylase (MTAP), and acireductone dioxygenase 1 (ADI1), found to be exclusively expressed in PRU cows, are involved in methionine regeneration from the polyamines [30]. The polyamines (putrescine, spermidine, and spermine) are key regulators of placental angiogenesis, and embryo development [31]. During the process of methionine regeneration from polyamines, the sulfur of 5'-methylthioadenosine (MTA) is recycled, synthesizing acireductone through the action of the enzyme MTAP [32]. The acireductone is then converted into methionine through the reaction catalyzed by ADI1 [32]. Taken together, the proteins exclusively expressed in PRU cows indicate an enriched pathway of methionine regeneration from polyamines. Kwon [33] reported a greater concentration of polyamines in the first third of gestation in ovine due to the intensive placental development, accompanied by its reduction as the pregnancy advances. Providing greater availability of protein in the intestines contributed to an increase in placenta angiogenesis, and blood flow during gestation through nitric oxide [34,35]. Therefore, the enrichment of this pathway at late gestation may be related to a greater concentration of polyamines, and consequently greater placental development during gestation.

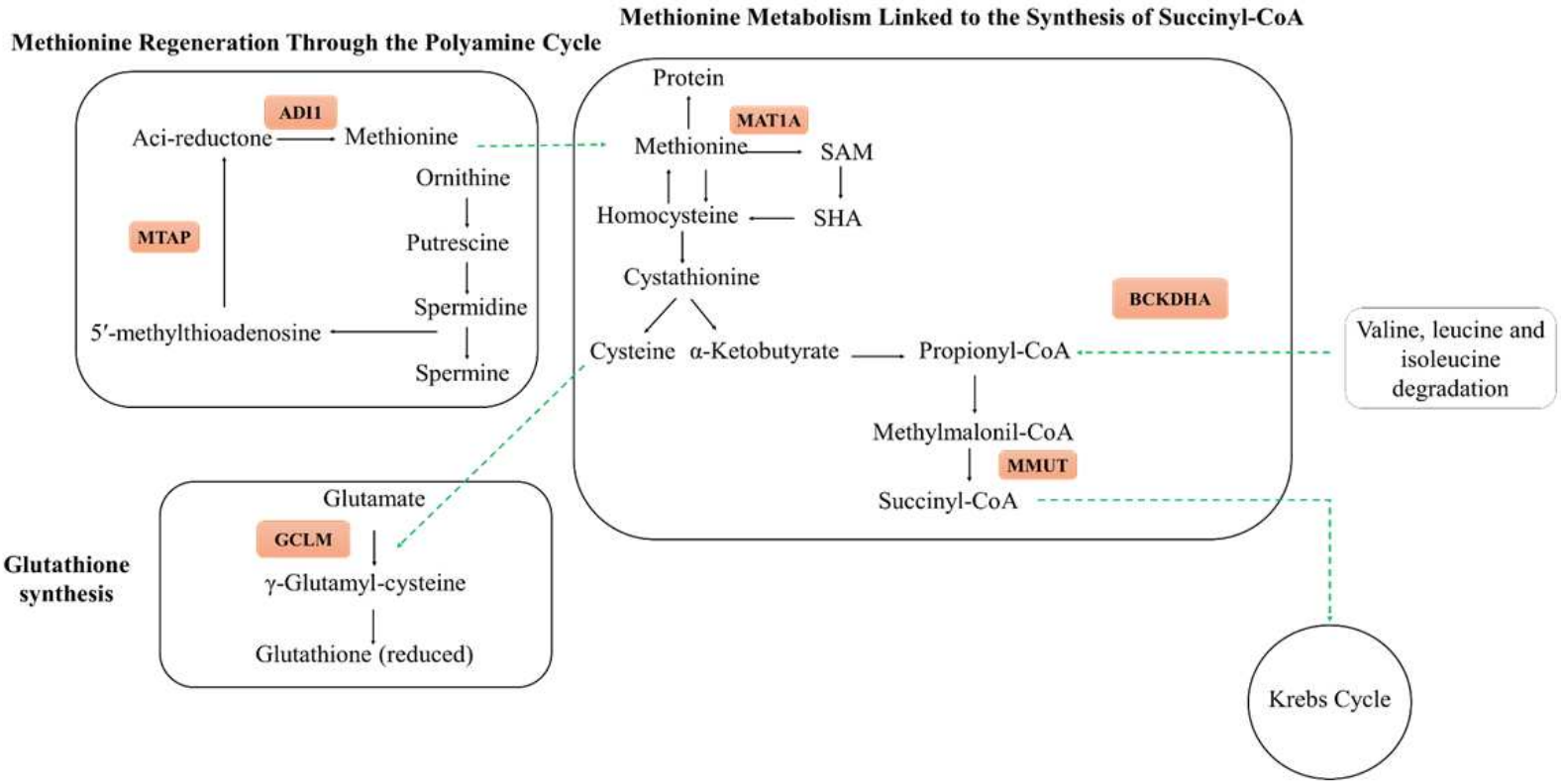

Fig 1. Enriched energy protein pathways of the exclusive proteins from treatment PRU. Summary of the protein metabolism pathways influenced by the experimental treatments. Proteins inside the orange square are positively regulated in treatment PRU (post-ruminal releasing of urea) compared to CON. ADI1: acireductone dioxygenase 1; MTAP: methylthioadenosine phosphorylase; GCLM: γ-glutamylcysteine synthase; BCKDHA: Branched chain keto acid dehydrogenase E1 subunit alpha; MMUT: methylmalonyl- CoA isomerase; MAT1A: S-adenosylmethionine synthase isoform type-1.

Evaluating the enriched pathways of the exclusively expressed proteins in PRU cows, we have identified pathways related to carbohydrate digestion, and absorption, glycolysis, pyruvate metabolism, oxidative phosphorylation, pentose phosphate pathway, and biosynthesis of amino acids (Figs 1 and 2). Therefore, our data indicate a change in the hepatic energy balance due to PRU supplementation. These findings can be explained by the improvement in ruminal metabolism by the post-ruminal nitrogen supply, as shown by Oliveira [10] which provided greater input of substrates to enter the metabolic pathways described above. Different isoforms of the protein Na+ /K+ ATPase (ATP1A), responsible for the ATP hydrolysis coupled to the active transport of Na+ and K+ across the plasma membrane [36] were identified exclusively in PRU cows. Wood [37] observed an increase in the abundance of ATP1A protein in the liver of pregnant cows, suggesting an increase in the metabolic rate during gestation. The action of ATP1A in the liver is related to the active transport of substrates, and the maintenance of ionic homeostasis [38]. Investigating the effects of increasing levels of forages in the diet of growing calves, Wang [39] detected a linear relationship between the abundance of liver ATP1A, and forage inclusion levels, suggesting that the production of short-chain fatty acids in rumen may influence liver ATP1A abundance. Souza [13] observed an increase in volatile fatty acids concentration, and lower pH in rumen when shifting urea release from rumen to the abomasum. This report supports our findings, in which an increase in the abundance of ATP1A in the liver of pregnant cows may be associated with the improvement in ruminal metabolism when supplemented with PRU.

Mitochondria act as a power supplier to the cells through the production of adenosine triphosphate (ATP) coupled to the electron transport via the respiratory chain complexes [40]. Electron transport mechanisms between the mitochondrial complexes may lead to the generation of reactive oxygen species (ROS), since the mitochondria electron transfer in the respiratory chain is not entirely efficient [41]. We observed an exclusive presence of proteins complexes of the electron transport chain; complex I (NDUFA8, NDUFAB1, NDUFS1, and NDUFV2), complex III (UQCRB, UQCRH, and UQCRC1), complex IV (COX72A, COX6B1, COX5B e MGC148714), and ATP synthase (ATP5D, ATP5F1, ATP5J, ATP5J2) in liver of PRU cows (Fig 2), indicating an increase in the oxidative phosphorylation pathway and greater production of ATP in PRU animals. Concomitantly, we found the γ-glutamylcysteine synthase (GCLM) protein exclusively expressed in treatment PRU cows. GCLM is involved in glutathione (GSH) synthesis [42] (Fig 1). The GSH plays an antioxidant role in acting against the reactive oxygen species (ROS) that causes protein degradation [43]. The balance of GSH is

closely connected to glucose metabolism through the pentose phosphate pathway, as this pathway generates NADPH used to maintain the reduced levels of GSH [43]. Moreover, the oxidative phosphorylation pathway, which was enriched in PRU treatment, is a potent source of ROS [43]. An imbalance between the increased production of ROS, and the availability of antioxidant defenses needed to reduce ROS accumulation during late gestation may expose cows to increased oxidative stress [44]. The oxidative stress is a significant underlying factor to dysfunctional host immune, and inflammatory responses that can increase the susceptibility of cows to a variety of health disorders, particularly during late gestation [45,46]. Therefore, our result indicates an increase in substrate availability in the liver, which contributed to greater metabolic efficiency in PRU cows. Furthermore, there was enhancement in GSH synthesis in PRU cows that may be reflect a responsive metabolic adaptation to support the increased ROS accumulation and decrease oxidative stress risk generated by increase in the synthesis of ATP in PRU cows.

We have also identified the protein aldolase (ALDOA), to be exclusively expressed in the liver of PRU cows. This protein participates in the last step of the energy-investment phase of glycolysis, catalyzing the conversion of fructose-1,6-bisphosphate into glyceraldehyde 3-phosphate, and dihydroxyacetone phosphate [47] (Fig 2). In the energy-generation phase of glycolysis the exclusively expressed proteins in PRU cows, phosphoglycerate mutase (PGAM1), and pyruvate kinase (PKLR) are involved in the conversion of 3-phosphoglycerate into 2-phosphoglycerate [48], and the conversion phosphoenolpyruvate into pyruvate [49], respectively (Fig 2). As such, our results suggest that the supplementation of PRU sources contribute for the formation of lactate, and acetyl-CoA from pyruvate. Moreover, lactate dehydrogenase (LDHA), also found to be exclusively expressed in PRU cows, catalyzes the conversion of lactate into pyruvate allowing the generation of reducing equivalents (NAD+) in anaerobic conditions (Fig 2). Whereas the subunit of the pyruvate dehydrogenase complex, the dihydrolipoamide acetyltransferase (DLAT), exclusively expressed in PRU cows, contribute for the synthesis of acetylCoA (Fig 2). In summary, our results indicate that supplementing cows during late gestation with PRU may cause an enhancement in glycolysis pathways, and contribute for the synthesis of acetyl-CoA, leading to the improvement in the liver energy balance.

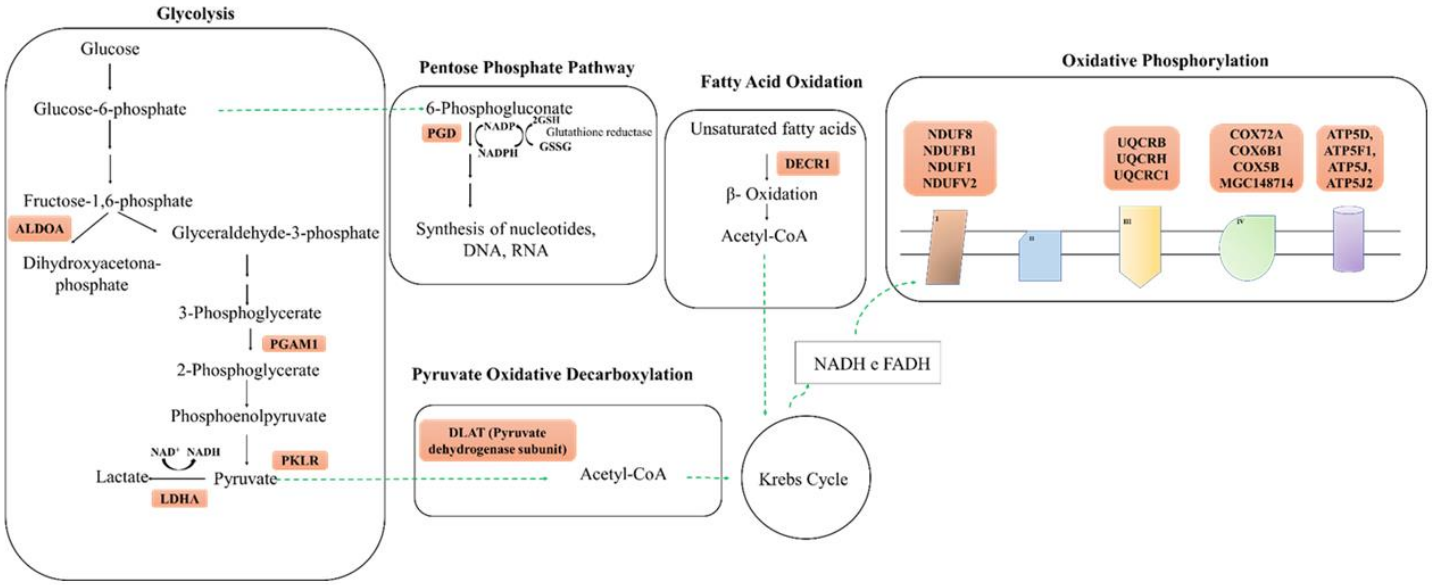

Fig 2. Enriched energy metabolism pathways of the exclusive proteins from treatment PRU.

Summary of the energy metabolism pathways influenced by the experimental treatments. Proteins inside the orange square are positively regulated in treatment PRU (post-ruminal releasing of urea) compared to CON. ALDOA: aldolase; PGAM1: phosphoglycerate mutase; PKRL: pyruvate kinase; LDHA: lactate dehydrogenase; PGD: 6-phosphogluconate dehydrogenase; DLAT: dihydrolipoamide acetyltransferase; DECR1: 2,4-dienoyl-CoA reductase; NDUFA8, NDUFAB1, NDUFS1 e NDUFV2: subunits of the complex I of the electron transport chain; UQCRB, UQCRH e UQCRC1: subunits of the complex III of the electron transport chain; COX72A, COX6B1, COX5B and MGC148714: subunits of the complex IV of the electron transport chain; ATP5D, ATP5F1, ATP5J, ATP5J2: subunits of the ATP synthase complex of the electron transport chain

The 6-phosphogluconate dehydrogenase (PGD) protein, exclusively expressed in PRU cows, play role in the decarboxylation of 6-phosphogluconate to ribulose 5-phosphate, reducing NADP into NADPH, and afterward the ribose-5-phosphate is synthesized (Fig 2). The NADPH is demanded to supply the reduction equivalents of most biosynthetic reactions, such as the fatty acid biosynthesis. Interestingly, the more abundant or exclusively expressed proteins in PRU compared to CON cows, such as methylmalonyl- CoA isomerase (MMUT), 2,4-dienoyl-CoA reductase 1 (DECR1), and branched chain keto acid dehydrogenase E1 subunit alpha (BCKDHA) are involved in energy metabolism, and lipid biosynthesis. The BCKDHA catalyzes the decarboxylation of the α-ketoacids from valine, leucine, and isoleucine, promoting the synthesis propionyl- CoA, whereas the enzyme MMUT regulate the conversion of methylmalonyl-CoA into succinyl- CoA entering the krebs cycle. The DECR1 is involved in the β-oxidation of the unsaturated fatty acids [50]. Taken together, our results suggest that the reduction equivalents synthesized in the pentose phosphate pathways may have been used in β-oxidation, promoting the formation of acetyl-CoA. While the production of ribose-5-phosphate from this pathway contributes to the synthesis of nucleotides, and nucleic acids. Thus, the current study provided evidence that the supplementation of PRU sources is beneficial in liver ATP synthesis, through the enriched pathways of glycolysis, and oxidative phosphorylation.

Protein supplementation has been the focus of the research on pregnant beef cows' nutrition [51]. When evaluating the partition of crude protein among maternal, and conceptus tissues in ewes during late pregnancy, a moderate net loss of carcass protein was observed over this period [4]. It is important mentioning that amino acid requirements increase in late gestation [1]. Under a scarcity of feedstuffs, maternal skeletal muscle mobilization occurs as an attempt to improve fetal access to amino acids [1]. In addition, part of the amino acids provided by maternal muscle breakdown may be used for the synthesis of milk protein or provide glucose through gluconeogenesis during the first weeks of lactation [52]. However, nutritional strategy that decreases the muscle breakdown in late gestation are recommended to optimize maternal metabolism and to meet fetal requirements. Our results revealed greater urinary level of 3-methyl histidine in CON compared to PRU cows. The 3-methyl histidine is an amino acid derived from the methylation of histidine resulting from the degradation of muscle proteins, and it cannot be reused for protein synthesis, and, consequently, it is excreted in urine [53]. Therefore, the greater urinary 3-methyl histidine in CON cows is an indicative of greater muscle catabolism compared to PRU cows. Therefore, our results suggest that supplementation with PRU reduced muscle catabolism in pregnant cows at late gestation, indicating greater amino

acid uptake PRU cows, thus reducing mobilization of maternal skeletal muscle. These findings can be explained by the increase in the ammonia pool for the synthesis of urea in the liver, which in turn generates greater uptake of recycled N by rumen microorganisms [9,10], contributing to the increase in microbial protein, and consequently reducing the demands for muscle protein mobilization. Although phenotypic differences were not observed between the experimental groups, it may cause a long term-effect in cows during the lactating period, and in their progeny performance, as previously described [54], where in the increase in the levels of abomasum infusion of urea contributed to a linear increase in milk protein and urea content that may be benefit calves' development through a better-quality milk intake. Therefore, supplementation with post-ruminal urea through the diet is an effective way to reduce the amplitude of maternal tissue mobilization during pregnancy and may lead to long-term benefits.

3.6. Conclusion

Shifting urea supply from the rumen to post-ruminal compartments decreases muscle catabolism in cows during late gestation. Our findings indicate that post-ruminal urea supplementation for beef cows at late gestation may improve the energy metabolism to support maternal demands. In addition, the post-ruminal urea release seems to be able to trigger pathways to counterbalance the oxidative stress associated to the increase liver metabolic rate.

Supporting information

S1 Dataset.

(XLSX)

Author Contributions

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Project administration: Marcio S. Duarte.

Resources: Javier Martín-Tereso, Isabela P. Carvalho.

Software: Thaís C. Costa, Tiago A. O. Mendes.

Supervision: Mateus P. Gionbelli, Marcio S. Duarte.

Validation: Tiago A. O. Mendes, Nick V. L. Serão, Mateus P. Gionbelli, Marcio S. Duarte.

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Writing – original draft: Marta M. Santos.

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4. CHAPTER 4

Nutrient supplementation of beef female calves at pre-weaning enhances the commitment of fibro-adipogenic progenitor cells to preadipocytes[3]

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4.1. Abstract

We aimed to evaluate the impact of nutrient supplementation of beef female calves at pre-weaning on adipogenic determination. Thirty-four female calves were assigned to two experimental treatments: Control (CON, n = 17), where animals were supplemented only with mineral mixture; Supplemented (SUP, n = 17), where animals received energy-protein supplement containing minerals (5 g/kg of BW per day) of their body weight. Animals were supplemented from 100 to 250 days of age, and muscle samples were biopsied at the end of the supplementation period. Regarding the performance variables, there were no differences between treatments for initial body weight (P = 0.75). The final body weight (P = 0.07), average daily gain (P = 0.07), rib eye area (P = 0.03), and rib fat thickness (P = 0.08) were greater in SUP female calves compared with CON treatment. The number of fibro-adipogenic progenitor cells (P = 0.69) did not differ between treatments, while a greater number of intramuscular pre-adipocytes were observed in SUP than CON female calves (P = 0.01). The expression of miRNA-4429 (P = 0.20) did not differ between treatments, while the expression of miRNA-129-5p (P = 0.09) and miRNA-129-2-3p (P = 0.05) was greater in CON than SUP female calves. Our results suggest that nutrient supplementation at early postnatal stages of development enhances the commitment of fibro-adipogenic progenitor cells into the adipogenic lineages allowing to an increase in intramuscular fat deposition potential of the animals later in life.

Keywords: Female calves, Marbling, microRNA, Preadipocytes, Transcriptional factor

4.2. Introduction

The understanding of the developmental dynamics of intramuscular adipose tissue is crucial to effectively enhance marbling in beef cattle. The formation of intramuscular adipocytes begins in the fetal period, occurring mainly during the late stage of gestation and extends until approximately 250 days of age in beef cattle, while the ability to generate new adipocytes decreases with animals age (Bonnet, CassarMalek, Chilliard, & Picard, 2010; Du et al., 2013). Moreover, other factors may affect the number of adipocytes, including physiological status, nutrition, and breeding conditions (Buonaiuto et al., 2022). It is reported that there is a time window period in which marbling is enhanced without the increase in other fat depots (Du et al., 2013). The intramuscular adipogenesis refers to the formation of intramuscular adipocytes that starts with the commitment of progenitor cells into preadipocytes

(determination), which proliferate and differentiate into cells capable of accumulating lipids (Tseng, 2010).

The intramuscular adipocytes are derived from a population of progenitor cells with dual differentiation capacity towards both fibrogenic and/or adipogenic lineage, forming the so-called fibro-adipogenic progenitor (FAP) cells. These cells express a membrane protein called plated-derived growth factor receptor alpha (PDGFRα) (Uezumi et al., 2011), which allows the protein PDGFRα to be used as a marker of FAP cells (Huang, Das, Yang, Zhu, & Du, 2012). The adipogenic commitment is characterized by the conversion of FAP cells into preadipocytes due to a transcriptional remodeling which leads to the activation of a variety of genes related to adipose tissue development (Kim, Min, Seo, & Kim, 2015). The delta-like homolog 1 (DLK1) is a transmembrane protein expressed in preadipocytes, extinguished during adipogenic differentiation, and therefore, not expressed in mature adipocytes (Wang & Sul, 2009). In addition to the role of DLK1, the transcriptional factor zinc finger (ZFP) 423 has been reported as a critical factor in adipogenic commitment and differentiation (Gupta et al., 2012). The ZFP423 stimulates the expression of peroxisome proliferator-activated receptor gamma (PPARγ), inducing the conversion of preadipocytes into mature adipocytes (Gupta et al., 2012). In addition, adipogenic determination may be regulated at the post-transcriptional level through microRNAs (Yu et al., 2020). MicroRNAs are small non-coding RNA molecules known to negatively regulate the translation of target genes by binding to mRNAs, leading to translational inhibition or mRNA degradation (Bassett et al., 2014).

Nutritional, environmental, and genetic factors shape progenitor cell differentiation (Du et al., 2013). Therefore, strategies aiming to enhance the number of intramuscular adipogenic cells that can differentiate into mature adipocytes at the finishing phase can be a tool to produce animals with greater marbling. As such, we aimed to evaluate the impact of nutrient supplementation of beef female calves at pre-weaning (100–250 days of age) on intramuscular adipogenic determination.

4.3. Material and methods

All experimental procedures were approved by the Ethical Committee on Animal Use of the Department of Animal Science at Universidade Federal de Viçosa, Minas Gerais, Brazil [protocol # CEUAP15/ 2021].

4.3.1. Animals and experimental diets

Thirty-four F1 Red Angus x Nellore nursing female calves, weighing 154Kg ± 24.4 kg were managed together with their respective dams in a grazing system (Uruchloa decumbens grass) and randomized into 8 paddocks (4 ha each; 4–5 female calves per paddock) with free access to waterers and feeders. Paddock was considered the experimental unit (n = 8). The experimental period started when the female calves reached 100 days of age and lasted until weaning (250 days of age). The experimental treatments were randomly assigned to the paddocks, which consisted of control (CON, n = 4) treatment, where the female calves received a mineral mixture ad libitum, containing dicalcium phosphate (500 g/kg), sodium chloride (472 g/kg), zinc sulfate (15 g/ kg), copper sulfate (7 g/kg), cobalt sulfate (0.5 g/kg), manganese sulfate (5 g/kg), sodium selenite (0.06 g/kg), potassium iodine (0.5 g/kg), and supplemented (SUP, n = 4) treatment where the animals received protein-energy supplementation (5 g/kg of BW per day) through a commercial supplement (Probeef Bambini Creep®,Cargill Nutriçao ˜ Animal, Itapira, SP, Brazil). In all treatments, the cow-calf pair was kept together, and the female calves received the supplement through a creep-feeding system. The nutritional composition of the energy-protein supplement is presented in Table 1. The pre-weaning period was chosen as the supplementation period considering the theoretical window of adipogenic determination proposed by Du et al. (2013). Once a month the female calves were weighed, and the supply of supplements was adjusted accordingly.

Table 1. Nutrient content of the energy-protein supplement used in the experiment.

Item	Levels
Dry matter[1]	826.2
Organic matter[2]	704.7
Crude protein[2]	237.9
NDFap[2,3]	307.0
Ether extract[2]	9.3

Assurance levels of minerals per kilogram of the supplement: 33 g of calcium (max), 18 g of calcium (min), 3.1 mg of cobalt (min), 60 mg of cooper (min), 1 mg of chromium (min), 3 g of sulfur (min), 2000 mg of fluoride (max), 6 g of phosphorus (min), 2.8 mg of iodine (min), 3000 mg of magnesium (min), 112 mg of manganese (min), 0.5 mg of selenium (min), 10 g of sodium (min), 181 mg of zinc (min).

[1]g/kg as fed

[2]g/kg dry matter

[3]Neutral detergent fiber corrected for ash and protein.

4.3.2. Animal performance data and feed samples chemical analysis

Animals were weighed at the beginning of the experiment and every 28 days throughout the experiment to be able to adjust the intake of supplements. The feed intake was monitored and adjusted daily by collecting and weighing the leftovers (if any) before morning feeding. At 250 days of age the female calves were weighed, and carcass ultrasound images were collected by using an Aloka SSD500 with a 3.5 Mhz 18 cm linear probe. The rib eye area and rib fat thickness were measured from the images captured at the Longissimus muscle between the 12th and 13th rib. Images were analyzed by using the Biosoft ToolBox for Bovine (Biotronics, Inc., Ames, USA).

Pastures samples were collected via manual grazing simulation every 28 days, dried in a forced-air circulation oven (Ar SL – 102; SOLAB®, Piracicaba, Sao ˜ Paulo, Brasil) at 55 ◦C to 60 ◦C for 72 h and then grounded with 1- and 2-mm knife mill type Willye (TE-680, SOLAB®, Sao ˜ Paulo, Brasil). Pasture samples were assessed according to AOAC (2005) for dry matter (DM, AOAC; method 934.01), crude protein (CP; AOAC; method 990.13), ether extract (EE; AOAC; method 920.39), neutral detergent fiber corrected for ash and protein (NDFap; using a heat stable α-amylase omitting sodium sulfite; Detmann, Souza, & Valadares Filho, 2012); methods INCT-CA F-002/1; N-004/1 e M-002/ 1). The nutrient content of the pasture is shown in Table 2.

Table 2. Nutrient content of the pasture throughout the experimental period.

Item (g/kg)	Months throughout the experimental period				
	February	March	April	May	Jun
Dry matter	240.1	251.3	321.9	298.3	320.0
Organic Matter	890.6	892.4	892.8	890.8	902.1
Crude Protein	109.6	104.2	63.1	73.4	83.1
NDFap[1]	518.7	549.8	606.9	595.9	579.5
iNDF[2]	184.7	192.4	236.8	317.1	287.1

[1]NDFap: Neutral detergent fiber correct for ashes and protein;
[2]iNDF: indigestible neutral detergent fiber

4.3.3. Skeletal muscle tissue sampling

At 250 days of age skeletal muscle samples were collected through a muscle biopsy. Samples were collected from the longissimus muscle located between the 12th and 13th rib. The area from where the muscle was biopsied was initially cleaned with 70% ethanol, and the

incision was performed 10 min after local anesthesia treatment (Lidocaine 2%). A sample with approximately 3 cm3 and divided into two sub-samples, one half was snap-frozen in liquid nitrogen for further total RNA, miRNA, and protein extraction, while the other half was immediately fixed in 10% (wt/vol) buffered formalin (pH = 7.4).

4.3.4 Immunofluorescence analysis of the skeletal muscle

Fixed skeletal muscle samples were dehydrated using crescent series of ethanol, diaphonized in HistoChoice®, and embedded in liquid Paraplast®. Transverse sections of 5 μm were obtained using a rotary microtome (RM 2265, Leica Biosystems, Nussloch, Germany) and placed in positively charged slides (Immuno slide, Easypath, Sao ˜ Paulo, Brazil). The antigen retrieval was performed in citrate buffer (pH 6.0) at 95 ◦C for 3 min. Sections were blocked with 2% bovine serum albumin (BSA, Sigma Aldrich) for 20 min and then incubated for 12 h at 4 ◦C in a moist chamber with the following antibodies: DLK1, a marker of preadipocytes, and PDGFRα, a marker of progenitor fibro-adipogenic cells diluted 1:400 in 2% BSA (Cell Signaling Technology) (Table 3). After the incubation with primary antibodies, the sections were then the sections were washed three times (5 min per washing) in TBS1x (Tris-Buffered Saline), and then incubated with the secondary fluorescent antibody (Alexa fluor™ 488 goat anti-rabbit IgG, Invitrogen, ThermoFisher) for 1 h at room temperature in a dark and moist chamber, following the incubation with the secondary antibody, the sections were washed three times (5 min per washing) with TBS1x. Slides were then mounted with ProLong® gold antifade reagent with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) (Molecular Probes, Life Technologies). Photomicrographs of all slides were obtained by using the EVOS M5000 Imaging System (Invitrogen, Thermo Fisher Scientific) and analyzed by using the ImageJ software (National Institutes of Health), to count the number of DLK1 and PDGFRα positive cells.

Table 3. List of antibodies used in immunofluorescence and western blot analyses.

Antibody	Content	Dilution	Manufacturer	Catalog number
DLK1	Rabbit monoclonal IgG	1:400	Cell signaling Technology®	2069
PDGFRα	Rabbit monoclonal IgG	1:400	Cell signaling Technology®	3174

PPARγ	Rabbit monoclonal IgG	1:500	Cell signaling Technology®	2443
ZFP423	Mouse monoclonal IgG	1:500	Cell signaling Technology®	Sc-514748

4.3.5. In silico experiment for functional analysis of DLK1 gene

To search for predicted protein-protein interaction of DLK1, the plugin GeneMANIA of Cytoscape was used (Montojo et al., 2010). The default networks using the WikiPathways attribute with auto-weighting were used. The transcriptional regulation of the genes involved in adipogenesis was explored, and the transcription factors (TF) identified through the TFM-Explorer software (http://bioinfo.lifl.fr/tfm-explorer /form.php), using the database from JAPAR weight matrices (Sandelin, Alkema, Engstrom, ¨ Wasserman, & Lenhard, 2004). To detect the potential interactions and extract the significant clusters, a score function was calculated as described by (Touzet & Varré, 2007). The list of the enriched TF was generated by Cytoscape (Shannon et al., 2003) using the Networks Gene Ontology (BiNGO) tool (Maere, Heymans, & Kuiper, 2005). To determine the significantly enriched GO terms, we assumed the standard statistical test and multiple test correction with a significance level of 0.05%. Based on the biological processes and a literature review, the main TF related to adipogenesis were selected and a gene-TF network was built using the NetworkAnalyzer tool in Cytoscape®. After the identification of the enriched genes of the gene-TF network, we used the online miRWalk® software (http://mirwalk. umm.uni-heidelberg.de/) to identify the possible miRNAs associated. Only the miRNAs with a minimum of 0.95 binding probability score and biding position (3UTR, CDS, 5UTR), were used as input. To identify the enriched miRNAs the NetworkAnalyzer tool from Cytoscape® was also used in this step. Thus, according to the number of binding sites between genes and miRNAs, it was possible to highlight the miRNAs through the gene-miRNAs network. Moreover, we were able to identify the main genes and miRNAs related to intramuscular adipose tissue development. According to these results, we performed the abundance analyses of mRNAs and miRNAs.

4.3.6. Total RNA extraction enriched with miRNA and cDNA synthesis

A total of 30 mg muscle tissue previously powdered in liquid nitrogen was homogenized using turrax (IKA ULTRA-TURRAX T18 digital, IKA, Staufen, Germany) for 10 s in lysis buffer of miReasy isolation kit (Qiagen, Hilden, Germany). Total RNA enriched with miRNA

was extracted using miReasy isolation kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. The concentration of miRNAenriched samples was estimated by NanoDrop LITE (Thermo Fisher Scientific, MA, USA), and their integrity was analyzed through 1% agarose gel electrophoresis. The reverse transcription of total RNAmiRNA enriched samples was performed using the miScript II RT kit (Qiagen, Hiden, Germany) following the manufacturer's instructions. The expression of both mRNA and miRNA was performed from the same cDNA sample to avoid possible bias due to differences in the extraction method.

4.3.7. Quantitative real-time PCR analysis

Quantitative RT-PCR (qPCR) was performed in a thermal cycler CFX96 Real-Time Detection Systems (Biorad, USA) using miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany). Oligonucleotides were designed based on the mature sequence of the identified miRNAs in the present study (Table 4). Mature sequences were obtained in the miRBase database (Kozomara & Griffiths-Jones, 2014) according to each miRNA name. Oligonucleotides used for the mRNA (Table 4) were designed using OligodT PrimerQuest software (IDT technologies, CA, USA) using sequences available on NCBI. The amplification efficiency ranged from 0.90 to 0.99. After amplification, a melting curve (0.01◦C/s) was used to confirm product purity. The qPCR reaction was performed as follows 1 cycle at 95◦C for 15min, 40cycles at 94◦C for 15s, 55◦C for 30s, 70◦C for 30s, and Melting Curve. The reference gene used for the mRNA expression was the 18S, while the ncRNA-U6 was used for miRNA expression. No differences between treatments were observed ($P > 0.05$) for the expression of 18S and ncRNA-U6. The expression of both mRNA and miRNA was calculated using the $2^{-\Delta Ct}$ method (Schmittgen & Livak, 2008).

Table 4. List of primers for the de mRNA e microRNA abundance analyses by RT-qPCR.

Item	Gene abbreviation	NCBI access code	Primer
Hepatocyte Nuclear Factor 4 Alpha	HNF4α	NM_001015557.1	F: TTCTGCCCGGAGCTATT R: GACTTCCTGCTTGGTGATG
Factor nuclear kappa β	NF-kappaβ	NM_001076409.1	F: GGATCACTCTGGGTGTAGTT R: CGTGACTGTGGTTACTCTCT
Peroxisome proliferator- activated receptor gamma	PPARγ	NM_181024.2	F: CTCCGTGGACCTTTCTATGA R: CATAGTGCGGAGTGGAAATG
Delta-like homolog1	DLK1	NM_174037.2	F: CTGTGCGGTCTGTTCTTATC R: TACGTTGGTTGCACACAC
18S ribosomal	18S	NR_036642.1	F: CCTGCGGCTTAATTTGACTC R: AACTAAGAACGGCCATGCAC
MicroRNA 4429	miR-4429	-	AAAAGCTGGGCTGAGAGGCG
MicroRNA 129-5p	miR-129-5p	-	CTTTTTGCGGTCTGGGCTTGCT
MicroRNA 129-2-3p	miR-129-2-3p	-	AAGCCCTTACCCCAAAAAGCAT
U6 snRNP small nuclear ribonucleoprotein	U6	-	GTGCTCGCTTCGGCAGCAC

4.3.8. Protein abundance quantification using western-blot analysis

Total protein from Longissimus thoracis muscle was extracted from 0.1 g of powdered tissue in 1 mL lysis buffer [10 mM Tris HCl, 100 mM NaCl, 0,5 mM DDT (dithiothreitol), 2.5 mM MgCl2, 0,5% triton X-100 and 1% of protease inhibitor cocktail (Sigma-Aldrich®)], the total protein content was estimated by Bradford protein assay (Bio-Rad, Hercules, CA, EUA) and stored at − 80 ◦C. The proteins were separated in SDSPAGE 10% gels loaded with 80 μg of protein per sample, transferred to PVDF (Polyvinylidene Difluoride) membranes, and blocked for 1 h at room temperature with 3% nonfat dried milk in TBS1x. The membranes were incubated for 12 h at 4 ◦C with the following primary antibodies: ZFP423 and, PPARγ (Table 3) diluted in TBS1x. After 12 h, the membranes were washed with Tris-Buffered Saline and 0,1% Tween® (TBSt) and incubated with secondary antibody (anti-rabbit IgG- Cell signaling® e anti- mouse IgG Sigma-Aldrich®) diluted 1:5000 in TBS1x for 1 h at room temperature. The membranes were rewashed with TBSt, revealed by ECL ClarityTM substrate (Bio-Rad, Hercules, CA), and the images were generated and quantified by densitometry using iBright FL1000 (Invitrogen, Thermo Fisher Scientific, MA, USA). Each SDS-PAGE gel was loaded with proteins extracted from all treatments. The internal control consisted of a pool of all samples.

4.3.9.Statistical analysis

Data were analyzed considering the completely randomized design of the experiment. The female calves were randomized across paddocks, and paddocks were randomized across treatments. Single measurements (including all analyses) were taken for each heifer. The statistical analyses were performed following the statistical model below:

$$yijk = \mu + \alpha i + pj{:}i + \varepsilon ijk$$

where yijk are the observed values, μ is the overall population mean, αi is the effect of the i th dietary treatment, pj:i is the random effect of the j th paddock within the ith treatment, and εijk is the random error, assumed iid N(0, σ2 ε). In this model, the εijk represented the effect of heifer nested within treatment × paddock. The approach for the statistical model used was based on St-Pierre (2007). The PROC MIXED of SAS® 9.2 (Statistical Analysis System Institute, Inc., Cary, NC, EUA) was used for all analyses. Significances were declared at $P < 0.05$ and trends were discussed when $0.10 < P \leq 0.05$. Before the final analyses, studentized residuals were removed

when not within ±3 standard deviations and normality (P-value >0.05) was assessed using Shapiro-Wilk's test (UNIVARIATE procedure of SAS).

4.4. Results

4.1.1. Animal performance

Regarding the performance variables (Table 5), there were no differences between treatments for initial body weight (P = 0.75). The final body weight (P = 0.07), average daily gain (P = 0.07), rib eye area (P = 0.03), and rib fat thickness (P = 0.08) were greater in SUP female calves compared with CON treatment.

Table 5. Performance of female calves from 100 to 250 days of age according to the experimental treatments.

Item	Control	Supplemented	*P*-value
Initial body weight, kg	157.6 ± 5.9	150.3 ± 5.9	0.75
Final body weight, kg	232.3 ± 3.6	243.4 ± 3.6	0.07
Average daily gain, kg/d	0.63 ± 0.02	0.72 ± 0.02	0.07
Rib eye area, cm^2	37.6 ± 1.56	43.7 ± 1.56	0.03
Rib fat thickness, mm	2.58 ± 0.2	3.2 ± 0.2	0.08

4.1.2. Presence of fibro-adipogenic cells and preadipocytes in skeletal muscle of female calves

The number of PDGFRα+ was not affected by nutrient supplementation at early postnatal stages (P = 0.69; Fig. 1). As shown in Fig. 2 number of DLK1+ cells were greater in the skeletal muscle of SUP compared with CON treatment (P = 0.01).

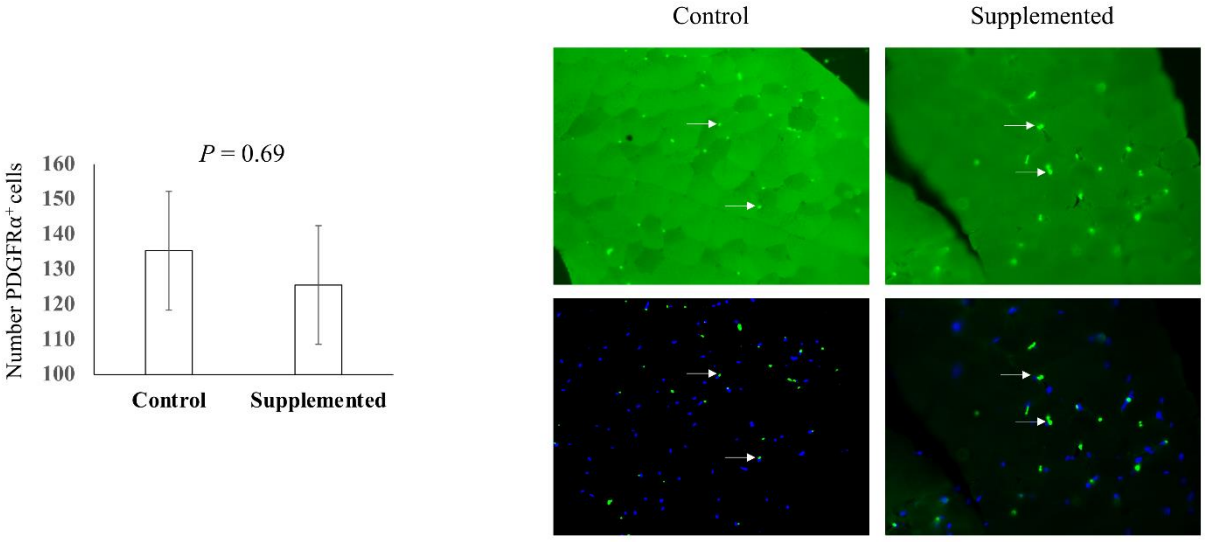

Figure 1 - Number of fibro-adipogenic (PDGFRα$^+$) cells in *Longissimus thoracis* from control (n = 17) and supplemented (n = 17) beef female calves supplemented from 100 to 250 days of age. Bars represent means ± SEM. In the representative images, green spots indicate PDGFRα$^+$ cells, while blue spots indicate cell nuclei (stained with DAPI). Photomicrographs were taken at 40x magnification.

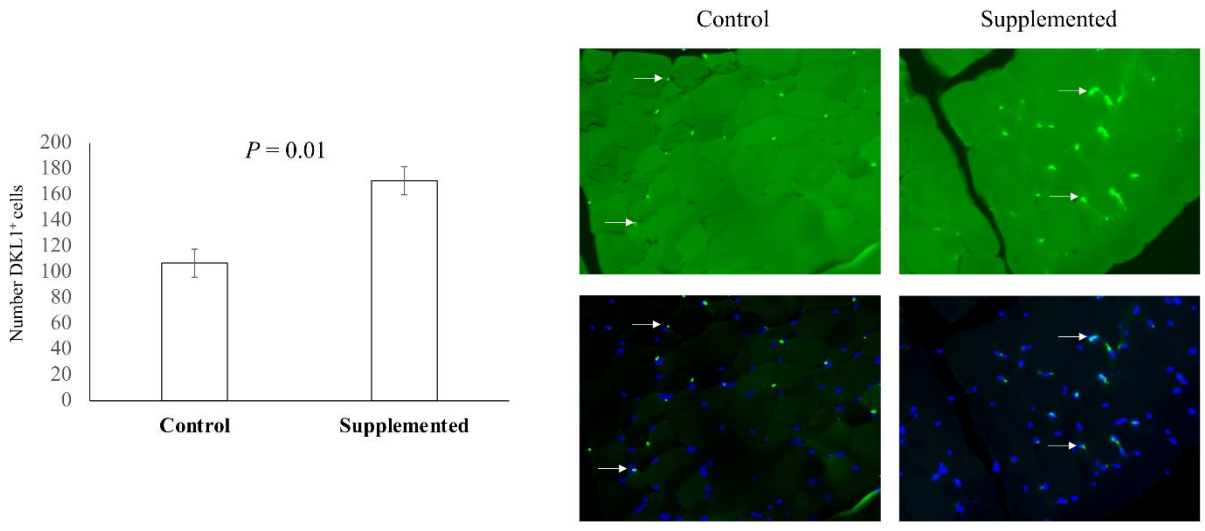

Figure 2 - Number of fibro-adipogenic (DLK1$^+$) cells in *Longissimus thoracis* from control (n = 17) and supplemented (n = 17) beef female calves supplemented from 100 to 250 days of age. Bars

represent means ± SEM. In the representative images, green spots indicate $DLK1^+$ cells, while blue spots indicate cell nuclei (stained with DAPI). Photomicrographs were taken at 40x magnification.

4.1.3. In silico functional analysis of DLK1 gene

In Table 6 are reported main regulatory elements of genes from adipogenesis pathway to according to the results in silico experiment. From DLK1 gene, we first searched for co-expressed genes (genes interaction network) followed by a regulatory elements (RE) search, which were used to build gene-transcription factors and gene-miRNA networks (Fig. 3).

Table 6. Main regulatory elements of genes from adipogenesis pathway, according to the network analyses with their biological processes (for transcription factors) and literature evidence.

Regulatory elements	Class*	Target	Biological Process	Literature
HNF4α	TF	WNT5B WNT10B WNT1 TWIST1 TGFB1 SMAD3 DLK1	Regulation of lipid metabolic process	Lipid homeostase (Hayhurst, Lee, Lambert, Ward, & Gonzalez, 2001)
NF-kappaβ	TF	WNT5B WNT10B SMAD3 MEF2D FZD1 FOXC2 DLK1	Regulation of lipid metabolic process, regulation of lipid storage	Related with lipid accumulation in dairy cow hepatocytes (Li et al., 2015)
PPARγ	TF	WNT5B WNT10B WNT1 TWIST1 TGFB1 SMAD3 MEF2D FZD1 DLK1	Regulation of lipid metabolic process, regulation of lipid storage	Regulator of adipogenesis and lipogenesis in adipocytes (Tontonoz, Hu, & Spiegelman, 1994)
miR-4429	miRNA	DLK1	-	Up-regulated in obese kids (Ferrante et al., 2015)
miR-129-5p	miRNA	DLK1	-	Potential Biomarker for Obesity (Fu et al., 2019)
miR-129-2-3p	miRNA	DLK1, SMAD3	-	Downregulated in adipose tissue under a obesogenic diet (Gracia et al., 2016)

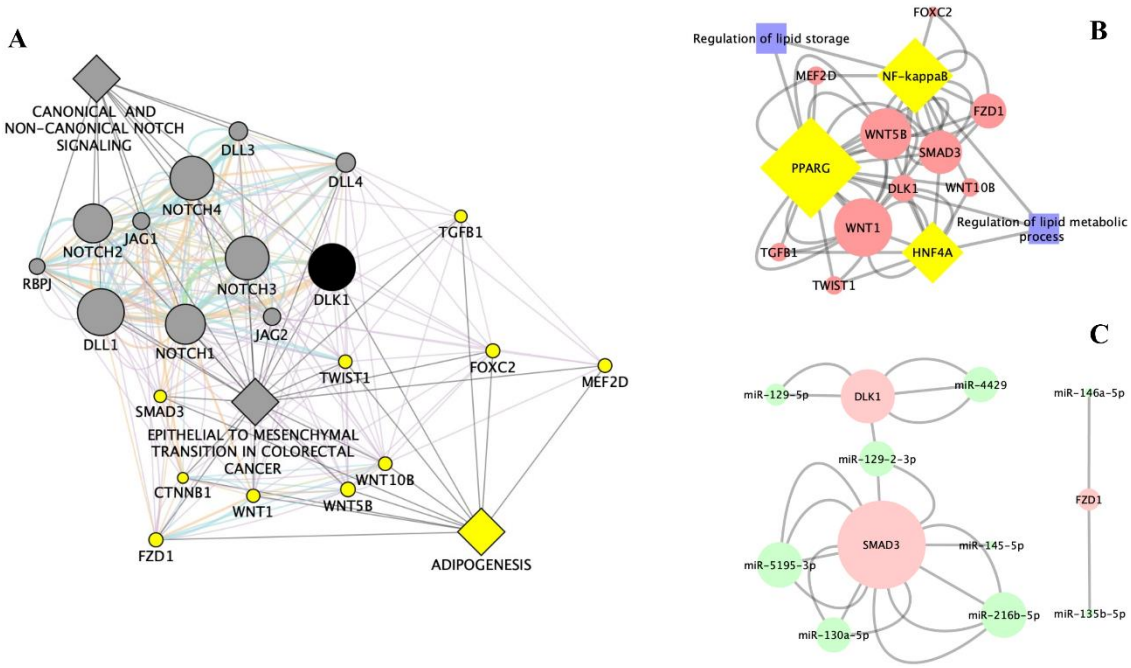

Figure 3 - Gene's interaction network. (A)Network of interactions for *DLK1* gene (black node) based on combined networks of WikiPathways (gray edges), co-expression (purple edges), predicted functional relationships between proteins (orange edges), co-localization (dark blue edges), genetic interactions (green edges), Pathways (light blue edges) and shared proteins domains (dark green edges). Yellow nodes are genes sharing the Adipogenesis pathway with *DLK1*. (B); Gene-transcription factor network. Adipogenesis pathway genes (pink nodes) and the main transcription factors related to lipids (Yellow nodes). Blue nodes are the related biological processes obtained via BinGO associated with lipids. (C); Gene-miRNA network. Adipogenesis pathway genes (pink nodes) and the main related miRNA (Green nodes).

4.1.4. Protein abundance and expression of miRNAs and target mRNAs controlling the intramuscular adipogenesis

As demonstrated in Fig. 4A, there were no differences between treatments for the expression of miR-4429 (P = 0.27). In addition, the mRNA abundance of DLK1 (P = 0.18), HNF4α (P = 0.18), and NFKβ (P = 0.15) (Fig. 4B) did not differ between treatments. The abundance of miR129–5p was greater in the skeletal muscle of CON than SUP female calves (P = 0.09; Fig. 4A). Similarly, the abundance of miR129–2-3-p was greater in the skeletal muscle of CON than SUP female calves (P = 0.05; Fig. 4A). In addition, we observed an increase in mRNA abundance of PPARγ (P = 0.04) in the skeletal muscle of CON compared to SUP female calves, while no

differences between treatments were found for the protein abundance of PPARγ (P = 0.31; Fig. 5A) and ZFP 423 (P = 0.42; Fig. 5B).

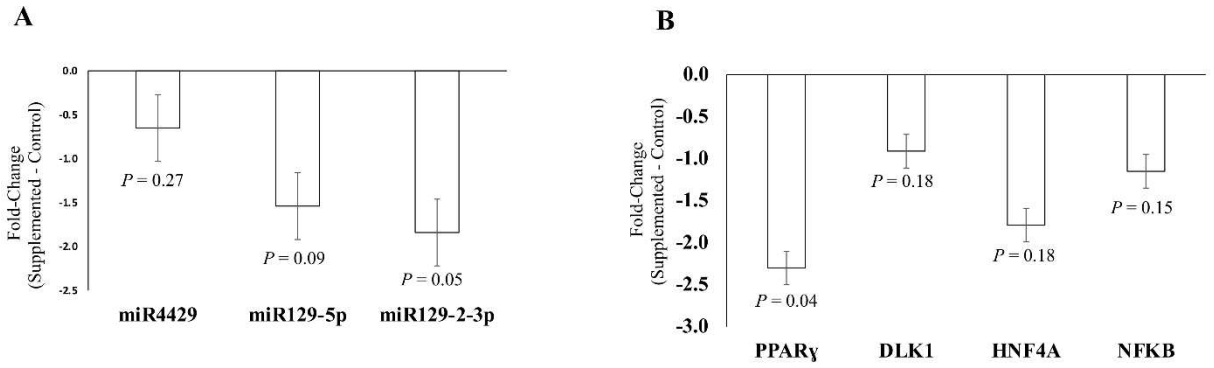

Figure 4 - Relative abundance of miRNAs targeting *DLK1* (A) and the mRNA abundance of the transcriptional factors involved in the intramuscular adipose tissue development (B) of female calves at 250 days old in treatment supplemented compared to control. Bars represent means ± SEM.

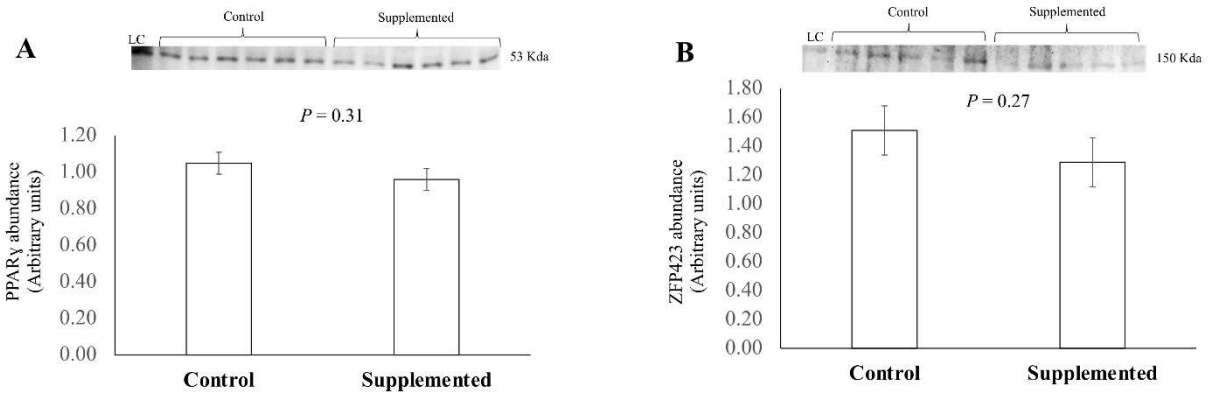

Figure 5 - Representative images of western-blot analysis and protein abundance for PPARγ (*P* = 0.31; A), and ZFP423 (*P* = 0.42; B). Protein abundance was measured in 250 days old female calves skeletal muscle from treatment control and supplemented. LC = Loading Control. Bars represent means ± SEM.

4.5. Discussion

The supplementation during the cow-calf phase is a common nutritional management practice used to meet the pre-weaned calves' requirements, allowing them to express their growth potential (Carvalho et al., 2019). Moreover, it has also been demonstrated that this practice may result in a greater marbling score at slaughter (Myers, Faulkner, Ireland, Berger, & Parrett, 1999; Shike, Faulkner, Cecava, Parrett, & Ireland, 2007). However, despite this increase in marbling scores in carcasses of animals that received a greater nutrient support at early stages of development, the mechanisms underlying the early intramuscular adipogenesis is not fully understood. In the present study we evaluated how intramuscular adipogenesis is affected by nutrient supplementation at early postnatal stages, using a typical timeframe that cow-calf operations start to supplement the female calves. Although in beef cattle, most of the efforts to enhance marbling deposition are focusing on the fattening phase (Campos et al., 2020; Carvalho et al., 2014; Fontes et al., 2021), it has been suggested that early stages of development would be an effective time-window to enhance intramuscular fat deposition (Du et al., 2013). To achieve this, supplementation of nutrients or other bioactive compounds may be applied to trigger the commitment of skeletal muscle mesenchymal progenitor cells to the fibro/adipogenic lineage (Du et al., 2013). Previous studies showed that feeding early-weaned calves a grain-based diet increases the hyperplasia of adipose tissue (Wertz et al., 2001; Wertz et al., 2002) and thus, increase the number of adipocytes which can be filled with lipids when animals undergo to finishing diets. In the present study, we first hypothesized that nutrient supplementation of beef female calves at preweaning (100 to 250 days of age) would enhance the number of fibro/ adipogenic (FAP) cells. Thus, we assessed the number of PDGFRα+ cells in the skeletal muscle of the female calves, which is known as a mesenchymal progenitor cell marker contributing to both adipogenesis and fibrogenesis. We found no differences between treatments for the number of PDGFRα+ cells between treatments. This result suggests that nutrient supplementation does not affect the abundance of FAPs in female calves' muscle.

The recruitment of FAPs cells to the adipogenic lineages is the initial step for intramuscular tissue development (F`eve, 2005). The protein DLK1 takes an important role in the initial process of adipogenic commitment by acting to maintain the pre-adipocitary state, preventing adipocyte differentiation (Hudak & Sul, 2013). As such, we speculated that nutrient supplementation would enhance intramuscular fat adipogenesis by increasing FAP differentiation to pre-adipocytes. Our

results demonstrated an increased number of DLK1+ cells in the skeletal muscle of female calves that received nutrient supplementation. Altogether, the lack of differences in the number of FAP cells and the increase in the number of pre-adipocytes observed in the skeletal muscle of supplemented female calves suggests that FAP cells are more responsive to nutritional interventions that cause an increase in their adipogenic commitment in beef female calves.

To further understand the role of DLK1 in intramuscular adipogenesis at the early development of beef female calves, we conducted an in-silico experiment to access putative transcription factors controlling the DLK1 expression. Based on our findings, we evaluated the mRNA expression of HNF4α, NFKβ, and PPARy on the skeletal muscle tissue from female calves of both treatments. Previous studies established that HNF4α and NFKβ positively regulate the Wnt signaling pathway in a synergical way (Chang et al., 2016; Herschlag & Johnson, 1993; Zhang, Wang, Shen, & Zhao, 2020). However, Wnt signaling pathway might also be regulated by different factors, such as several transcription factors, proteins, and miRNA (Moon, Kohn, De Ferrari, & Kaykas, 2004). Wnt signaling maintains preadipocytes in an undifferentiated state through inhibition of the adipogenic transcription factors CCAAT/ enhancer binding protein a (C/EBPα) and PPARy (Ross et al., 2000). Thus, DLK1 and Wnt signaling pathways seem to have the same effect on pre-adipocytes, it may establish connections between those signaling pathways in the skeletal muscle. However, the mRNA expression of HNF4α and NFKβ did not differ between treatments, suggesting that the transcription factors HNF4α and NFKβ were not individually related to the increased number of DLK1+ cells in the skeletal muscle of female calves that received a great amount of nutrients.

It has been shown that the mRNA expression and the protein abundance of DLK1works as waves, in which is greater in preadipocytes, decreases during the adipocyte's differentiation, and it is absent in mature adipocytes (Wang & Sul, 2009), indicating that downregulation of DLK1 is necessary for differentiation to occur (Smas & Sul, 1993). These findings are in accordance with our results once the low expression of the mRNA PPARγ was observed in SUP treatment. The differentiation of committed preadipocytes to adipocytes is controlled by PPARγ and several other transcription factors (Farmer, 2006). The zincfinger protein (ZFP423) was identified as a transcriptional factor inducing PPARγ mRNA expression (Gupta et al., 2010). It has been demonstrated that mRNA expression of DLK1 is reduced in the presence of ZFP423 transcription factor (Gupta et al., 2012). Thus, we further evaluated if there were differences in the abundance

of ZFP423 between treatments that would be associated with the lower mRNA expression of PPARγ. However, despite the greater number of DLK1+ cells and the lower mRNA expression of PPARγ observed in the skeletal muscle of SUP female calves, no differences were observed in the abundance of ZFP423 between treatments. We have also assessed the protein abundance of PPARγ and did not observe differences between treatments. Collectively, these results suggest that differences observed in the number of DLK1+ positive cells and mRNA lower expression PPARγ in SUP group were not associated with changes in ZFP423 levels.

Despite the greater number of pre-adipocytes (DLK1+ cells) in SUP treatment, the mRNA expression of DLK1 did not differ between treatments. Based on these results, we speculated that lower nutrient supply in the CON group may trigger post-transcriptional regulation that resulted in lower abundance of the protein DLK1, decreasing the adipogenic commitment. It has been suggested that miRNA expression is influenced by nutrition (Ross & Davis, 2014), and it is involved in the regulation of adipogenic determination (Yu et al., 2020). miRNAs are small non-coding RNA molecules known to negatively regulate the translation of target genes by binding to mRNAs, leading to translational inhibition or mRNA degradation (Bassett et al., 2014). Thus, we first performed an in-silico study to identify miRNAs targeting DLK1. Based on the results, we selected the three highest-scored miRNA candidates that target DLK1 and assessed their abundance in the skeletal muscle tissue from female calves of both treatments. We observed a greater abundance of miR129–5-p and miR129–2-3-p in the skeletal muscle of the CON group. It has been reported that the expression of the miR-129 family is associated with the downregulation of cell proliferation (Deng, Tang, & Wang, 2021), while Yu et al. (2020) reported the expression of miR-129-5p in bovine preadipocytes targeting the DLK1 gene. Moreover, Li et al. (2019) reported that greater expression of miR-129 family represses the Wnt signaling pathway. As discussed previously, DLK1 and Wnt signaling pathways inhibit preadipocyte to adipocyte transition (Tseng et al., 2005) and increase preadipocyte proliferation (Hudak & Sul, 2013). Interestingly, our data suggest that a greater miR-129-5p and miR129–2-3-p expression in CON group negatively regulated the number of DLK1+ positive cells by inhibiting the translation of DLK1 protein and possibly inhibiting the Wnt signaling pathway, which may have lowered the recruitment of FAPs cells to the adipogenic lineage. However, we have not investigated the relationship between DLK1 and Wnt signaling pathway in the adipogenic commitment in our study, but our findings may warrant further investigation.

4.6. Conclusion

Nutrient supplementation of beef female calves at pre-weaning (100 to 250 days of age) enhances the number of pre-adipocytes in the skeletal muscle preparing intramuscular adipose tissue for fat deposition during the postweaning period. Our results suggest that this increase in the number of pre-adipocytes is caused not due to an increase in mesenchymal stem cells but due to an increase in the adipogenic commitment of fibro-adipogenic progenitor cells, which is associated with post-transcriptional regulation through miR-129 family.

CRediT authorship contribution statement

M.M. Santos: Conceptualization, Methodology, Software, Validation, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **T.C. Costa:** Conceptualization, Methodology, Software, Validation, Investigation, Writing – review & editing. **W. Silva:** Software, Validation, Formal analysis. **L.Z. Pistillo:** Software, Validation, Formal analysis. **D.V. Teixeira Junior:** Software, Validation, Formal analysis. **L.L. Verardo:** Software, Validation, Formal analysis, Data curation. **P.V.R. Paulino:** Conceptualization, Methodology, Investigation, Resources, Data curation. **C.B. Sampaio:** Conceptualization, Methodology, Investigation, Resources, Data curation. **M.P. Gionbelli:** Validation, Formal analysis, Investigation, Data curation, Writing – review & editing. **M. Du:** Writing – review & editing. **M.S. Duarte:** Conceptualization, Methodology, Investigation, Resources, Data curation, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

Data availability

Data will be made available on request.

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5. CHAPTER 5

5.1. General conclusions

In summary, the current study showed evidence that nutritional strategies during the fetal and pre-weaning stages improve maternal metabolism and enhance the number of pre-adipocytes in the skeletal muscle preparing intramuscular adipose tissue for fat deposition during the postweaning period. Our findings indicate that post-ruminal urea supplementation for beef cows at late gestation may improve the energy metabolism to support maternal. Shifting urea supply from the rumen to post-ruminal compartments decreases muscle catabolism in cows during late gestation. In addition, the post-ruminal urea release seems to be able to trigger pathways to counterbalance the oxidative stress associated to the increase liver metabolic rate. Moreover, nutrient supplementation of beef female calves at pre-weaning (100–250 days of age) increase in the adipogenic commitment of fibro-adipogenic progenitor cells, which is associated with post-transcriptional regulation through miR-129 family.