THAÍS CORREIA COSTA

FETAL PROGRAMMING AND MOLECULAR CHARACTERIZATION OF THE SKELETAL MUSCLE DEVELOPMENT IN RUMINANTS

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

Co-adviser: Tiago Antônio de Oliveira Mendes

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

(Marie Curie)

BIOGRAPHY

THAÍS CORREIA COSTA, filha de Edilaine Correia Costa e Silvio Ricardo Acevile Costa, nasceu em Santo André – SP, em 5 de Outubro de 1993.

Em Março de 2011, ingressou no curso de Zootecnia na Universidade Estadual Paulista "Júlio de Mesquita Filho", campus Botucatu-SP, onde graduou-se em 4 de Dezembro de 2015.

Em Agosto de 2016, 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 Julho de 2018.

Em Agosto de 2018 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. Durante o doutorado sanduíche (2020-2021), realizou parte de sua pesquisa no *Nutrigenomics and Growth Biology Laboratory*, na *Washington State University*, sob orientação do Prof. Dr. Min Du.

ABSTRACT

COSTA, Thaís Correia, D.Sc., Universidade Federal de Viçosa, July, 2022. **Fetal programming and molecular characterization of the skeletal muscle development in ruminants.** Adviser: Marcio de Souza Duarte. Co-adviser: Tiago Antônio de Oliveira Mendes.

The intrauterine conditions modulate the skeletal muscle development and establish, at least partially, the postnatal body composition, influencing the final meat quality. Therefore, it is important that the maternal plane of nutrition meets the requirements or maternal body reserves may be mobilized in order to allow an adequate fetal growth. Although, many studies have reported the effects of maternal nutrition on fetal skeletal muscle development, the insight into the molecular pathway's regulation needs further investigation. Thus, the current study was developed based on three experiments. The aim of the first study was to investigate the impact of maternal protein restriction during mid-gestation on the skeletal muscle composition of the offspring. From day 100 to 200 of gestation, the cows were exposed to a diet containing a protein supplement (CON, n = 9) or not (RES, n = 9). Samples from calves' *Longissimus thoracis* were taken at two time point postnatally, at 30d and 450d of age. RES diet triggered a decrease ($P < 0.01$) in muscle fiber number in both stages of evaluation, while collagen content was higher at 30d of age ($P < 0.05$). Although, no difference ($P > 0.05$) was observed in the expression of *MHC2X* at 450d, calves at 30d of age had greater expression of this gene. These results clearly shows that maternal protein restriction during a critical period of gestation permanently impact muscle fiber formation, alter the initial fiber metabolism and collagen accumulation. However, an adequate postnatal environment may contribute to muscle fiber type switching. The second and third studies were developed considering the same treatments and experimental units aiming to elucidate the effects of maternal feed restriction during different stages (first or last half) of gestation on the transcriptome and proteome profile in the skeletal muscle of the newborn goat. Fourteen pregnant goats, gestating males were assigned into one of the experimental treatments: animals fed at 50% maintenance requirements from 8-84d of gestation and then fed at 100% of maintenance requirement from day 85 of gestation to parturition (RM, n = 6), or fed at 100% of maintenance requirement from 8–84 d of gestation and then fed at 50% of maintenance requirement from day 85 of gestation to parturition (MR, n = 8). The offspring (n = 14) were slaughter at birth, the *Longissimus thoracis* muscle was sampled and the transcriptome and proteome profiles were evaluated. Transcriptome analysis

showed a total of 20,359 transcripts, where 766 and 1146 were found as exclusively expressed in treatments RM and MR, respectively. From the 66 differentially expressed (DE) genes (FDR < 0.05), 6 and 60 genes were up and downregulated in treatment RM compared to MR, respectively. The upregulation of genes in treatment RM are involved with the maintenance of satellite cells, possibly due to the impairment in myoblast differentiation during the prenatal period. Moreover, the upregulation of genes related to low marbled carcasses, may conduct the reduction in the deposition of intramuscular fat in the offspring. Maternal feeding restriction during the last half of gestation (MR) provided an upregulation of genes related with the balance between glucose anabolism and catabolism, in addition to an activation of protective mechanisms against the excessive cell oxidative stress. Proteomic analysis identified a total of 415 sarcoplasmic proteins, and after filtering, 181 and 46 proteins were found exclusively in treatments RM and MR, respectively. From the 159 proteins present in both treatments, only 13 were deemed differentially abundant proteins (DAPs) considering FDR < 0.05. Through the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses, it was identified enriched biological processes (BP) and signaling pathways (SP) related with the energy-investment phase of glycolysis, in addition to the increase in the usage of glycogen and fatty acids storages in RM compared to MR. While the proteins found in MR treatment showed enriched BP and SP associated with the energy-generation phase of glycolysis, and an enhancement in the biosynthesis of glutamine. Altogether the results generated by transcriptome and proteome analyses suggest that maternal feed restriction alter the abundance of this molecules, mainly altering the skeletal muscle composition and the overall energy metabolism of the newborns' skeletal muscle.

Keywords: Beef cattle. Caprine. Developmental programming. Proteome. Skeletal Muscle. Transcriptome.

RESUMO

COSTA, Thaís Correia, D.Sc., Universidade Federal de Viçosa, julho de 2022. **Programação fetal e caracterização molecular do desenvolvimento muscular esquelético em ruminantes.** Orientador: Marcio de Souza Duarte. Coorientador: Tiago Antônio de Oliveira Mendes.

As condições intrauterinas modulam o desenvolvimento muscular esquelético e definem, pelo menos parcialmente, a composição corporal pós-natal, influenciando a qualidade final da carne. Desta forma, é importante que o plano nutricional materno atenda as exigências, ou as reservas corporais maternas possam ser mobilizadas para permitir um crescimento fetal adequado. Embora muitos estudos tenham reportado os efeitos da nutrição materna sobre o desenvolvimento muscular esquelético fetal, a compreensão sobre a regulação de vias moleculares precisa de mais investigações. Assim, o presente estudo foi desenvolvido com base em três experimentos. O objetivo do primeiro estudo foi investigar o impacto da restrição de proteína durante o terço médio da gestação sobre a composição do musculo esquelético da progênie. Do dia 100 ao 200 de gestação, as vacas foram expostas a uma dieta com (CON, n = 9) ou sem (RES, N = 9) suplementação de proteína. Amostras do musculo *Longissimus thoracis* dos bezerros foram coletadas em dois momentos no pós-natal, aos 30 e 450 dias de idade. A dieta RES desencadeou uma diminuição ($P < 0.01$) no número de fibras musculares em ambos os estágios de avaliação, enquanto o conteúdo de colágeno foi maior aos 30 dias de idade ($P < 0.05$). Embora não tenha sido observada diferença ($P > 0.05$) na expressão de *MHC2X* aos 45 dias, os bezerros de 30 dias de idade apresentaram maior expressão desse gene. Estes resultados demonstram que a restrição de proteína durante um período crítico da gestação impacta permanentemente na formação das fibras musculares, altera o metabolismo das fibras e o acúmulo de colágeno no primeiro mês de vida dos bezerros. No entanto, o ambiente pós-natal favorável pode contribuir para a mudança do tipo de fibra muscular. O segundo e o terceiro estudo foram desenvolvidos considerando os mesmos tratamentos e unidades experimentais, com o objetivo de elucidar os efeitos da restrição alimentar materna durante diferentes estágios (primeira ou última metade) de gestação sobre o perfil do transcriptoma e do proteoma do musculo esquelético de cabritos recém-nascidos. Quatorze cabras prenhes, gestando machos, foram designadas a um dos tratamentos experimentais: cabras alimentadas com 50% dos requerimentos para mantença do dia 8 ao 84 da gestação e depois alimentadas com 100% dos requerimentos de mantença do dia 85 de gestação até o parto (RM, n = 6), ou cabras alimentadas

de forma a atender 100% dos requerimentos de mantença do dia 8 ao 84 de gestação e depois restritas de modo atender 50% dos requerimentos nutricionais do dia 85 de gestação ao parto (MR, n = 8). Os cabritos (n = 14), foram abatidos ao nascer, o musculo *Longissimus thoracis* foi amostrado e os perfis do transcriptoma e do proteoma foram avaliados. A análise do transcriptoma identificou um total de 20.359 transcritos, onde 766 e 1146 foram encontrados expressos exclusivamente nos tratamentos RM e MR, respectivamente. Dos 66 genes diferencialmente expressos (DE, FDR < 0.05), 6 e 60 foram up e downregulados no tratamento RM comparado ao MR, respectivamente. Os genes upregulados no tratamento MR estão envolvidos com a manutenção de células satélites, possivelmente devido à falha na diferenciação dos mioblastos durante o período pré-natal. Além disso, a upregulação dos genes relacionados com carcaças com pouco marmoreio, podem conduzir a redução da deposição de gordura intramuscular na progênie. A restrição alimentar materna durante última metade da gestação (MR) proporcionou a upregulação de genes envolvidos com o balanço entre o anabolismo e o catabolismo de glicose, além de uma ativação de mecanismos que protegem a célula contra o excessivo estresse oxidativo. A análise do proteoma identificou um total de 415 proteínas sarcoplasmáticas, e após a filtragem, 181 e 46 proteínas foram encontradas exclusivamente nos tratamentos RM e MR, respectivamente. Das 159 proteínas presentes em ambos os tratamentos, apenas 13 foram consideradas proteínas diferencialmente abundantes (DAPs) considerando FDR < 0.05. Através das análises de Ontologia Gênica (GO) e Kyoto Encyclopedia of Genes and Genomes (KEGG), foram identificados processos biológicos (BP) e vias de sinalização (SP) relacionadas com a fase de investimento da glicólise, além do aumento na utilização das reservas de glicogênio e ácidos graxos no tratamento RM comparado ao MR. Enquanto as proteínas encontradas no tratamento MR apresentaram BP e SP associadas com a fase de pagamento da glicólise, e com o aumento da biossíntese de glutamina. No geral, os resultados gerados a partir das análises do trancriptoma e do proteoma sugerem que a restrição alimentar materna altera a abundância destas moléculas, principalmente modificando a composição do musculo esquelético e o metabolismo energético no musculo esquelético do recém-nascido.

Palavras-chave: Bovinos de corte. Caprinos. Musculo Esquelético. Programação do Desenvolvimento. Proteoma. Transcriptoma

SUMMARY

1. CHAPTER 1

1.1. General Introduction

The concept of fetal programming is based on the hypothesis that an specific challenge during a certain period of gestation may modulate fetal development causing long-term effects in progeny's life (Barker, 1992). In livestock this manipulation may occur in a typical manner, due to the exposure of pregnant ruminants to challenges of seasonal variations and alteration in the amount and nutritional values of the pasture. Although the intrauterine manipulation of the embryonic and fetal development has been often associated as a disadvantage, programmed events can hold positive outcomes by preparing the offspring to face the postnatal environment (Caton et al., 2020). In addition, the mechanisms that driven the final outcome are derived from the adaptative responses of the offspring, through their genetic and epigenetic potential, to maternal nutritional challenge (Caton et al., 2020).

The strategies of breeding and parturition season are guided by the physiological status of the animals, as well as the weather, considering the availability of feeding sources. Thus, the parturition and lactation periods are managed to occur during the rainy season to maximize the intake of high-quality pasture. Nonetheless, critical period of gestation overlaps with the dry season, and the supply bellow maternal requirement compromise maternal body condition score, impairing the embryonic and fetal development. For instance, during early gestation many critical events that assure conceptuses' survival, including, placental development (Funston et al., 2010), organogenesis (Meyer et al., 2010), and the initial myogenesis of the skeletal muscle (Du et al., 2010). While, mid-to-late gestation is characterized by the active processes of secondary myogenesis, muscle hypertrophy, adipogenesis and fibrogenesis (Du et al., 2010). Because of the greater fetus's development during late gestation, maternal requirements increase accompanied by the greater utilization of glucose and amino acids by the gravid uterus (Bell et al., 2005). Once the maternal requirements are not attended, mechanisms of maternal protein mobilization are enhanced in order to meet the demand by the growing fetuses (Bell and Ehrhardt, 2000).

Because meat production is linked to animal's muscle growth potential and body composition, and the main events associated to the formation of this tissue occur in the intrauterine period, strategies of maternal supplementation during crucial periods of gestation are consider an important topic of investigation. In a recent review, the effects of maternal protein and/or energy supplementation showed variable effects on offspring's phenotype,

possibly due to the divergencies on the duration of supplementation and evaluation period among the studies (Moriel et al., 2021). In-depth studies identified changes in many biological processes related to energy metabolism in the skeletal muscle of the offspring resulting from maternal diet (Paradis et al., 2017; Sanglard et al., 2018). Depending on the study objective, the analyses using target approaches to identify specific biological processes may be useful to respond a determined hypothesis. However, in cases where there is an ample hypothesis to be tested and many biological processes could be being regulated, sequencing-based approaches for profiling the molecules (RNA, proteins, metabolites) represents an advantageous alternative.

In this context, RNAseq allows the identification in large-scale of the transcripts differentially expressed between treatments, and their involvement in the regulation of the metabolic pathways related to tissue composition (Benítez et al., 2017). Although, RNAseq has been a valuable tool for fetal programming experiments, there are a poor correlation between the expression levels of transcriptome and its respective proteins (Abreu et al., 2009; Payne, 2015). These divergencies occur due to the number of required and connected processes that reflects protein abundance, such as mRNA processing and degradation, localization, protein modification, among others (Vogel and Marcotte, 2012). Thus, the integration of transcriptome analysis and proteomic approaches offer an opportunity to assess a more functional molecular landscape (Vogel and Marcotte, 2012; Payne, 2015).

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 effects of maternal protein restriction during mid-gestation in beef cattle on the short and long-term skeletal muscle composition of the offspring.

3 – Investigate how feed restriction at 50% of maintenance requirements during different stages of gestation affects the transcriptome of the newborn goats' skeletal muscle.

4 – Elucidate the effects of maternal plane of nutrition during distinct times of gestation on the proteome profile of the skeletal muscle of the newborn goat.

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2. CHAPTER 2

Fetal programming in ruminant animals: understanding the skeletal muscle development to improve meat quality[1]

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Review Article

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2.1. Implications

- The intrauterine environment is crucial for the skeletal muscle formation, which depends on maternal supplies for an adequate growth and development.
- Disturbs involving maternal feed restriction or overfeeding directly affects the offspring's skeletal muscle composition, influencing the final meat quality.
- The nutritional manipulation during the intrauterine period contributes to achieving desirable meat quality traits, such as marbling and tenderness.
- Metabolism plays an important role in providing metabolites that are used as substrates in epigenetics mechanisms, which can contribute to phenotypes that are more desirable and establish phenotype inheritance across generations.

Keywords: epigenetic modifications, livestock, maternal nutrition, skeletal muscle development.

2.2. Introduction

The efficiency of livestock production has been largely demanded by population growth, in addition to cost reduction per unit of productive resources, food safety and quality. The biggest challenges for the meat industry are to produce meat in quantity, which meets the global demand and causes less environmental impact as possible. One of the ways to reach those goals is to shorten the cycle of animal production.

Since the skeletal muscle, which provides meat, begins to develop during the intrauterine stage, manipulating fetal muscle development may help improve the efficiency of production. However, the muscle has lower priority in nutrient partitioning compared to vital tissues during the prenatal development (Long et al., 2009; Meyer et al., 2010). Therefore, its sensitivity to maternal nutrient supply and changes in the intrauterine environment, affects the composition of muscle, such as lean muscle mass, connective tissue and fat, and may contribute to the final meat quality. Early to mid-gestation is a crucial timeline for increasing the number of muscle fibers, which occurs exclusively during the prenatal period (Du et al., 2010). Concomitantly, the adipose tissue begins to develop during mid to late gestation, and although fat cells continue to form after birth, the ability to form new fat cells decreases over time (Du et al., 2010).

Altering the intrauterine environment may also change the proportion of muscle fibers, which have different metabolic properties (Zhu et al., 2006; Aragão et al., 2014). The type of substrates preferred by the muscle fibers may affect the final meat quality. Due to the forage quantity and quality fluctuation throughout the year, pregnant ruminants raised in pasture are commonly exposed to an undernutrition environment (Duarte et al., 2013a), impairing fetal development, and causing last-long effects. Therefore, in this review we summarize the impacts of fetal programing on the skeletal muscle development, in addition to management strategies that impact in final body composition and meat quality focusing on ruminant animals.

2.3. Skeletal muscle development

The skeletal muscle is the major component of the carcass and its components, such as muscle fibers, connective and adipose tissue (Figure 1) affect the quality of meat. The processes, by which muscle fibers, adipose and connective tissue are formed, are termed myogenesis (formation of muscle fibers), adipogenesis (formation of fat cells), and fibrogenesis (formation of connective tissue), respectively.

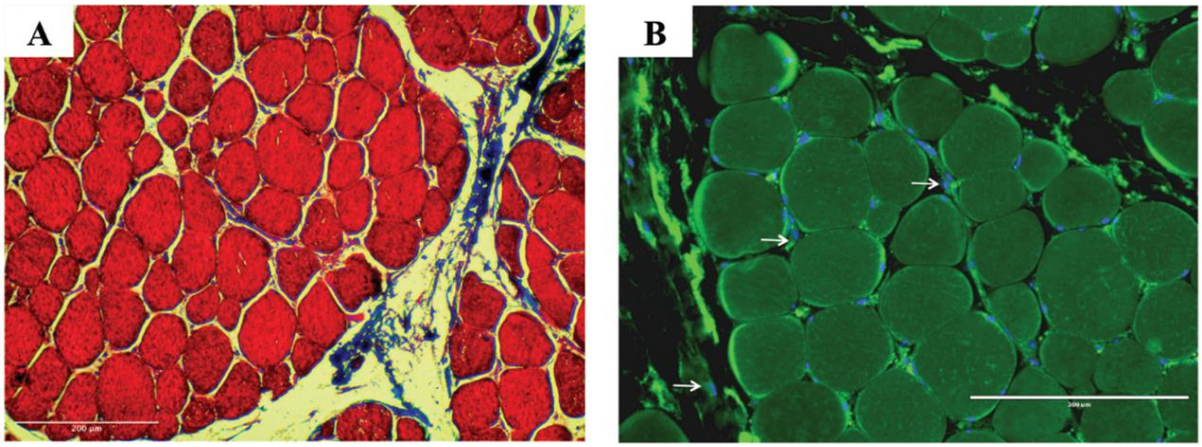

Figure 1. (A) Photomicrograph (20X of magnification) of skeletal muscle of beef cattle showing the muscle fibers (red), and intramuscular collagen (blue). **(B)** Photomicrograph (20X of magnification) of skeletal muscle of beef cattle showing preadipocytes and adipocytes (indicated by white arrows) within muscle fibers.

2.3.1. Myogenesis

The formation of muscle fibers occurs in the prenatal stage and is commonly divided into primary and secondary myogenesis. In cattle, the primary muscle fibers are formed during the embryonic period, within two months after conception, while secondary muscle fibers are formed during the fetal stages between the 2nd to 7th months of gestation (Du et al., 2010). Although the secondary muscle fibers represent the majority of fibers in adults, primary muscle

fibers are used as a template for the formation of secondary muscle fibers during the fetal stage (Swatland, 1973).

Muscle mass is determined by the number (hyperplasia) and size (hypertrophy) of muscle fibers. Hyperplasia occurs exclusively in the prenatal period, and the number of muscle fibers is fixed at birth. Moreover, muscle hypertrophy also begins during the prenatal period and extends to after the of pubertal stage. During the course of myogenesis, a population of myogenic cells become quiescent and locates surrounding muscle fibers in mature muscle, which are termed satellite cells. The proliferation and fusion of satellite cells with existing muscle fibers contribute to postnatal muscle fiber hypertrophy (Kuang et al., 2007). Therefore, the establishment of greater number of muscle fibers at birth and increase in the number of satellite cells, may positively impact meat production efficiency, which can be achieved through an adequate nutritional and environmental support during the intrauterine stage.

2.3.2. Adipogenesis

Similar to myogenesis, the adipogenesis involves cell determination and differentiation. As a competitive process, the same population of embryonic stem cells which can develop into muscle fibers can also undergo differentiation into fibro-adipogenic progenitors, a group of undifferentiated cells which can further develop into mature adipocytes and fibroblasts (Uezumi et al., 2010). In ruminants, adipogenesis begins around mid-gestation, concomitantly with the secondary myogenesis (Du et al., 2010). Unlike myogenesis, the adipogenesis is not limited to prenatal stages, however hyperplasia potential of this tissue decreases over time (Du et al., 2013). Visceral fat hyperplasia extends until the neonatal stage, while subcutaneous and intermuscular fat extends until early weaning, and intramuscular fat formation extends until approximately 250 days of age (Du et al., 2013). After the respective period of hyperplasia, the adipocytes undergo hypertrophy contributing for fat storage.

2.3.3. Fibrogenesis

Fibrogenesis begins during the late fetal stage, accompanied with adipogenesis; both fat cells and fibroblasts are developed from fibro-adipogenic progenitor cells. Fibroblasts are responsible for secreting components of connective tissue, including collagen and enzymes that catalyzes collagen cross-linking, which occurs slowly, increases with age and contribute to the background toughness of meat (Zhao et al., 2019).

Since adipose and connective tissue are originated from a common source of progenitor cells during fetal stage, there is an opportunity for manipulation in this period in a way that favors the increase of intramuscular fat cells and decrease of fibroblasts and the accumulation of connective tissue, improving meat quality (Du et al., 2013).

2.4. Muscle fiber types and meat quality

2.4.1. Skeletal muscle metabolism

Skeletal muscle mainly utilizes carbohydrate and fat as energy sources. In ruminants, fermentation by ruminal microbiota produces short chain fatty acids, such as, acetate, propionate, and butyrate. The proportion of these short chain fatty acid is influenced by feed sources, mainly by the roughage:concentrate ratio. Ruminal degradation of diets rich in forages provides an adequate environment for producing high proportion of acetate. While diets rich in grains, favors the production of propionate. Through the hepatic gluconeogenesis, propionate is converted into glucose that can be used as carbon sources for many processes. For lipid synthesis, the subcutaneous adipose tissue primarily uses acetate, while the intramuscular adipose tissue uses high proportion of glucose (provided by propionate in ruminants) (Smith et al., 2018). Although, the intramuscular fat is a desirable meat quality parameter, the achievement of this fat depot without the enhancement in other fat depots remains a challenge (Du et al., 2013).

Another important factor that affects meat quality traits is the energy metabolism of the muscle fibers. Muscle fibers are classified by their contractile and metabolic properties. Type I fibers are characterized by the slow speed of contraction, the oxidative (aerobic) metabolism, which uses fat as the main energy source, possess great amount of mitochondria, and are rich in myoglobin that confer red color to the meat (Listrat et al., 2016). Based on the greater abundance of intracellular lipid stores, accompanied by the more active hormone-sensitive lipase (HSL) and the efficiency in trans-sarcolemmal transport in type I muscle fibers, there are a greater utilization of free fatty acid, favoring the fat accumulation in comparison with type 2 fibers (for review see: Schiaffino and Reggiani, 2011). Moreover, type I muscle fiber has increased insulin sensitivity, greater capacity of fatty acid uptake and triglyceride storage (Dyck et al., 1997). Evaluating the correlation of muscle fiber type and meat quality parameter of beef cattle, Joo et al. (2017) reported a positive correlation between intramuscular fat, fiber number percentage and fiber area percentage of type I muscle fiber compared with type II, demonstrating that muscle fiber type is an important factor that contributes to fat accumulation.

Type II muscle fibers are classified as fibers with fast speed contraction, which uses glucose as the main energy source, and have low myoglobin content, making meat with a white color (Listrat et al., 2016). Besides the meat color, the type of muscle fibers also influences meat tenderness and water holding capacity (Ryu and Kim, 2005). Moreover, the relative proportion of these muscle fiber types varies depending on species, muscle function, breed, gender, age, among others. Genetic selection for increased muscle mass favors the establishment of greater proportion of type II fibers in non-ruminant animals (Hocquette et al., 1998). Increased number of fast (type II) muscle fibers were observed in 14 days old lambs born from ewes fed restricted during the 30-70 days of gestation, regardless the sex of the offspring, and consequently, increased lean:fat ratio was also observed (Daniel et al., 2007), impairing fat deposition.

2.5. Epigenetic regulating muscle growth and development

The epigenetic study how the patterns of gene expression are altered, without the changes in DNA sequence (Feil, 2006). The epigenetic mechanism is very sensitive for nutritional changes, and includes, but is not restricted to, DNA methylation, histone modifications, and non-coding RNAs (Ibeagha-Awemu and Zhao, 2015) (Figure 2).

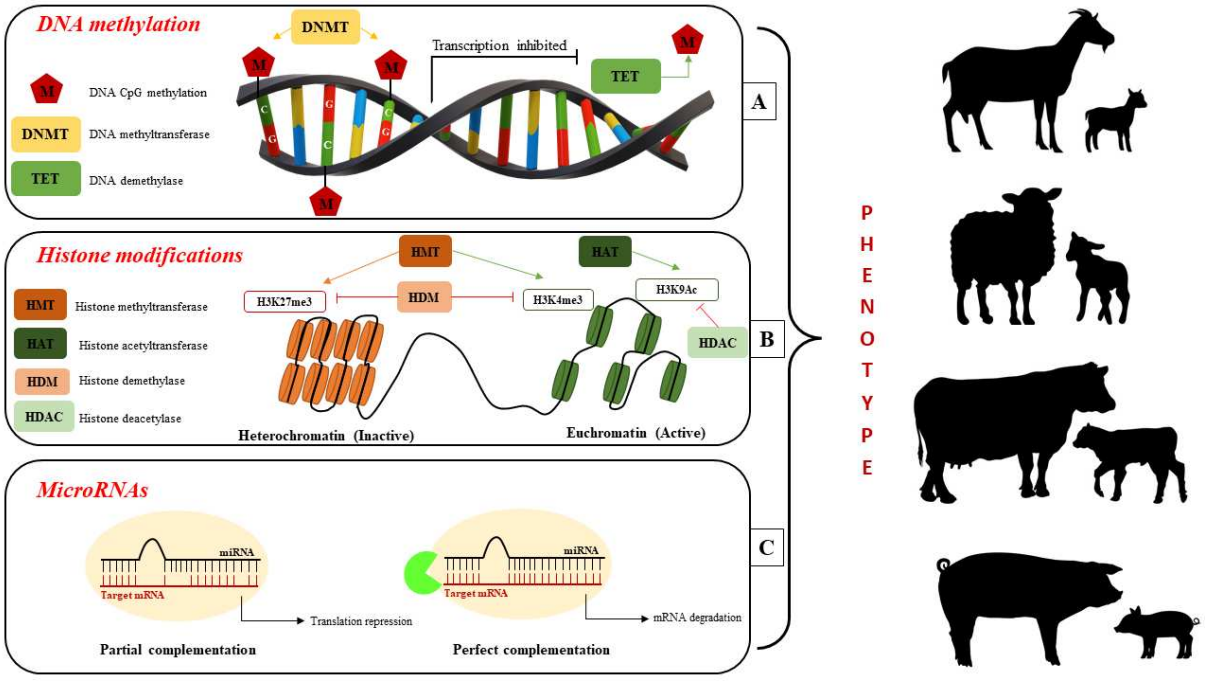

Figure 2. (A) DNA methylation in CpG islands catalyzed by enzymes DNA methyltransferases (DNMTs) leads to gene silencing, which can be reverted by DNA demethylases, represented by the enzyme ten-eleven translocation (TET). **(B)** Histone modification influencing the chromatin structure. The mark H3K27me3 result in a condensed chromatin (heterochromatin) and consequently gene silencing, while the marks H3K4me3 and H3K9Ac result in an unpacked chromatin (euchromatin) facilitating transcriptional factors to bind and proceed

gene transcription. HMTs and HATs transfer the methyl and acetyl group to the residues of amino acids in the histone tails, respectively. While HDM and HDAC remove the methyl and acetyl group, respectively. **(C)** Inhibitory role of microRNAs (miRNAs) on the target mRNA. In case of unperfect base pairing with the target mRNA, miRNAs aim to inhibit translation and consequently protein synthesis, while the perfect complementation cause the degradation of the target mRNA

The DNA methylation consist in the inclusion of a methyl group on the 5' position of the cytosine residues located at the CpG islands in the promoter region of the gene, blocking the transcription factor to bind and consequently causing gene transcription repression (Osorio et al., 2017). The enzymes DNA methyltransferases (DNMT) catalyzes the transfer of a methyl group from the methyl donor S-adenosylmethionine (SAM), synthesized in the methionine cycle from several dietary precursors (Triantaphyllopoulos et al., 2016). In contrast the α-ketoglutarate (α-KG) dependent ten-eleven translocation (TET) family of proteins catalyzes the demethylation and reverting the gene transcriptional silencing (Ito et al., 2010). In a maternal obesity model, Yang et al. (2013) reported the enhancement on the expression of the early adipogenic marker *Zfp423* in fetal tissues from obese mothers, regulated by the hypomethylation on the promoter region of this gene. Moreover, DNA methylation regulated by nutrient restriction intervention can potentially and permanently influence muscle development. Evaluating maternal nutrient restriction during mid-to-late gestation in ewes, Paradis et al. (2017) observed that the skeletal muscle from the maternal nutrient restricted offspring presented hypermethylation levels in the promoter region of *IGF2*, which is an important inducer of cell differentiation.

The histones proteins are responsible for packing the genomic DNA and thus forming the chromatin structure. The N-terminal tails of histone are target of several post-translational modification (PTM), including acetylation, methylation, phosphorylation, among others (Triantaphyllopoulos et al., 2016). The combination of different PTM in a determined histone can be called histone code (Jenuwein and Allis, 2001). Depending on the PTM a determined histone receives, different response will be generated in a way that activate or silence gene expression. These modifications directly affect the chromatin structure, taking over the structure of heterochromatin or euchromatin, associated with a compacted (repressing gene expressing) and relaxed (activating gene expressing) state, respectively (Jenuwein and Allis, 2001). The marker histone 3 lysine 27 trimethylation (H3K27me3) is associated with gene silencing and is catalyzed by the Polycomb repressive complex 2 (PCR2). Maternal obesity trigged the decrease in the modification H3K27me3 in fetal mice tissues (fetal tissues free from head, heart, lung, liver, gelatinous tissue, spinal cord and primordial bones), resulting in the repression of gene transcription, that combined with the decrease in the DNA methylation in

the promoter region of Z*fp423*, enhanced adipogenesis (Yang et al., 2013). In contrast to the H3K27me3 modification that causes gene silencing, the marks histone 3 lysine 9 acetylation (H3K9Ac) and the histone 3 lysine 4 trimethylation (H3K4me3) activates gene transcription (Jia et al., 2016). Throughout the increase in H3K9Ac and H3K4me3 in the promoter region of myostatin, its expression was enhanced in the skeletal muscle of the offspring from maternal fed low protein diets during pregnancy and lactation (Jia et al., 2016), implying in reduction in muscle mass and muscle fiber size.

MicroRNAs (miRNAs) are a single-strand RNAs belonging to the class of small non-coding RNAs, playing roles inhibiting gene expression post-transcriptionally. The inhibitory mechanisms of the miRNAs involve base pairing with the targeted mRNA and cause distinct types of repression, such as blocking the translation, and recruiting complexes to degrade the target mRNA. It has been shown that obesogenic diets during ewe's gestation, contributes for the decrease in the expression of the miRNA *let-7g* in fetal muscle, which contribute to enhance the expression of adipogenic markers and the number of adipocytes (Yan et al., 2013). Similarly, pro-adipogenic miRNAs (miR-103 and miR-21) were upregulated, while the anti-adipogenic miRNA (miR-34a) was downregulated in the skeletal muscle of calves resulting from cows submitted to a medium plan of nutrition during late-gestation (Moisá et al., 2016), implying in a possible increase in the adipogenic potential throughout the progeny life.

2.6. Fetal programming and the efficiency for the deposition of muscle and fat

2.6.1. Muscle fiber number, size, and composition

As previously mentioned, the potential of muscle growth is based on adequate formation of muscle fibers during the prenatal period, since there is no increase in muscle fiber number postnatally. The nutrient delivery for embryonic and fetal development are exclusively provided by the dam and therefore assured by a sufficient maternal nutrition. For pregnant ruminants raised in pasture, feed restriction due to forage seasonality may impair the development of the offspring. Under this situation, maternal nutrients are prioritized for the formation of fetal vital tissues instead of secondary tissues, such as skeletal muscle. The impairment caused by maternal protein restriction in beef cattle during mid-gestation, imply in a long-lasting reduction of muscle fiber number in the offspring, in addition to alter muscle metabolism to greater proportion of glycolytic type of fiber early in life,

which can be reverted depending on the postnatal environment (Costa et al., 2021a). Similarly, the energy restriction during late gestation contributes for the downregulation of genes involved in the oxidative metabolism, and consequently favoring the less efficient glycolytic metabolism in the skeletal muscle of calves (Sanglard et al., 2018). Although no phenotypic differences were observed in the skeletal muscle of the newborn goats, resulting from maternal feed restriction at different stages of gestation (Costa et al., 2019), the skeletal muscle transcriptome profile was altered, resulting in differentially expressed genes that are involved with skeletal muscle development and energy metabolism (Costa et al., 2021b).

In order solve feed or nutrient restriction issues, strategies of supplementation during certain periods of gestation may represent an alternative to improve the offspring's skeletal muscle development, in addition to maintain the balance in maternal metabolism. For example, maternal protein supplementation during mid-gestation in cows tend to increase the pregnancy rate in the subsequent breeding season (Rodrigues et al., 2021), while the supplementation during late gestation reduced maternal tissues mobilization (Lopes et al., 2020), and may improve cows' reproductive parameters. Although the costs of supplement are relatively high in a livestock production system, an alternative for decrease the feeding and labor costs may be achieved by reducing the frequency of energy-protein supplementation during prepartum, without causing negative effects in maternal performance and metabolism (Moura et al., 2020).

Regarding the effects of maternal protein supplementation during different stages of gestation on the offspring's performance, Marquez et al. (2017) reported an increased in the number muscle fibers when the supplementation was applied during mid-gestation and despite the lack of difference in body weight, calves born from protein supplemented dams during mid or late gestation presented greater ribeye area. Moreover, protein supplementation during mid-gestation for cows may increase offspring's body weight at birth and provide greater adipogenic potential in their skeletal muscle (Rodrigues et al., 2021). Taken together, these recent findings show that nutrient supplementation during gestation, ensure the adequate fetal development and may influence the postnatal performance.

2.6.2. Meat marbling and tenderness

The intramuscular fat or marbling is an important parameter that influences meat quality, affecting taste, juiciness, tenderness, and aggregate economic value to the final product. Selecting breeds for lean growth, impairs the intramuscular fat deposition, and consequently reduce meat quality in terms of marbling parameters (Du et al., 2013). Moreover,

the attempting to increase intramuscular fat, without increase the overall body fat accumulation remains challenging for beef producers (Du et al., 2013). The other factor accounting for meat quality is tenderness, which is mainly influenced by the combination of collagen fibrils and the intermolecular cross-linking in the connective tissue, conferring the background toughness of the meat (Zhao et al., 2019).

Both under and overnutrition during pregnancy affects adipogenesis and fibrogenesis and may alter fat deposition potential postnatally, the final carcass marbling, and the overall composition. Maternal protein restriction during mid-gestation may have caused reduction in the intramuscular collagen content in calves' skeletal muscle early in life, in addition to an inefficient collagen remodeling at the finishing phase (Costa et al., 2021a). Overnutrition during mid-gestation may also affect the skeletal muscle adipogenesis, by increasing the expression of adipogenic markers and tended to increase the expression of the fibrogenic marker (COL1) in the skeletal muscle of the crossbred (beef and dairy breeds) fetuses (Gionbelli et al., 2018). In addition, evaluating the effects of maternal overnutrition during different time points of gestation, Duarte et al. (2014) reported the enhancement in the adipogenic markers (*Zfp423*, *C/EBPα*, and *PPARγ*) accompanied by the accumulation of intramuscular collagen in the fetuses' skeletal muscle from overnurished cows.

Due to the presence of fibro-adipogenic progenitor cells in postnatal period, diet intervention in this period may slightly contribute for improve marbling and tenderness. Feeding cull cows with high-energy diets tended to increase the intramuscular fat content and contributed to increase meat tenderness (Fontes et al., 2021). In contrast, feeding beef cattle with vitamin A during the fattening phase, impaired the lipid biosynthesis in the skeletal muscle and consequently decreased the intramuscular fat deposition (Campos et al., 2020), negatively contributing for meat quality parameters.

Not only dietary interventions may affect the intramuscular fat deposition potential, but also the animal's breed attributes. Comparing distinct beef cattle breeds, Duarte et al. (2013b) showed that the high potential of marbling in Wagyu cattle is accompanied by the enhancement in connective tissue, possibly due to greater abundance of fibro-adipogenic progenitor cells compared with Angus cattle. Similarly, the abundance of fibro-adipogenic progenitor cells may account for difference between Angus and Nellore cattle in marbling fat development. Due to high proportion of these cells, Angus present higher adipogenic potential than Nellore cattle, while the fibrogenesis is not different between these breeds (Martins et al., 2015), which could explain a high level of marbling in Angus.

2.7. Conclusions and prospects

The embryonic and fetal stages are crucial for the formation of muscle fibers, adipose and connective tissues. Understanding the specific stages of gestation that affects the final offspring's carcass composition, help to improve beef production and meat quality. Maternal nutrition during gestation may alter muscle fiber composition and the lean:fat ratio, leading to the production of a desirable body composition. Marbling and tenderness are critical quality characteristics of the meat. The combination of maternal nutrition, breed and post-natal supplementation in a so-called marbling window, can result in the elevated number of intramuscular fat cells, leading to more marbled carcasses.

Despite the increasing number of studies and data generated from trials evaluating the effects of maternal nutrition on offspring's development and meat quality, some divergences are still found, possibly due to the time period of nutrient intervention, type of intervention (diet or nutrient specific), breed, multiple pregnancy in case of sheep and goats, and fetal sex. The integration of these data through systematic review and meta-analysis would improve the future practical decision in the field. Moreover, there is a lack of studies on epigenetic mechanisms regarding intrauterine manipulation in ruminant animals. The development of these studies, combined with other omics tools in a system biology would be beneficial for the overall knowledge of cellular mechanisms involved in muscle development and meat quality traits.

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3. CHAPTER 3

Skeletal muscle development in postnatal beef cattle resulting from maternal protein restriction during mid-gestation[2]

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3.1. Simple summary

The intrauterine period plays a major role in skeletal muscle development and metabolism, including the formation of muscle fibers and adipose and connective tissue. Since the embryo and fetus depend on maternal nutrition to develop and grow, understanding the effects and finding potential strategies of skeletal muscle manipulation may be a valuable alternative to enhance beef cattle performance postnatally. In the present study, we evaluated the effects of maternal protein restriction during mid-gestation on the short and long -term skeletal muscle composition of the offspring. Our results suggest that the detrimental effects of maternal protein restriction during mid-gestation were associated with decrease in muscle fibers formation and may have contributed to the increase in collagen content in the skeletal muscle of the offspring. Although the changes in muscle fiber metabolism were not persistent, maternal protein restriction may contribute to such a short-term alteration. Our findings highlight the importance of an adequate nutritional plane for pregnant beef cows, to improve offspring's performance, and consequently, the meat quality.

3.2. Abstract

We aimed to investigate the effects of maternal protein restriction during mid-gestation on the skeletal muscle composition of the offspring. In the restriction treatment (RES, n = 9), cows were fed a basal diet, while in the control (CON, n = 9) group cows received the same RES diet plus the protein supplement during mid-gestation (100–200d). Samples of *Longissimus thoracis* muscle were collected from the offspring at 30d and 450d postnatal. Muscle fiber number was found to be decreased as a result of maternal protein restriction and persisted throughout the offspring's life ($p < 0.01$). The collagen content was enhanced ($p < 0.05$) due to maternal protein restriction at 30d. *MHC2X* mRNA expression tended to be higher ($p = 0.08$) in RES 30d offspring, however, no difference ($p > 0.05$) was found among treatments at 450d. Taken together, our results suggest that maternal protein restriction during mid-gestation has major and persistent effects by reducing muscle fiber formation and may slightly increase collagen accumulation in the skeletal muscle of the offspring. Although maternal protein restriction may alter the muscle fiber metabolism by favoring the establishment of a predominant glycolytic metabolism, the postnatal environment may be a determinant factor that establishes the different proportion of muscle fiber types.

Keywords: bovine; dietary protein restriction; maternal effects; mid-gestation; muscle tissue

3.3. Introduction

In tropic regions, due to seasonal variations of dry and rainy period, beef cattle reproduction and lactation is synchronized with the rainy season to maximize the intake of high-quality forages. Nonetheless, in some stages of gestation, such as, mid-gestation, the quantity and quality of the pastures is limited [1], characterized by high insoluble fiber and lignin but low nitrogen content [2]. As the pregnancy advances, cows' nutrient requirements increase, and the gravid uterus requires greater amounts of glucose and amino acids to foster fetal growth and development [3]. In a protein deficiency scenario, commonly experienced by the dams during mid-gestation, proteins are mobilized from maternal tissues (especially skeletal muscle) in an attempt to provide gluconeogenic precursors for glucose production needed to support fetal development [4].

Hence, strategies of maternal supplementation during certain periods of gestation [5] or offspring's post-natal supplementation [6] are adopted in order to minimize the damages caused by maternal restriction during gestation. However, since the number of muscle fibers are fixed at birth, offspring's supplementation may be limited in improving beef cattle potential for muscle growth in the postnatal stages of life. Besides myogenesis, adipogenesis and fibrogenesis begin around mid-gestation as a competitive process since these cellular lineages are originated from a common ancestor cells, named mesenchymal stem cells [7]. Although adipogenesis is not limited to prenatal stages, its hyperplasia potential decreases over time [8]. Thus, maternal feed restriction directly affects fetal development by changing skeletal muscle composition, and ultimately impacts postnatal performance.

Meat quality is defined by multifactorial parameters, roughly affected by extrinsic and intrinsic factors**.** Among the factors affecting meat quality, the composition of muscle fibers [9], intramuscular fat (marbling level) [10] and connective tissue structure [11] may be, at least partially, manipulated during the prenatal stage in order to produce a desirable final body composition. Although many studies have reported the negative effects of maternal restriction during gestation on offspring skeletal muscle [12–14], there are only few studies addressing the long-term effect of maternal protein restriction during mid-gestation in beef cattle. Moreover, it remains unclear whether the negative changes can be partially rescued during postnatal stages.

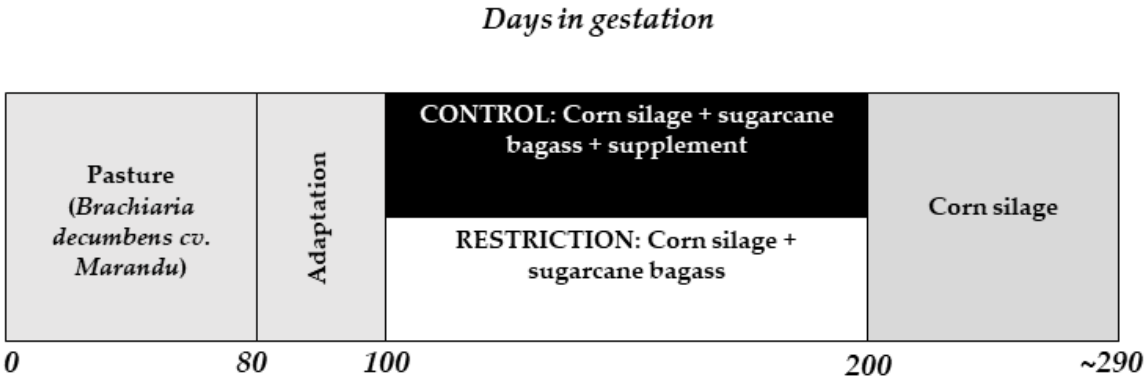
In the current study, we hypothesized that maternal protein restriction during mid-gestation would alter the skeletal muscle development in the offspring which would persist until the finishing phase. Thus, the skeletal muscle composition in two distinct times of offspring

cattle was examined in order to monitor the impacts of mid-gestation nutrient deficiency on muscle growth of female and male calves.

3.4. Materials and Methods

3.4.1. Animal Husbandry, and Experimental Design

All animal care and handling procedures were approved by the Animal Care and Use Committee of the Department of Animal Science at the Universidade Federal de Lavras, Lavras, Minas Gerais, Brazil (protocol No. 015/17). Eighteen pregnant Tabapuã (*Bos indicus)* cows weighing 480.11 ± 82.97 kg and 5.69 ± 4.14 years of age (mean ± SD), gestating males (n = 8) and females (n = 10), were randomly assigned at day 80 of gestation into two treatments, according to the Scheme I.



Scheme I. Scheme showing the period of the dietary treatments according to the gestational timeline**.**

The cows were confined in individual pens (20 m^2) and submitted to an adaptation period of 20 days. The treatments were applied from day 100 of gestation to day 200, representing the mid-gestation. Experimental diets were offered in the form of total mixture ration (TMR) and consisted in treatment control (CON; female = 4; male = 5), where the cows were fed a basal diet plus a protein supplement at the level of 0.35% of the body weight (3.5 g/kg/day); and treatment restricted (RES, female = 6; male = 3), where the cows were fed a basal diet ad libitum of roughage (corn silage and sugarcane bagasse) containing low crude protein (CP) and a high neutral detergent fiber (NDF) content. The protein supplement consisted of a 50: 50 mixture of soybean meal and a commercial supplement (40% CP; Probeef Proteinado Sprint®, Cargill Nutrição Animal, Itapira, SP, Brazil). Table 1 contain the composition of the diets.

Table 1. Chemical composition (mean) of the diets offered to the pregnant cow during mid-gestation and finishing diet offered to the offspring.

Item	Mid-Gestation [1]		Offspring [2]	
	Basal Diet	Supplement	Corn Silage	Concentrate
DM (g/Kg)	418	881	458	907
OM (g/Kg DM)	951	957	960	885
CP (g/Kg DM)	53.3	400	99.1	351
NDFap (g/Kg DM) [3]	631	213	468	167
NFC (g/Kg DM)	242	342	367	340
EE (g/Kg DM)	24.1	41.2	25.8	25.5

[1] Experiemtal diets were offered to pregnant cows from day 100 to 200 of gestation; [2] Diets offered to the offspring during the finishing phase; DM = dry matter; OM = organic matter; CP = crude protein; NDFap = neutral detergent fiber corrected for ash and protein; NCF = non-fiber carbohydrate; EE = ether extract; [3] NFC = 100 − (%NDF + %CP + %EE + %Ash).

From 200 days of gestation until parturition all the cows were fed ad libitum with corn silage (DM = 35.2%; CP = 7.2%, and NDF = 54.9%) and a commercial mineral mixture. The offspring were submitted to the same nutritional conditions after birth. At the weaning phase, all calves were kept with its dams in a high-quality tropical pasture (Brachiaria decumbens cv. Marandu; DM = 29.7 %; CP = 13.0 %; NDF = 62.6%) in an intensive grazing management. Weaning was performed 200 days after birth. After weaning, the animals were kept in pasture for 60 days to promote group uniformity. At 260 days of age, calves were assigned to feedlot with individual pens and fed with rising levels of concentrate until harvest (~450d). Heifers and young bulls had free access to water and were fed twice daily (0700 h and 0100 h). The finishing phase were performed from 390 to 450 days of age. The diet offered during the finishing phase (Table 1) was composed of corn silage (268 g/kg DM basis), ground corn (582 g/kg DM basis), and commercial supplement (150 g/kg DM basis), considering the Nutrient Requirements of Zebu and Crossbred Cattle (BR-CORTE) [15].

3.4.2. Skeletal Muscle Sampling

Samples of *Longissimus* muscle, located at the level of 10^{th} and 11^{th} ribs, were collected through biopsy from the calves at day 30 after birth and after feedlot period (~450d). Briefly, the *Longissimus thoracis* area was cleaned with 70% ethanol, and the incision was performed 10 min after local anesthesia treatment (Lidocaine 2%) approximately 3 cm^3 of tissue was collected and part of tissue was snap frozen in liquid nitrogen and stored at −80 °C, and the

other part was fixed in fresh 10% (wt/vol) buffered formalin for further histological analysis. The local of collection was sutured and the animals were accompanied and treated with antibiotics and anti-inflammatory drug. The sutures were removed two weeks after biopsy.

3.4.3. Morphometric Evaluation by Histochemical and Image Analysis

Skeletal muscle samples previously fixed in fresh 10% (wt/vol) buffered formalin in phosphate buffer was dehydrated using a crescent ethanol series and embedded in paraffin. Sections of 5 μm were obtained using a rotary microtome (RM 2265, Leica Biosystems, Nussloch, Germany) and stained with Masson's trichrome [16]. For observation of the number and area of muscle fibers, ten digital images of muscle sections per animal were obtained at 40× magnification and analyzed using the ImageJ software (National Institute of Health, Baltimore, MD, USA). To measure intramuscular collagen content (stained in blue), images obtained at 20× magnification were converted into grayscale, threshold to the same extent to highlight and quantify the collagen area, which was expressed as the percentage of the total area [17].

3.4.4. Total RNA Extraction and mRNA Expression Analysis

The frozen samples were powdered in liquid nitrogen and total RNA extracted from 0.1 g of tissue using Trizol® (InvitrogenTM, Thermo Fisher Scientific®, Oregon, USA) using the manufacturer's recommendations. Total RNA was quantified with a NanoDrop spectrophotometer. The RNA samples were reverse transcribed into cDNA using the GoScriptTM Reverse Transcription System Kit (Promega Corporation, Madison, WI, USA), and quantified [absorbance (A) ratio at 260 and 280 nm] using a NanoDrop spectrophotometer, with an optimal 260/280 ratio between 1.8 and 2.0. The primers of the genes (Table 2) were designed using PrimerQuest software (www.idtdna.com/Scitools/Applications/PrimerQuest; access date: 05/2020) with sequences obtained in GenBank (www.ncbi.nlm.nih.gov; access date: 05/2020), through the *Bos taurus* reference genome (ARS-UCD1.3).

Table 2. List of primers for relative gene expression by RT-qPCR

Gene Name	**Gene Abbreviation**	**Primer**
Paired box 7	*PAX7*	F: CGGGCATGTTTAGCTGGGAGA
		R: TCTGAGCACTCGGCTAATCGAAC
Platelet-derived growth factor receptor A	*PDGFRα*	F: GCTCCCTTCCTTTGTCTCTTAC
		R: CGATTTGTCCTCCCATCTAACC
Myosin heavy chain 1	*MHC1*	F: ATCGCTGAATCCCAGGTCAA
		R: ACCAAGATGTGGCACGGCTA
Myosin heavy chain 2A	*MHC 2A*	F: CACCCTGGAGCAGACAGAGA
		R: TCCCTGGATTTGCGTGATG
Myosin heavy chain 2X	*MHC 2X*	F: TTTCCAGACCGTGTCTGCTC
		R: GGGATGATGCAGCGTACAAAG
Zinc finger protein 423	*ZFP423*	F: CCAGATGACCTTCGAGAATGAG
		R: CACTAGCTGTAGCAGGACAATAA
CCAAT-enhancer-binding protein alpha	*C/EBPα*	F: GGCAACGACTTTGACTACC
		R: CTCGTACAGAGGCTCCAG
Peroxissome proliferator activated-receptor gamma	*PPARγ*	F: TCCACTCCGCACTATGAG
		R: GGGATACAGGCTCCACTT
Transforming growth factor beta	*TGFβ*	F: AACCTGTGTTGCTCTCTCGG
		R: GAGGTAGCGCCAGGAATTGT
Fibronectin	*FN1*	F: CTGAGACCACCATCACCATTAG
		R: CTCGGAACTGTAAGGGTTCTTC

Collagen type I, alpha 1	*COL1A1*	F: CCACCCCAGCCGCAAAGAGT
		R: ACGCAGGTGACTGGTGGGATGTC
Collagen type III, alpha 1	*COL3A1*	F: GGCCCCCTGGAAAGGACGGA
		R: CCCCGCCAGCACCACAACAT
Lysyl oxidase	*LOX*	F: CAGAAGATCCAATGGGAGAACA
		R: TGGCATCAAGCAGGTCATAG
Prolyl 4-Hydroxylase Subunit Alpha 1	*P4HA1*	F: GGATGAGTGGGACAAGCCTC
		R: ACCGTCTCCAAGTCTCCTGT
Matrix metalloproteinase-2	*MMP2*	F: CGGCAAGTATGGCTTCTG
		R: CTCCTCCTGTGGGTCTTC
TIMP metallopeptidase inhibitor 1	*TIMP1*	F: CAGAGAGGCTACACCAGAG
		R: CACAACCAGCAGCATAGG
TIMP metallopeptidase inhibitor 2	*TIMP2*	F: GAAGGAGGTGGACTCTGG
		R: CCGGAGAGGAGATGTAGC
18 S ribosomal	*18S*	F: CCTGCGGCTTAATTTGACTC
		R: AACTAAGAACGGCCATGCAC

Real-time quantitative PCR was performed in the thermal cycler ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using the detection method SYBR Green (Applied Biosystems—Foster City, CA, USA) and GoTaq® qPCR Master Mix kit (Promega Corporation, Madison, WI, USA) using the following cycle parameters: 95 °C for 2 min and 40 cycles at 95 °C for 15 s and 60 °C for 60 s. The results are expressed relative to 18S using $\ln(2^{-\Delta\Delta Ct} + 1)$ [18].

3.4.5. Statistical Analyses

Data from stage 1 representing the 30d old offspring were analyzed separately from data from stage 2 that represent 450d old offspring, through the model:

$$Y_{ijk} = \mu + N_i + S_j + (NS)_{ij} + e_{ijk}$$

where, Y_{ijk} is the observed measurement; μ is the overall mean; N_i is the fixed-effect of the *i*th level of maternal nutrition; S_j is the fixed-effect of the *j*th level of offspring sex; NS_{ij} is the interaction between N and S; and the e_{ijk} is the random error associated with Y_{ij} with e_{ijk}~N(0,σ_e^2). Prior to the final analyses, extreme data were removed when Studentized residuals within ± 1.5 standard deviations, and normality ($p > 0.05$) was assessed using Shapiro–Wilk's test. Least square means were estimated for all effects and compared using Tukey's method adopting α= 0.05 of probability for type I error and between α = 0.05 and 0.10 of probability for trends. All statistical procedures were performed using the software R.

3.5. Results

3.5.1. Maternal Intake and Performance

The protein supplementation (CON) between 100 and 200 days of gestation increased ($p = 0.03$) the cow's total daily dry matter intake by 26% (7.44 kg/day in CON vs. 5.88 kg/day in RES) compared to RES cows. There was no effect of fetal sex ($p = 0.89$) or interaction between fetal sex and nutritional treatment ($p = 0.20$) on maternal feed intake. The diet consumed between 100 and 200 days of gestation met the equivalent of 83% and 104% of the energy requirements and 59% and 104% of the protein requirements of RES and CON cows, respectively (calculated according to the nutrient requirements for pregnant Zebu cows [19]). Protein supplemented (CON) cows had greater ($p = 0.03$) average daily gain (ADG) than RES cows (0.262 vs. −0.184 kg/d), as well as the cows pregnant with female calves which had greater ($p = 0.04$) ADG than cows pregnant with male calves. No interaction effect ($p = 0.19$) was observed for nutritional treatment and fetal sex on maternal ADG between 100 and 200 days of gestation.

3.5.2. Muscle Cell Area, Number, and Collagen Content

There was no interaction effect ($p > 0.05$) between maternal nutrition and sex regarding the histological variables, muscle cell area, number, and collagen content evaluated in the current study.

There was no difference ($p > 0.05$) in muscle fiber area between treatments, in both stages evaluated (Figure 1). However, muscle fiber hyperplasia was impaired by maternal protein restriction during mid-gestation. The muscle fiber number was reduced in restricted calves at 30d ($p < 0.01$), which persisted to the finishing stage (450d; $p < 0.01$), as well as the number of fibers in the rib eye area ($p = 0.02$; Figure 1B). Moreover, the offspring cattle of protein restricted cows had higher ($p < 0.01$) collagen content at 30d, compared with the control group (Figure 1A). No difference ($p > 0.05$) in collagen content was observed in 450d old animals (Figure 1B).

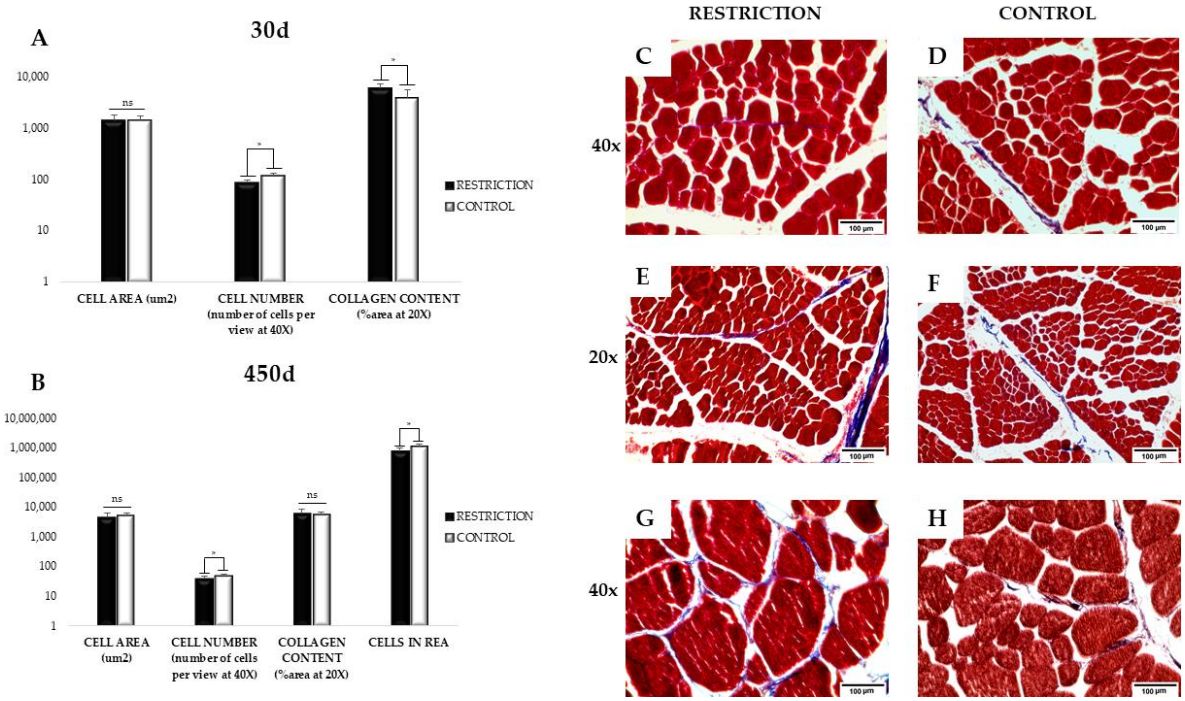


Figure 1. Cell area (um^2), cell number (number/view), collagen content (%area) of the skeletal muscle of the offspring at 30d and 450d of age resulting from maternal protein restriction and control group (**A**,**B**), and number of muscle cells in rib eye area (REA) measured at 450d (**B**). Bars represent means ± SEM. Histological images from the skeletal muscle of the offspring from restriction (**C**,**E**) and control (**D**,**F**) group at 30d. Histological images from the skeletal muscle of the offspring from restriction (**G**) and control (**H**) group at 450d. To combine different measurement units into the graph, the data were transformed into log10.

3.5.3. mRNA Expression of Adipogenic Markers

For the expression of the early adipogenic marker *Zfp423* there was a tendency ($p = 0.08$) of an interaction effect between maternal nutrition and sex at 30d, however, males and females did not differ due to the maternal dietary treatments (Figure 2A).

The expression of the late adipogenic markers *CEBPα and PPARγ* did not differ between treatments in either stages evaluated ($p > 0.05$; Tables 3 and 4).

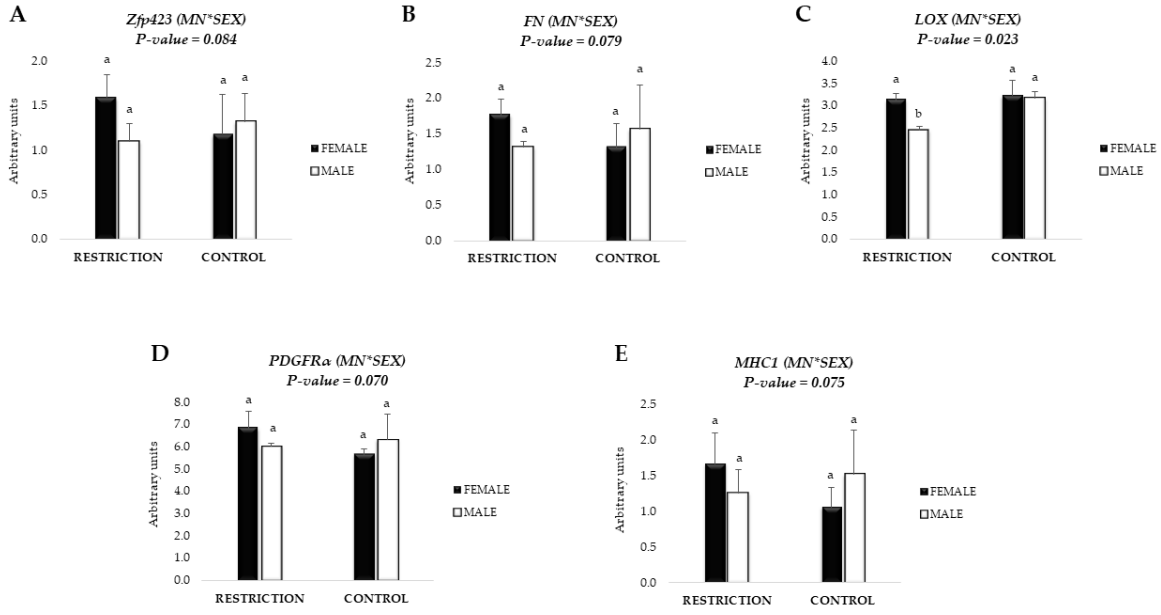


Figure 2. Interaction effect between maternal treatment (CON vs. RES) and offspring's sex (FEMALE vs. MALE) on the relative gene expression of *Zfp423* (**A**), *FN* (**B**), *LOX* (**C**), *PDGFRα* (**D**), and *MHC1* (**E**) at 30d. Different letters indicate differences among groups. Tendency was considered when $0.10 < p > 0.05$ and significant differences when $p < 0.05$.

Table 3. Least square means ± standard errors for mRNA expression of adipogenic, fibrogenic, collagen crosslinking, collagen remodeling, and satellite cells markers evaluated on the skeletal muscle of the 30d offspring according to maternal nutrition and sex.

Gene	Maternal Nutrition [1]		Sex		*p*—Value [2]		
	Restriction	Control	Female	Male	MN	S	MNxS
mRNA expression of adipogenic markers (arbitrary units)							
C/EBPα	6.20 ± 1.52	5.95 ± 2.40	6.46 ± 1.36	5.57 ± 2.53	0.796	0.403	0.596
PPARγ	8.25 ± 1.04	7.43 ± 0.98	7.96 ± 1.13	7.69 ± 1.01	0.116	0.876	0.360
mRNA expression of fibrogenic markers (arbitrary units)							
TGFβ	1.36 ± 0.13	0.99 ± 0.21	1.02 ± 0.18	1.00 ± 0.16	0.629	0.970	0.472
COL1	0.92 ± 0.15	0.93 ± 0.20	0.97 ± 0.16	0.88 ± 0.20	0.920	0.358	0.120
COL3	1.28 ± 0.16	1.13 ± 0.38	1.19 ± 0.28	1.23 ± 0.30	0.300	0.618	0.135
mRNA expression of collagen crosslinking markers (arbitrary units)							
P4Ha1	1.83 ± 0.52	1.62 ± 0.65	1.70 ± 0.62	1.76 ± 0.47	0.411	0.690	0.256
mRNA expression of collagen remodeling markers (arbitrary units)							
TIMP1	8.17 ± 0.60	8.30 ± 0.65	8.32 ± 0.68	8.13 ± 0.52	0.670	0.496	0.181
TIMP2	3.05 ± 0.16	2.75 ± 0.53	3.02 ± 0.27	2.76 ± 0.52	0.132	0.278	0.405
MMP2	2.32 ± 1.25	2.72 ± 1.33	2.61 ± 1.27	2.42 ± 1.34	0.537	0.667	0.330
mRNA expression of satellite cells marker (arbitrary units)							
PAX7	5.55 ± 0.26 [a]	5.23 ± 0.37 [b]	5.44 ± 0.39	5.33 ± 0.29	0.099 [†]	0.623	0.501

[1] RES = restriction and CON = control cows. [2] The main effects of maternal nutrition (MN), sex (S) and their interaction (MNxS); [a,b] Within a variable, means differences ($p < 0.05$) or tendency (p- value between 0.05 and 0.10).

Table 4. Least square means ± standard errors for mRNA expression of adipogenic, collagen crosslinking, collagen remodeling, satellite cells,and fibro-adipogenic progenitor cells marker evaluated on the skeletal muscle of the 450d offspring (finishing phase) according to maternal nutrition and sex.

Gene	Maternal Nutrition [1]		Sex		*p*–Value [2]		
	Restriction	Control	Female	Male	MN	S	MNxS
mRNA expression of adipogenic markers (arbitrary units)							
ZFP423	2.52 ± 0.57	2.68 ± 0.62	2.54 ± 0.50	2.67 ± 0.71	0.598	0.718	0.139
C/EBPα	8.39 ± 1.91	8.82 ± 3.61	8.84 ± 3.27	8.31 ± 2.30	0.762	0.658	0.353
PPARγ	9.64 ± 1.64	8.74 ± 1.25	9.64 ± 1.47	8.49 ± 1.21	0.208	0.167	0.134
mRNA expression of collagen crosslinking markers (arbitrary units)							
LOX	4.14 ± 0.84	4.25 ± 1.31	4.42 ± 1.17	3.92 ± 0.91	0.845	0.314	0.294
P4Ha1	2.51 ± 0.26	2.69 ± 0.38	2.60 ± 0.34	2.58 ± 0.30	0.289	0.696	0.156
mRNA expression of collagen remodeling markers (arbitrary units)							
TIMP1	6.64 ± 2.97	5.93 ± 2.65	6.57 ± 2.66	6.01 ± 3.02	0.634	0.808	0.723
TIMP2	3.33 ± 0.54	3.04 ± 0.64	3.41 ± 0.57	2.94 ± 0.55	0.310	0.162	0.205
mRNA expression of satellite cells marker (arbitrary units)							
PAX7	6.66 ± 1.02	6.46 ± 0.89	6.95 ± 0.85 [a]	5.98 ± 0.76 [b]	0.638	0.042 *	0.412
mRNA expression of fibro-adipogenic progenitor cells marker (arbitrary units)							
PDGFRα	6.61 ± 2.47	7.08 ± 1.51	6.75 ± 2.48	6.98 ± 1.35	0.649	0.901	0.433

[1]RES = restriction and CON = control cows. [2] The main effects of maternal nutrition (MN), sex (S) and their interaction (MNxS); [a,b] Within a variable, means differences ($p < 0.05$) or tendency (p- value between 0.05 and 0.10).

3.5.4. mRNA Expression of Fibrogenic Markers and Collagen Content

There was a tendency (p = 0.08) of an interaction effect between maternal nutrition and sex at 30d regarding the expression of *FN*, however, males and females did not differ due to the maternal dietary treatments (Figure 2B). The expression of the fibrogenic related growth factor *TGFβ,* and the genes coding the components of the extracellular matrix (ECM), *COL1,* and *COL3* did not differ between treatments at 30d (p > 0.05; Table 3).

In the finishing phase (450d), there was a tendency of interaction between maternal nutrition and sex regarding the expression of *FN* (p = 0.08) and *TGFβ* (p = 0.09), however, males and females did not differ due to the maternal dietary treatments (Figure 3A,B). Furthermore, there was a significant interaction effect among maternal nutrition and sex regarding the expression of *COL1* (p = 0.04) and *COL3* (p = 0.02), without differences between females and males from both treatments (Figure 3C,D).

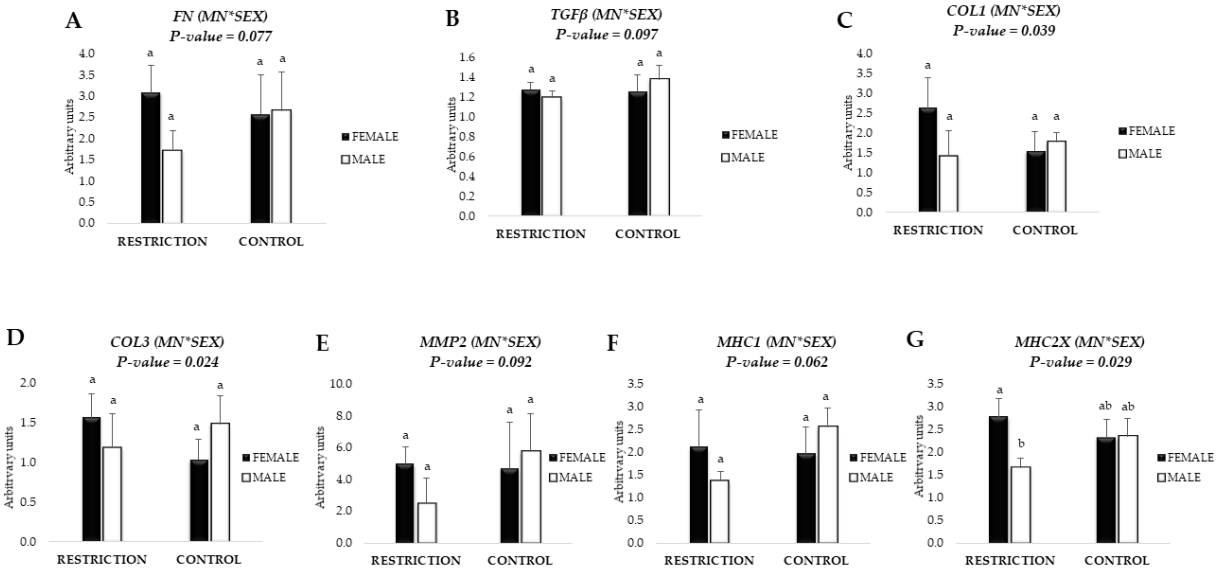


Figure 3. Interaction effect between maternal treatment (CON vs. RES) and offspring's sex (FEMALE vs. MALE) on the relative gene expression of *FN* (A), *TGFβ* (B), *COL1* (C), *COL3* (D), and *MMP2* (E), *MHC1* (F), and *MHC2X* (G) at 450d. Different letters indicate differences among groups. Tendency was considered when 0.10 < p > 0.05 and significant differences when p < 0.05.

3.5.5. mRNA Expression of Markers related to Collagen Crosslinking and Remodeling

There was a significant interaction effect between maternal nutrition and sex regarding the *LOX* expression (p = 0.02) at 30d, where females from restriction group (RES) had higher expression of *LOX* compared with males from the same experimental group (Figure 2C). No difference was observed in the expression of *P4Ha1* (p = 0.41) between treatments at 30d (Table 3) In the finishing stage (450d), no differences (p > 0.05) were observed in the expression of the markers related with collagen crosslinking, *LOX* and *P4Ha1* (Table 4).

At 30d, there was no difference ($p = 0.54$) in the expression of the marker of collagen remodeling *MMP2* (Table 3). A tendency ($p = 0.09$) of interaction effect between maternal nutrition and sex was observed in the expression of *MMP2* at 450d, despite minor changes due to either treatment or sex (Figure 3E).

In both stages evaluated, there were no differences ($p > 0.05$) in the expression of the markers of collagen remodeling, *TIMP1*, and *TIMP2* between treatments (Tables 3 and 4).

3.5.6. mRNA Expression of Markers Related with Satellite Cell and Fibro-Adipogenic Progenitor (FAP) Cells

The expression of *PAX7* tended ($p = 0.09$) to be higher in RES compared with CON at 30d (Table 3). In the finishing stage (450d), the expression of *PAX7* only differed between the sexes, where females had higher expression ($p = 0.04$) than males (Table 4).

There was a tendency ($p = 0.07$) of interaction effect between maternal nutrition and sex at 30d regarding the expression of *PDGFRα*, however, males and females did not differ due to the maternal dietary treatments (Figure 2D).

In the finishing phase (450d), there was no difference ($p = 0.65$) in the expression of *PDGFRα* between treatments (Table 4).

3.5.7. mRNA Expression of Myosin Heavy Chain Isoforms

There was a tendency in interaction effect between maternal nutrition and sex regarding the expression of *MHC1* ($p = 0.08$) at 30d, without differences between females and males from both treatments (Figure 2F). At 30d, the expression of *MHC2X* tended to be higher ($p = 0.08$) in RES compared with CON, while the expression of the other isoform, *MHC2A* did not differ between treatments ($p = 0.40$; Figure 4A).

No differences ($p = 0.60$) between treatments were observed in the finishing stage, regarding the expression of *MHC2A* (Table 3; Figure 4B). There was a tendency ($p = 0.06$) in the interaction effect between maternal nutrition and sex regarding the expression of *MHC1* in finishing phase (450d), without differences between females and males from both treatments (Figure 3F). An interaction effect was also observed in the expression of *MHC2X* ($p = 0.03$) at 450d, where females from treatment RES presented higher expression of *MHC2X* than males, while in the CON group no differences were observed between females and males (Figure 3G).

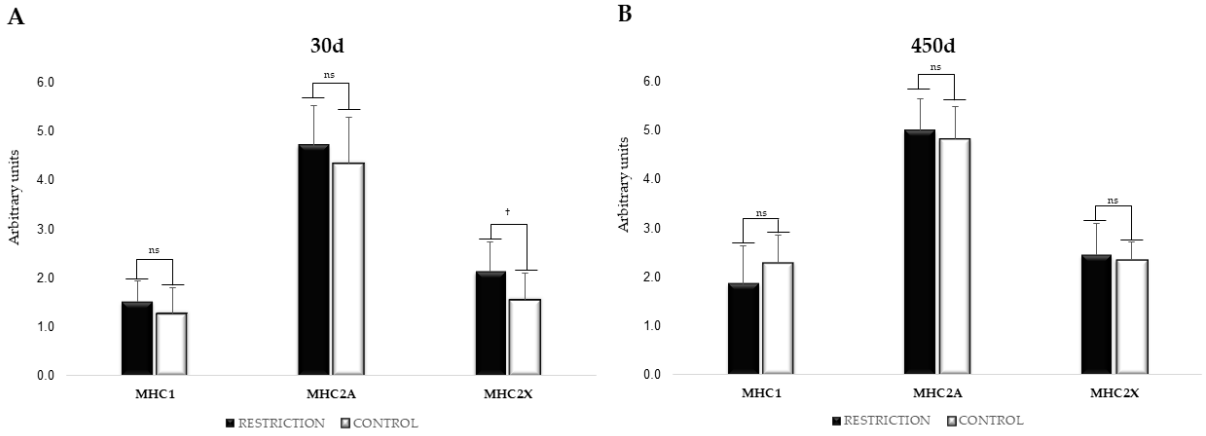


Figure 4. Relative gene expression (means ± SEM) of Myosin Heavy Chain (MHCs) isoforms in the skeletal muscle of the offspring resulting from maternal protein restriction and control group evaluated at 30d (**A**) and 450d (**B**); ns = not significant ($p > 0.10$); † tendency when $0.10 < p > 0.05$.

3.6. Discussion

The meat industry demands high quality meats, seeking high marbled carcasses, which can be partially achieved by manipulating the skeletal muscle composition from prenatal stages to the postnatal life. Although it is well established that maternal restriction during gestation impairs the skeletal muscle development of the offspring [20–22], the mechanism and the long-term effects still need to be more studied. Through the evaluation of the skeletal muscle composition in two distinct stages of offspring's life, the present study enabled the identification of differences in the number of muscle fibers, which were lower in the resulting offspring from maternal protein restriction during mid-gestation and this impairment persisted until the finishing phase. Consistent with previous studies, maternal restriction before the 210d of gestation has a major impact in muscle fibers number, due to myogenesis that begins during the embryonic stage (primary myofibers), persists in fetal stages (secondary myofibers), and slows until late gestation [7]. While maternal restriction after 210d of gestation may affect the muscle fiber size [23], which was not affected by the maternal dietary treatment in the current study. Although, muscle fiber number cannot increase after birth [24], a population of quiescent satellite cells may contribute to support postnatal growth and repair [25]. PAX7-positive cells are markers of satellite cells, which indicates the presence of undifferentiated myoblasts that have not reached their terminal differentiation [26]. Under the demand of hypertrophy, these cells may proliferate and fuse with existing muscle fibers [27]. Previous studies have shown that maternal feed restriction did not alter the expression of *PAX7* in goats [14], as well as the number of satellite cells in sheep [28]. A prenatal low protein diet resulted in reduced cell number, but the expression of *PAX7* and myogenic markers were unaltered in the skeletal muscle of young and adult rats [29]. In the current study, the expression of *PAX7* tended to be

greater in RES at 30d of age, however, no difference was observed at 450d. Similarly, maternal undernutrition tended to increase *PAX7* expression in the offspring's *Psoas major* muscle [30]. The inconsistency among studies may be due to the differences in the time of nutrient restriction. Satellite cells are formed during the late gestation [31], where no nutrient restriction was applied in our study. Another possible explanation is the diversity of cells that are able to express *PAX7,* such as vascular, immune [32], and central nervous system cells [33].

Besides muscle fiber formation, adipose and connective tissue are established during the prenatal period. The population of fibro-adipogenic progenitor (FAPs) cells holds the capacity of differentiate into adipocytes and fibroblasts, therefore, both processes may have antagonistic effects [34,35]. Marbling is a desirable meat quality parameter that depends on adipogenesis to enhance the number of intramuscular adipocytes. In cattle, adipogenesis and fibrogenesis initiate concomitantly with the secondary myogenesis during mid-gestation, however, most of adipocytes and fibroblast develops in late gestation [7]. The transcriptional factor that regulates late adipogenesis, C/EBPα and PPARγ [36] play roles on targeting and enhancing the expression of genes responsible in adipocyte gene program [37]. Since the maternal protein restriction was applied during mid-gestation in the current study, adipogenesis might not be affected in the skeletal muscle of the offspring in both stages evaluated.

Although, the expression of the cell surface marker *PDGFRα,* expressed by FAP cells [38,39], was not different between groups, fibrogenesis may be impaired in the skeletal muscle of the offspring. Fibroblasts are responsible for secreting components of connective tissue, including collagen and enzymes that catalyzes collagen cross-linking [31]. The process of collagen crosslink occurs slowly, increasing with age and contributing to the background toughness of meat [40]. The TGF-β signaling pathway is the most studied pro-fibrotic factor involved in tissue fibrosis [41,42]. After the binding of TGF-β to its receptor, the Smad complex translocates to the nucleus, recognizes the Smad-binding elements and initiates the transcription of the components of the extracellular matrix (ECM) [43], including *FN*, *COL1*, and *COL3*. At 30d the skeletal muscle of offspring resulting from maternal protein restriction, presented higher collagen content, despite the lack of difference in the expression of fibrogenic key genes. Collagen accumulation, in RES offspring at 30d may have occurred due to an adaptative mechanisms acquired in the restricted environment, which allowed the increase in this accumulation while in non-restricted conditions. Such adaptations include alteration in maternal metabolism and placenta in order to improve the efficiency of nutrient transfer to the fetus (for review, see [44]).

For further investigation of the mechanisms related to collagen content, we analyzed the key enzymes regulating collagen crosslinking and remodeling. Studies have shown that collagen content is positively correlated with collagen crosslinking and negatively correlated with collagen turnover [45]. Such correlation regarding collagen crosslinking was not observed in the present study, where the expression of the enzyme responsible for collagen crosslinking did not differ between treatments in addition to the expression of *LOX* that appears to be sex dependent at 30d animals. This could indicate that despite the accumulation, the crosslinking of collagen fibers was not altered as a result of maternal protein restriction. Collagen remodeling and consequently ECM homeostasis is mediated by the enzymes MMPs and TIMPs [46], both of which regulate collagen synthesis and degradation. MMPs degrade the majority of the ECM proteins [46,47], while the TIMPs inhibit the activity of MMPs [47], therefore balancing the tissue metabolism. The maternal restriction during the mid-gestation did not affect the overall collagen remodeling in the skeletal muscle of offspring at both stages evaluated, however the maternal treatment effect in the expression of fibrogenic markers in the skeletal muscle of the offspring at the finishing phase (450d) may be sex dependent. Moreover, the lack of visible differences in collagen content at 450d may have occurred due to a partial inefficiency in collagen remodeling.

In response to the restricted intrauterine environment, not only may the cell commitment of the skeletal muscle components be altered, but also the use of energy sources. The relative proportion of muscle fiber types varies depending on species, muscle function, breed, gender, age, among others [48]. Type I fibers are characterized by the slow speed of contraction, and by the oxidative (aerobic) metabolism, which uses fatty acids as the main energy source [49]. Meanwhile, type II fibers are classified as fast speed contraction fibers, which use glucose as the main energy source [49]. In the current study, maternal protein restriction during mid-gestation tended to increase the expression of *MHC2X* in the skeletal muscle of the offspring at 30d, implying a predominantly glycolytic metabolism, however at 450d there were no differences in the expression of *MHC2X* compared to CON group. These data are consistent with previous findings [50–52], where maternal undernutrition contributed for the increase in type II muscle fibers in the skeletal muscle of the offspring. However adequate postnatal diet and environment allowed the fiber type switching to occur [28,50].

3.7. Conclusions

In conclusion, our results demonstrate that maternal protein restriction during mid-gestation impairs muscle fiber formation in the skeletal muscle of offspring, by decreasing the muscle fiber number and persisting until the finishing phase. The expression of the genes that regulate adipogenesis in the skeletal muscle of the offspring was not impaired by maternal protein restriction during mid-gestation, however, the enhancement of fibrogenesis may have an impact on collagen content accumulation accompanied by a non-altered and sex dependent collagen crosslink on 30d old offspring, in addition to an inefficient collagen remodeling at the finishing stage. Moreover, protein restriction of cows during mid-gestation may have a short-term effect on offspring's muscle fiber metabolism, which does not have lasting effects on cattle at harvest.

Author Contributions:

Conceptualization, M.P.G. and M.d.S.D.; methodology, K.B.N., M.C.G., J.A.M.M. and T.C.C.; software, T.C.C. and E.B.S.; validation, T.C.C. and E.B.S.; formal analysis, T.C.C.; investigation, T.C.C., M.D. and M.S.D.; resources, M.D., M.P.G. and M.d.S.D.; writing—original draft preparation, T.C.C.; writing—review and editing, T.C.C.; visualization, T.C.C., M.D., K.B.N., M.C.G., J.A.M.M., E.B.S., M.P.G., and M.d.S.D.; supervision, M.D., M.P.G. and M.d.S.D.; project administration, T.C.C.; funding acquisition, M.D., M.P.G. and M.d.S.D. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Animal Care and Use Committee of the Department of Animal Science at the Universidade Federal de Lavras, Lavras, Minas Gerais, Brazil (protocol 015/17).

Data Availability Statement

Data will be made available, upon reasonable request to the corresponding author.

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Conflicts of Interest:

The authors declare no conflict of interest.

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4. CHAPTER 4

Transcriptome changes in newborn goats' skeletal muscle as a result of maternal feed restriction at different stages of gestation[3]

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4.1. Abstract

We investigated how feed restriction at 50% of maintenance requirements during different stages of gestation affects the transcriptome of newborn goats' skeletal muscle. Fourteen pregnant dams were randomly assigned into one of the following dietary treatments: animals fed at 50% of maintenance requirement from 8-84 d of gestation and then fed at 100% of maintenance requirement from day 85 of gestation to parturition (RM, n = 6), and animals fed at 100% of maintenance requirement from 8-84 d of gestation and then fed at 50% of maintenance requirement from day 85 of gestation to parturition (MR, n = 8). At birth, samples of offspring's *Longissimus* muscle were collected for total RNA extraction and sequencing. Our data showed 66 differentially expressed (DE) genes (FDR < 0.05). A total of 6 genes were upregulated and 60 downregulated (FDR < 0.05) in the skeletal muscle of the newborns resulting from treatment RM compared with MR. Our results suggest that the DE genes upregulated in newborn goats' skeletal muscle from the RM group compared to MR, included genes related to satellite cells, and genes that indicates impaired insulin sensitivity and changes in the composition of intramuscular fat. The DE genes upregulated in newborn goats' skeletal muscle from the MR group compared to RM, are also related to impaired insulin sensitivity, as well as a predominantly oxidative metabolism and cellular oxidative stress. However, protective mechanisms against insulin sensitivity and oxidative stress may have been augmented in the skeletal muscle of offspring from MR treatment compared to RM, in order to maintain cellular homeostasis.

Keywords: Caprine, fetal programming, RNA-seq, muscle metabolism, undernutrition

4.2. Introduction

Fetal programming is based on the hypothesis that environmental stimuli or insults during critical periods of prenatal development may result in permanent changes in the structure and metabolism of an organism, leading to long term consequences (Barker, 1992). Previous studies have shown that the skeletal muscle tissue is affected by maternal feed restriction during gestation (He et al., 2013; Zhang et al., 2015; Paradis et al., 2017). Also, skeletal muscle is one of the most dynamic and plastic tissues (Frontera and Ochala, 2015), extremely susceptible to variations in nutrient supplies during the prenatal stage compared to other vital tissues. The early to mid-gestation is an important period for fetal skeletal muscle development, due to the increase of muscle cells number (hyperplasia), which develops exclusively during the prenatal stage. Concomitantly with secondary myogenesis, adipogenesis begins during mid to late

gestation, and although there is evidence of an increase in fat cells after birth, the density of pluripotent cells decreases over time (Du et al., 2010).

Energy metabolism in the offspring's skeletal muscle may also be impaired by maternal nutrition during gestation (Yang et al., 2016), leading to changes in metabolic flexibility, altering the substrate sources for ATP synthesis. These skeletal muscle adaptations are consistent with the hypothesis of the "thrifty phenotype", which postulates that when fetal nutrition is poor, an adaptive response occurs that leads to altered metabolism (Hales and Barker, 2001). For example, Selak et al. (2003) observed a mitochondrial deficiency, which contributes to the decrease of ATP synthesis and compromises glucose transport and utilization in the skeletal muscle of intrauterine growth-retarded rats.

Besides the numerous studies demonstrating alteration in several signaling pathways in the offspring's skeletal muscle, changes in maternal nutrition occurring at different gestational stages can lead to different physiological outcomes in the offspring (Moisá et al., 2015). In this context, real-time qPCR tools are frequently used to determine the relative expression of specific target genes. However, this targeted approach, does not allow for the identification of a global set of genes. Thus, sequencing-based methods, such as RNA-sequencing (RNA-seq) are powerful tools which allow us to evaluate a set of transcripts (transcriptome) in a given tissue, and quantify the differentially expressed (DE) genes during development and under different conditions (Wang et al., 2009). Therefore, RNA-seq can be an advantageous tool that can provide more comprehensive knowledge and identify potentially new mechanisms altered by maternal feed restriction observed in the offspring's skeletal muscle.

Our previous study did not identify phenotypic differences in the offspring resulting from maternal feed restriction between the experimental groups (Costa et al., 2019), however, molecular mechanisms may have sustained compensatory growth and contributed to the lack of physical differences. In this context we hypothesized that maternal feed restriction during different stages of gestation alters the transcriptome profile in the skeletal muscle of newborn goats. Thus, the objective of this study was to utilize RNA-seq to identify the global differential gene expression profiles and biological processes of the skeletal muscle in newborn goats affected by maternal feed restriction during the first and second half of gestation.

4.3. Material and methods

4.3.1. Animals and sampling

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 number 09/2017).

The experiment was a continuation of a study investigating the effects of maternal feed restriction during different stages of gestation on the skeletal muscle of the offspring previously described in Costa et al. (2019). Briefly, 14 nulliparous dairy goats (Saanen), weighing 50 ± 13 kg, at 19 ± 7 months of age (mean ± SD) were submitted to estrus synchronization and artificially inseminated using semen from a single male (Saanen). The day of insemination was considered day 0 of gestation. The dams were housed in individual pens and submitted to an adaptation period of 7 d receiving the experimental diet and water *ad libitum*. After the adaptation period, the dams were randomly assigned into one of the following dietary treatments: animals fed at 50% of maintenance requirement from 8-84 d of gestation and then fed at 100% of maintenance requirement from day 85 of gestation to parturition (term ~ 150d; RM, n = 6), and animals fed at 100% of maintenance requirement from 8-84 d of gestation and then fed at 50% of maintenance requirement from day 85 of gestation to parturition (MR, n = 8). Experimental diets were adjusted weekly based on the body weight and gestational age of the dams and consisted in 111.6 g/kg of crude protein (CP) and 676 g/kg of total digestible nutrients (TDN) on dry matter (DM) basis, composed of corn silage (723 g/kg DM basis), soybean meal (96 g/kg DM basis), ground corn (165 g/kg DM basis) and mineral mixture (16 g/kg DM basis), considering the nutritional requirements for pregnant dairy goats (NRC, 2007).

After birth, male newborn goats (n = 14) were immediately separated from the dams and following approved guidelines stunned using a non-penetrating captive bolt pistol, and exsanguinated. In the case of twins, we selected the heaviest newborn goat. Skeletal muscle samples were collected from the *Longissimus thoracis* muscle and stored in liquid nitrogen for further RNA extraction and sequencing.

4.3.2. RNA extraction, library generation and sequencing

Total RNA was extracted using RNeasy Fibrous Tissue Mini Kit (Qiagen Inc., Germantown, MD, USA) following the manufacturer's recommendation. The RNA quantification and integrity were determined by Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). Samples with RNA integrity number (RIN) higher

than 8 (n = 14) were sent to Novogene (Sacramento, CA, USA) for library preparation and sequencing on an Illumina HiSeq4000 instrument, following the 150 bp paired-end protocol. An average of approximately 23 million raw reads/sample (150 bp paired-end reads) were generated (Table 1).

4.3.3. Quality control and assembly

The quality control of raw sequences was evaluated by the FastQC program (Andrews, 2010) and poor sequences were filtered with the Trimmomatic software version 0.36 (Bolger et al., 2014). The illumina adapters were removed, allowing 2 seed mismatches, a palindrome clip threshold of 30, and a simple clip threshold of 10. To remove low quality reads, we used a sliding window trimming that scanned 4 bases each and removed them when the Phred score average was bellow 15. Short reads were also removed. Reads 50 bases or longer were retained after trimming. After the quality control measures, an average of 21 million trimmed reads/sample remained, corresponding to 93% of raw reads (Table 1).

Trimmed reads were aligned to the goat reference genome (*Capra hircus*, assembly ARS1) available at NCBI (www.ncbi.nlm.nih.gov) with ID GCA_001704415.1 using TopHat 2.1.1 software (Trapnell et al., 2009) with Bowtie2 (Langmead and Salzberg, 2012) for alignment. An average of 76% of trimmed reads were successfully mapped (Table 1).

Table 1. Summary of sequenced reads, trimmed reads and mapped reads to the goat reference genome.

Treatment	Sample	Total Reads	Trimmed Reads	% Trimmed Reads	Mapped Reads	% Mapped Reads
MR	1	22,656,017	21,344,634	94.2	F: 17,401,220 R: 16,797,503	81.5 78.7
	2	23,128,719	20,057,993	86.7	F: 15,560,823 R: 15,051,444	77.6 75.0
	3	21,494,772	20,199,413	94.0	F: 15,395,237 R: 14,913,742	76.2 73.8
	4	28,790,577	27,141,345	94.3	F: 20,624,787 R: 19,967,572	76.0 73.6
	5	21,449,097	20,295,123	94.6	F: 17,479,414 R: 16,903,700	86.1 83.3
	6	18,219,469	17,046,120	93.6	F: 12,508,113 R: 4,842,492	73.4 28.4
	7	25,875,331	24,188,442	93.5	F: 19,890,066 R: 19,251,243	82.2 79.6
	8	27,355,478	25,047,818	91.6	F: 18,877,288 R: 18,189,586	75.4 72.6
RM	1	20,195,795	19,068,867	94.4	F: 15,238,057 R: 14,766,121	79.9 77.4
	2	23,001,254	21,695,246	94.3	F: 17,309,988 R: 16,718,451	79.8 77.1
	3	21,389,732	20,072,421	93.8	F: 16,067,259 R: 15,505,893	80.0 77.2
	4	21,483,827	20,191,419	94.0	F: 16,017,808 R: 15,489,336	79.3 76.7
	5	25,402,784	23,967,043	94.3	F: 19,699,811 R: 19,017,998	82.2 79.4
	6	25,070,539	23,443,609	93.5	F: 18,109,868 R: 17,540,294	77.2 74.8

MR = treatment maintenance-restriction; RM = treatment restriction-maintenance; F = forward; R = reverse

4.3.4. Differential expression analysis

The Cuffdiff tool (Cufflinks 2.2.1) was used to count reads, normalize transcript expression by fragments per kilobase of transcript model per million reads mapped (FPKM), and identify the differentially expressed genes between treatments. Input included the mapped reads, the indexed genome (generated by the Bowtie2 software) and the reference annotation file. Cuffdiff uses a model for fragment counts based on the beta negative binomial distribution to control cross-replicate variability and read mapping ambiguity. The *P*-values reported by Cufffdiff were corrected for multiple testing by using the false discovery rate (FDR) method (Benjamini and Hochberg, 1995), and differentially expressed (DE) genes were deemed significant when FDR < 0.05. The data quality assessment of principal component analysis (PCA) from the RNAseq analysis was performed using R package (v 3.4.4) (Team, 2013) and plotted using the GraphPad Prism software (version 5.01 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com).

4.3.5. Gene ontology (GO) and network analyses

Gene Ontology (GO) enrichment and network analyses were built using the ClueGo 2.5.3 (Bindea et al., 2009) application from Cytoscape 3.7.2 (https://cytoscape.org). We assessed the enriched biological processes shared by the DE genes identified in both treatments based on an unilateral hypergeometric test and Benjamini-Hochberg correction (Bei and Hong, 2013; Lewin and Grieve, 2006). Only the biological processes and molecular functions identified with a FDR < 0.05 were considered. The edges connecting the nodes were based on the Kappa statistic (Kappa score = 0.4) (Huang et al., 2009; McHugh, 2012).

4.4. Results

4.4.1. Differential gene expression in newborn goats' skeletal muscle

From a total of 20,359 transcripts obtained in the skeletal muscle of newborn goats, 3.8% (766) and 5.6% (1,146) of the transcripts were found exclusively in the treatments RM and MR, respectively. These exclusive genes were not explored in the present study, due to the fact that the GO, pathways, and network analyses showed that the same pathway (KEGG pathway) was enriched in both treatments, while no GO was associated to the exclusive genes. Despite the difference in the pools of exclusive genes between the treatments RM and MR, the pathway analysis indicated that neuroactive ligand-receptor pathways (oas04080) is enriched in both treatment groups (RM FDR = 0.0013; MR FDR = 2.35E-06). For differential expression analyses, we tested a total of 18,447 transcripts (90.6% from the total) corresponding to the common transcripts present in both treatments. By using a FDR adjusted cutoff of 0.05, a total

of 66 genes showed differential expression between the treatments (Table 2). Particularly, six genes were upregulated and 60 downregulated in RM compared with MR (Table 2; Fig. 1)

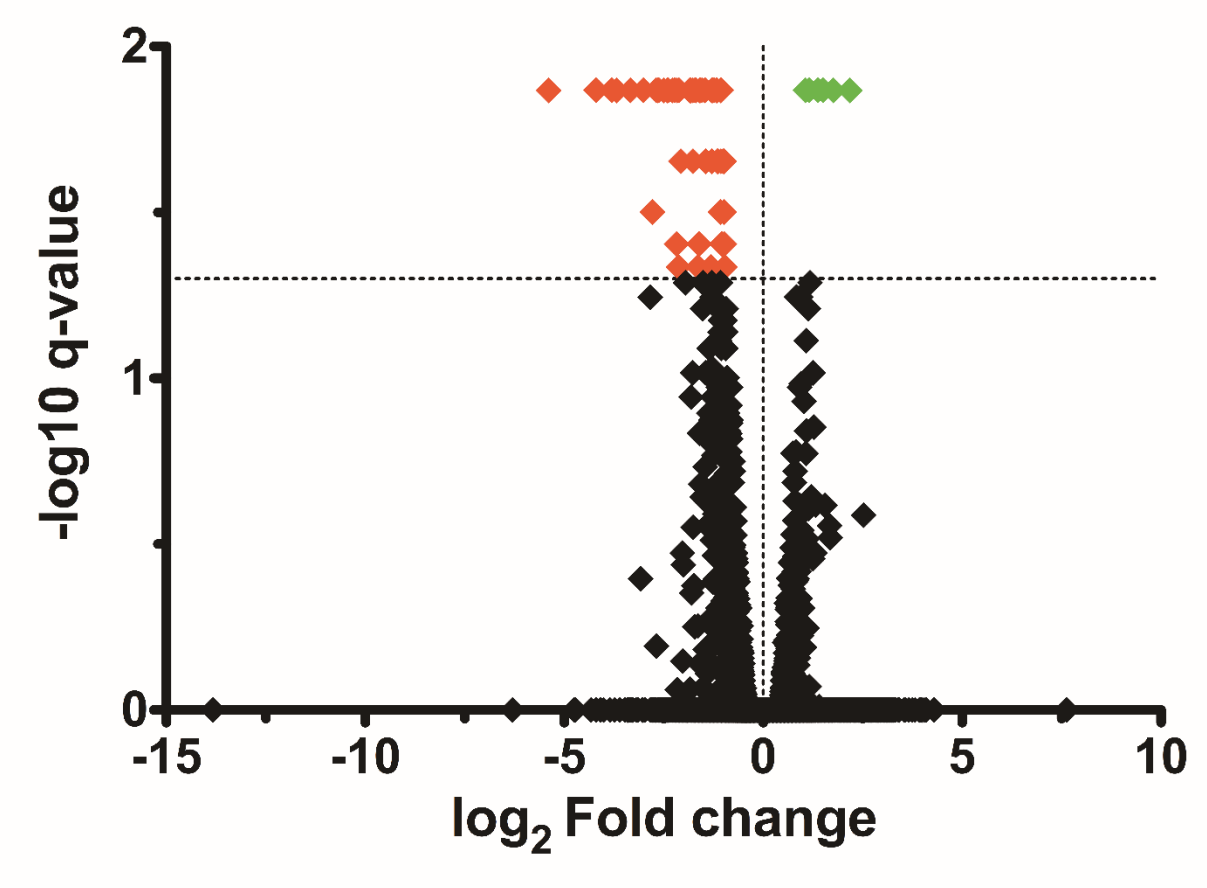

Fig. 1 Volcano plot comparing gene expression fold changes (FC) between treatments (ratio RM /MR). Differentially expressed (DE) genes (FDR < 0.05) are highlighted in red and green. DE genes highlighted in green are upregulated and DE genes highlighted in red are downregulated**.**

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Table 2. Differentially expressed genes in the skeletal muscle of the offspring.

Gene symbol	NCBI ID	Gene description	MR (FPKM)	RM (FPKM)	log2(fold change)[1]	FDR[2]
CYTL1	102190869	cytokine like 1	1.06	4.78	2.18	1.36E-02
LOC102180330	102180330	myelin P2 protein	2.21	7.51	1.77	1.36E-02
UGT8	102174638	UDP glycosyltransferase 8	0.69	1.96	1.51	1.36E-02
NPNT	102172699	nephronectin	1.49	3.86	1.38	1.36E-02
LOC108637521	108637521	uncharacterized	2.54	5.68	1.16	1.36E-02
LOC108637838	108637838	glutathione S-transferase theta-1	28.37	59.34	1.06	1.36E-02
RAB11FIP5	102186696	RAB11 family interacting protein 5	12.38	6.35	-0.96	4.62E-02
JUNB	102181201	JunB proto-oncogene, AP-1 transcription factor subunit	30.97	15.74	-0.98	3.15E-02
SLC35E4	102170260	solute carrier family 35 member E4	86.10	43.72	-0.98	2.22E-02
KLF15	102179715	Kruppel like factor 15	100.24	50.77	-0.98	3.93E-02
TRIB1	102172644	tribbles pseudokinase 1	20.20	10.21	-0.98	2.22E-02
SOX18	106502775	SRY-box transcription factor 18	43.41	21.50	-1.01	2.22E-02
HYAL2	102172212	hyaluronidase 2	27.15	13.20	-1.04	3.93E-02
ID1	102182889	inhibitor of DNA binding 1, HLH protein	46.45	22.37	-1.05	1.36E-02
MAFF	102182388	MAF bZIP transcription factor F	67.94	32.57	-1.06	2.22E-02
CREM	100861259	cAMP responsive element modulator	67.23	32.03	-1.07	1.36E-02
ADRB2	102179381	adrenoceptor beta 2	10.28	4.88	-1.08	3.15E-02
ZSWIM4	102174646	zinc finger SWIM-type containing 4	3.51	1.60	-1.14	2.22E-02
SGK1	102176607	serum/glucocorticoid regulated kinase 1	8.53	3.84	-1.15	2.22E-02
METRNL	102173882	meteorin like, glial cell differentiation regulator	26.59	11.96	-1.15	1.36E-02
PPARD	102174518	peroxisome proliferator activated receptor delta	26.81	11.86	-1.18	1.36E-02
LOC108634753	108634753	collagen alpha-1(I) chain	49.65	21.18	-1.23	1.36E-02
SIK1	102191654	salt inducible kinase 1	48.79	20.41	-1.26	1.36E-02
CNN1	102190599	calponin 1	12.88	5.37	-1.26	1.36E-02
LOC102188626	102188626	cytospin-B	11.62	4.79	-1.28	1.36E-02
LOC102184306	102184306	ras GTPase-activating protein 4	3.77	1.54	-1.29	2.22E-02
BTG2	102187789	BTG anti-proliferation factor 2	32.44	13.27	-1.29	1.36E-02
ATF3	102183991	activating transcription factor 3	100.35	40.49	-1.31	4.62E-02
SNAI3	102188886	snail family transcriptional repressor 3	3.44	1.27	-1.44	2.22E-02

ITPRIP	102190930	inositol 1,4,5-trisphosphate receptor interacting protein	13.30	4.84	-1.46	1.36E-02
RFLNB	102173226	refilin B	5.95	2.15	-1.47	1.36E-02
C15H11orf96	102181701	chromosome 15 C11orf96 homolog	63.85	22.02	-1.54	1.36E-02
PCK2	102179952	phosphoenolpyruvate carboxykinase 2, mitochondrial	6.34	2.14	-1.57	1.36E-02
FOS	102171520	Fos proto-oncogene, AP-1 transcription factor subunit	133.51	44.15	-1.60	1.36E-02
HIP1R	102178295	huntingtin interacting protein 1 related	9.33	3.05	-1.61	3.93E-02
CREB5	102190378	cAMP responsive element binding protein 5	4.32	1.39	-1.64	4.62E-02
CSRNP1	102186348	cysteine and serine rich nuclear protein 1	32.27	10.05	-1.68	1.36E-02
AMPD3	102176807	adenosine monophosphate deaminase 3	21.78	6.74	-1.69	1.36E-02
HMOX1	100860951	heme oxygenase 1	65.29	19.88	-1.72	1.36E-02
HSPA6	102185412	heat shock protein family A (Hsp70) member 6	2.46	0.73	-1.76	2.22E-02
PSPH	102184397	phosphoserine phosphatase	10.42	3.07	-1.77	1.36E-02
RRAD	102175153	RRAD, Ras related glycolysis inhibitor and calcium channel regulator	301.21	85.57	-1.82	1.36E-02
NR4A3	102188043	nuclear receptor subfamily 4 group A member 3	3.64	1.02	-1.84	1.36E-02
TNFRSF12A	102177022	TNF receptor superfamily member 12A	234.70	65.34	-1.84	1.36E-02
LOC102168687	102168687	interferon-induced protein with tetratricopeptide repeats 1	0.93	0.22	-2.07	2.22E-02
METTL21C	102180054	methyltransferase like 21C	5.87	1.35	-2.12	1.36E-02
RUNX1	102178020	RUNX family transcription factor 1	2.52	0.58	-2.13	4.62E-02
ADAMTS4	102176281	ADAM metallopeptidase with thrombospondin type 1 motif 4	2.13	0.48	-2.16	1.36E-02
TMEM154	102188161	transmembrane protein 154	1.84	0.41	-2.17	3.93E-02
SLC16A10	102181685	solute carrier family 16 member 10	8.20	1.76	-2.22	1.36E-02
TRIM63	102172216	tripartite motif containing 63	758.52	162.15	-2.23	1.36E-02
HSD17B7	102179058	hydroxysteroid 17-beta dehydrogenase 7	3.00	0.62	-2.28	1.36E-02
ARID5A	102174482	AT-rich interaction domain 5A	9.82	1.88	-2.38	1.36E-02
LOC102171344	102171344	uncharacterized	4.41	0.84	-2.40	1.36E-02
NTSR2	102186972	neurotensin receptor 2	3.85	0.73	-2.41	1.36E-02
NR4A2	102169808	nuclear receptor subfamily 4 group A member 2	6.98	1.24	-2.50	1.36E-02
RAB20	102183802	RAB20, member RAS oncogene family	41.48	6.74	-2.62	1.36E-02
LOC102175876	102175876	hemoglobin subunit beta-A-like	7.70	1.23	-2.64	1.36E-02
TNFRSF6B	102169607	TNF receptor superfamily member 6b	6.18	0.96	-2.69	1.36E-02
PTX3	102179601	pentraxin 3	10.87	1.58	-2.78	3.15E-02
TRIB3	102181514	tribbles pseudokinase 3	3.52	0.44	-3.01	1.36E-02
LOC102188072	102188072	metallothionein-2	75.95	7.52	-3.34	1.36E-02

CHAC1	102173008	ChaC glutathione specific gamma-glutamylcyclotransferase 1	6.89	0.54	-3.68	1.36E-02
BDNF	102180782	brain derived neurotrophic factor	5.55	0.40	-3.81	1.36E-02
SESN2	102175632	sestrin 2	5.48	0.30	-4.20	1.36E-02
FOSL1	102172040	FOS like 1, AP-1 transcription factor subunit	8.81	0.21	-5.39	1.36E-02

MR = Maintenance-restriction; RM = Restriction-maintenance; FPKM = Fragments per kilobase of transcript model per million reads [1]Positive and negative log2 (fold change) indicates genes up and downregulated in the treatment RM compared to MR. [2]FDR: False discovery rate; Adjusted P-value for multiple testing with the Benjamini–Hochberg procedure

4.4.2. Gene set enrichment analyses

We performed enrichment analyses of the DE genes, in order to assess the biological processes (BP), and molecular functions (MF) that could be differentially regulated between treatments. Upregulated genes (FDR < 0.05) did not share common BP or MF, and therefore, individual gene function will be further discussed. The six upregulated (FDR < 0.05) genes in RM in relation to MR, were: *CYTL1* (cytokine like 1), *LOC102180330* (myelin P2 protein), *LOC108637521* (uncharacterized), and *LOC108637838* (glutathione S-transferase theta-1), *NPNT* (nephronectin), and *UGT8* (UDP glycosyltransferase 8).

From the 60 downregulated genes (FDR < 0.05) in RM in relation to MR, 21 genes were assigned to BP, sub BP, and MF, according to the previous established criteria in ClueGO (FDR< 0.05; Kappa score = 0.4). Significant BP (FDR < 0.05; Table 3) are related to skeletal muscle tissue development (*ATF3, BTG2*, *FOS, MAFF*, and *METTL21C*), muscle cell proliferation (*CNN1, HMOX1, NR4A3*, *PPARD*, and *TRIB1*), gluconeogenesis (*ATF3*, *LOC102191654, PCK2*, and *SESN2*), cellular response to metal ion (*FOS*, *HMOX1*, *JUNB*, and *LOC102184306*), regulation of extrinsic apoptotic signaling pathway via death domain receptor (*ATF3*, *HMOX1*, and *ITPRIP*) and positive regulation of myeloid leukocyte differentiation (*FOS*, *TRIB1*, and *RUNX1*; Fig. 2). The enriched sub BP (FDR < 0.05; Table 3) are related to fatty acid oxidation (*NR4A3, PPARD*, and *SESN2*), glucose transmembrane transport (*NR4A3, SESN2*, and *TRIB3*), regulation of smooth muscle cell proliferation (*CNN1*, *HMOX1, NR4A3*, and *TRIB1*), regulation of extrinsic apoptotic signaling pathway (*ATF3*, *HMOX1*, *HYAL2*, *ITPRIP*, and *TNFRSF12A*) and positive regulation of extrinsic apoptotic signaling pathway (*ATF3*, *HYAL2*, and *TNFRSF12A*; Fig. 2). The enriched MF (FDR < 0.05; Table 3) are related to nuclear receptor activity (*NR4A2, NR4A3*, and *PPARD*), and protein kinase inhibitor activity (*HYAL2*, *ITPRIP*, and *TRIB1;* Fig. 2). The downregulated genes, represented as triangles, bonded with their respective enriched biological process and molecular function, represented as circles, can be better visualized in Fig. 2.

Table 3. Biological processes and Molecular functions associated with the downregulated genes in the skeletal muscle of the offspring from treatment RM compared to the treatment MR.

GO ID	GO Term	FDR[1]	Associated Genes[2]
Biological Process			
GO:0033002	muscle cell proliferation	1.54E-04	[CNN1, HMOX1, NR4A3, PPARD, TRIB1]
GO:0048660	regulation of smooth muscle cell proliferation	2.08E-04	[CNN1, HMOX1, NR4A3, TRIB1]
GO:0006094	gluconeogenesis	2.29E-04	[ATF3, LOC102191654, PCK2, SESN2]
GO:0007519	skeletal muscle tissue development	2.29E-04	[ATF3, BTG2, FOS, MAFF, METTL21C]
GO:1902041	regulation of extrinsic apoptotic signaling pathway via death domain receptors	3.81E-04	[ATF3, HMOX1, ITPRIP]
GO:2001238	positive regulation of extrinsic apoptotic signaling pathway	3.94E-04	[ATF3, HYAL2, TNFRSF12A]
GO:0071248	cellular response to metal ion	4.08E-04	[FOS, HMOX1, JUNB, LOC102184306]
GO:2001236	regulation of extrinsic apoptotic signaling pathway	4.21E-04	[ATF3, HMOX1, HYAL2, ITPRIP, TNFRSF12A]
GO:0002763	positive regulation of myeloid leukocyte differentiation	6.95E-04	[FOS, RUNX1, TRIB1]
GO:0019395	fatty acid oxidation	1.33E-03	[NR4A3, PPARD, SESN2]
GO:1904659	glucose transmembrane transport	1.33E-03	[NR4A3, SESN2, TRIB3]
Molecular Function			
GO:0004860	protein kinase inhibitor activity	3.98E-04	[HYAL2, ITPRIP, TRIB1]
GO:0004879	nuclear receptor activity	3.98E-04	[NR4A2, NR4A3, PPARD]

[1]FDR: False discovery rate; Adjusted P-value for multiple testing with the Benjamini–Hochberg procedure.[2]Downregulated genes in treatment RM compared to MR treatment.

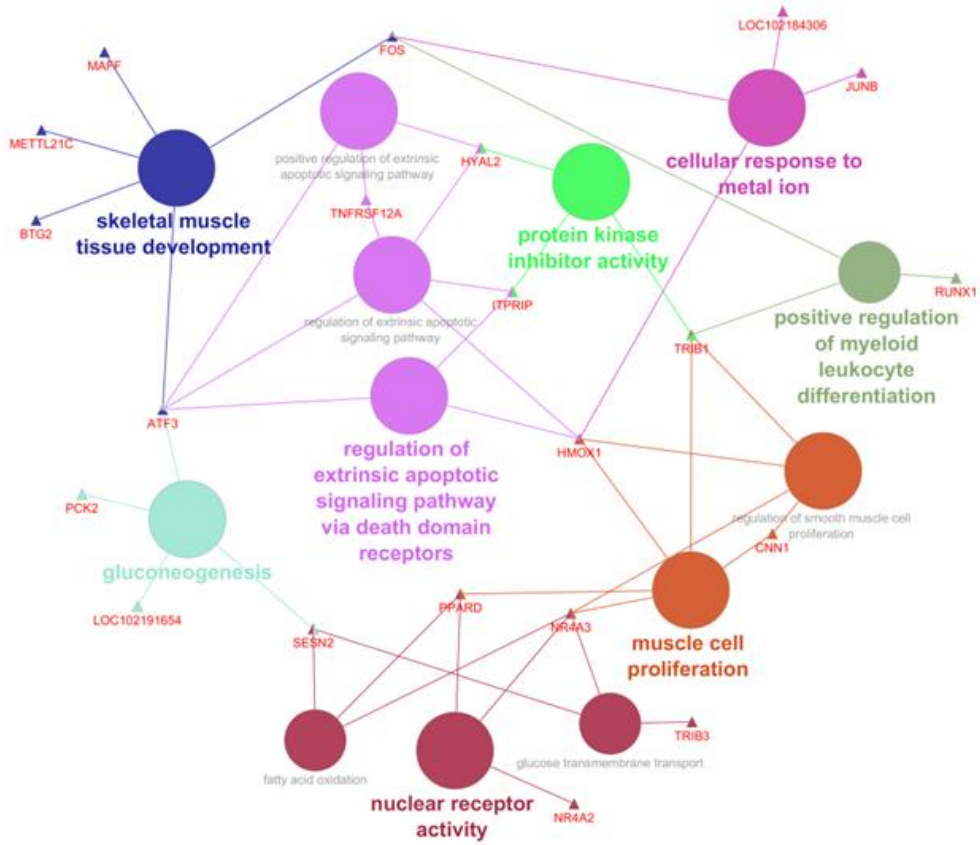

Fig. 2 Analyses of the enriched biological process and molecular function the downregulated genes in treatment RM compared to treatment MR. Network connecting the downregulated genes (triangles) with the enriched biological processes (circles). The node colors represent the functional group and nodes size represents the term enrichment significance. The most significant (FDR < 0.05) term in the group is labeled with colorful and bold letters, while sub biological processes are labeled with black and small letters.

4.5. Discussion

Maternal plane of nutrition programs fetal growth and development, through epigenetic modulation, leading to changes in gene or protein expression. Changes in the offspring phenotype may vary according to the type and extent of maternal insult during gestation. Studies have suggested that an adequate plane of nutrition during gestation after a period of restriction may sustain a state of compensatory growth and normalize the phenotype between restricted and non-restricted offspring (Costa et al., 2019; Gonzalez et al., 2013). Although there may be no apparent fetal phenotypic responses to maternal feed restriction, the offspring may show adaptation mechanisms at molecular levels (Paradis et al., 2017). In the current study, the RNA-seq technology allowed the identification of DE genes in the skeletal muscle of the offspring from dams which experienced two different feed restriction protocols during gestation. The effect of maternal feed restriction experienced by the experimental units of the current study, was previously reported by Costa et al. (2019). The dams which experienced feed restriction during the first or second half of gestation presented loss in the average daily gain of maternal tissues in the respective periods of restriction (Costa et al., 2019). Since the newborns were harvested soon after birth for sampling, the discussion is based on sequencing data of the newborns' skeletal muscle, in order to understand which molecular mechanisms are affected depending on the feeding restriction period and possibly predict postnatal phenotype.

Among the DE genes, *CYTL1*, *NPNT,* and *UGT8* were upregulated in the skeletal muscle of the offspring from treatment RM compared to MR. *CYTL1* is widely expressed in a variety of cells, including CD34+ hematopoietic stem cells (Yang et al., 2018). Beauchamp et al. (2000) showed that CD34 is also present in the majority of quiescent skeletal muscle satellite cells. When required, satellite cells undergo the process of activation, proliferation, differentiation, and fusion (Chargé and Rudnicki, 2004). During the process of differentiation, the abundance of NPNT, an extracellular matrix protein involved in cellular adhesion is increased and it stimulates the myoblast fusion (Sunadome et al., 2011). The upregulation of *CYTL1* in the offspring resulting from mothers that experienced feed restriction during early-to-mid gestation (RM) in comparison to MR may have provided greater number of quiescent satellite cells and the upregulation of *NPNT* may contribute to myoblast fusion and consequently form new myofibers later in life. Moreover, it was also demonstrated that *CYTL1* and *NPNT* may contribute to the level and composition of fatty acid in the skeletal muscle. Low marbled meat presented high expression of *NPNT* (Clark et al., 2011). While the downregulation of *CYTL1* was observed in broilers that displayed high polyunsaturated fatty

acids percentage in the skeletal muscle (Yang et al., 2018). Thus, the upregulation of *CYTL1* and *NPNT* may contribute to the decrease in the intramuscular fat deposition accompanied by the decrease in polyunsaturated fatty acid concentration in the skeletal muscle of the offspring from RM compared to MR group.

Evaluating the effects of calorie restriction in rats' skeletal muscle, Obanda et al. (2015) observed that, although there was no reduction of lipid deposition in the skeletal muscle of calorie-restricted rats, sphingolipids metabolism was altered and caused effects in insulin sensitivity. The enzyme UDP glycosyltransferase 8 (*UGT8*) catalyzes the transfer of galactose to ceramide to synthesize the sphingolipids galactosylceramide (GalCer) (Marcus and Popko, 2002), which is negativelly correlated with insulin sensitivity and positively with pro-inflammatory responses (Pillon et al., 2018). In this context, the upregulation of *UGT8* in the skeletal muscle of RM offspring compared to MR group, could cause insulin resistance, which can be characterized for many factors including impaired glucose transport, glucose phosphorylation, and reduced glucose oxidation and glycogen synthesis (Abdul-Ghani and Defronzo, 2010). The enzyme responsible for glucose phosphorylation in the skeletal muscle is HKII. Although, insulin resistance would lead to decrease HKII activity, our previous study (Costa et al., 2019) showed that *HKII* mRNA abundance was increased in the treatment RM compared to MR. Due to such inconsistency the main role of the gene *UGT8* in the skeletal muscle of feed restricted offspring needs further investigations.

Among the downregulated genes in the skeletal muscle of the offspring from treatment RM compared to MR, methyltransferase like 21C (*METTL21C*) is involved in the biological process of skeletal muscle tissue development. The protein methyltransferases perform multiple functions in the muscle, modifying histones and cytoplasmic proteins (for review see: Clarke, 2013). Elevated abundance of the protein METTL21C regulates protein turnover during various situations, such as, hypertrophy and starvation (Wiederstein et al., 2018). There is evidence that *METTL21C* expression is absent during myogenesis, is limited to mature type I myofibers (Wang et al., 2019a), and also the protein encoded by the gene *METTL21C* correlates positively with the amount of slow type I fibers (Wiederstein et al., 2018), which have oxidative characteristics. The lack of difference between treatments regarding the expression of the marker of slow type I fibers observed in our previous study (Costa et al., 2019) may be due to the molecular markers that was chosen (*MYH1*), since *METTL21C* expression is restricted to slow MYH7-positive muscle fibers (Wiederstein et al., 2018). Regarding muscle fiber types, the nuclear transcription factor peroxisome proliferator-activated delta (*PPARδ*),

downregulated in skeletal muscle of the offspring from treatment RM compared to MR participates in biological processes related to muscle cell proliferation and fatty acid oxidation. Specifically, the protein encoded by *PPARδ* regulates the skeletal muscle fiber phenotype, coordinating the increase in oxidative enzymes and mitochondrial biogenesis, and, consequently, an increase in the proportion of slow type I fibers (Wang et al., 2004). Moreover, PPARδ regulates the transcription of the mitochondrial phosphoenolpyruvate carboxykinase 2 (*PCK2*) (Idrees et al., 2019), also downregulated in the skeletal muscle of the offspring from treatment RM compared to MR. *PCK2* is related to the gluconeogenesis biological process and is an important enzyme that catalyzes the conversion of mitochondrial oxaloacetate to phosphoenolpyruvate, which is involved in glucose synthesis (gluconeogenesis) or in generating precursors for triglyceride synthesis (glyceroneogenesis) (Beale et al., 2007). Hence, PCK2 plays an important role in both energy metabolism and lipid homeostasis. Taken together, the skeletal muscle of the offspring resulting from maternal feed restriction during mid-to-late (MR) gestation, may have a greater proportion of oxidative myofibers compared to RM. Moreover, the percentage of number and area of type I fibers was positively correlated with intramuscular fat (IMF) content (Joo et al., 2017), which would possibly contribute for increase in IMF content and consequently the improvement in meat quality in MR offspring compared to RM group.

Among the downregulated genes in RM compared to MR, tribbles homolog 3 (*TRIB3*) and tribbles homolog 1 (*TRIB1*) genes are related with the glucose transmembrane transport and muscle cell proliferation biological processes, respectively. Both increases and decreases in glucose concentration in a skeletal muscle cell line were capable of enhancing the expression of tribbles homolog 3 (TRIB3), considered an energy sensor (Liu et al., 2010). Additionally, TRIB3 overexpression inhibits the downstream insulin signaling pathway, reducing the glucose transport system (Liu et al., 2010). As a consequence of impaired glucose oxidation, TRIB3, and TRIB1 contribute to the modulating of skeletal muscle differentiation and lipid metabolism (Prudente et al., 2012). Unlike *TRIB3*, other mechanisms that optimize glucose utilization in order to return to energy homeostasis appear to be activated in the skeletal muscle of the offspring in response to maternal feed restriction during mid-to-late gestation (MR). This is the case for the orphan nuclear receptor subfamily 4 group A member 3 (*NR4A3*) gene, downregulated in RM compared to MR, which is involved in the regulation of genes that control glucose and fatty acid utilization in the skeletal muscle, in a way that improves insulin sensitivity, glucose tolerance and transport and also results in reduced fat deposition (Zhang et

al., 2014). Thus, the mechanisms involved by *TRIB3* of reducing glucose transport and oxidation accompanied by the action of *NR4A3* which in contrast improves glucose utilization may characterize an attempt to reach energy balance in the skeletal muscle of the offspring from MR compared to RM group.

The high metabolic capacity of skeletal muscle makes it susceptible to oxidative stress, characterized by an imbalance between reactive metabolites (ROS) and antioxidants, leading to cellular damage and altering production of macromolecules including lipids, DNA, and proteins (Meng and Yu, 2010). Also, increased ROS production impairs the regenerative capacity of satellite cells (Fulle et al., 2004). A number of downregulated genes in RM compared to MR are associated with oxidative stress response and protective mechanisms that ensure cell survival. The expression of B-cell translocation 2 (*BTG2*), and Tumor necrosis factor (TNF) receptor superfamily member 12A (*TNFRSF12A*) appears to be associated with the increase in cellular oxidative stress, while Sestrin2 (*SESN2*), Heme oxygenase-1 (*HMOX1*), and the transcriptional factors *MAFF*, *FOS* and *JUNB* are known to be activated in response to oxidative stress and their activation results in decreased ROS production and promote cell survival. BTG2 expression is upregulated during oxidative stress (Imran and Lim, 2013; Rouault et al., 1996), has an antiproliferative role, controlling cell-cycle, apoptosis, and differentiation (Yuniati et al., 2019). Apoptosis mediated by BTG2 appears to take place through activation of the nuclear factor kappaB (NFκB) pathway (Imran and Lim, 2013). Upstream of the NFκB pathway, signaling transduction can be initiated by the activation of the transmembrane family receptors TNFRSF12A (Blanco-Colio, 2014). In differentiating muscle cells, NFκB and activating protein -1 (AP-1) transcription factor family, that consists of JUN, FOS, MAF and ATF are activated in response to oxidative stress (Zhou et al., 2001). The induction of SESN2 mediated by oxidative stress, DNA damage, hypoxia, and nutritional stress, triggers homeostatic mechanisms such as the downregulation of ROS accumulation (Wang et al., 2019b). Moreover, the biological effects of HMOX1 are associated with a decrease in ROS production, improvement of cell survival, and proliferation under oxidative stress (Kozakowska et al., 2012). Due to the increased expression of established markers of cellular protective pathways against excessive oxidative stress in the skeletal muscle of MR newborns, we hypothesize that they experience higher levels of oxidative stress, and therefore activate protective mechanisms against it, when compared to the skeletal muscle of newborns resulting from maternal feed restriction during early-to-mid gestation.

4.6. Conclusion

In summary, the findings of the present study demonstrate that maternal feed restriction during different stages of gestation alters the transcriptome of the newborn goats' skeletal muscle. It is important to highlight that feed restriction, at some point, affected the expression of genes associated with the skeletal muscle development and metabolism. Specifically, feed restriction in the first half in comparison to feed restriction in the second half of gestation may have resulted in reduced myoblast differentiation resulting in greater number of satellite cells in the postnatal period. Also, based on the upregulated genes, maternal feed restriction during the first half of gestation may have impact in insulin sensitivity, compromise intramuscular fat accumulation and fatty acid composition in the skeletal muscle of the offspring, in comparison to feed restriction in the second half of gestation.

Compared to feed restriction during the first half of gestation, the offspring resulting from maternal feed restriction during the second half of gestation also may have impaired insulin sensitivity in the skeletal muscle, however, it appears that protective mechanisms may be activated in order to improve insulin sensitivity and glucose tolerance. Moreover, feed restriction during the second half of gestation may have resulted in increased oxidative metabolism as compared to feed restriction during the first half of gestation, and generated a response against the oxidative stress, promoting cell survival and homeostasis in the skeletal muscle of the offspring.

CRediT authorship contribution statement

TCC: Methodology, Software, Validation, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing. **TAOM:** Methodology, Software, Validation, Formal analysis, Resources, Data Curation, Writing - Review & Editing. **MMSF:** Writing - Review & Editing. **MML**: Investigation, Writing - Review & Editing. **MD:** Writing - Review & Editing. **NVLS:** Investigation, Resources, Writing - Review & Editing. **LMPS:** Investigation, Writing - Review & Editing. **FB:** Writing - Review & Editing. **MFR:** Investigation, Resources, Writing - Review & Editing. **FFS:** Investigation, Resources, Writing - Review & Editing. **MPG:** Methodology, Writing - Review & Editing. **MSD:** Conceptualization, Methodology, Investigation, Resources, Supervision, Project administration, Funding acquisition.

Conflict of interest

The authors declare that they have no competing interests.

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5. CHAPTER 5

Impact of maternal feed restriction at different stages of gestation on the proteomic profile of the newborn skeletal muscle[4]

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5.1. Simple Summary

Due to forage seasonality, pregnant ruminants raised in pasture are commonly subjected to a period of feed restriction, which may lead to changes in the skeletal muscle metabolism of the offspring. Such alteration may have long-term consequences for the offspring's performance and carcass composition. In the current study, we evaluated the effects of feed restriction at different stages of gestation on the proteomic profile in the skeletal muscle of the offspring. The results showed that both periods of restriction, whether applied in the first or last half of gestation, influence muscle energy metabolism of the progeny. Specifically, the restriction in the first half of gestation influences muscle glycogen utilization, fatty acid oxidation, and the energy-investment phase of glycolysis, while the restriction in the second half of gestation influences the energy-generation phase of glycolysis and the production of glutamine in the skeletal muscle of the offspring.

5.2. Abstract

We aimed to investigate the effects of the maternal plane of nutrition during gestation on the proteome profile of the skeletal muscle of the newborn. Pregnant goats were assigned to the following experimental treatments: restriction maintenance (RM) where pregnant dams were fed at 50% of their maintenance requirements from 8–84 days of gestation, and then feed of 100% of the maintenance requirements was supplied from 85–parturition (n = 6); maintenance restriction (MR) where pregnant dams were fed at 100% of their maintenance requirements from 8–84 days of gestation, and then experienced feed restriction of 50% of the maintenance requirements from 85–parturition (n = 8). At birth, newborns were euthanized and samples of the *Longissimus thoracis* muscle were collected and used to perform HPLC-MS/MS analysis. The network analyses were performed to identify the biological processes and KEGG pathways of the proteins identified as differentially abundant protein and were deemed significant when the adjusted p-value (FDR) < 0.05. Our results suggest that treatment RM affects the energy metabolism of newborns' skeletal muscle by changing the energy-investment phase of glycolysis, in addition to utilizing glycogen as a carbon source. Moreover, the RM plane of nutrition may contribute to fatty acid oxidation and increases in the cytosolic α-KG and mitochondrial NADH levels in the skeletal muscle of the newborn. On the other hand, treatment MR likely affects the energy-generation phase of glycolysis, contributing to the accumulation of mitochondrial α-KG and the biosynthesis of glutamine.

Keywords: *Capra hircus*; energy metabolism; maternal nutrition; muscle tissue; proteome; undernutrition

5.3. Introduction

Improvement in the efficiency of livestock production has been largely demanded due to population growth, in addition to food safety, and quality. Feed restriction followed by realimentation commonly occur postnatally in ruminants as a consequence of the variability of food resource resulting in an event called compensatory growth, promoting the increase in the animal's growth rate [1]. However, feed restriction during certain periods of gestation may negatively impact the embryo and fetus development, leading to long-term consequences that may impact postnatal growth and health [2,3]. Specifically, the early gestational period in utero development is markedly characterized by fetal organ development, as well as the beginning of skeletal muscle development where primary and secondary myogenesis occurs, while the majority of intramuscular adipogenesis and fibrogenesis occur in the last half of gestation [4]. Moreover, it is well-established that muscle hyperplasia and most of the fibro-adipogenic progenitor cells are limited to the prenatal period [4]. Hence, depending on the period in which maternal feed restriction is applied, distinct biological processes may be activated or deactivated by the action of transcripts, proteins, or metabolites, modifying the whole course of cell fate in the skeletal muscle of the progeny.

Commonly, pregnant ruminants raised in pasture are subjected to a period of feed restriction, resulting from the lack of quantity and quality of forages. Hence, under this situation, maternal metabolism prioritizes the delivery of nutrients for the formation of vital tissues instead of secondary tissues, such as skeletal muscle, which is a disadvantage in animals raised for meat production [5,6]. Studies evaluating the impacts of maternal undernutrition on offspring's development, health, and performance have been reported, using targeted approaches [7] as well as sequencing-based approaches for profiling mRNAs (RNAseq) [3,8]. Although RNAseq provides important information about differentially expressed genes related to biological events comparing different experimental scenarios, the expression of mRNAs does not reflect its translation in a functional protein [9], as the mRNA transcription is only partially associated with the abundance of proteins [10]. Therefore, proteomic approaches have been demonstrated to be a useful tool in livestock studies that provides the identification of differentially abundant functional proteins, allowing the establishment of its interaction and enriched molecular pathways [11].

Given the importance of meat production for human consumption, the mechanisms involved in skeletal muscle development have been widely studied over the past years [12]. Muscle proteins are classified based on their function and solubility and can be divided into sarcoplasmic, myofibrillar, and structural proteins [13]. The sarcoplasmic fraction plays roles in regulating cellular metabolism, where the glycolytic enzymes comprise the majority of sarcoplasmic proteins [14]. Because the utilization of substrate for producing energy for skeletal muscle development is dependent on maternal nutritional status, we hypothesized that maternal feed restriction programs the skeletal muscle proteome of the offspring, based on alterations of sarcoplasmic proteins. Moreover, fractioning the protein extract allows the identification of proteins of interest that are often less expressed and hard to detect [15,16]. Besides the expansive potential of proteomics, studies linking the effects of maternal nutrition at different stages of gestation with alteration of the offspring's skeletal muscle proteome are still little explored in livestock animals. Thus, in the present study, we aimed to investigate the impact of the maternal feed restriction at different stages of gestation (first or second half) on the proteomic profile of the skeletal muscle of newborns.

5.4. Materials and Methods

5.4.1. Animals and Sampling

The Ethical Committee on Animal Use of the Department of Animal Science at Universidade Federal de Viçosa, Minas Gerais, Brazil (protocol number 09/2017) approved all the procedures prior to the beginning of the experiment.

The detailed description of the experimental management practices was previously described in [17]. Briefly, 14 nulliparous pregnant dairy goats were randomly assigned to one of the following dietary treatments: treatment RM (n = 6) consisted of feed at 50% of maintenance requirement from 8–84 days of gestation followed by feed at 100% of maintenance requirement from day 85 of gestation to parturition (~150 days), while the treatment MR (n = 8) consisted of animals fed at 100% of maintenance requirement from 8–84 days of gestation and then fed at 50% of maintenance requirement from day 85 of gestation to parturition. It is worth mentioning that effects of maternal nutrient restriction compared to feed adhering to the nutrient requirements result in the impairment of the offspring's skeletal muscle development [2,8,18,19]. Thus, the choice of the experimental design was based on the aim of evaluating what metabolic processes were likely affected by varying the maternal plane of nutrition when the restriction occurs during the gestational period.

The dams were weighed once a week and the week of gestation was taken into consideration for the adjustment of the dry matter intake, in order to meet the objective of the experimental treatments. The same diet was offered to the dams for both treatments and consisted of 111.6 g/kg of crude protein (CP) and 676 g/kg of total digestible nutrients (TDN) on a dry matter (DM) basis, which was composed of corn silage (723 g/kg DM basis), soybean meal (96 g/kg DM basis), ground corn (165 g/kg DM basis), and mineral mixture (16 g/kg DM basis). The amount of dry matter offered to the dams adhered to the nutritional requirements for pregnant dairy goats [20], meeting either 50% or 100% of their requirements according to the experimental treatments.

After birth, male newborn goats (n = 14) were immediately separated from the dams, stunned using a non-penetrating captive bolt pistol, and exsanguinated. In the case of twins (4 in RM and 5 in MR), the heaviest newborn goat was selected to be sampled. Skeletal muscle samples (~1 g) were collected from the *Longissimus* muscle and stored in liquid nitrogen for further protein extraction.

5.4.2. Protein Extraction

Sarcoplasmic protein fraction of *Longissimus* muscle was extracted from 0.1 g of tissue in 1 mL lysis buffer (20 mM Tris HCl pH 8, 5 mM EDTA, 1% 2-mercaptoethanol, and 1% protease inhibitor cocktail (Sigma-Aldrich®, San Luis, MO, USA), homogenized using a shaft-type homogenizer (LabGEN 125, Cole-Parmer, Bunker Hill, IL, USA). The homogenate was centrifuged at 20,200× *g* for 20 min at 4 °C. The supernatant was collected, aliquoted, and stored at −80 °C. Sarcoplasmic protein content was estimated by Bradford Protein Assay (Bio-Rad, Hercules, CA, USA).

5.4.3. Protein Identification and Data Processing

Proteins were digested using a solution containing 50 mM ammonium bicarbonate and 20 ug trypsin (Promega, Madison, WI, USA) overnight at 37 °C. Tryptic peptides were then dried using a SpeedVac centrifuge (AG-22331, Eppendorf, Hamburg, Germany), resuspended with trifluoroacetic acid (TFA), and desalted using Zip-Tip, according to the manufacturer's protocol. Samples were again dried in SpeedVac centrifuge (AG-22331, Eppendorf, Germany) and sent to Central Analítica of IQ-USP (São Paulo, Brazil) for protein identification and quantification performed in a NanoAquity high-performance liquid chromatographer (HPLC) coupled with a maXis 3G high-resolution Q-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). The raw data were processed with MaxQuant (v. 1.6.3.3) software with

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. For the calculation of the label-free quantification (LFQ) protein intensity, the LFQ mode was added and at least two unique peptide ratios (min LFQ ratio count = 2) were considered. The goat reference proteome used was obtained from UniProt database (ID: UP000291000) available in (www.uniprot.org) (accessed on 27 September 2019).

5.4.4. Network Analyses

The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enrichment analysis was built with String version 11.0 (string-db.org) (accessed on 1 January 2020). The interaction network of the exclusive proteins from the treatments MR and RM was obtained using the available interaction map from the closest species (*Ovis aries*), with the default option (medium confidence given by score of 0.4) and connections defined according to statistical evidence. KEGG pathways were considered significantly enriched based on adjusted FDR [21] using p-value < 0.05. For the differentially abundant proteins (DAPs), the network analysis was built in ClueGo 2.5.3 [22], a Cytoscape 3.7.2 application. The biological processes shared by the DAPs were identified based on a unilateral hypergeometric test also adjusted by FDR [23,24]. Only the biological processes identified with the adjusted p-value < 0.05 were considered for posterior analysis involving protein networks. In this context, the node colors represent the functional group; whereas the node size represents the term enrichment significance, being the additional inferences on edges connection based on the Kappa statistic (Kappa score = 0.4) [25,26].

5.4.5. Statistical Analysis

Statistical analysis was performed based on a peptide-based model through the MSqRob software (version 0.7.6) [27,28]. The peptide intensities were log2-transformed and normalized based on the robust linear regression (RLR) method. Prior to analysis, proteins identified by site, reverse, and potential contaminants were filtered and removed. The statistical model considered the fixed effects of treatments (RM and MR), the fixed effect of the number of offspring (1 or 2), and the random effect of sequence. The random effect was included to provide fixed effects estimation independently of the peptide effect. Since we aimed to investigate the treatment effects, the contrasts were made between treatments comparing RM

vs. MR, with significance based on ANOVA and posterior differentially abundant proteins (DAPs) adopting *p*-value (FDR) < 0.05.

5.5. Results

5.5.1. Differentially Abundant Proteins

Initially, a total of 415 proteins were identified (Table S1) in the skeletal muscle of newborn goats. However, after removing the proteins only identified by site, potential contaminants, and reverse sequences, a total of 386 proteins were maintained. We found 181 and 46 exclusive proteins in the treatments RM and MR, respectively (Figure 1). For DAPs, 159 proteins corresponding to common proteins present in both treatments (intercept) were tested (Figure 1).

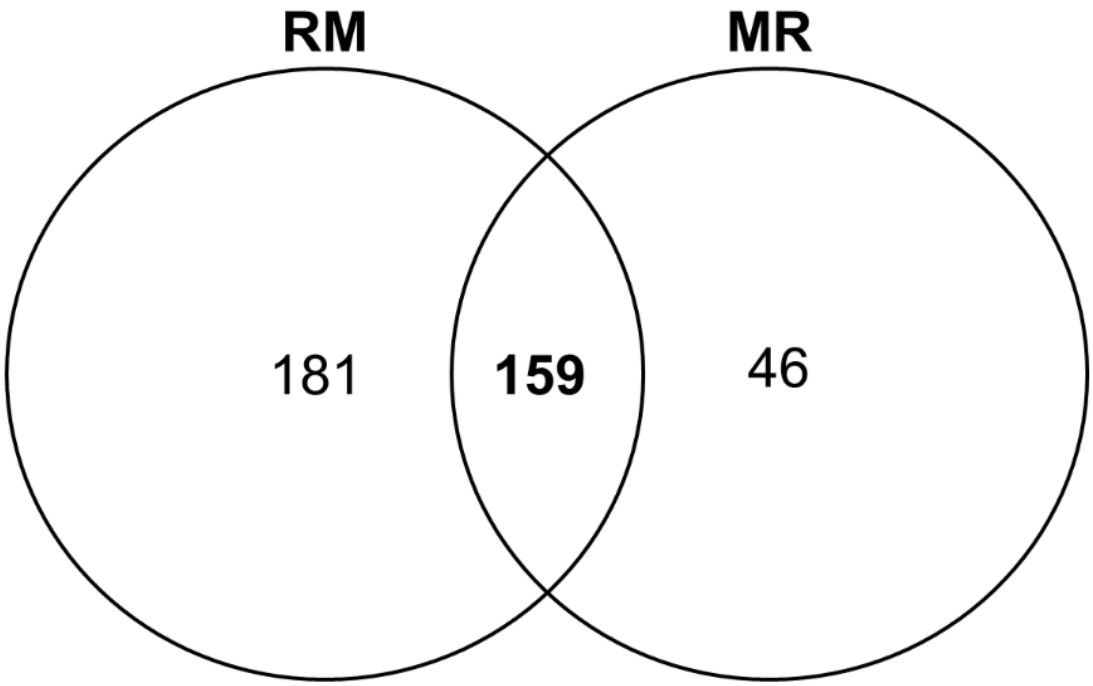

Figure 1. Venn diagram of the proteins identified. Number of proteins identified in each treatment (exclusive), the intercept containing the number of proteins common in both treatments. RM = restriction maintenance; MR = maintenance restriction.

Controlling the adjusted *p*-value (FDR) < 0.05, a total of 13 proteins were differentially abundant between treatments (Figure 2, Table 1). Among them, ATP-dependent 6-phosphofructokinase (PFKM), dihydrolipoyl dehydrogenase (DLD), lipoyl-binding domain-containing protein (DLST), S-formylglutathione hydrolase (ESD), IF rod domain-containing protein (DES), triosephosphate isomerase (TPI1), and 5 uncharacterized proteins corresponding to the genes *FLNC*, *HSPA9*, *SELEMBP1*, and *MYOM1* were more abundant in RM compared with MR (FRD < 0.05; Table 1). Calponin homology (CH) domain-containing protein

(SMTNL1) and pyruvate kinase (PKM) were less abundant in RM compared to MR (FDR < 0.05; Table 1).

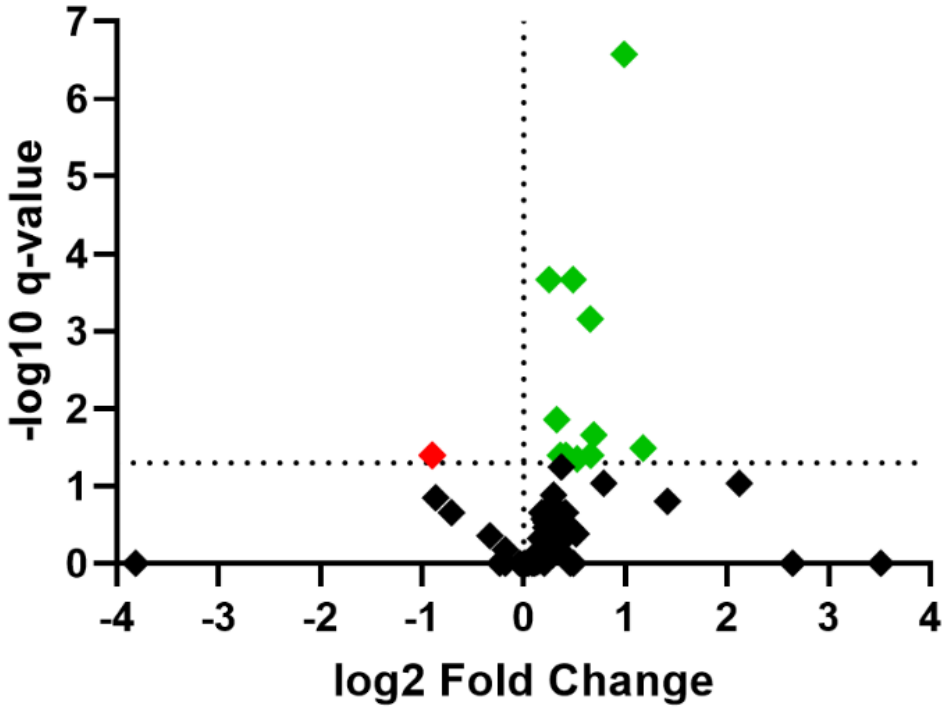

Figure 2. Volcano plot comparing protein abundance fold changes (FC) between treatments (ratio RM/MR). Differentially abundant proteins (DAPs; FDR < 0.05) are highlighted in red and green. DAPs highlighted in green are upregulated and DAPs highlighted in red are downregulated. The black squares represent the non-significant proteins (FDR > 0.05).

Table 1. Differentially abundant proteins (DAPs) in the skeletal muscle of the offspring.

Accession	Protein Name	Gene Name	FDR [1]	Fold Change [2] (RM vs. MR)
A0A452DPE6	Calponin homology (CH) domain-containing protein	SMTNL1	0.0398564	−0.90
A0A452ET82	Pyruvate kinase	PKM	0.0398564	−0.90
A0A452FHP7	Uncharacterized protein	FLNC	0.0002162	0.25
A0A452FBM2	Uncharacterized protein	HSPA9	0.0138994	0.33
A0A452G4K3	ATP-dependent 6-phosphofructokinase	PFKM	0.0398564	0.36
A0A452FWD9; A0A452FWC6	Dihydrolipoyl dehydrogenase	DLD	0.0398564	0.42
A0A452FX48I; A0A452FWP3	Uncharacterized protein	AGL	0.0002162	0.49
A0A452ESM1; A0A452ERW8; A0A452ERN3	Lipoyl-binding domain-containing protein	DLST	0.0446152	0.53
A0A452EYA9; A0A452EYB9; A0A452EY59	Uncharacterized protein	SELENBP1	0.0006971	0.66
A0A452FIG7	S-formylglutathione hydrolase	ESD	0.0398564	0.66
A0A452EJS4	Uncharacterized protein	MYOM1	0.0219753	0.69
A0A452DWL1	IF rod domain-containing protein	DES	0.0000003	0.99
A0A452ET55	Triosephosphate isomerase	TPI1	0.0321358	1.18

RM = restriction maintenance; MR = maintenance restriction; [1] FDR= false discovery rate; [2] negative and positive fold change indicates the less and more abundant proteins in the treatment RM compared to MR.

5.5.2. Protein-Protein Interaction Network and KEGG Pathways of the Exclusive Proteins

The interaction network of the exclusive proteins from treatment RM was significant (*p*-value (FDR) < 1 c 10^{-16}), while in the treatment MR, the *p*-value was higher than RM (*p*-value (FDR) < 1.8×10^{-5}); however, it was still significant, indicating that the proteins are at least partially biologically connected.

KEGG analysis from the exclusive proteins of treatment RM indicate pathways of interest related to tight junction, carbon metabolism, valine, leucine, and isoleucine degradation, mRNA surveillance pathway, fatty acid degradation, RNA transport, long-term potentiation, tryptophan metabolism, regulation of actin cytoskeleton, and citrate cycle (TCA cycle) (Table 2).

Although the proteins exclusive to MR treatment showed fewer interactions between them, interesting pathways were enriched, such as glycolysis/gluconeogenesis, citrate cycle (TCA cycle), biosynthesis of amino acids, carbon metabolism, metabolic pathways, HIF-1 signaling pathway, pyruvate metabolism, arginine and proline metabolism, and phagosome and 2-oxocarboxylic acid metabolism (Table 2).

Table 2. Enriched metabolic pathways of the exclusive proteins from treatment RM and MR.

KEGG ID	Description	FDR [1]	Protein Names [2]
			Treatment RM
oas04530	Tight junction	9.44 x 10^{-8}	**ACTN1,ACTN4, EZR, LOC443340, MSN, MYH1, MYH13, MYH4, MYH8, MYL6, OMYHC2A**, PPP2R1A**, PPP2R1B, RDX, RHOA, YWHAQ**
oas01200	Carbon metabolism	7.73 x 10^{-7}	**ACAT1, ACO1, ADH5, DLAT, ECHS1**, HADHA**, IDH1, OGDH, OGDHL ,PGD**
oas00280	Valine, leucine, and isoleucine degradation	2.98 x 10^{-5}	**ACAT1, ALDH2, ECHS1**, HADHA, **HIBADH**, HSD17B10, OXCT1
oas03015	mRNA surveillance pathway	5.52 x 10^{-5}	**EIF4A3, PABPC1, PABPC4, PPP1CA, PPP1CB, PPP1CC**, PPP2R1A, **PPP2R1B**
oas00071	Fatty acid degradation	6.03 x 10^{-5}	**ACAT1, ADH5, ALDH2, ECHS1,** ECI1, HADHA
oas03013	RNA transport	6.03 x 10^{-5}	**EEF1A2, EIF4A1, EIF4A2, EIF4A3**, EIF4EBP1, **PABPC1, PABPC4**, SEC13, SUMO2, SUMO3
oas04720	Long-term potentiation	6.03 x 10^{-5}	**PPP1CA, PPP1CB, PPP1CC,** PPP1R1A, PPP3CA, PPP3CB, PPP3CC
oas00380	Tryptophan metabolism	9.75 x 10^{-5}	**ACAT1, ALDH2, ECHS1,** HADHA, **OGDH, OGDHL**
oas04810	Regulation of actin cytoskeleton	1.30 x 10^{-4}	**ACTN1, ACTN4, EZR, LOC443340, MSN, PPP1CA, PPP1CB, PPP1CC, RDX, RHOA**
oas00020	Citrate cycle (TCA cycle)	1.80 x 10^{-4}	**ACO1, DLAT, IDH1, OGDH, OGDHL**
			Treatment MR
oas00010	Glycolysis / Gluconeogenesis	2.68 x 10^{-5}	**AKR1A1, ALDH7A1, ENO2, PDHA1, PDHA2, GAPDH**
oas00020	Citrate cycle (TCA cycle)	2.68 x 10^{-5}	**IDH3A,** IDH3G, **PDHA1, PDHA2**
oas01230	Biosynthesis of amino acids	2.68 x 10^{-5}	ARG1, **ENO2, GLUL, IDH3A**, IDH3G, **GAPDH**
oas01200	Carbon metabolism	9.08 x 10^{-5}	**ENO2, IDH3A**, IDH3G, **PDHA1, PDHA2, GAPDH**
oas01100	Metabolic pathways	0.00064	**AKR1A1, ALDH7A1,** ARG1, **ENO2, GLUL, IDH3A,** IDH3G, LAP3, MTHFD1, PDHA1**, PDHA2, GAPDH**
oas04066	HIF-1 signaling pathway	0.00074	EIF4E**, ENO2, PDHA1, PDHA2, GAPDH**
oas00620	Pyruvate metabolism	0.0012	**ALDH7A1, PDHA1, PDHA2**

oas00330	Arginine and proline metabolism	0.0019	**ALDH7A1,** ARG1, **LAP3**
oas04145	Phagosome	0.0033	**RAC1, TUBB, TUBB1, TUBB2A**
oas01210	2-Oxocarboxylic acid metabolism	0.0055	**IDH3A,** IDH3G

RM = restriction maintenance; MR = maintenance restriction; [1] FDR= false discovery rate; [2] bold font are the proteins that are present in each treatment that are connected into the protein-protein interaction network.

5.5.3. Interaction Network and Functional Enrichment of Differentially Abundant Proteins

Due to the presence of uncharacterized proteins identified between the DAPs, we built a network based on gene names. Moreover, all DAPs (more or less abundant proteins) were combined into the same network. The DAPs network revealed enriched biological processes and sub-biological processes related to negative regulation of hematopoietic stem cell differentiation (HSP9A), protein succinylation (DLD, DLST), ribose phosphate metabolic process (PFKM, PKM, TFI1, DLD, DLST), generation of precursor metabolites and energy (AGL, TFL1, PKM, PFKM), formaldehyde catabolic process (ESD), pyruvate kinase activity (PKM), and various sub-processes of interest, such as, positive regulation of protein secretion (MYOM1), glycolytic process (TFL1,PFKM,PKM), and lysine metabolic process (DLST), among others (Figure 3).

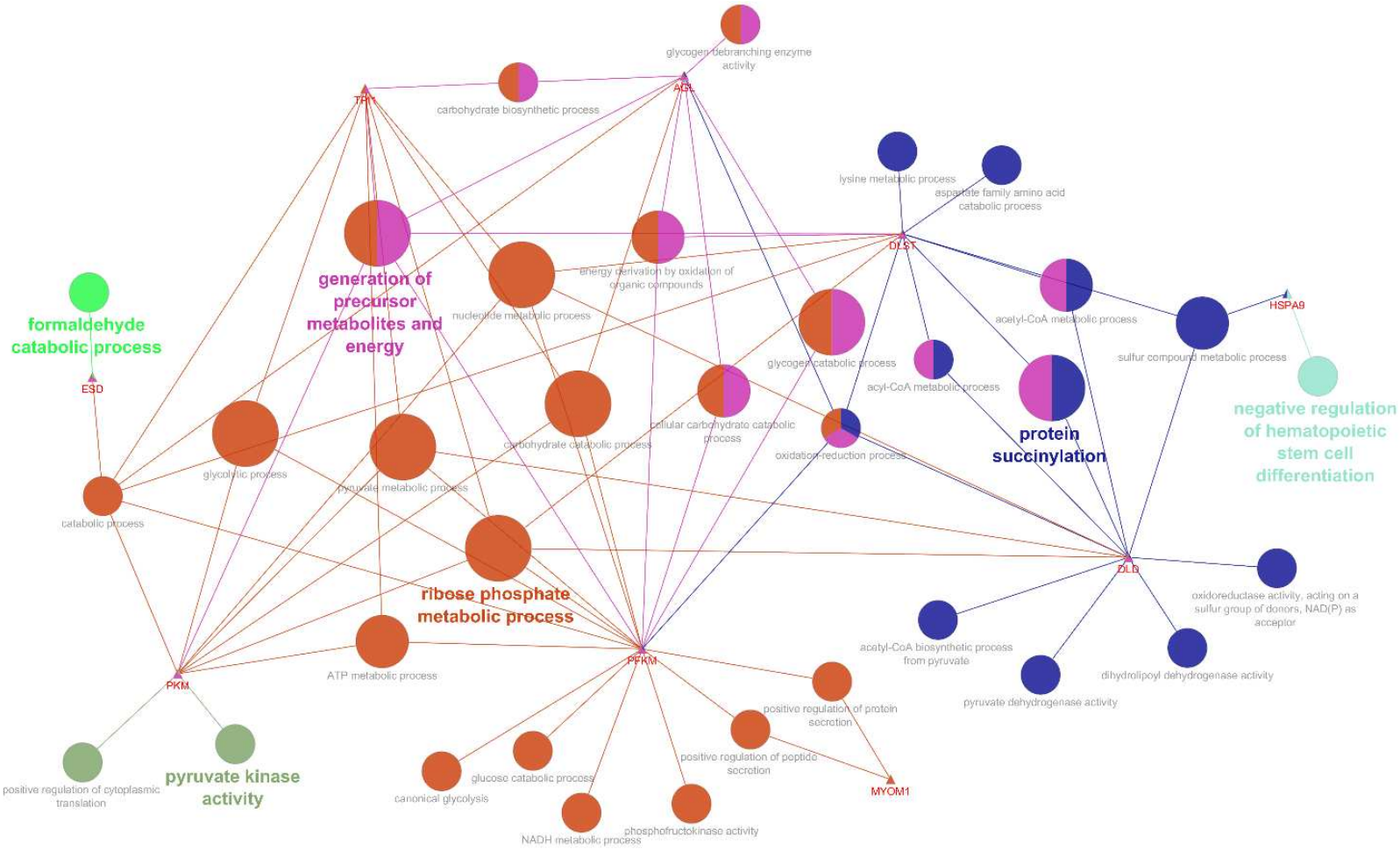

Figure 3. Analyses of the enriched biological process from the differentially abundant proteins (DAPs). The triangles represent the protein names, while the circles represent the biological processes. Different node color means distinct functional group, and node size means the significance. The most significant (FDR < 0.05) term is labeled with bold letters.

5.6. Discussion

Through the differences previously reported for the average daily gain of maternal tissues between treatments [17], the effectiveness and the objective of the experimental treatments in causing feed restriction at different time-points of gestation were reached. As such, we were successfully able to assess the consequences maternal feed restriction that occurred only in the first or last half of gestation for the proteome profile of the newborn skeletal muscle.

Maternal feed restriction impairs the offspring's skeletal muscle development, causing long-lasting impact throughout the animals' productive life. The mechanisms of placenta adaptation in the face of a scarce intrauterine environment ensure fetus survival, and may cause permanent structural and functional changes by programming its metabolism through changes in transcripts, proteins, and metabolite profile [29]. Given the importance of vital tissues for fetuses (i.e., organs and viscera), the lack of nutrients during gestation prioritizes nutrient delivery for this purpose, while skeletal muscle development may be impaired. Thus, with the employment of proteomic approaches in the current study, we have identified the differentially abundant proteins (DAPs) and exclusively expressed proteins in the skeletal muscle of the offspring, resulting from maternal feed restriction during the first or last half of gestation. Specifically, the current study identified 13 DAPs involved in general biological processes mostly related to muscle energy metabolism. From the exclusive expressed proteins (181 in RM and 46 in MR), we performed a network analysis and the identification of KEGG pathways revealed enriched pathways related to fatty acid degradation, glycolysis/gluconeogenesis, citrate cycle (TCA cycle), and biosynthesis of amino acids, among others. Although the enriched biological processes and pathways were similar between treatments, the protein abundance inherent to each treatment affects different steps of the pathway and thus influences the muscle energy balance.

The skeletal muscle characteristics of plasticity allow its metabolism to change and adapt according to the environment, such as calorie and nutrient intake [30]. In terms of metabolic properties, skeletal muscle is the primary site of glucose uptake and storage [31]. Glucose is oxidized to generate ATP through two major pathways: the oxidative (aerobic) and the glycolytic (anaerobic) pathway. Through the aerobic pathway, glucose undergoes glycolysis to generate pyruvate, followed by its conversion into acetyl-coA, which enters the citrate cycle, producing substrates for the electron transport chain which results in ATP synthesis.

The proteins found to be differentially abundant and exclusive in our experimental treatments appear in biological processes (BP) and signaling pathways (SP) associated with energy metabolism, such as glycolytic process (BP), pyruvate metabolism (SP), glycolysis/gluconeogenesis (SP), and citrate cycle (TCA cycle; SP) (Figure 4).

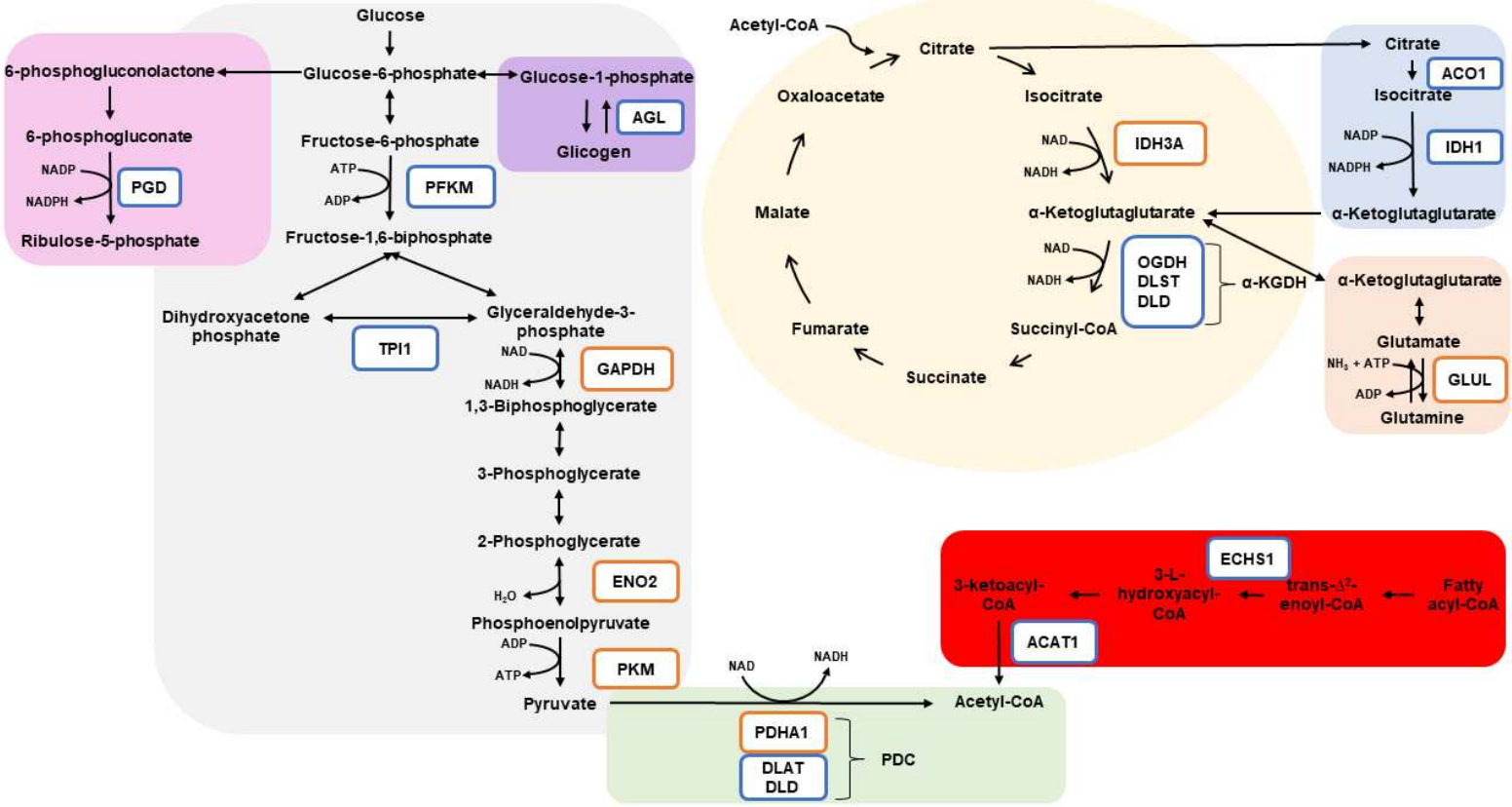

Figure 4. Summarized metabolic pathways influenced by the experimental treatments. Glycolysis is represented in gray, glycogenolysis in purple, pentose phosphate pathway in pink, oxidative decarboxylation of pyruvate to form acetyl-CoA in green, citrate cycle in yellow, cytoplasmic conversion of citrate into α-ketoglutarate in blue, glutamine synthesis in orange, and fatty acid degradation in red. Proteins inside orange squares were exclusively expressed in MR or were more abundant in MR compared to RM. Proteins inside blue squares were exclusively expressed in RM or were more abundant in RM compared to MR. α-KGDH: α- ketoglutarate dehydrogenase complex; ACAT1: acetoacetyl-CoA thiolase; ACO1: aconitase 1; AGL: glycogen debranching enzyme; DLAT: dihydrolipoyl transacetylase; DLD: dihydrolipoamide dehydrogenase; DLST: dihydrolipoyllysine-residue succinyltransferase; ECHS1: enoyl CoA hydratase, short chain 1; ENO2: enolase 2; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GLUL: glutamine synthetase; IDH1: isocitrate dehydrogenase 1; IDH3A: isocitrate dehydrogenase 3 catalytic subunit alpha; OGDH: 2-oxoglutarate dehydrogenase; PDC: pyruvate dehydrogenase complex; PDHA1: pyruvate dehydrogenase E1 subunit alpha 1; PFKM: phosphofructokinase; PGD: 6-phosphogluconate dehydrogenase; PKM: pyruvate kinase; TPI1: triosephosphate isomerase.

5.6.1. Glycolysis

Glycolysis is commonly divided into the energy-investment and energy-generation phases [32–34]. Among the more abundant proteins in RM compared to MR, PFKM and TPI1 play roles in the energy-investment phase of glycolysis. The enzyme PFKM uses ATP to catalyze the irreversible conversion of fructose-6-phosphate into fructose-1,6-bisphosphate (FBP). FBP is then cleaved into glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP). DHAP is a precursor of lipid synthesis, while GAP is utilized in the next steps of glycolysis. Moreover, DHAP and GAP can be reversibly converted by the reaction involving the enzyme TPI1 [35]. A recent study showed that DHAP may function by signaling the availability of glucose to activate the complex of proteins mTORC1 [36], which is an important regulator of cellular growth, protein synthesis, autophagy, and lipogenesis [37]. Studies evaluating post-mortem glycolysis and meat quality parameters observed that TPI1 abundance was associated with beef tenderness [38,39] and was positively related to the rate of pH decline, which may partially be associated with meat color [40–43]. Therefore, since TPI1 may influence the abundance of both metabolites (DHAP and GAP), and consequently, the synthesis of lipids and ATP, the greater abundance of TPI1 in the skeletal muscle of animals born from dams that were feed-restricted in the first half of gestation compared to those born from dams feed-restricted in the last half of gestation may not be attributed to a specific pathway (lipid or ATP synthesis). However, we speculate that the greater abundance of TPI1 in the skeletal muscle of offspring from RM treatment would negatively impact the conversion from muscle into meat upon slaughter later in life, with consequences for meat color and tenderness parameters.

According to Akram [32], when glycolysis is initiated from the glycogen breakdown (glycogenolysis), fewer ATPs are consumed in the first phase of glycolysis, because in this case, the first step of glucose activation is dispensable. In this context, the protein AGL, which is an important protein in the process of glycogenolysis, was more abundant in the treatment RM compared to MR. Such an observation may indicate that, despite the ATP utilization in the reaction catalyzed by the PFKM, the skeletal muscle of the offspring from treatment RM may use glycogen as its main carbon source, and consequently, more ATP would be spared from the first step of glycolysis.

The second phase of glycolysis, named the energy-generation phase, is characterized by the oxidative conversion of GAP to pyruvate and the formation of ATP and NADH [34]. Three enzymes that participate in this phase of glycolysis were exclusively expressed in the treatment

MR or were less abundant in RM compared to MR. GAPDH, which was exclusively detected in treatment MR, catalyzes the reversible conversion of GAP into 1,3-biphosphoglycerate, in addition to generating NADH from this reaction [32]. An important glycolytic enzyme, ENO2, was also exclusively expressed in the treatment MR. Although ENO2 (gamma-enolase) is one of the enolase isoforms found to be predominantly located in the neuron and neuroendocrine tissues [44], ENO2 was found to be expressed in human and rat cultured muscle cells [45]. The reversible reaction that converts 2-phosphoglycerate into the energy-rich phosphoenolpyruvate (PEP) is catalyzed by ENO2 [32,46]. After that, PEP is dephosphorylated in a reaction catalyzed by the enzyme PKM, downregulated in RM compared to MR, leading to the final glycolytic step where pyruvate and ATP are formed [46]. Thus, the results related to glycolysis indicate that the treatment RM may cause an enhancement in the energy-investment phase of glycolysis, while treatment MR may have a major function favoring the energy-generation phase of glycolysis.

Once formed, pyruvate may follow different pathways. In our current study, we identified proteins in both treatments that participate in the same destiny as pyruvate. The oxidative decarboxylation of pyruvate to form acetyl-CoA, CO2, and NADH is catalyzed by a well-orchestrated complex called pyruvate dehydrogenase complex (PDC). The PDC is composed of three catalytic subunits: pyruvate dehydrogenase (PDH), dihydrolipoamide acetyltransferase (DLAT), and dihydrolipoamide dehydrogenase (DLD) [47,48]. Intriguingly, PDHA1, which is a component of the PDH subunit, was exclusively expressed in the treatment MR. On the other hand, the proteins that are components of the subunit DLAT were exclusively expressed in treatment RM, while proteins that are components of DLD were more abundant in RM compared to the MR group. Because our experimental treatment was based on feed restriction, it is possible that some vitamins may have been differentially metabolized by the dams, and consequently, their availability to the fetuses was different between treatments. For instance, vitamin B1 is a cofactor of the subunit PDH [49], while vitamin B5 is the key precursor for the biosynthesis of CoA [49], utilized as a cofactor in subunit DLAT, and the precursors of FAD and NAD are vitamin B2 [49] and B3 [50], respectively, which serve as cofactors in the subunit DLD.

5.6.2. Citrate Cycle (TCA)

Citrate cycle (TCA) initiates catabolizing of acetyl-CoA molecules through the steps involving many enzymes that produce reducing equivalents NADH and FADH, which will be further driven to ATP production in the electron-transport chain [46]. The first step of TCA

produces citrate, and like pyruvate, citrate may follow a variety of pathways both inside and outside the mitochondria matrix, in addition to regulating intermediate metabolites. When cellular iron levels are high, the enzyme ACO1, which was found to be exclusively expressed in treatment RM, catalyzes the reversible conversion of the cytosolic citrate into isocitrate [51,52]. Cytosolic isocitrate is then metabolized by the enzyme IDH1, also found to be exclusively expressed in treatment MR, generating α-ketoglutarate (a-KG) and NADPH [52], which is an important cofactor involved in lipid and cholesterol metabolism [53,54]. Moreover, findings from Moreno et al. [55] showed that ACO1 was positively associated with adipogenic markers, linking the iron metabolism with the adipogenic potential of the adipose tissue. Thus, our results may suggest that, compared to treatment MR, maternal feed restriction during the first half of gestation may influence the iron metabolism in the skeletal muscle of the offspring, which may result in enhancement of the protein abundance of ACO1 and IDH1 and likely increase the biosynthesis of lipids.

Treatment RM may also influence the pathways downstream a-KG, since the components of the a-KG dehydrogenase (α-KGDH) complex were found to be exclusively expressed in treatment RM (OGDH) or were more abundant in RM compared to MR (DLAT and DLST). The a-KGDH complex function catalyzed the conversion of a-ketoglutarate to succinyl-CoA and produced NADH [56]. Thus, based on these results, RM treatment, not only influences the a-KG production in cytosol but also its downstream reaction on the mitochondria, leading to the enhancement of NADH synthesis.

5.6.3. Glutamine

Regarding the cytosolic formation of α-KG, in contrast to our findings in treatment RM, treatment MR may have influenced the increase in mitochondrial α-KG, since the enzyme responsible for the conversion of the mitochondrial isocitrate into α-KG (IDH3A) was exclusively expressed in treatment MR. The amount of α-KG in mitochondria is dependent on the state of oxidation reduction (redox). For example, high levels of NAD over NADH lead to the conversion of α-KG into succinyl-CoA, while high levels of NADH over NAD lead to α-KG accumulation and consequently its participation in several other biological processes, such as the biosynthesis of amino acids. To prevent the excess of α-KG in the cells, reactions associated with the production of glutamine may occur [57]. The protein GLUL, exclusively expressed in the treatment MR, uses ammonia to catalyze the conversion of glutamate into glutamine [58,59]. When required, skeletal muscle releases glutamine into the blood, supplying the energy and protein requirements of other cells and tissues [60]. Moreover, glutamine

oxidation is crucial for energy production and survival of pluripotent stem cells, in addition to donating nitrogen in de novo nucleotide synthesis [61]. Taken together, the exclusive expression of the proteins IDH3A and GLUL in the skeletal muscle of the offspring from treatment MR may indicate the accumulation of mitochondrial α-KG and its fate of glutamine synthesis, increasing the availability of this amino acid, which can be further utilized in several biological processes.

5.6.4. Fatty Acid Degradation

Under the aerobic conditions, ATP production is obtained through three key biological pathways, including the citrate cycle and fatty acid degradation. As previously discussed, our experimental treatments may have affected the overall skeletal muscle energy metabolism. The proteins ACAT1 and ECHS1 found to be exclusively expressed in treatment RM are involved in the fatty acid beta-oxidation signaling pathways. ECHS1 catalyzes the second reaction, while ACAT1 catalyzes the last reaction, ending in acetyl-CoA synthesis. Our results were contrary to the study which reported the downregulation of the corresponding genes in bulls subjected to compensatory growth conditions (restriction followed by realimentation) [62], reinforcing that the results may depend on the period of feed restriction (prenatal vs. postnatal) or post-transcriptional factors. Interestingly, ACAT1 can acetylate the lysine residues of PDHA1, causing inhibition of the PDC in some conditions and favoring cell proliferation [63]. Hence, the exclusive expression of PDHA1 in treatment MR in particular may be related to the exclusive expression of ACAT1 in RM, which is able to inhibit this protein. Taken together, these findings may result in increased fatty acid degradation in the skeletal muscle of the offspring from treatment RM.

5.6.5. Pentose Phosphate Pathways

Interestingly, the nucleotide metabolism may have been affected by our experimental treatment. The protein PGD was exclusively expressed in treatment RM, and it links the cytosolic carbohydrate metabolism with protein secretion [64]. PGD is a NADP-dependent enzyme that catalyzes the decarboxylation of 6-phosphogluconate to ribulose 5-phosphate (Ru5P), producing NADPH in the aerobic stage of the pentose phosphate pathways [46]. NADPH is mainly used in the processes of cellular redox balance and antioxidant defense [65], while the Ru5P is used for the synthesis of nucleotides and nucleic acids [46]. Therefore, the greater production of NADPH generated by the reaction involving PGD may be required in the skeletal muscle of the offspring, resulting from maternal feed restriction at the first half of gestation.

5.7. Conclusions

In conclusion, our data indicate that feed restriction at different stages of gestation affects the overall proteins related to energy metabolism in the skeletal muscle of the newborn. Specifically, our results showed that maternal feed restriction during the first half of gestation followed by non-restriction regulates proteins related to the energy-investment phase of glycolysis. On the other hand, the energy-generation phase of glycolysis is steeper in the skeletal muscle of the offspring resulting from dams that were not feed-restricted at the first half of gestation and were feed-restricted at the second half of gestation. Moreover, carbon sources may be largely provided by glycogen in the skeletal muscle of the offspring born from dams feed-restricted at the first half of gestation. Indeed, skeletal muscle of newborns from dams feed-restricted at the first half of gestation had an increase in cytosolic α-KG; however, it appears that α-KG is used to produce NADH in mitochondria. On the other hand, in the newborns from dams feed-restricted at the second half of gestation, the α-KG accumulation in mitochondria appears to have deviated for the biosynthesis of glutamine. Furthermore, proteins involved in fatty acid degradation were enhanced by feed restriction at the first half of gestation, in addition to influencing the NADPH production by regulating an enzyme that participates in the pentose phosphate pathways. However, it is possible that offspring from a dam undergoing feed restriction during the first half of gestation likely experience extensive use of glycogen and fatty acid storage. Although the long-term effects of maternal nutrition on skeletal muscle development and metabolism have been exhibited in previous studies, if the changes observed in our trial persist in the skeletal muscle throughout the progeny's life, they need to be further investigated.

Supplementary Materials

The following supporting information can be downloaded at: https: //www.mdpi.com/article/10.3390/ani12081011/s1, Table S1: Sheet containing all the proteins identified, its respective molecular weight, and the LFQ intensities of each replicate.

Author Contributions

Conceptualization, M.d.S.D. and M.P.G.; methodology, T.C.C., T.A.d.O.M., and L.L.D.; software, T.C.C., T.A.d.O.M., and L.L.D.; validation, T.A.d.O.M. and T.C.C.; formal analysis, T.C.C. and T.A.d.O.M.; investigation, T.C.C. and T.A.d.O.M.; resources, M.d.S.D., T.A.d.O.M., and M.P.G.; data curation, T.C.C. and T.A.d.O.M.; writing—original draft preparation, T.C.C.; writing—review and editing, T.C.C., T.A.d.O.M., and M.d.S.D.;

visualization, T.C.C., L.L.D., T.A.d.O.M., M.M.d.S., R.V., M.P.G., and M.d.S.D.; supervision, M.d.S.D. and T.A.d.O.M.; project administration, T.C.C.; funding acquisition, M.d.S.D. and M.P.G. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki, and was approved by the Animal Care and Use Committee of the Department of Animal Science at the Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil (protocol 09/2017).

Informed Consent Statement

Not applicable.

Data Availability Statement

The raw data are provided in the supplementary table. Any additional data will be made available upon reasonable request to the corresponding author.

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Conflicts of Interest

The authors declare no conflict of interest.

5.8. References

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6. CHAPTER 6

6.1. General conclusions

In summary, the current study showed evidence that maternal restriction during crucial periods of gestation contributed to regulate and define skeletal muscle composition and metabolism in a time-dependent manner. Specifically, muscle hyperplasia is sensitive to the prenatal environment. Indeed, protein restriction during mid-gestation in cows, reduced muscle fiber number permanently in beef progeny. The connective tissue and muscle metabolism are also sensitive to the intrauterine condition; however, it may suffer further modifications depending on the post-natal environment. The in-depth evaluation of the complete set of transcripts and proteins in the skeletal muscle of the newborn goat showed that both plane of nutrition modify the overall energy metabolism. However, feed restriction in the first half of gestations appear to bring more detrimental effects, since the usage of storages sources are enhanced, accompanied by the non-activation against the excessive oxidative stress.