Nutritional Epigenetic Modifications in Beef Cattle

by

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Abstract

Nutrition of the beef cow during pregnancy influences fetal development and potential changes in phenotype. On average 9-10% of beef cows are below the optimal condition score of 2.5/5 at both pre-breeding and pregnancy tests, indicating potential nutritional stress during gestation (Waldner and García Guerra, 2013). Epigenetic modifications are reported to regulate the changes in phenotype due to maternal nutrition during gestation. Prior research on the impact of gestational feed restriction during early to mid-gestation on semen methylation, reproductive development, and carcass characteristics in cattle progeny revealed differentially methylated regions (DMRs) in the semen as well as influences on growth trends and carcass characteristics in bull and steer progeny. The objective of the current study is to evaluate further the influence of maternal nutrition during gestation on molecular mechanisms regulating the observed changes in semen, *Longissimus dorsi* (LD), *Semimembranosus* muscle (SM), and liver (LV) at slaughter in cattle progeny (steers and bulls) and validate the DMRs identified in the semen methylation analysis.

DNA and RNA were extracted from the progeny and categorized based on the diet provided to their respective dams (Moderate or low diet) during gestation and genetic potential for residual feed intake (High-RFI or Low-RFI). DNA methylation analysis was conducted in LD, SM, and LV in steers and semen in bull progeny using EpiTYPER technology to validate DMRs identified in semen WGBS analysis and assess the influence of prenatal nutrition and RFI on the methylation at slaughter. The influence of prenatal nutrition and/or RFI on gene expression was also conducted in LD, SM, LV, and testis in the progeny at slaughter using nCounter element Tagset carried out by NanoString technologies.

In the bull study, the DMR validation study showed a difference in the methylation trend between average methylation (EpiTYPER) and mean methylation difference (whole-genome

bisulfite sequencing [WGBS]). However, semen CpG methylation analysis showed that prenatal diet increased methylation in moderate diet (Mdiet) progeny in DMRs associated with testicular development and reproductive development (*ALDH3B1* and *INSL3*) as well as genomic imprinting and growth development (*IGF2R*-DMR2 and *GRB10*). Additionally, Low diet (Ldiet) bulls displayed higher expressions of the growth-promoting gene *PDPK1* in LD and SM muscle.

In steers, DMR methylation due to prenatal nutrition in LD, SM, and LV tissues mostly did not corroborate the findings in semen WGBS analysis. However, DMRs, *INSL3,* and *IGF2R* showed similar methylation trends in semen and SM due to prenatal nutrition while *GRB10* displayed similar methylation trends in SM and LV tissues in the current study. Also, RFI had a greater influence on methylation in LD, SM, and LV tissues while prenatal nutrition had a higher influence on methylation in SM muscle compared to LD and LV. The findings from this project show potential molecular modifications as a result of prenatal nutrition and selection for feed efficiency and provide a further understanding of epigenetic mechanisms regulating fetal programming phenomenon.

Preface

This thesis is an original work of Mumuni Gibril. The research studies of this project involved the use of Angus heifers received ethics approval by the University of Alberta Animal care and Use Committee (AUPs 135, 457, 483, and 877) in accordance with the guidelines issued by the Canada Council on Animal Care.

The research was conducted under the leadership and supervision of Drs. Carolyn Fitzsimmons at the University of Alberta. Animal treatment and experimental design for study 1 and 2 were conducted by Aidin Foroutan Naddafi and Meale Sarah Jade under supervision of Drs. Carolyn Fitzsimmons.

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Dedications

I dedicate this work to the Almighty Allah and my mother Hajia Sahadatu Alidu

for the support, endless prayers, and encouragement.

I am eternally GRATEFUL

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To my supervisor, Dr. Carolyn Fitzsimmons, I would like to express my gratitude for this opportunity and for providing a great learning environment for me to conduct this research. I have acquired not skills not only in academics but in addition, some interpersonal skills that has shaped me into becoming an upright individual.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
ADG	Average daily gain
AI	Artificial insemination
BCS	Body condition score
C/EBPs	CCAAT/enhancer-binding proteins
CpG	Cytosine-guanine dinucleotide
DM	Dry matter
DMI	Daily dry matter intake
DMRs	Differentially methylated regions
Dnmts	DNA methyltransferases
DOHAD	Developmental Origin of Health and Diseases
FBXO32	F-box only protein 32
FW	Final weight
GIT	Gastrointestinal tract
GS	GrowSafe System®
IFN- τ	Interferon- τ
IP	Immunoprecipitation
IUGR	Intrauterine growth restriction
LD	*Longissimus dorsi*
LV	Liver
MDWT	Midpoint weight
MEF2A	Myocyte enhancer factor 2A
MRFs	Myogenic regulatory factors
MWT	Metabolic bodyweight
NGS	Next generation sequencing
PGC	Primordial germ cells

$PGF_{2\alpha}$	Prostaglandin F2-alpha
PPARγ	Peroxisome proliferator-activated receptor gamma
RFI	Residual feed intake
HPA	Hypothalamic-pituitary-adrenal
RRBS	Reduced representation bisulfite sequencing
SM	*Semimembranosus muscle*
TGFβ	Transforming growth factor-beta
TRIM63	Tripartite motif-containing protein 63
TS	Testis
WGBS	Whole-genome bisulfite sequencing

Chapter 1. Introduction and Literature Review

1.1. Introduction

The intrauterine environment provides conditions necessary for the growth and development of the fetus during pregnancy, making fetal growth and development highly susceptible to its conditions. During pregnancy, aberrations in the utero-environment impair the development of the placenta as well as organ systems and tissue (Burton et al., 2010). The placenta serves as a transport system for the supply of nutrients and other substrates for the formation and development of organs and systems that are required for the normal functioning of an individual. The fetal growth trajectory is largely dependent on maternal nutritional storage and supply and is also directly related to the morphology and efficiency in the functional capability of the placenta (Redmer et al., 2004). This implies that maternal prenatal nutritional status dictates the intrauterine conditions for fetal development (Funston et al., 2010).

Several epidemiological studies in humans and livestock have elucidated the importance of prenatal maternal nutrition to the development and performance of offspring both in pre-and postnatal stages (Mi et al., 2000). In humans, maternal undernutrition and overnutrition during pregnancy predispose offspring to metabolic and cardiovascular disorders and increase the risk of perinatal mortality and morbidity (Barker et al., 1990). In livestock studies, suboptimal nutrient supply to the fetus could lead to stunted development of organs and tissues that are deemed economically important, increased morbidity and mortality of offspring, impairment in postnatal development, and reduced feed efficiency, which consequently can reduce the general productivity of the livestock (Funston & Summers, 2013; Wu et al., 2006).

The process by which the fetus perceives and responds to both intrinsic and extrinsic signals during critical stages of development is known as fetal programming (Marciniak et al., 2017).

Intrinsic factors influencing fetal development are related to the placental functions while quality and quantity of maternal nutritional supply, environmental stress, and age during gestation serve as extrinsic factors that affect fetal development (Funston & Summers, 2013).

In beef cattle production, the productivity of cattle is dependent on genotype as well as production management. In a bid to maximize economic gains, cattle producers may compromise production factors such as quality or quantity of feed, which accounts for about 70% of total production costs (Lawrence et al., 2008). Also, due to extreme changes in climatic weather conditions over the production period, the quality and quantity of feed available for cattle are not always optimum. Furthermore, the feed and water intake of beef cattle exposed to extreme weather conditions are compromised resulting in increased stressed situations (National Research Council (US) Subcommittee on Environmental Stress, 1981)

These factors could lead cattle to be underfed at critical stages of production such as the breeding season, gestation, as well as the development of their offspring (Dunlap et al., 2015). The objective of this chapter is to describe and discuss the processes that occur during gestation in cattle, fetal development, and programming, the impact of prenatal nutrition on productivity, and mechanisms underlying fetal programming.

1.2. Pregnancy in cattle and fetal developmental processes

Maternal cognizance of pregnancy is influenced by the interaction between the embryo, its membrane, and the uterus. The recognition of pregnancy in cattle initiates with the production of progesterone which results from inactivation of the process of luteolysis when fertilization occurs, allowing the corpus luteum to persist (Caleb et al., 2014). According to Peters (1996), failure of luteolysis inactivation, and thus insufficient progesterone secretion accounts for approximately 60-65% fall in pregnancy rates. The embryo inhibits the luteolytic process by preventing the

development of oxytocin receptors in the uterus. Oxytocin stimulates the secretion of the hormone prostaglandin F2-alpha ($PGF_{2\alpha}$). The presence of the $PGF_{2\alpha}$ hormone is a luteolytic signal that indicates ongoing estrus activities and stalls the activities of the corpus luteum. The recognition of pregnancy requires the release of an anti-luteolytic signal, interferon- τ (IFN- τ) by the conceptus (Spencer et al., 1996). The release of biochemical signals by the conceptus in the uterus is termed maternal recognition of pregnancy (Spencer & Bazer, 2004). The interaction between the maternal membrane, the embryo, and/or corpus luteum to prevent the regression of progesterone production signifies a successful recognition of pregnancy (Bazer et al., 1997).

The ovum begins to divide mitotically approximately 30 hours after fertilization leading to the formation of a 16 to 32 cluster of cells known as a blastomere (Valdão *et al.,* 2018). The blastomere will subsequently hollow out by day 8, forming a blastocyst consisting of trophoblast and an inner cell mass. The trophoblast is formed from the surface cells of the blastomere. It develops into the yolk sac and part of the placenta, eventually becoming the main nutritional source for the embryo (Figure 1.1). The inner cell mass eventually develops into the embryo and subsequently the fetus.

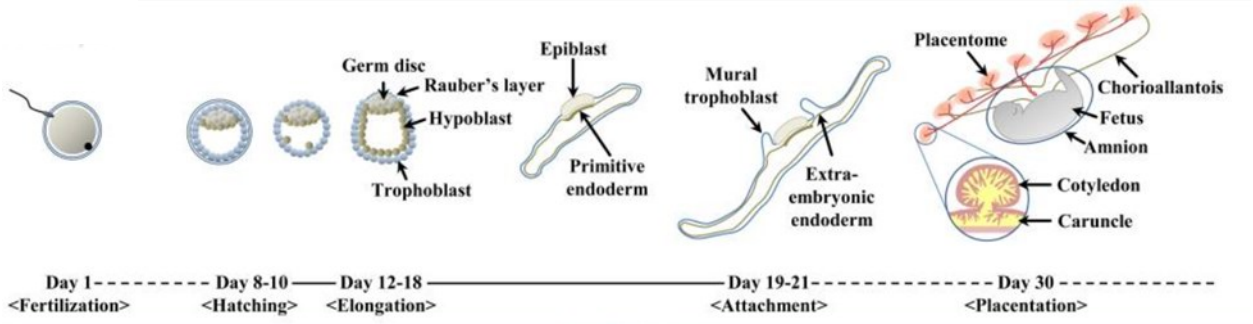

Figure 1.1 The development of a bovine embryo from fertilization to placentation

In cattle, the embryo establishes contact with the uterus by five weeks after fertilization in a process known as implantation. Major organs, systems, and tissues start to develop during the embryonic stage. As the developmental process progresses, the nutritional requirements of the embryo also increase leading to the formation of an extra-embryonic membrane. This extra-embryonic membrane creates a conduit for the transfer of nutrients, exchange of gases, and discharge of fetal waste, and is known as the placenta. The formation of the placenta creates an intimate connection between the fetal and maternal uterine membranes for physiological exchange. The blood flow within the placenta influences the efficiency to which nutrients and metabolites are transferred to the fetus (Reynolds & Redmer, 1995). Fetal growth and metabolism are also enhanced by placental steroidogenesis. Hormones including progesterone and estrogens help in the maintenance of pregnancy, hence proving a suitable intrauterine environment for fetal growth during mid to late gestation (Conley & Mason, 1990; Perin & Maluf, 2015).

1.2.1. Embryogenesis

The period between fertilization to the completion of organogenesis is known as embryogenesis. The period usually begins from day 1 to about day 42 of pregnancy and is characterized by a process known as gastrulation (Papaioannou, 2004). Gastrulation is the development three germ layers namely endoderm, ectoderm, and mesoderm, which eventually give rise to various organs and systems. The endoderm is the innermost part of the germ layers, it develops into part of the digestive system, respiratory tract, majority of endocrine glands, and the urinary tract (Hogan & Zaret, 2002; Perin & Maluf, 2015; Wlizla & Zorn, 2015). The mesoderm is the mid-germ layer, and it is functionally associated with the endoderm and ectoderm. Mesoderm differentiates and evolves into parts of cranial tissues, muscle, cardiac, and circulatory systems. By its association with the endoderm, mesoderm aids in the formation of tissues such as the liver, pancreas, and

trachea. The mesoderm in association with ectoderm forms part of the dermis (Papaioannou, 2004). The mesoderm germ layer also gives rise to the excretory and respiratory systems. The ectoderm is the outer germ layer that mainly evolves into the nervous system and the integumentary system (Nowotschin & Hadjantonakis, 2020).

1.2.2. Skeletal muscle development

The development of muscle fibers, which are the main constituent of skeletal muscle, is one of the more important prenatal developmental processes in beef cattle research as skeletal muscle is a major trait of economic importance. Therefore, the development of muscle fiber during gestation is crucial to the productivity of the offspring. The process of muscle development during the gestation period is known as myogenesis (Yan et al., 2013).

Muscle development takes place during the prenatal and postnatal stages of life. However, the most important aspect of the muscle developmental process occurs during the prenatal stage i.e., both embryonic and fetal periods of gestation. The embryonic and fetal muscle developmental process is known as primary and secondary myogenesis, respectively. Postnatally, muscle fiber tends to undergo hypertrophy with no new formation of muscle fiber (Rehfeldt et al., 2000). As mentioned earlier, the mesoderm is the germ layer involved in the development of muscle. The myogenic process is regulated by proteins Wingless and Int, paired box gene *Pax 3* and *Pax 7*. Wingless and Int initiate embryonic myogenesis by activating myogenic signaling pathways and *Pax 3* and *Pax 7* aid in the activities of myogenic regulatory factors (MRFs), Myf-5, Myo-D, and myogenin (Cisternas et al., 2014; Yan et al., 2013). These MRFs regulate myogenic progression and lineage (von Maltzahn et al., 2012). Myogenic precursor cells originating from mesenchymal are regulated by MRFs and converted into myoblasts. The myoblasts multiply, differentiate, and

fuse into multinucleated cells known as myotubes. These myotubes subsequently form muscle tissues (Du et al., 2010a; Rehfeldt et al., 2000).

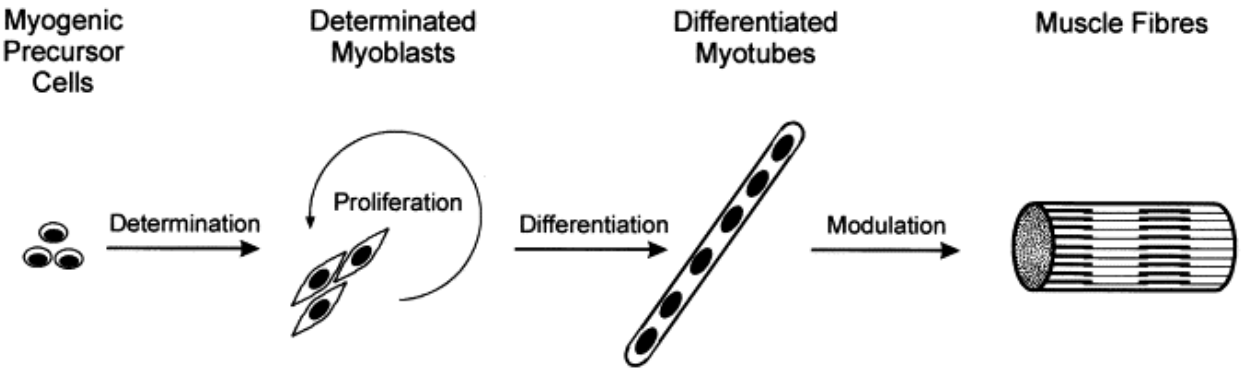

Figure 1.2 The process of myogenesis

Primary myogenesis occurs during the first 2 months of gestation in cattle and produces a small number of muscle fibers. Many muscle fibers are formed during the secondary stage of myogenesis during the fetal stage and occur between the 2^{nd} to 7^{th} months of fetal development of gestation.

1.2.3. Adipogenesis

Mesenchymal stem cells also differentiate into adipocytes in a process known as adipogenesis. The formation of adipocytes occurs during mid-gestation coinciding with myogenesis. Preadipocytes are formed in the initial stages of the process and differentiate into mature adipocytes in late gestation. Transcription factors peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding proteins (C/EBPs) regulate adipogenesis. Intramuscular adipocytes are formed during adipogenesis, and this is known as marbling. Marbling is a major criterion for meat quality due to its role in the tenderness, juiciness, and flavor of the

meat (Tong et al., 2008). These meat quality factors influence consumer acceptance and palatability of the meat (Du et al., 2010a; Miller, 2014).

1.2.4. Fibrogenesis

Fibrogenesis, a process whereby connective tissues are formed, occurs during mid-gestation in association with adipogenesis. Fibrogenesis also arises from mesenchymal cells (Du et al., 2011) through the expression of growth factor and cytokine, transforming growth factor-beta (TGFβ) (Yan et al., 2013). Fibrogenesis plays a role in meat marbling and tenderness (Du et al., 2011).

1.2.5. Fetal growth

The fetal stage of pregnancy begins after the end of the embryonic stage up until day 280. During this period, the fetus begins to increase in size. In cattle, fetal weight increases daily by 10 g during day 70 to 100 days of gestation (Eley et al., 1978). The growth exponentially increases to approximately 200-300 g/day during day 200 to 250 of gestation and eventually declines to about 100 g/day as parturition approaches. The extent of growth is influenced by maternal, placental and fetal factors (Vorherr, 1982). Maternal genetic and non-nutritional factors contribute about 25% and 55% variance in fetal growth respectively (Vorherr, 1982). Non-nutritional maternal factors includes age and parity (Ferrell & Ferrell, 1993). Placental efficiency which is defined partly by its weight is determined prior to the fetal stage, also contributes to fetal growth. Transplacental flow of nutrient and hormones is regulated by uteroplacental vascular development (Reynolds et al., 2010). Fetal factors that contribute to fetal growth are mainly its genotype which are inherited by both paternal and maternal genomes (McGrath & Solter, 1984). The physiological changes in fetal development increase its susceptibility to conditions within the intrauterine environment, which is influenced by factors such as placental insufficiency, maternal nutritional, and metabolic status.

The process of parturition is characterized by hormonal and physiological changes including enlargement of mammary glands and abdomen. In cattle, parturition begins at approximately 280 days of gestation. However, factors including breed of sire and dam, age and parity of the cow, calf sex, and diet affect the onset of parturition (Zaborski et al., 2009). Therefore, gestation length could differ, and parturition could begin days before or after the 280 days.

1.3. Effect on prenatal nutrition on the fetal developmental processes

The importance of the quality and quantity of maternal dietary intake heightens during critical gestational periods. Conceptus development relies on histiotrophic and hemotrophic nutritional exchanges to survive and evolve from early gestation to parturition. Both histiotrophic and hemotrophic exchange involve a transfer of nutritional material from maternal membrane to conceptus to facilitate development. Histiotrophic exchange takes place during early embryonic development before the placenta is formed while hemotrophic exchange involves blood-borne exchange and occurs after placentation (Burton et al., 2020). Epidemiological studies on the origins of adult diseases revealed that suboptimal maternal prenatal nutrition affects pathological processes including placentation, glucose tolerance, and lipid metabolism of the developing fetus (Barker, 2004a). The impact on these processes independent of the postnatal environment can result in adult cardiovascular diseases (Barker, 1995). In a similar vein, prenatal nutrition influences the developmental processes of eutherian livestock such as goats, sheep, and cattle, and consequently postnatal performance. Undernutrition and suboptimal supply of nutrients for fetal development have been a focal point of animal agriculture research (Lucas, 1998).

Livestock research undertaken to evaluate the impact of prenatal nutrition on fetal development has implicated prenatal undernutrition as leading to undesirable effects on economic traits in production (Funston et al., 2010; Long et al., 2010). There are common instances in livestock

production that could lead to undernutrition or suboptimal feeding during gestation. For example, in the beef, cattle industry producers are usually faced with issues of calving difficulty due to large fetal size and feed unavailability, in both intensive and extensive systems, respectively (Noya et al., 2019). Incidence of dystocia could render the cattle enterprise unproductive and uneconomical, as a result of increased risk of calf morbidity and mortality (Dhakal et al., 2013; Wallace et al., 1996). In pasture-based as well as intensive system productions, high stocking rate and grouping strategy can influence feed intake due to increased competition for feed by more dominant animals. Pregnant cows when grouped with more dominant and/or non-pregnant ones, can have limited access to feed. This creates a situation of undernutrition for the pregnant cows and eventually impacts fetal developmental (Beck et al., 2016; Grant & Albright, 2001). In extensive systems of production, seasonality of feed availability coupled with little to no supplementation also increases the incidence of suboptimal nutrition at different production stages. Feed restriction during gestation leads to intrauterine growth restriction (IUGR). Fetal growth restrictions can have diverse consequences depending on the timing, and level of the restriction (Belkacemi et al., 2010; Hyatt et al., 2008). Suboptimal nutrition in addition to hindering the smooth transition from an embryo to a fully developed neonate can also manifest its effects long-term by increasing offspring susceptibility to diseases, altered metabolic functions of tissues, and in some cases affecting the subsequent reproductive ability of the dam (Mossa et al., 2015). Therefore, adequate nutrition from conception to parturition is essential in not only ensuring successful fetal development but also improving the economic viability and sustainability of the livestock production enterprises. The succeeding sub-chapters discuss the effect of prenatal nutrition during the three stages of gestation on fetal developmental processes and postnatal performance.

1.3.1. First trimester of gestation

Nutritional requirements of the conceptus have been reported to be almost negligible in the first trimester. However, histiotrophic nutritional exchange is essential for processes such as placentation to occur to facilitate other developmental processes successfully (Burton et al., 2020). Fetal growth is perturbed during the latter stages of gestation when the transplacental exchange is hindered due to improper formation of the placenta. The efficiency of the placenta in regulating the circulation of nutritional materials to a developing fetus is largely dependent on placental and fetal angiogenesis (Reynolds & Redmer, 2001). Angiogenesis involves the formation of vascular beds, which is a determinant of placental blood flow. This process is essential in ensuring an optimal transplacental exchange when hemotrophic nutrition begins. As gestation progresses, this ensures adequate uterine-umbilical blood flow (Bairagi et al., 2016; Reynolds et al., 2006). The process of placentation is affected by many factors including maternal age, genotype, heat stress, and maternal undernutrition (Reynolds & Redmer, 2001). Embryonic organogenesis begins to take place concurrently as germ layers differentiate and evolve into various organs including the brain, heart, and liver. An aberration in the development of these organs increases the susceptibility to fetal mortality or offspring morbidity. About 30 to 50% of embryonic loss occurs during early pregnancy, and perturbed placentation and embryonic organogenesis are cited as causative factors (Reynolds & Redmer, 2001). In livestock studies, maternal feed restriction during the first trimester of gestation affected the development of the utero-fetal membrane in heifers and consequently affected the growth trajectory of the fetus (Micke et al., 2010). A 50% feed restriction from day 28 to 78 of gestation in sheep affected placenta vascularity, limited the transplacental supply of glucose and affected organs such kidney, lungs, and liver of fetuses on day 78 of gestation (Vonnahme et al., 2003). Parr & Williams (1982), made similar observations when ewes

were provided differing feeding regimes for the first 35 days of gestation; embryonic liver weight and plasma glucose concentrations of feed restricted ewes were reduced. Fetal weight at day 90 of gestation was also significantly reduced when ewes were fed to lose 12% of body weight by the end of the first trimester (Everitt, 1964). In guinea pigs, placental efficiency was affected when feed was restricted to 40% of requirements during early gestation (Dwyer et al., 1992). In contrast, some studies observed no significant differences in either placental or foetal membrane development between feed restricted and non-restricted foetuses during early gestational stages (Heasman et al., 1998; Steyn et al., 2001; Wallace, 1948).

The first phase of the biphasic muscle formation process initiates during the first trimester. Primary myofibres are predominantly formed during early gestation and are setting the foundation on which secondary myofibres are formed in subsequent stages (Wilson et al., 1992). The number of myofibers formed in the primary phase is limited and is mainly influenced by genetic factors more so than extrinsic factors like maternal nutrition (Picard et al., 2002). Wilson et al. (1992) reported that primary myofibres make up only 2% of the total muscle fibers in an adult sheep. Due to the limited number of muscle fibers formed during primary myogenesis, and the fact that the process is highly influenced by genetics, the impact of nutrient restriction may not be severe postnatally, but it still may decrease the number of secondary myofibres. A 50% feed restriction from day 28 to 78 of gestation decreased the number of secondary myofibres in *Longissimus dorsi* muscle in sheep (Zhu et al., 2006). Also, Quigley et al., (2005) observed that feed restriction during early gestational period decreased secondary myofibres by 20% in the feed restricted ovine fetuses by day 75 of gestation. Although prenatal nutrition may not directly impact primary myogenic processes, research has shown that it could indirectly do so by influencing the fetal expression of genes relating to muscle development. Skeletal muscle development involves intricate activities

that require the activation of genes (Gossett et al., 1989). Foroutan et al. (2021) reported that restricting prenatal nutrition from day 30 of gestation influenced the expression of muscle development gene myocyte enhancer factor 2A (*MEF2A*) in adult bulls. It is essential, therefore, that the provision of diet to pregnant dams during early gestation is adequate to avoid potential long-term negative consequences.

1.3.2. Second trimester of gestation

The second trimester is a period in which most skeletal muscle formation takes place. Secondary myofibers are developed as well as the initiation of adipogenesis (Du & Zhu, 2009). Nutrients are partitioned into tissues according to their metabolic rate (Bauman & Currie, 1980). In the presence of suboptimal feed supply or restriction, priority supply of nutrients is given to vital organs including the brain, heart, and liver. This makes tissues with a lower metabolic rate such as skeletal muscle vulnerable to its impacts (Close & Pettigrew, 1990; Redmer et al., 2004; Zhu et al., 2006). Unlike the primary phase of myogenesis, the second phase taking place during mid-gestation is more influenced by extrinsic factors like maternal nutrition (Picard et al., 2002). Secondary myogenesis is a period where a greater number of muscle fibers are formed (Yan et al., 2013), and a severe feed restriction during mid-gestation would have a greater effect on fetal growth by limiting the number of myofibers formed. Ithurralde et al. (2020) evaluated pasture-based induced feed restriction and observed a decrease in the ratio of secondary to primary fibers in muscle tissues including *Gluteus medius, Gluteobiceps, Longissimus lumborum, and Semimembranosus.* Cafe et al. (2006) similarly conducted a pasture-based study where pregnant cows were restricted to either a low or a high plane of nutrition pasture starting mid-gestation until parturition. The results from this study indicated that prenatal nutritional treatment influenced progeny birth weight where calves born by cows exposed to low plane pastures had low birth weight, low growth rate, and low

weaning weight compared to those of cows provided with high plane pastures. Additionally, high birth weight progenies were 10% heavier at feedlot entry, displayed higher growth feedlot growth rate, and feed intake (Cafe et al., 2009). In a follow-up study to determine the long-term consequences on carcass characteristics, Greenwood et al. (2006), evaluated the carcass characteristics of calves from Cafe et al. (2006) at age of 30 months and observed that calves who had low birth weight had lighter hot carcass weight (~8% lower) and less overall retail beef yield as compared to their counterparts with high birth weight.

The overall acceptance of meat by consumers depends on carcass characteristics including tenderness juiciness, and flavor (Du et al., 2010b; Miller, 2014). These characteristics are predetermined by the various myogenic processes including adipogenesis, and fibrogenesis (Du et al., 2010a; Zhao et al., 2019). These processes have also been shown to be nutritionally programmed similar to myogenesis (Symonds et al., 2004). A 40% feed restriction from early to mid-gestation, increased the deposition of adipose tissue in the latter stages of gestation. There was also an increase in the mRNA abundance of *IGF1* and *IGF2* receptors (Bispham et al., 2003). Both *IGF1* and *IGF2* are involved in stimulating adipose deposition and this is usually accompanied by increased susceptibility of growth of adipose tissue to anabolic effects (Lorenzo et al., 1993). The increased anabolic effects on adipose tissue increase fat deposition and morbidity rate as a consequence of maternal prenatal nutrition. Since all muscle fibers are formed from early to mid-gestation, provision of quality and adequate feed should be a top priority in meat-based livestock production. Añez-Osuna et al. (2019), also made similar observations with a tendency of increasing *IGF2* mRNA abundance in *Longissimus dorsi* muscle of newborn calves whose dams were given low fat during mid to late gestation. The authors explain that limited fat supplementation most likely resulted in a lower plane of nutrition, and therefore reduced level of

nutrient uptake by the placenta due to occurrence of major developmental processes such as formation of essential tissue and organs during the second trimester of gestation, there is therefore the possibility of prenatal nutrition inducing a long-term impact of progeny performance postpartum.

1.3.3. Third trimester of gestation

Feed restriction during the late gestational stage in almost all species has been shown to decrease fetal growth and subsequent birth weight. The third trimester is a period of fetal muscle hypertrophy (McCoard et al., 2001). As gestation progresses, the transfer of nutritional materials needs to increase significantly in line with the increasing growth of fetal membranes as well as the increasing demand for nutritional supplies (Greenwood & Cafe, 2007; Molina et al., 1991; Vonnahme, 2012). About 75% of fetal weight is obtained during the third trimester (Bauman & Bruce Currie, 1980; Robinson et al., 1980). Essentially, an appropriate combination of macro-and micronutrients is key in ensuring adequate growth and development in the last stages of development (McMillen & Robinson, 2005). Energy-based feed restriction in the last trimester has been shown to impact fetal growth trends (Boyd et al., 1987). Maternal energy intake restricted to 70% during the last 40 days of gestation reduced the expression of genes involved in muscle development and metabolism in skeletal muscle of steers (Sanglard et al., 2018). Similarly, Radunz et al. (2012) demonstrated that feeding differing energy-sources during the late gestation period affected the metabolism of calves, and in the long run implicated their carcass characteristics and quality.

The supply of a high protein diet during late gestation is also essential to muscle hypertrophy. This is because muscle development is regulated by protein synthesis and degradation (Brown, 2014; Murach et al., 2018; Tipton & Phillips, 2013). Sandoval et al. (2020)

reported that protein degradation genes F-box only protein 32 (*FBXO32*) and Tripartite motif-containing protein 63 (*TRIM63*) were upregulated in the skeletal muscle fetus in sheep during the last stage of gestation, which indicates a potential influence of restricting maternal diet (50%) on the expression of these genes and a potential association to the observed reduction in muscle hypertrophy during the last stage of gestation. As parturition approaches, fetal maturation does not only encompass skeletal muscle growth but also leads to fully developed vital organs to help transition the fetus to a postnatal environment (Gluckman et al., 1999). During the late gestation period, dietary supplementation is essential not only to meet the high nutrient demand of the fetus but also crucial in transitioning the dam to the lactating phase postpartum. A well-conditioned dam during late gestation ensures adequate milk production, which is essential to the postnatal performance of offspring (Stalker et al., 2006). A 42% protein supplementation during the final stages of gestation improved the reproductive performance of heifers compared to their counterparts whose dams were not supplemented (Martin et al., 2007).

The birth weight of neonates is the first phenotypic manifestation of the events of gestation (Rehfeldt & Kuhn, 2006). Birth weight is an important measure in livestock production as it factors in the survivability and postnatal performance of offspring (Donald et al., 1999; Quiniou et al., 2002; Vonnahme, 2012). Offspring with higher birth weight tend to have a greater survival rate and perform better postnatally than those born with low birth weight (Funston et al., 2010). Nutrition during late gestation is essential in determining birth weight due to the spike in nutrient demand and exponential growth of the fetus. Provision of adequate feed and or nutrient supplements to pregnant dams could help avoid any detrimental consequence that may arise due to restricted feed or inadequate nutrient supply. Restricting the diet of ewes during the last 6 weeks of gestation resulted in the birth weight of their ram lambs being significantly lower compared to

those that received adequate feed (Khanal et al., 2014). Similarly, LeMaster et al. (2017) reported that the birth weight of calves from nutrient-restricted and nutrient-restricted but protein-supplemented dams was reduced compared to the control group when feed was restricted during the last 100 days of gestation. These observations are akin to those of Bellows & Short (1978), who observed that the birth weight of calves was affected by decreasing feed levels during the last 90 days of gestation to control calving difficulty. Also, supplementing protein into the diet of dams during late gestation produced steer progenies that were heavier compared to those whose dams did not receive protein supplementation (Larson et al., 2009). In humans, nutrient supplementation at the start of the third trimester of gestation resulted in a significant increase in the birth weight of male offspring (Mora et al., 1979).

In terms of survivability, there is an inverse relationship between birth weight and the risk of neonatal mortality i.e., the higher the birth weight, the lower the risk of mortality and vice versa (Christian, 2010; Vilanova et al., 2019). Feed restriction during the last 100 days of gestation increased the mortality rate in calves from feed-restricted dams as compared to their counterparts (Corah et al., 1975). Manning & Vehaskari (2001) induced intrauterine growth restriction by restricting protein supplementation during mid-gestation until birth in rats. Results from the study revealed that offspring from the low-protein group had 15% low birth weight and a 31% lower survival rate compared to offspring from the control group. In swine, intrauterine growth restriction due to inadequate maternal diet and litter size could lead to variations in birth weight within the litters (Foxcroft et al., 2006). Milligan et al. (2002) showed that individuals within a litter with a birth weight below the average of their respective litter had a low survival rate and recorded a higher pre-weaning death rate.

Although there is compelling evidence of the effect of gestational nutrition, birth weight, and the correlation between birth weight and survival rate, it is important to acknowledge that the impact of prenatal nutrition could manifest much later during production. This is especially true when feed restriction occurs earlier during gestation as vital organs and economically important issues were developing. Therefore, the provision of adequate feed and nutrients in their right proportions to pregnant dams throughout all stages of gestation is essential in optimum growth, health, and performance of offspring pre-and postnatal.

1.4. Effect on prenatal nutrition on offspring postnatal performance

A common thread through most fetal programming research is the association of birth weight to fetal growth retardation. The use of birth weight as a marker of fetal programming has been challenged in recent times with some researchers citing that early and/or mid nutrient restriction during fetal development may not necessarily manifest its effect on the birth weight (Barker, 2004b). This absence of an effect is usually the case when nutrient supply is restored to an adequate level in late gestation (Gopalakrishnan et al., 2004; Khanal & Nielsen, 2017). However, the formation of tissues and organs that play a role in essential biological function could also be programmed without necessarily manifesting in low birth weight but impedes proper physiological function postpartum (Gluckman et al., 2008; Sangild, 2006). Suboptimal development during gestation leads to several physiological immaturities such as gastrointestinal immaturity and metabolic homeostatic immaturity (Fowden et al., 1994; Gluckman et al., 1999). These consequences will hinder a successful transition from intrauterine to the extrauterine environment (Avila et al., 1989).

In livestock production, the postnatal performance of offspring is determined by fetal uterine experiences during gestation coupled with postnatal health and lactational performance of

dam, and quality of nutrients supplied after weaning (Greenwood et al., 2010; Robinson et al., 2013). Hence, ensuring an adequate body condition score (BCS) of the dam prepartum impacts the health and lactation performance of the dam as well as enhances neonatal survival and performance postnatally. The sustainability and economic viability of a livestock production enterprise depends on the postnatal performance of offspring in areas of growth, and reproductive performance (Caton et al., 2020). This section of the literature review discusses the impact of prenatal nutrition in areas considered to be of economic importance to beef cattle production.

1.4.1. Fetal organ development and growth trajectory

A well-developed digestive, metabolic and endocrine systems are pre-requisite for optimum postnatal growth performance (Greenwood et al., 2005). Growth restricted offspring can be metabolically immature due to the programming of the gastrointestinal tract (GIT). Neonatal piglets growth-restricted during gestation had lower levels of metabolic regulatory proteins in the small intestines, jejunum, and liver (Wang et al., 2008). In ruminants especially, low expressions of protein particularly in the small intestines may be detrimental to the efficiency of nutrient absorption and utilization as it is the site for terminal digestion (Trotta & Swanson, 2021; Wu, 1998). Similarly, Wang et al. (2005), reported evidence where the morphology of GIT including the stomach and small intestines of IUGR piglets was developmentally impaired when examined between 2 to 4 hours postpartum. In sheep, gastrointestinal growth was compromised when fetal growth restriction was induced by maternal feed restriction and carunclectomy (Trahair et al., 1997). Furthermore, Avila et al. (1989) reported that the total weight of small intestines and gut of IUGR fetuses was significantly reduced compared to non-IUGR fetuses. On the other hand, Liu et al. (2016) showed that increasing energy content in maternal diet during gestation influenced intestinal development of offspring, where piglets from high-energy diet sows had higher intestinal

and villus weight and height. Other evidence of morphological change in GIT due to prenatal nutrition has been reported in rats (Baserga et al., 2004). Conversely, in bovine, maternal nutrient restriction between day 30 to 125 days of gestation did not affect fetal visceral organ development, and by day 245, there was significant growth in the visceral organ of fetuses from nutrient restricted but alimented dams after day 125 of gestation. Additionally, for calves, maternal feed restriction did not significantly affect the majority of gastrointestinal development. However, the weight of the small intestines was negatively impacted (Duarte et al., 2013). Major organogenesis occurs in the early stages of gestation (Meyer et al., 2010) and research conducted on the influence of feed restriction on GIT development has been carried out when feed is restricted during either mid and/or late gestation. This findings implies that there may not be a prenatal nutritional effect on the neonatal stomach and its characteristics (Duarte et al., 2013). Hence, resulting in some variation in the observation of the influence of gestational maternal nutrition on early fetal development. Postnatally, the GIT and liver utilize about half of the total energy expenditure (Caton et al., 2000). Programming of the GIT affects factors influencing growth performance including feed intake, average daily gain (ADG), and growth rate (Bolhuis et al., 2009). Feed intake of female pups exposed to feed restriction during fetal development was significantly lower than the control counterparts at 100 days postpartum (Zambrano et al., 2006). These observations were in agreement with Schinckel & Short (1961), who reported a decrease in feed intake of lambs from feed-restricted ewes during the first 4 months postpartum. Low intake of feed affects growth rate in subsequent stages of production. Houssin & Davicco (1979) demonstrated that digestibility for dry matter, energy, crude protein organic matter, and calcium of neonates with lower birth weight were lower compared to those with higher birth weight. Therefore, an impairment of the GIT sets up situations where offspring nutrient utilization is affected. Inefficiency in nutrient

utilization results in a slower and/or impaired postnatal growth trajectory at various stages of production.

In terms of weight gain, Taplin & Everitt (1964) reported that lambs from feed-restricted ewes were smaller in weight compared to their counterparts within the first 20 weeks postpartum. However, there was significant catch-up on weight gain by lambs from ewes that were feed restricted during the latter stages of gestation. According to Greenwood et al. (1998), the average daily gain (ADG) in low birth-weight lambs was significantly decreased compared to those with higher birth weight. At weaning, the average daily gain (ADG) of sired calves from feed-restricted cows from birth to weaning, weight gain, and live weight at weaning were lower than their counterparts from well-fed cows during pregnancy (Cafe et al., 2006). Likewise, at weaning, nutrient restriction during gestation negatively affected the weaning weight of calves (Larson et al., 2009). At slaughter, the weight of pigs from nutrient-restricted sows weighed less than their counterparts from protein-supplemented sows (Schoknecht et al., 1993). Similarly, lambs from feed-restricted ewes were 9% smaller in weight compared to the lambs from non-feed restricted ewes at maturity (Schinckel & Short, 1961). IUGR was also reported not to affect growth parameters including the bodyweight of foals (Allen et al., 2004). Impaired postnatal growth increases the time for livestock offspring to reach market weight, raising the cost of production and negatively impacting the economic viability of the livestock enterprise. This evidence shows that prenatal programming of metabolic organs and tissues has the potential to negatively influence the sustainability of livestock production.

1.4.2. Postnatal muscle growth

Skeletal muscle is an economically important tissue in raising livestock especially for meat-type enterprises and also an integral part of growth performance as it constitutes about 40% of body

weight (Alexander & Bell, 1975; Khanal & Nielsen, 2017; Thornton, 2019). Postnatal development of skeletal muscle is an accretion of the muscle fibers formed during early to mid-gestation as muscle fiber numbers are mostly fixed at birth (Buttery et al., 2009; Rehfeldt et al., 1999) and is influenced by genetic factors (Rehfeldt et al., 2000). The rate of growth and function of skeletal muscle is determined by the muscle fiber-type composition in terms of metabolism and contraction i.e., oxidative, or glycolytic, and slow or fast fibers respectively (Rehfeldt et al., 2011). Postnatally, most primary muscle fibers formed during gestation transform into slow muscle fiber types while secondary muscle fibers are transformed into fast fibers (Picard et al., 2002). Most muscle fibers are formed during the secondary myogenesis phase, hence secondary muscle fibers contribute significantly to growth performance (Picard et al., 2002). Both epidemiological and livestock research has provided evidence of prenatal nutrition having an impact on these processes. A nutrient restriction during the mid-gestation stage resulted in a decrease in the number of muscle fibers in *Longissimus dorsi* of beef progeny, which persisted throughout offspring's life (Costa et al., 2021). Lamb offspring subjected to nutrient restriction during gestation when their dams were provided 50% of nutrient requirements had fewer myofibres at 8 months postnatal. According to Fahey et al. (2005), neonatal lambs from feed-restricted ewes had fewer fast fibers for *Vastus lateralis* and *Longissimus* muscles as compared to those from unrestricted ewes. The ratio of secondary: primary (S:P) muscle fibers was impacted in pigs at 5 weeks postnatal; pig progeny from supplemented dams had a significantly greater S:P ratio during different phases of fiber hyperplasia, compared to the control group. Consequently, the supplemented group had an increased gain: feed ratio and faster growth rate (Dwyer et al., 1994).

The activities of satellite cells are another factor in muscle hypertrophy of established myofibres (Oksbjerg et al., 2013). Satellite cells merge with muscle fibers to increase DNA content

and protein synthesis (Rehfeldt et al., 2011). The rate of protein synthesis should exceed degradation for optimum skeletal growth (Brameld et al., 1998). The nutritional status of animals programmed due to IUGR and low birth weight can influence the activities of these cells and by default impact muscle growth (Thornton, 2019). The growth of semitendinosus muscle was slower as well a lower abundance of both DNA and RNA in the muscle was observed in lambs with low birth weight (Greenwood et al., 2000).

The expression of insulin-like growth factors *IGF1* and its receptor *IGF1R* regulate the activities of satellite cells in postnatal muscle growth (Chargé & Rudnicki, 2004). Both in-vitro and in-vivo studies have elucidated the positive effect of both *IGF1* and *IGF1R* on skeletal muscle development in postnatal stages of life by altering muscle-specific transcription factors and increasing DNA and protein content respectively (Adams & McCue, 1998; Coleman et al., 1995; Musarò et al., 2001). In humans, Verkauskiene et al. (2005) reported that the IGF1 axis of adults growth-retarded during gestation was programmed and resulted in decreasing concentration of plasma IGF1 and its binding protein IGFBP. The influence of IUGR on skeletal muscle development particularly in beef production not only impacts growth but also plays a role in other aspects of progeny performance such as carcass characteristics.

1.4.3. Effect of carcass composition and meat quality

The impact of prenatal nutrition on carcass composition and quality is determined by how severe feed restriction influenced fetal growth, subsequent effect on feed efficiency, and postnatal growth performance (Greenwood & Bell, 2019). These effects influence the body composition of livestock at slaughter. Hence, the quality of meat is determined by final body composition including skeletal muscle, fiber number, fat, and connective tissues (Wu et al., 2006).

Low-weight pigs at birth had higher levels of subcutaneous fat relative to high-birth-weight pigs as well as lower lean meat content at slaughter. In addition, total fiber number and meat tenderness were low in low-weight pigs for *Semitendinosus* muscle (Gondret et al., 2005). Likewise, Karunaratne et al. (2005) reported that the smallest littermates had an increased proposition of fat compared to the largest littermates, plus increased intramuscular fat and low meat tenderness scores. A 35% decrease in maternal nutrition during early gestation resulted in fatter carcasses, impaired meat tenderness, and affected meat color in adult bulls (Noya et al., 2022). Similarly, restricting maternal diet during early to mid-gestation may have altered gestational myogenic and adipogenic processes resulting in restricted steer progeny having significant lower dressing percentage compared to those from dams provided with moderate diet (Meale et al., 2021). Calves born from protein supplemented cows during gestation were shown to have higher meat quality grades compared to nutrient restricted offspring (Larson et al., 2009). On the other hand, postnatal studies reported no significant effect of prenatal nutrition on either carcass measurements and/or meat quality. For example, prenatal nutrition did not influence carcass characteristics including hot carcass weight and empty body weight, dressing percentage, and marbling score (Long et al., 2010). Also, Long et al. (2012) observed that while nutrient restriction did not affect carcass measurement in steers, there was an increase in adipocyte size in the nutrient-restricted steers. This increase could be an indication of altered metabolism; however, the animals were slaughtered as calves and long-term consequences were not investigated. Similarly, Sen et al. (2016) reported that there was no difference in meat quality measurement in lambs of nutrient restricted, moderately-fed, and over-nourished dams during the early to mid-gestation period.

1.4.4. Interaction of prenatal nutrition and genetic potential for feed efficiency

In cattle production, feed efficiency plays a significant role in profitability of the enterprise. Feeding accounts for at least 60% of production cost and therefore the use of feed-efficient cows aids in making cattle production sustainable (Elolimy et al., 2018). Efficient animals are determined by their residual feed intake (RFI) value, which is the difference between an animal's actual and expected feed intake required for maintenance and growth (Basarab et al., 2003). RFI has also been shown to affect feeding behaviour including daily feed intake, which could eventually affect weight gain and body condition score. Hence, this trait could interact with prenatal nutrition and impact fetal development and postnatal performance (Kelly et al., 2010). A study conducted by Foroutan et al. (2021) reported that maternal genetic potential for RFI influenced the expression of muscle-regulatory genes *MEF2A* in adult bull progeny. In steers, it was observed that there was an RFI-prenatal diet interaction on dressing percentage of progeny, where steer offspring from feed restricted dams with a high genetic potential for RFI (Ldiet-HRFI) had lower dressing percentage. Additionally, marbling content was also reported to be influenced by RFI for the study (Meale et al., 2021). Therefore, it is important to assess the influence of RFI in conjunction with prenatal diet on fetal development as well as potential long-term effect on performance.

1.4.5. Effect of reproductive performance

Livestock production relies on the reproductive ability of the animals to sustain production year-in and year-out. For beef cattle in Canada, a breeding age female should give birth to and successfully raise a calf to weaning every year. Hence, reproductive development of progeny is essential in sustaining the beef production industry. As in skeletal muscle, nutrition for the development of reproductive tissues during gestation is at the short-end of nutrient partitioning in

cases of feed restriction (Vonnahme, 2012). In male offspring, reproductive performance can be affected when maternal prenatal nutrition hinders the development of the testes during early gestation (Mccoski et al., 2021). Testicular size has been used to quantitatively predict the production of spermatozoa in bulls (Willett & Ohms, 1957), ram lambs (Yarneyi et al., 1990), and humans (Arai et al., 1998). All these studies show a positive correlation between the size of testicles, hormone production, and the amount of semen produced. According to Bielli et al. (2002), restricting metabolizable energy intake of Merino ewes during gestation significantly decreased the number of Sertoli cells in neonatal lambs. There were also differences in the weight of paired testis, where lambs from non-restricted ewes had heavier paired testes. Sertoli cell number is highly correlated with testicular size and sperm production (Hochereau-de Reviers et al., 1995). A similar finding of a reduced number of Sertoli cells as well as Leydig cells due to prenatal energy restriction in swine was made by Lin et al. (2019). A reduced number of these cells could be an indication of potential negative effects on semen production in adulthood. The reproductive ability of bulls showed some implications with maternal feed restriction during the early gestation period. Sperm quality was lowered and increasing days in reaching puberty. Subsequently, the concentration of follicle-stimulating hormone was also lowered by days 330 and 438 postnatal (Copping et al., 2018). Although there are some impacts of prenatal nutrition on semen production and its quality, this may not provide enough evidence for the overall effect on male fertility (Cameron et al., 1984).

In female progeny prenatal maternal feed restriction during delayed fetal ovarian follicular development (Rae et al., 2001), which could have implications at later stages of life. In subsequent research, Rae et al. (2002) reported that the ovulation rate of female ewes from feed-restricted dams was reduced compared to those from the moderately fed dam during gestation. A 50% feed

restriction during mid-gestation in sheep was reported to decrease large corpora lutea in restricted female progeny at 10 months of age (Kotsampasi et al., 2009). Furthermore, Mossa et al. (2013) showed that restricting prenatal maternal diet during gestation resulted in their heifers possessing compromised ovarian reserve and a potential suboptimal fertility. Similarly, Hoffman et al. (2018) reported that ovarian follicle formation, uterine weight, and number of endometrial glands in the uterus of female progeny whose dams were feed restricted during late gestation were lower relative to their control counterparts.

In contrast, some researchers have observed a lack of discernable effects of prenatal feeding regime on reproductive factors in offspring. Testicular size and semen quality were not affected in male offspring at either 6 weeks or 20 months postnatal age when ewes were feed restricted by 50% (Rae et al., 2002). In ewe lambs, supplementing metabolizable protein during gestation did not affect the reproductive performance of the F1 generation (Van Emon et al., 2015). Also, Cracco et al. (2021), showed that providing differing levels of feed to dams during gestation had no influence on the reproductive development and performance of heifers at 18 months of age. As with other research on fetal programming, variations in results could be attributed to timing, the severity of feed restriction, as well as postpartum husbandry management.

1.5. Molecular mechanisms of fetal programming

Data from both epidemiological and livestock studies have corroborated the impacts of uterine programming of the fetus through maternal insults. Although an extensive amount of research has been carried out on fetal programming, the mechanisms underlying its processes are still in the early stage of investigation (Vo & Hardy, 2012). However, mediators of intrauterine programming include the actions of glucocorticoids, genetic polymorphisms, and epigenetics (Gicquel et al., 2008; Harding et al., 2010). As gestation progresses into the last trimester and fetal maturation

surges, increasing levels of glucocorticoids in the maternal-fetal membrane play a significant role in the developmental switch of organs such as lungs, brain, and kidneys (Moisiadis & Matthews, 2014a), and regulate fetal gene transcription (Fowden et al., 2005). These changes are essential for postnatal survival (Fowden et al., 1998). However, in situations where the dam is exposed to stress factors including undernutrition, the levels of glucocorticoids exceed the normal threshold leading to an increase in the levels in the fetal membrane (Harding et al., 2010). Higher glucocorticoid levels affect fetal growth through their influence on fetal hypothalamic-pituitary-adrenal (HPA) axis activity (Chapman et al., 2013; Seckl & Holmes, 2007). The HPA axis is involved in regulating several functions including metabolic, cardiovascular, and reproductive function (Braun et al., 2013; Moisiadis & Matthews, 2014b) and influences response to stressful conditions (Fowden et al., 2005). Therefore, altered HPA axis activity affects fetal growth trajectory and possible alterations in postnatal physiological abilities (Gicquel et al., 2008). The association of the actions of glucocorticoids with the activities of the HPA axis and fetal growth trajectory makes it a possible mechanism regulating fetal programming (Harding et al., 2010).

Another possible mechanism influencing fetal programming is the somatotropic axis, which includes the IGF gene family and the genes encoding their receptors including *IGF1, IGF1R, IGF2*, and *IGF2R* (Bauer et al., 1995; Gicquel & Le Bouc, 2006; Ong et al., 2015). The somatotropic axis is mainly regulated by maternal nutritional status and in the case of the fetus, the state of its uterine environment (Bauer et al., 1995). The IGF family has been shown to play a significant role in fetal growth (Hattersley & Tooke, 1999). The *IGF2* gene regulates early embryonic growth and development while *IGF1* regulates growth during the final stages of gestation (Baker et al., 1993; Bloomfield & Harding, 1998). Therefore, genetic polymorphism of *IGF1* and *IGF2* may elucidate the effect of maternal undernutrition on fetal growth and possibly

postnatal performance (Gicquel et al., 2008). Heude et al. (2007) reported that polymorphism of *IGF2* was associated with height in human adults. Similarly, polymorphism of *IGF2* has been demonstrated to be associated with body mass index in middle-aged men (Gaunt et al., 2001). Although nutrition affects the circulation of both IGFs, levels of IGF1 appear to be more affected compared to IGF2 (Fowden, 2003). This implies that feed restriction would affect its concentration and consequently alter fetal growth trajectory. Low *IGF1* concentration has been shown to lead to lower birth weight (Arends et al., 2002). Vaessen et al. (2002) also reported that IGF1 polymorphism caused a 215 g decrease in the birth weight of diabetic individuals, suggesting that the IGF family is a mediator fetal programming.

Perhaps the most studied mechanism regulating fetal programming are epigenetic modifications, which could also be the mechanism influencing the activities of HPA and somatotrophic axis on fetal programming. Epigenetic modifications involve the regulation of gene expression beyond the genetic make-up of an individual. Epigenetic modifications can be influenced by the external environment including diet, stress, and trauma. Both epidemiological and livestock studies have linked epigenetics to fetal programming as well as the etiology of diseases including cancer, cardiovascular, and neurobehavioral diseases (Choi & Friso, 2010). The succeeding sections of this literature review will focus mainly on epigenetics, its mechanisms, and its influence on postnatal development and performance of offspring.

1.5.1. Overview of epigenetic modifications

The genome of an organism constitutes its genetic make-up, which dictates how cells within the organism function through the process of DNA transcription and protein translation. Chemical compounds can attach to the DNA within a genome, controlling its function within a cell. It does so by influencing when a protein is produced, the rate of production, and possibly how a protein

is produced, without altering the DNA sequence. These chemical compounds are known as the epigenome or "epigenetic marks" (NHGRI, 2019.; Tiffon, 2018). This phenomenon implies that the manifestation of a phenotype is not only dictated and influenced by the genetic make-up of an organism, but also the epigenome. The activities of the epigenome allow for translation of the same genetic information into variable phenotypes (Agarwal & Weinstein, 2018). Epigenetics is defined as the heritable alterations of gene expression independent of its underlying genomic sequence (Agarwal & Weinstein, 2018). Developmental biologist, Conrad Waddington in 1942 first coined the word epigenetics to describe the complexities of developmental processes underlying the link between genotype and its resulting phenotype using *Drosophila melanogaster* (Waddington, 2012; Watson, 2014). Since then, interest in epigenetics and its influences on phenotypes has increased. In the field of health, epigenetics has been associated with diseases such as cancer and developmental disorders including Prader-Willi and Angelman syndromes (Choi & Friso, 2010; Esteller, 2008; Watson, 2014).

Epigenetic modifications play a role in embryonic development by initiating and maintaining the cellular differentiation process leading to tissue and cell-specific gene expression (Delcuve et al., 2009). Therefore, a change in the epigenetic modification leads to a change in the expression of a gene and subsequent variations in phenotype or diseased conditions (Esteller, 2008; Lillycrop et al., 2005; Tiffon, 2018). External environmental factors including stress, pathogens, drugs, and nutrition influence epigenetic processes by affecting the epigenome (Cropley et al., 2006; Tiffon, 2018). Due to fetal dependence on maternal nutritional status for the supply of nutrients for its development, prenatal nutrition affects the epigenetic constitution of the fetus, altering its gene expression and phenotypic expressions.

1.5.2. Role of epigenetics in fetal programming

The Developmental Origin of Health and Diseases (DOHAD) hypothesis has provided evidence that metabolic disorders in adulthood are not caused exclusively by genetic factors but in large part are influenced by fetal intrauterine factors (Zhu et al., 2019). From the plasticity of the epigenome to factors that influence intrauterine environment makes, the epigenetic modification is a prime mechanism behind fetal programming. Furthermore, epigenetic modifications can be transgenerationally inherited through mitosis and meiosis (Turek-Plewa & Jagodziński, 2005; Waterland & Michels, 2007). During embryonic development, inherited parental genomes undergo a series of processes to reprogramme the epigenetic landscape of the developing embryo (Hsu et al., 2015). The reprogramming enables the revision of previously acquired epigenetic modifications in the parental genome, a phenomenon known as genomic imprinting (Surani et al., 1993). However, some of these epigenetic modifications that control the expression of specific genes could be passed on to the embryos transferring undesirable traits to subsequent generations, and this is known as germline epigenetic inheritance (Rakyan et al., 2001).

1.5.3. Mechanisms of epigenetics

Epigenetic mechanisms include DNA methylation, histone modification, chromatin remodeling, and non-coding RNA (Choi & Friso, 2010). DNA methylation studies were previously conducted by our research group and part of the objective of the current study is to validate the results from those studies. Therefore, the following section discusses the mechanisms of DNA methylation and its implications on gene expression and phenotype in livestock production.

1.6. DNA methylation

DNA methylation is the most studied and well-characterized of the epigenetic mechanisms. It involves the covalent addition of a methyl group to the 5-carbon position of a cytosine base in a

cytosine-guanine dinucleotide (CpG) region (Bird, 1978; Santi et al., 1983). In mammals, about 60-90% of CpGs are methylated and the remaining unmethylated CpGs cluster into regions known as CpG islands (Suzuki & Bird, 2008). A CpG island is approximately 200 bp in length and contains over 50% cytosine-guanine dinucleotide (Janitz & Janitz, 2010). The CpG islands tend to be present near the promoter or first exon region of a tissue-specific genes (Bird, 1986; Li et al., 2019; Turek-Plewa & Jagodziński, 2005). This property allows the methylated CpGs to induce an effect on gene transcription by either inhibiting the binding of transcription factors to the promoter, or to attract methylation binding proteins (methyl-CpG binding protein, MBPs) that then bind to the DNA and thus block transcription factors from binding to the DNA (Kass et al., 1997). Hence, methylation of the CpGs within the CpG island is generally linked to the silencing of genes and repressing their expression. Additionally, DNA methylation is also associated with other functions such as X chromosome modification, genomic imprinting, chromatin modification, and normal embryonic development (Sulewska et al., 2007). DNA methylation is a tissue-specific process whereby the methylation of CpGs within each tissue differs from others allowing for the expression of the gene that is essential to the functions of the tissue (Razin & Riggs, 1980; Razin & Szyf, 1984).

DNA methylation is catalyzed by enzymes known as DNA methyltransferases (Dnmts) which include Dnmt1, Dnmt2, Dnmt3a, and Dnmt3b. These methyltransferases perform different and specific roles in the DNA methylation process. Dnmt1, Dmmt3a, and Dnmt3b are involved in methylation processes during gametogenesis, embryogenesis, and the development of somatic tissues in mammals (Rakyan et al., 2001). Dnmt3a and Dnmt3b are involved in *de novo* methylation processes during embryogenesis while Dnmt1 regulates the maintenance of methylation patterns (Hermann et al., 2004; Turek-Plewa & Jagodziński, 2005). Dnmt2 on the

other hand has been shown to have low methylation activity (Li, 2002). Some studies in mice have shown the necessity of these methyltransferases in embryonic development. Hirasawa et al. (2008) used conditional knockout mice and demonstrated that the absence of maternal and zygotic Dnmt1 resulted in complete demethylation of imprinted loci in the blastocyst of the mice. DNA methylation has emerged a leading epigenetic mechanism potentially inducing transcriptional changes to genes and subsequent variations in phenotype. Hence, deviation in methylation-regulated developmental processes, such as genomic imprinting could modify fetal development and postpartum performance.

Nutrition is a major regulator of the DNA methylation process. Nutrients such as methionine and folates which are involved in one-carbon metabolism, serve as methyl group donors (Anderson et al., 2012). These nutrients can also influence enzymes that activate DNA methylation (Choi & Friso, 2010; Cyr & Domann, 2011; Kim et al., 2009). Other nutrients such as zinc, selenium, and Vitamin C have been associated with DNA methylation. The deficiency of these nutrients resulted in either hypo or hyper-methylation in cells (Davis et al., 2000; Dreosti, 2001; Halliwell, 2001). The activities of the Dnmts have been shown to also be susceptible to external factors including stress. This implies that the environmental conditions in which an individual develops in could determine their DNA methylome. Furthermore, DNA methylation is potentially reversible since Dnmt activities can be regulated by external factors. Therefore, some modification factors make it possible to reverse the effects of the methylation process (Szyf, 2009). As mentioned in earlier sections of this literature review, there are possibilities of pregnant cows to be nutritionally stressed and studies have shown that such stress could influence fetal DNA methylation processes (Li et al., 2021). It is therefore crucial to understand the potential influence

of maternal gestational nutrition on DNA methylation and its regulation of fetal and progeny development.

1.6.1. DNA methylation and fetal development

The embryonic stage of development is a period where an organism's DNA methylome and its patterns are first established. The methylation reprogramming of a developing fetus occurs in two phases (Figure 1.3). The first phase begins immediately after fertilization, where the paternal genome is demethylated rapidly while demethylation of the maternal genome takes a more gradual replication-dependent approach (Rougier et al., 1998). Methylation pattern are reestablished and remain stable after differentiation of somatic cells (Bommarito & Fry, 2019; Monk et al., 1987). The second phase of methylation programming takes place within primordial germ cells (PGC) (Figure 1.3) in a sex-specific approach during gametogenesis (Allegrucci et al., 2005). An example is the DNA methylation pattern in X-chromosome inactivation. X-chromosome inactivation is a mechanism whereby one of the X chromosomes of a female embryo is inactivated in cells excluding the oocytes (Matias et al., 2020). Yasukochi et al. (2010) reported that inactive X-chromosomes are hypermethylated at the CpG islands that are located within promoter regions of a gene, silencing their expression. Additionally, genomic imprinting is also an essential event that occurs during development. Imprinting refers to the expression of genes based on their parental origin. Examples include genes such as *H19*, *IGF2, GRB10,* and *IGF2R* (Deshpande et al., 2021). A study by Li et al. (1993) indicates that expression of *H19*, *IGF2,* and *IGF2R* was affected in mutant mice embryos as a result of impaired activities of Dnmts. Post-gastrulation development requires the *de novo* methylation patterns attained for normal development. An aberration in the methylation reprogramming results in impaired development and some situations could lead to mortality (Bommarito & Fry, 2019).

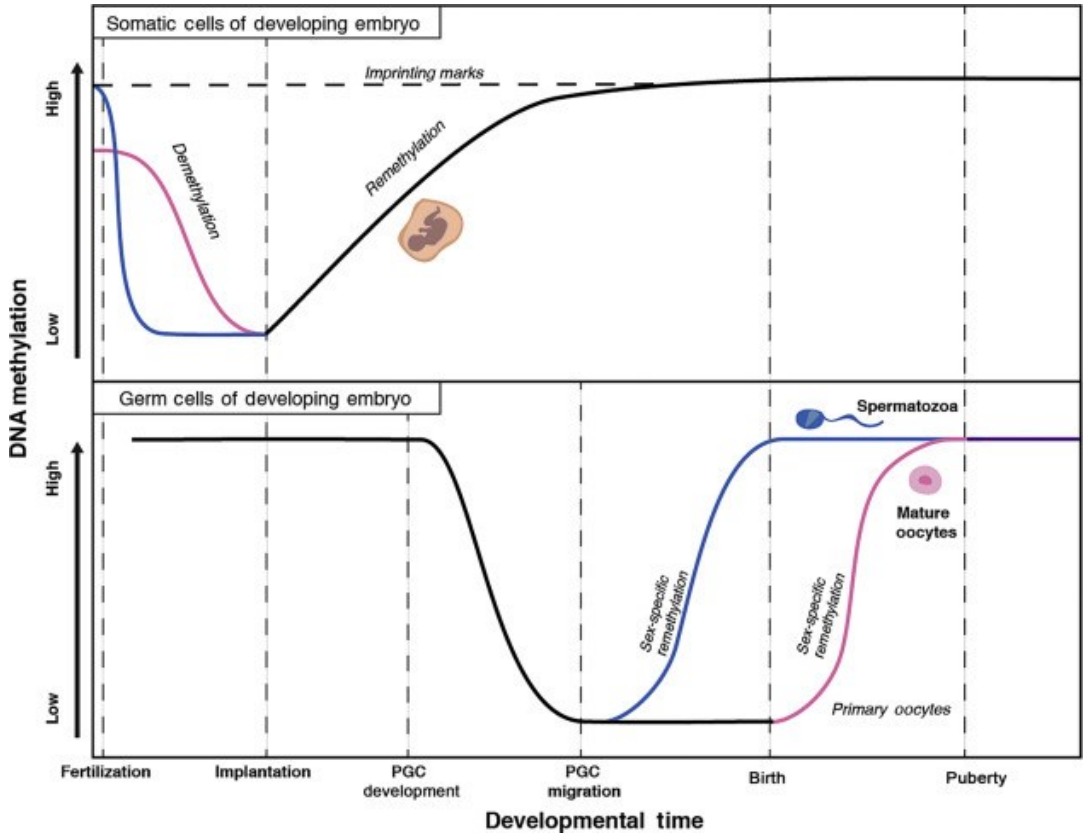

Figure 1.3 Schematic of DNA methylation programming. Initial phase of the programming takes place after fertilization during embryogenesis. Parental genome is first demethylated and then remethylated. The succeeding phase takes place after implantation in the primordial germ cell to develop sex-dependent methylation patterns

1.6.2. Effect of prenatal nutrition on DNA methylation

For developing fetuses, maternal nutrition affects DNA methylation processes consequently affecting the fetuses' DNA methylome. This may influence fetal developmental processes and phenotypic characteristics postnatally, independent of their genomic constitution (Dolinoy et al., 2007). The agouti mouse is an example of an organism possessing an epigenetically determined phenotype, its coat color. The allele (A^{VY}) controlling the expression of the *agouti* gene is susceptible to epigenetic modifications and its expression is associated with endocrine and physiological disorders (Cooney et al., 2002). Waterland & Jirtle (2003) supplemented *a/a* dams

with extra folic acid, choline, betaine, and vitamin B_{12} throughout pregnancy. This resulted in increased methylation of the A^{VY} loci and altered the coat color phenotype of A^{VY}/a progeny (Figure 1.4).

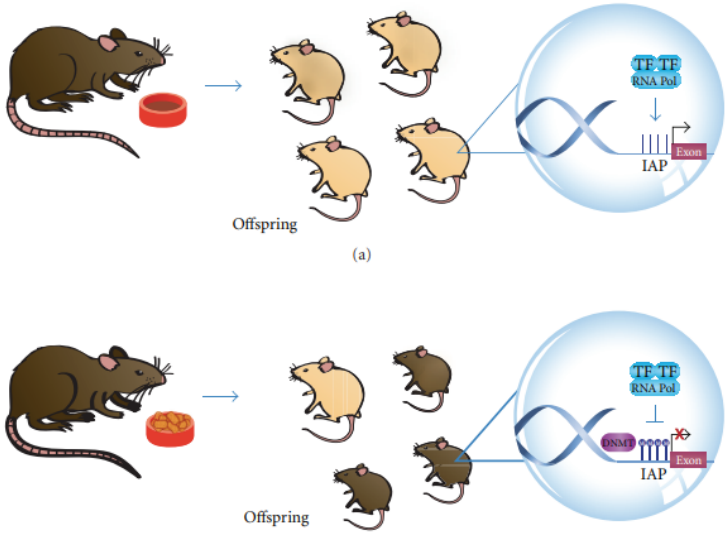

Figure 1.4 Diagram showing the change in coat color in progeny of agouti mouse when their dams are supplemented with diet enriched with methyl donor

Furthermore, a study in sheep showed that providing differing feed regimes ranging from restricted to overfeeding to pregnant ewes resulted in variations in cellular proliferation as well as DNA methylation patterns between the fetuses (Peterson et al., 2021). In bovine studies, restricting dams' feed in mid-to late gestation was reported to influence methylation in *IGF2*-DMR2 in fetal muscle tissue (Paradis et al., 2017). Devos et al. (2021) reported that maternal feed restriction during early to mid-gestation influenced DNA methylation in steer progeny where the progeny displayed differential methylation patterns in muscle and liver tissues at birth, weaning and at slaughter, with respect to prenatal diet. Also, in humans, maternal exposure to hunger and its resulting consequences have been associated with DNA methylation changes. A prime example is

evidence from the Dutch famine in the winter of 1944-45 which resulted in a surge in adult cardiovascular diseases in progeny. In 2008, Heijmans et al. reported that those individuals exposed to the famine had differential methylation patterns for the *IGF2* imprinted gene compared to their unexposed counterparts.

As mentioned earlier, in the absence of external factors influencing DNA methylation, progeny could inherit methylation patterns during embryonic development and lead to phenotypes that are associated with this modification. Although there has been extensive research conducted on DNA methylation, studies on DNA methylation effects in ruminant livestock are scarce. Therefore, more research is needed to gain more understanding of the relationship between DNA methylation, prenatal nutrition, and progeny gene expression.

1.6.3. Methods of measuring DNA methylation

There are several factors to consider when choosing a particular technology for analyzing DNA methylation including the aim of the study, sensitivity and specificity requirement of the DNA sample, availability of bioinformatics, specialized equipment and reagents, and cost (Kurdyukov & Bullock, 2016). There are three methods for detecting and measuring methylation patterns: bisulfite conversion, restriction enzyme-based, and affinity capture or enrichment approach (Laird, 2010; Šestáková et al., 2019). These methods serve as a pretreatment of the DNA and it involves the treatment of genomic DNA with a methylation-dependent step followed by the use of a molecular biology technique to identify and quantify methylated regions in terms of genome-wide, specific, or global methylation (Laird, 2010; Mansego et al., 2013).

1.6.3.1. Bisulfite conversion approach

The bisulfite methylation analysis approach employs the conversion of genomic DNA as the first step. Sodium bisulfite treatment of DNA deaminates the region of unmethylated cytosine into

uracil, leaving methylated cytosines unconverted (Hayatsu et al., 1970). The bisulfite conversion approach has been referred to as the gold standard of the methylation approaches as the approach is known to quantify methylation status both qualitatively and efficiently at single-base pair resolution (Li & Tollefsbol, 2011). There are however some disadvantages that come with the approach, some of which include; possible incomplete conversion of unmethylated cytosines, degradation of DNA after chemical treatment, and a relatively high amount of starting DNA sample required (Mansego et al., 2013; Wang et al., 2017). Another major limitation for the bisulfite conversion approach is the inability to differentiate between 5-hydroxymethylcytosine (5hmC) and 5-methylcytosine (5mC) (Huang et al., 2010). The 5hmC is a relatively novel cytosine modification dominant in embryonic stem cells and has been reported to be an intermediate modification for DNA demethylation (Shen & Zhang, 2013; Tahiliani et al., 2009). The antibodies developed in assessing 5mC methylation via bisulfite approach have been shown to not effectively assess 5hmC methylation (Huang et al., 2010).

After bisulfite treatment, methylation-based differences are converted into DNA sequence-based differences. This allows for the quantification of methylation status across the entire genome using various PCR-based methods (Kurdyukov & Bullock, 2016). Examples of PCR-based methods include bisulfite sequencing PCR, droplet digital PCR, bisulfite pyrosequencing, EpiTYPER, and methylation-sensitive high-resolution melting (MS-HRM) (Wani & Aldape, 2016).

1.6.3.2. Restriction enzyme-based approach

A restriction enzyme-based approach to the detection of methylation patterns involves the use of restriction enzymes such as HpaII, NotI, SmaI, or MspI to specifically digest and cleave either methylated or unmethylated regions (Pajares et al., 2021). HpaII and MspI have been used in

conjunction to detect methylation patterns. Both HpaII and MspI detect CCGG sequences and can either digest or cleave these sequences based on their methylation status. HpaII is usually blocked and unable to cleave the sequence in situations where the second cytosine in the CCGG sequence is methylated (i.e., $C^{m}CGG$) while MspI can cleave the sequence irrespective of its methylation status (Tost, 2016). After the use of restriction enzymes, methylation status can be detected using PCR methods or southern blot hybridization methods (Fraga & Esteller, 2002). This approach is especially limited by the specificity of the restriction enzymes used in the analysis. Some methods in association with restriction enzyme, quantifies DNA methylation including MSRE-southern blot and MSRE-PCR (Pajares et al., 2021)

1.6.3.3. Affinity enrichment approach

The affinity enrichment approach utilizes antibodies or methyl-binding proteins to detect methylation status. This approach involves the immunoprecipitation (IP) of nonspecific DNA fragments by anti-5mC antibodies to identify methylcytosine or methyl-binding protein beads used to purify the DNA resulting in an enrichment for methylated sequences (Thu et al., 2010). Specifically, genomic DNA is fragmented and divided into an IP fragment and an untreated genome reference. The IP fragment is denatured and incubated with the anti-5mC antibody or a methyl-binding protein (Thu et al., 2010). The enrichment step is followed by labeling the IP fragment and reference genomic DNA with fluorescent dyes and then co-hybridized onto a microarray chip (Zuo et al., 2009). The methylation status of a gene is detected using the ratio of fluorescent intensities of enriched to total genomic DNA. The affinity enrichment approach is easy, sensitive, and specific, and suitable for genome-scale methylation analysis (Pajares et al., 2021). However, the approach does not provide quantitative DNA methylation values but enrichment values for the status of methylation over the genomic region. Another limitation is that due to the

high specificity of the approach tailored for dense methylated CpG regions, the approach may not be suitable for low methylated CpG regions (Zuo et al., 2009).

1.6.3.4. Profiling DNA methylation

The approaches of identifying methylation status allows for profiling DNA methylation on a whole genome or specific fragment level. These approaches distinguish between methylated and unmethylated DNA regions followed by techniques to quantify the methylation status. Some of these techniques includes high throughput technologies such as next generation sequencing (NGS) and array hybridization techniques. While the approaches discussed above could be used in assessing methylation at whole-genome level, each approach does have its pros and cons. Bisulfite approach allows for methylation status identification at single nucleotide resolution while requiring a relatively low DNA input compared to the affinity enrich approach. The restriction enzyme approach has been reported to be prone to false-positive result due to incomplete digestion (Fan & Chi, 2016). Also, bisulfite conversion can be used to interrogate methylation status at whole-genome and specific locus level unlike the affinity enrichment approach. Examples of such techniques are whole-genome bisulfite sequencing (WGBS), and reduced representation bisulfite sequencing (RRBS), and EpiTYPER. Compared to the bisulfite approach, the affinity enrichment is cheaper. Therefore, the choice of approach in interrogating DNA methylation status could depend on level of methylation information required, cost, and quantity of DNA sample.

1.7. Concluding remarks

Prenatal nutrition plays a significant role in fetal development. The development of tissues and/or organs essential to the functions and performance of offspring in postnatal life is dependent on maternal nutrient supplies, and therefore maternal feed restriction at critical stages of gestation affects fetal development. Furthermore, the selection for feed efficiency could also influence

feeding behavior and consequently interact with gestational diet to regulate fetal development. Impaired fetal development affects postnatal development in areas of growth, reproduction, carcass composition, and meat quality. The phenomenon of maternal experiences during gestation influencing fetal development is known as fetal programming. Epigenetic modifications have been speculated to be the mechanism underlying the fetal programming process. This implies that the provision of adequate nutrition to dams is essential in the sustainability of every livestock production. Therefore, this study seeks to elucidate the effects of prenatal feed restriction at day 30 to 150 of gestation and genetic potential for RFI on DNA methylation patterns and the expression of genes of metabolic importance. The results from this study will aid in explaining the molecular mechanisms, specifically DNA methylation, and their relation to some phenotypic variations in cattle progeny.

1.8. Thesis objectives

The objectives of this study are:

1. To Determine if differential methylation patterns discovered from WGBS of bull semen can be verified by another method in semen.
2. To Determine if differential methylation patterns discovered from WGBS of bull semen can be found in other tissues of the same and other animals belonging to the same experiment.
3. To determine the effect of prenatal feed restriction from day 30 to 150 of gestation and genetic potential for RFI on DNA methylation in semen and gene expression *in Longissimus dorsi, Semimembranosus* muscle, liver, and testis tissues between bull offspring: and

4. To determine the effect of prenatal feed restriction from day 30 to 150 of gestation and genetic potential for RFI on DNA methylation and gene expression in *Longissimus dorsi, Semimembranosus* muscle, and liver tissues between steer offspring.

1.9. General thesis hypotheses

This thesis proposes the following general hypotheses

1. EpiTYPER quantification of DNA methylation in this study would validate the DMRs identified using WGBS technique.
2. Similar DMRs identified in semen due to prenatal restriction would be present in *Longissimus dorsi*, *Semimembranosus* muscle and liver tissues in steer progeny.
3. Prenatal feed restriction during day 30 to 150 of gestation alters DNA methylation pattern in *Longissimus dorsi, Semimembranosus* muscle and liver tissues of steers and semen in bull offspring; and
4. Prenatal nutrition feed restriction during day 30 to 150 days of gestation influences the expression of genes in *Longissimus dorsi, Semimembranosus* muscle and liver tissues of steer offspring and *Longissimus dorsi, Semimembranosus* muscle, liver, and testis tissues of bull offspring.

Chapter 2. Effect of prenatal feed restriction and RFI on semen methylation and tissue gene expression in bull progeny

2.1. Introduction

Global beef cattle production is expected to increase by 6% and account for 9% of total protein consumption by 2030, compared to 52% projected contribution by poultry (OECD/FAO, 2021). The slow growth in beef production is attributed to increasing production costs (OECD/FAO, 2021). Feeding constitutes the largest input expenditure accounting for approximately 60% of the total production cost in a beef production enterprise (Greenwood, 2021). Cattle producers can reduce feed costs by practicing a semi-intensive or extensive system of production that is highly reliant on pasture availability (Noya et al., 2019). However, the quality and quantity of the pastures are dependent on climatic conditions which can vary extremely throughout the production stages (Greenwood, 2021). Ultimately, variations in the availability and quality of feed result in situations of underfeeding arising in the cattle herd. Furthermore, factors such as herd dynamics introduce competition for feed within the herd, where socially and physically dominant individuals tend to have more access to feed than others (Grant & Albright, 2001). Potentially, these situations could result in cases of underfeeding that may coincide with gestational periods and consequently affect fetal development (Long et al., 2010).

Prenatal maternal nutrition regulates fetal growth and subsequent postnatal development of progeny (Du et al., 2011). Fetal development is a nutrient-dependent process that relies on maternal nutritional supplies via the placenta to ensure their efficiency (Wang et al., 2012). The formation of the placenta can be impaired by nutrient restriction in the early gestational stages, which disrupts the materno-fetal exchange of nutrients (Belkacemi et al., 2010; Greenwood et al., 2017; Toschi & Baratta, 2021). Nutrient restriction at different stages of gestation affects the formation of vital tissues and organs regulating postnatal development dictating the economic

potential of a livestock enterprise (Wallace, 1948). Hence, limited or restricted nutrition at critical periods of gestation results in fetal intrauterine growth retardation (IUGR) (Wu et al., 2006). In a study by Long et al. (2009), feed restriction during early gestation negatively impacted the growth of bovine fetuses by day 125 of gestation. Furthermore, Meyer et al. (2010) reported that early to mid-gestation feed restriction negatively affects the formation of fetal visceral organs and gastrointestinal tract development. Thus, nutrient absorption and utilization in subsequent stages of production could be perturbed. Similarly, Andrés et al. (2020), showed that growth-retarded fetuses have lower birthweights, which potentially could impair postnatal development. Therefore, ensuring adequate and quality nutrient supply to pregnant dams to produce well-developed offspring is prudent and essential for maximum progeny performance and economic benefit.

As compared to other livestock species such as poultry and swine, cattle are less efficient due to relatively high energy requirements for maintenance, and slow reproductive rates (Basarab et al., 2003). Feed efficiency of beef cattle influences production cost as well as the profitability of the enterprise. Thus, the use of feed-efficient cattle could reduce input costs and can improve the economic prospects of production (Arthur & Herd, 2008; Elolimy et al., 2018). Residual feed intake (RFI) is a feed efficiency measurement employed by cattle producers to determine and select feed-efficient animals for production (Koch et al., 1963). RFI is the difference between the actual and expected feed intake needed for the growth and maintenance of livestock (Arthur et al., 2001; Basarab et al., 2003). The heritability of the genetic potential for RFI has been estimated to range from moderate (0.21) to high (0.60) (Hoque & Suzuki, 2009; Seabury et al., 2017). Hence, cattle producers also employ the use of RFI to produce genetically feed-efficient progeny for subsequent production (Herd et al., 1997). Although selection for feed efficiency has its advantages, it may interact with other production traits in both positive and negative ways. Selection for RFI has been

associated with reproductive impairment in livestock species (Hagger, 1994; Kerr & Cameron, 1995). In bulls, Fontoura et al. (2016) reported decreased sperm motility in high feed efficient groups, indicating potential influences on fertility. Similarly, Behrouzi et al. (2022) also reported that selection for high feed efficiency (LRFI) impacted pregnancy rates in heifers. In contrast, Hafl et al. (2012) and Kowalski et al. (2017), reported similar reproductive traits and performance irrespective of RFI potential. Further interrogation on the impacts of RFI on production traits is needed to address the growing concerns of its interference in the efficiency of some economic characteristics.

Epigenetic modification is the molecular mechanism hypothesized to regulate the effect of maternal metabolic status on fetal development (Bernal & Jirtle, 2010). It is defined as heritable changes in gene expression without a change in the underlying DNA sequence (Casadesús & Noyer-Weidner, 2013). Mechanisms of epigenetic modifications include DNA methylation, histone modification, and microRNA (Lee, 2015). DNA methylation is the most studied epigenetic modification and it involves the covalent addition of a methyl group to the 5' carbon position of cytosine in a cytosine-guanine (CpG) dinucleotide (Bird, 1992; Moore et al., 2013). DNA methylation is an integral part of fetal development and plays a significant part in X-chromosome inactivation and genomic imprinting (Bird & Wolffe, 1999). Methylation in the promoter region of a gene generally downregulates its transcriptional activities, thereby potentially regulating biological pathways involved in physiological functions. Maternal nutritional status during gestation could alter the methylation process of DNA in the developing offspring, leading to variation in phenotype of economic traits. Prenatal feed restriction has been shown to lead to differential methylation of genes involved in cellular processes, and immune or reproductive function in fetuses of restricted dams (Andrés et al., 2021). Tobi et al. (2014) showed that maternal

exposure to hunger during the Dutch famine was associated with differential DNA methylation in regulatory genes involved in organ development, growth, and metabolism in exposed offspring. There is limited knowledge on the molecular mechanisms regulating the effect of prenatal nutrition on gene expression within different tissues of the offspring during postnatal stages of development.

Previously, our research group identified differentially methylated regions (DMRs) associated with prenatal diet in beef cattle via whole genome bisulfite sequencing (WGBS) of DNA isolated from semen obtained from progeny of feed-restricted dams (Foroutan et al., 2021). In the current study, we employed the use of quantitative individual fragment bisulfite sequencing analysis (EpiTYPER) to interrogate the results from WGBS. Therefore, our first objective is to determine if DMRs identified by our WGBS can also be identified via EpiTYPER in the same tissue (semen). Additionally, a study conducted by Johnson et al. (2019) investigated the effects of prenatal nutrition and RFI on the reproductive potential of the same bull offspring. Results from this study indicated a prenatal diet and time interaction effect on weight of the bull progeny, where prenatal feed restricted groups had a faster growth rate between 10 to 16 months of age. Furthermore, a potential influence of RFI on the fertility of bull progeny was reported. This prompted us to investigate possible molecular mechanisms underlying the epigenetic control of growth in these bulls, and the genetic control of RFI. Utilizing the DMR results from WGBS, we prioritized genes involved in to investigate for expression differences within in *Longissimus dorsi* (LD), *Semimembranosus muscle* (SM), liver (LV), and testis (TS) tissues resulting in altered gene expressions.

2.2. Materials and methods

2.2.1. Animals and experimental design

The experimental protocol for this study was approved by the Animal Care and Use Committee at the University of Alberta (AUPs 877 and 1129), under the Canadian Council on Animal Care

(CCAC) guidelines on: the care and use of farm animals in research, teaching and testing (Canadian Council on Animal Care, 2009). Sixty purebred Angus heifers previously measured for RFI using the GrowSafe System® automated feed recording system (Vytelle® LLC., Calgary, Alberta, Canada) were used for the experiment. Housing of heifers and dietary test were carried out at the University of Alberta Roy Berg Kinsella Research Ranch (Kinsella, Alberta, Canada). The population from which these heifers originated was described previously by Mao et al. (2013). The animals and experimental design of this study is detailed by Johnson et al. (2019) and Foroutan et al. (2021), and the following description of the initial RFI test for virgin heifers, estrous-synchronization and artificial insemination, pregnancy detection and allocation of pregnant heifers into prenatal diet treatment groups, conduct of the prenatal feeding trial, and subsequent husbandry of both dams and their calves until slaughter of steer progeny is detailed in these publications, but is included here for the convenience of the reader.

Heifers whose age ranged between 9 and 12 months were housed in dry-lot pens that contained feed bunks monitored by the GrowSafe System® (GS), as described by Basarab et al. (2003) and Mao et al. (2013). The RFI test was carried out over 74 days in late winter and early spring of 2013, and was described previously by Johnson et al. (2019) and Foroutan et al. (2021). The test diet consisted of 70% silage and 30% barley grain, and a 21-day acclimatization period was allowed for heifers to adjust to the diet and GS feeding system before the intake measurements were officially recorded. Daily feed intake was measured using the GrowSafe software and used to calculate total feed intake over the entire test. Feed samples were collected weekly and pooled monthly, and from the pooled samples dry matter (DM) and energy content analysis (Table 2.1) were determined using wet chemistry by Parkland laboratories (Red Deer, AB, Canada), in accordance with recommended methods of professional association of official Analytical

Chemists, and the National Forage Testing Association. Heifers were weighed twice at the beginning and end of the test, and once every 14-day interval during the test. An Aloka SSD-210 portable ultrasonographic scanner (Aloka Co., Tokyo, Japan), was used to measure rib fat thickness ($12/13^{th}$ rib fat depth and longissimus thoracis (LT area) at the end of the test.

Table 2.1 Nutrient analysis of ration (%DM) fed to heifers during the initial GrowSafe trial, 30-150 days of treatment and 150 days to parturition

Diet composition (%DM)	**GrowSafe trial ration**	**30-150 days ration**		**150 days to parturition**
Feed type	TMR[1]	Brome grass hay	Oats	Hay
DM[2] (%)	62.0	81.8	89.7	81.4
CP[3] (%DM)	11.9	10.3	11.9	18.7
ADF[4] (%DM)	28.6	49.5	15.0	32.3
NDF[5] (%DM)	45.7	70.1	29.9	39.1
TDN[6] (%DM)	67.5	52.8	77.0	64.8
Ca (%DM)	1.67	0.65	0.13	1.67
P (%DM)	0.39	0.25	0.37	0.26
K (%DM)	0.91	2.55	0.59	2.12
Mg (%DM)	0.23	0.20	0.15	0.33
Na (%DM)	0.42	0.01	0.02	0.04
Salt (%DM)	1.05	0.04	0.04	0.10

[1]Total mixed ration
[2]Dry matter
[3]Crude protein
[4]Acid detergent fibre
[5]Neutral detergent fibre
[6]Total digestible nutrient

Daily feed intake was calculated by dividing the total feed intake over the test period obtained from GS, by the number of days of the test. Daily dry matter intake (DMI) of each heifer was calculated using DM content (62%) of diet and the calculated daily feed intake. DMI was standardized to 10 MJ per kg DM based on the energy content of the diet, to obtain standardized daily DMI. Average daily gain (ADG) of each heifer was calculated based on their plotted serial weights over time and calculated linear regression of weight over time (days). Growth was normal amongst all heifers, as indicated the coefficient of determination (R^2) of their growth curve, which was not less than 95% for any heifer. Midpoint weight (MDWT) of each heifer was calculated as their initial weight in kg, plus the product of ADG multiplied by half of the days on test. Metabolic bodyweight (MWT) was calculated from midpoint bodyweight ($MDWT^{0.75}$). A linear regression model was fit using PROC GLM in SAS (SAS institute, Inc, Cary, NC, USA) to generate regression coefficients to predict expected DMI of a heifer with respect to its body weight and growth. The model was:

$$\mathbf{Y}_i = \boldsymbol{\beta}_0 + \boldsymbol{\beta}_1\mathbf{ADG}_i + \boldsymbol{\beta}_2\mathbf{MWT}_j + \boldsymbol{\beta}_3\mathbf{FUFAT}_k + \mathbf{e}_{ijk} \qquad \textbf{(1)}$$

Where Y_i is the standardized daily DMI for the *i*th heifer, β_0 is the intercept, β_1 is the partial linear regression coefficient of ADG, β_2 is the partial linear regression coefficient of MWT, β_3 is the partial regression coefficient of final ultrasound rib fat thickness (FUFAT), and e_i is residual error for the *i*th heifer. RFIf (RFI corrected for FUFAT) for each heifer was computed as the difference between the standardized daily DMI, and the expected DMI that was predicted using the regression intercept and regression coefficients generated from the model. Heifers were categorized based on their calculated RFIf as either high residual feed intake (HRFI) or low residual feed intake (LRFI) by ordering heifers from high to low RFIf and allocating them into two equal groups. No statistical

differences in other traits such as bodyweight, ADG, or age, were detected between the HRFI and LRFI heifer groups (data not shown).

Heifers categorized based on their RFI were bred to sires identified to be of the same RFI group i.e., HRFI heifers were bred to HRFI sires (n=2) and LRFI were bred to LRFI sires (n=2), as per Johnson et al. (2019) and Foroutan et al. (2021). RFI estimated breeding value (EBV) of HRFI sires and LRFI sires at the time of selection were +0.174, +0.140 kg DMI/day, and -0.230 and -0.482 kg DMI/day, respectively. EBV accuracies were 0.859, 0.874, 0.805, and 0.712, respectively. The sires' RFI EBV and accuracy were predicted based on a traditional pedigree-based Best Linear Unbiased Prediction method with the numerator relationship matrix defined from the pedigree as previously described (Chen et al., 2015). AI sires were also selected so that EBV for other recorded traits were similar across all four sires. Heifers from each RFI category were subdivided into two groups by stratified random sampling, and sub-groups were tested for any bias in RFIf and weight. The heifers' pedigree was examined to ensure they were not related to any of the sires, and then each subgroup was then assigned one of the sires with the same RFI group for breeding via oestrous synchronization and artificial insemination.

Free-choice hay was fed to the heifers after the GrowSafe test until mid-May. Heifers were allowed to graze on perennial mixed sown and native grass during oestrus-synchronization and artificial insemination (AI) until pregnancy was confirmed. Breeding of heifers also was described previously by Johnson et al. (2019) and Foroutan et al. (2021). It followed two estrous synchronization protocols. The first AI was timed, whereas the second was performed 12 h after detection of oestrus. During the first round of the estrous synchronization, a CIDR® (controlled internal drug release, Eazi-Breed^{TM}, Zoetis Animal Health, Kirkland, QC, Canada) was inserted into the vagina of each heifer (day 0). Simultaneously, 100 μg of gonadotrophin releasing hormone

(GnRH) (GnRH; Fertiline®, Vétoquinol Canada Inc., Lavaltrie, QC, Canada) was delivered to the heifer by intramuscular (IM) injection. On day 7, the CIDR® was then removed and 500 μg cloprostenol (Kirkland, Quebec, Canada) was administrated IM. The first round of the estrous synchronization concluded with a second injection of GnRH administered intramuscularly 55 hours after CIDR® removal, and the heifer was artificially inseminated (timed AI). In the beginning of the second synchronization phase, a CIDR® was again inserted vaginally into each heifer on day 22 relative to the start of the first synchronization. Due to the possibility of heifers being pregnant after first insemination, there was no administration of GnRH or cloprostenol in the second round of estrous synchronization. CIDR® s were removed 7 days after insertion (day 29), and a KAMAR Heatmount™ detector (Kamar Inc., Steamboat Springs, CO, USA) was mounted anterior to each heifer tailhead with a liquid adhesive and aided in oestrus detection. Visual observation of the occurrence of oestrous was preformed three times daily from Day 30 to 36. Heifers observed to be in oestrus either by a fully activated KAMAR, or in standing oestrous, were inseminated using the AM-PM rule (if heat first observed in the morning, cows were inseminated in the evening and vice versa).

Detection of pregnancy was performed ~28 days after AI via transrectal ultrasonography (Aloka-500-V scanner equipped with a 7.5-MHz linear transducer; Aloka Co., Tokyo, Japan). Pregnant heifers were assigned to either one of two dietary treatments as previously described by Johnson et al. (2019) and Foroutan et al. (2021). Briefly, heifers were stratified (considering RFIF, body weight, rib fat depth, AI sire, and conception date), and randomly placed into prenatal diet treatment groups. They remained in these groups from day 30 to day 150 of gestation. One of the diets (moderate diet or Mdiet) was formulated to provide growth of 0.7 kg/day ADG (roughly 100% of NRC recommendations for growing pregnant heifers). The diet consisted of brome grass

hay and supplemented with oats (Tables 2.1 and 2.2). The second diet (low diet) which consisted of mainly hay with no supplementation, was formulated to provide 75% of the growth potential of the Mdiet. The diets were adjusted monthly after heifers were weighed to account for heifer growth and increasing conceptus growth. Feed samples of both the hay and the oats were collected weekly, and pooled monthly before being submitted for nutrient analysis (Parkland Laboratories, Red Deer, AB, Canada). Table 1 shows the nutrient analysis of the hay and oats provided to the heifers during the trial period. Cobalt Iodised Salt (Windsor Salt Ltd, Pointe Claire, QC, Canada) was provided free-choice with a vitamin premix (Vitamin A, D, E-10M Vitamin Premix for Livestock Feeds, Hi-Pro Feeds LP, Sherwood Park, AB, Canada) at a rate of one cup per gallon of salt.

Table 2.2 Dietary adjustment of moderate and low diets over the course of the trial (as fed)

Adjustments	Moderate		Low	
	Hay (kg/day)	Oats (kg/day)	Hay (kg/day)	Oats (kg/day)
Initial[1]	6.94	3.45	9.71	0.00
1st adjustment[2]	7.73	3.75	9.33	0.00
2nd adjustment[3]	9.21	3.95	11.29	0.00
3rd adjustment[4]	7.14	5.65	12.84	0.00
4th adjustment[5]	7.02	6.80	10.78	2.61

[1]Initial: Jul. 24, 2013
[2]1st adjustment: Aug. 19, 2013
[3]2nd adjustment: Sept. 11, 2013
[4]3rd adjustment: Oct. 8, 2013
[5]4th adjustment: Nov. 8, 2013

After 150 days of gestation, all heifers were housed together and offered free-choice hay until ~2 months after parturition, when they then grazed mixed tame and native grass pasture until weaning in the fall (Johnson et al., 2019). Nutrient analysis of the free-choice hay fed from the end of the feeding trial until the pasture season is shown in Table 2.1.

Post weaning management practices for bull offspring (n = 23; Mdiet-HRFI = 6, Mdiet-LRFI = 3, Ldiet-HRFI = 9, Ldiet-LRFI = 5) were carried out in accordance with industry standards for potential replacement breeding bulls (Johnson et al., 2019). Bulls were weaned on the 12th of November 2014 and were housed in a drylot setting until slaughter. Free-choice grass hay was initially provided to the bulls for 1 month, after which an extra 12 lbs/day of oats was offered, as fed. Beginning in mid-February the bulls were slowly transitioned to a barley silage plus barley grain-based diet consisting of 50.4% barley silage, 46% barley grain and 3.2% of a feedlot supplement, which consisted of 32% crude protein, 440 mg/kg of monensin, trace minerals and vitamin (Beef grower supplement, Cargill Inc., Minneapolis, MN, US). At the end of April 2015, the bulls were transported to Agriculture and Agri-Food Canada Lacombe Research Centre (Lacombe, AB), allowed to acclimatize to a new feedlot pen and final diet consisting of 55% barley silage and 45% barley grain (Johnson et al., 2019). This diet was fed to the bulls until slaughter in August 2015. Nutrient analysis of both the transition diet and final diet are found in Table 2.3.

Table 2.3 Nutrient analysis of ration (%DM) fed to bulls during the transition stage and final feeding period.

Diet composition	Transition diet	Final diet
DM[1]%	52.5	56.1
CP[2] (%DM)	14.9	14
ADF[3] (%DM)	21.4	25.25
NDF[4] (%DM)	34.2	40.5
Ca (%DM)	1.26	0.94
P (%DM)	0.41	0.34
K (%DM)	0.97	1.38
Mg (%DM)	0.24	0.23
Na (%DM)	0.24	0.13
FE (PPM)	772	336
Mn (PPM)	121	70
Zn (PPM)	163	61

[1]DM: Dry matter
[2]CP: Crude protein
[3]ADF: Acid detergent fibre
[4]NDF: Neutral detergent fibre
[5]TDN: Total digestible nutrients

2.2.2. Semen collection, carcass characteristics, and tissue sample collection

Collection of semen and tissue samples as well as carcass characteristics for bull offspring was performed and detailed by Johnson et al. (2019) and (Foroutan et al., 2021). Briefly, the semen was collected at 4-week intervals via electroejaculation between the ages of 13 to 16 months. Morphological evaluation of the semen was carried out by making a semen smear using eosin-nigrosine stain as described by Barth and Oko, 1989. After an initial motility and quality check (subjective assessment at X 400), samples with 50% motility were diluted with equal volumes of semen extender (AndroMed; Minitube, Ingersoll, ON, Canada) and transferred to 15-mL tubes. These tubes were placed in 250-mL beakers with 200 mL water maintained at the same temperature as semen (35ºC) and placed at 4ºC for a minimum of 4 h for chilling and equilibration (Krishnakumar et al., 2011). Post-chill motility and preservation of semen were carried out as described by Johnson et al. (2019).

Bulls were maintained in the feedlot until ~17 months of age and then slaughtered at the Agriculture and Agri-Food Canada, Lacombe Research Centre Abattoir, AB, Canada. The entire process was conducted over 4 days, with an average of seven bulls slaughtered per day. Slaughter dates were continuous except for a 1-week separation between the 2nd and 3rd dates. As much as possible, all prenatal nutrition and RFI groups were included on each slaughter date. On the day of slaughter, bulls were weighed to obtain their final weight (FW). At slaughter, they were first stunned by a captive bolt and then exsanguinated. Between 5 to 10 g of tissue samples from LT muscle (from the left side of the bull, between the 12th and 13th ribs), SM muscle (left), liver, and testis were collected approximately 30-45 min after exsanguination. The tissue was immediately frozen in liquid nitrogen and then stored at -80ºC until RNA isolation.

2.2.3. Selection of DMR-associated genes

Results of the WGBS experiment conducted on DNA isolated from semen samples obtained from the bull progeny (Foroutan, 2020) were used to identify DMRs to analyze for methylation differences via EpiTYPER technology. All bioinformatic analysis of WGBS data were carried-out and detailed by Foroutan (2020). Specifically, data filtering and quality assessment, methyl-cytosine identification and DMRs associated with prenatal diet treatment (Mdiet and Ldiet) identification. Potential DMRs were assessed for significance using Mann-Whitney-U (MWU) at q-value < 0.05 and minimum mean methylation difference ≥ 0.1 (Foroutan, 2020). DMRs were prioritized and selected on the basis of their biological functions related to growth and metabolism (Table 2.4).

2.2.4. Semen DNA extraction and methylation analysis

The following procedures were performed as a part of the current investigation. The semen DNA extraction followed the procedure outlined by Foroutan, (2020). Specifically, frozen semen was thawed at room temperature and 150 µL of each semen straw was transferred to a 1.5 mL Eppendorf tube. One mL of 1X STE buffer (100mM Tris; pH=8, 10mM EDTA; pH=8, 1M NaCl) was added to the tube, and after a brief vortex, the tube was centrifuged at 7000 ×g for 5 min at room temperature. The supernatant was poured off and the remaining pellet was washed (to separate clumps of cells from semen extender) again twice with 1 mL 1X STE buffer. After the final wash, the supernatant was again poured off and the pellet was resuspended in 336 µL 1X STE. The tube was vortexed, then 40 µL of 10% SDS (final concentration ~1%), 20 µL of 20 mg/mL Proteinase K, and 14 µL of 1 M DTT (final concentration ~35 mM) were added to the tube. The tube was incubated in a rotating hybridization oven at 56°C overnight for 24 hrs. Two MaXtract tubes (Qiagen, Hilden, Germany) were prepared by pulse centrifugation at 15,000 ×g for 20-30 sec. Then 1 mL of phenol:chloroform: isoamyl alcohol (PCI = 25:24:1) was added to

one set of MaXtract tubes. The sperm cell lysate from the overnight incubation was poured into the same MaXtract tube, mixed by inversion for 5 min, and centrifuged at 15,000 ×g for 5 min at 4 ℃. A total of 400 µL of chloroform: isoamyl alcohol (CI = 24:1) was added to the other set of MaXtract tubes. The aqueous layer (top) was transferred from the PCI tube to the CI tube, mixed by inversion for 5 min, then centrifuged at 15,000 ×g for 5 min at 4℃. Then 0.1 volume of 3 M sodium acetate (~40 µL) was added to a new 1.5 mL tube, and 400 µL of the aqueous layer was transferred into the tube containing sodium acetate. Two volumes of 100% ethanol (~880 µL) were added, and the tube was frozen at -80℃ for 2 hrs. Following the freezing process, the tube was left at room temperature for 3 min until the liquid inside melted. After melting, the tube was centrifuged at 15,000 ×g for 15 min at 4℃. The ethanol supernatant was poured off into a waste flask, and 1 mL of 70% ethanol was added to the pellet, which was followed by centrifugation at 15,000 ×g for 5 min at 4℃. The pellet was washed with 70% ethanol for a second time and then the pellet was resuspended in 100 µL AE buffer (10 mM Tris-Cl, 0.5 mM EDTA; pH=9.0) (Qiagen). Total DNA was quantified using a Nanodrop-2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, NC, USA) and the sample was then stored at -20 °C. DNA samples were then shipped to Genome Quebec Innovation Centre (Montreal, Canada) for DNA methylation analysis.

DNA methylation analysis was performed by Genome Quebec using the EpiTYPER MassARRAY technology provided by Agena Bioscience (San Diego, CA, USA). A total of 15 target amplicons (Table 2.4) were analyzed. Genomic DNA (~1µg) was treated with sodium bisulfite using an EZ-DNA methylation Gold kit (Zymo Research, Irvine, CA, USA). The bisulfite-treated DNA samples (1.5 µL) were placed into a 384-well microplate for PCR amplification using a T7-promoter-tagged reverse primer with the following conditions: 1X PCR

Buffer (QIAGEN, Hilden, Germany), 1.25 mM $MgCl_2$, 0.5 mM dNTPs, 0.25 µM of each forward and reverse primers, QIAGEN HotStar Taq 0.1 U/µL, plus RNAse free water to equal a final total volume of 5 µL. The PCR cycling conditions were: 95 ℃ for 15 min, then 45 cycles of 95 ℃ for 30 sec, 56 ℃ for 90 sec, 72 ℃ for 2 min, and a final 72 ℃ for 10 min. Thereafter the reaction was held at 4 ℃. A shrimp alkaline phosphatase (SAP) (Agena Bioscience) treatment of the PCR product was then performed to discard unincorporated DNA nucleotides with the following conditions: 2 µL of a 0.15 U/µL mixture of the SAP enzyme and RNAse free water was added to 5 µL of PCR. The temperature cycling conditions for the SAP reaction were: 37 ℃ for 10 min, 85 ℃ for 5 min, and then the reaction was held at 4 ℃. The PCR products were then transcribed into single-stranded RNA and cleaved by RNAse A at uracil residues using the following reaction conditions: 0.64 X of T7 Polymerase buffer, 3.4 µL of T cleavage Mix, 3.14 mM of DTT, 3.15 U/µL of T7 RNA & DNA Pol, 0.09 U/µL RNase A plus RNase Free water to make a total of 5 µL/reaction. Two µL of PCR/SAP reaction was transferred to a new plate to which 5 µL of cleavage mix was added. Cleavage temperature conditions were as follows: 37 ℃ for 3 hours, followed by a hold at 4℃. The EpiTYPER reactions were dispensed onto a SpectroCHIP 384 Array using a Nanodispenser RS 1000 (Agena Bioscience), and data were acquired using a MassARRAY MALDI-TOF mass spectrometer (Agena Bioscience). The data was analyzed using the EpiTYPER software (Agena Biosciences).

Table 2.4 Gene and chromosomal location of the 15 DNA fragments selected for the methylation analysis

Gene name	Gene symbol	Chromosomal location[1]	CpG Analyzed[2]
Acyl-CoA dehydrogenase very long chain	*ACADVL*	19:26938525-26939292	9/10
Aldehyde dehydrogenase 3 family member B1	*ALDH3B1*	29:45537643-45538425	24/28
Collagen like tail subunit of asymmetric acetylcholinesterase	*COLQ*	1:152537871-152538635	29/32
Dipeptidyl peptidase like 6	*DPP6*	4:116520561-116521160	17/18
Growth factor receptor-bound protein 10	*GRB10*	4:5274311-5275031	20/20
Histone deacetylase 4	*HDAC4*	3:118258228-118259046	30/33
Insulin-like growth factor 2 receptor-DMR2	*IGF2R-DMR2*	9:96220873-96221574	24/25
Insulin-like growth factor 2 receptor-WGBS	*IGF2R-WGBS*	9:96278463-96279243	26/35
Immunoglobulin-like and fibronectin type III domain containing 1	*IGFN1*	16:79717001-79717767	8/10
Insulin like 3	*INSL3*	7:5326081-5326799	15/16
3-phosphoinositide dependent protein kinase 1	*PDPK1*	25:2062070-2062783	25/26

Phosphorylase kinase regulatory subunit alpha 2	*PHKA2*	X:124497025-124497732	14/16
Protein kinase AMP-activated non-catalytic subunit gamma 2	*PRKAG2*	4:114315277-114316030	17/19
Ras protein-specific guanine nucleotide releasing factor 1	*RASGRF1*	21:25311128-25311844	16/17
Stearoyl-CoA desaturase 5	*SCD5*	6:97506791-97507486	15/15

[1] Chromosomal location is within genome assembly ARS-UCD1.2/bosTau9

[2] CpG Analyze: Analyzable CpG sites/Total CpG sites in each amplicon

2.2.5. RNA extraction and NanoString gene expression analysis

Frozen LD, SM, and LV samples were grounded in liquid nitrogen using a mortar and pestle. Approximately 100 mg of each sample (n=23 for each tissue type) were aliquoted into 2 ml micro-centrifuge tubes. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions with the following modifications: the LD and SM samples were incubated at room temperature for 20 minutes during the phase separation step. During RNA precipitation phase, 250 µl of isopropanol and high salt solution (1.2M NaAc, 0.8M NaCl) were used and all RNA pellet washing steps were performed using 80% ethanol, centrifuged at 8300 rpm for the first wash, and nucleic acid was re-precipitated with 10 µl sodium acetate, 100% ethanol and incubated for an hour in a -80ºC freezer. The final RNA pellets were air dried on ice and resuspended in 50 µl nuclease-free water. The total RNA (1 µl) was quantified using the NanoDrop 2000C spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) and 2200 TapeStation system (Agilent Technologies, Santa Clara, CA, USA). RNA extraction for TS tissues was performed and detailed by Foroutan (2020) .

Gene expression analysis was performed on 100 ng of total RNA (30–50 ng/µL) extracted from LD, SM, LV, and TS using the nCounter RNA gene expression assay (NanoString Technologies, Seattle, Washington, USA) and their Elements TagSets technology. Genes (n =30) selected on the basis of differential methylation of associated DMRs as identified by WGBS, or their metabolic and growth-related functions, were selected for the expression analysis (Table 2.5). Probe A and B for each transcript were synthesized by Integrated DNA Technologies (IDT) using publicly available bovine sequences listed in Table 4. The RNA samples were mixed with Elements Tagset and tagged with a biotinylated capture probe and a fluorescent molecular barcode reporter probe to form a target probe complex. A hybridization buffer was used to incubate the target probe complex at 67 °C for 16–21 h using a thermocycler. Excess and unbound probe were

removed after purification on a nCounter prep station. The remaining RNA hybrid and probe complex were immobilized and electro-stretched on a streptavidin-coated cartridge. Molecular barcodes were subsequently counted using an automated nCounter digital analyzer which represents relative target gene expression within each sample. The data is reported as counts of unique barcodes associated with each gene.

Table 2.5 List of genes analyzed by NanoString nCounter genes expression analysis in LD, SM, and LV.

Gene	Symbol	Accession No.[1]	Position[2]
Acyl-CoA dehydrogenase very long chain	*ACADVL*	NM_174494.2	656-755
Acyl-CoA thioesterase 7	*ACOT7*	NM_001075682.2	769-868
Aldehyde dehydrogenase 3 family member B1	*ALDH3B1*	NM_001075518.1	1621-1720
Collagen like tail subunit of asymmetric acetylcholinesterase	*COLQ*	NM_001035297.4	1009-1108
Dipeptidyl peptidase like 6	*DPP6*	NM_174040.2	1873-1972
Growth factor receptor-bound protein 10	*GRB10*	NM_001192586.1	1651-1750
Histone deacetylase 4	*HDAC4*	XM_024990281.1	4436-4535
Insulin-like growth factor 1	*IGF1*	NM_001077828.1	346-445
Insulin-like growth factor 1 receptor	*IGF1R*	NM_001244612.1	2767-2866
Insulin-like growth factor 2	*IGF2*	NM_174087.3	401-500
Insulin-like growth factor 2 receptor	*IGF2R*	NM_174352.2	8126-8225
Immunoglobulin-like and fibronectin type III domain containing 1	*IGFN1*	XM_024976573.1	801-900
Inositol polyphosphate-5-phosphatase A	*INPP5A*	XM_024986036.1	758-857
Insulin like 3	*INSL3*	NM_174365.2	109-208
Insulin receptor	*INSR*	XM_002688832.2	1818-1917
Potassium voltage-gated channel interacting protein 1	*KCNIP1*	NM_001013604.1	276-375
Myocyte enhancer factor 2A	*MEF2A*	NM_001083638.2	1751-1850
Myostatin	*MSTN*	NM_001001525.3	703-802
Myogenic factor 5	*MYF5*	NM_174116.1	661-760
Phosphofurin acidic cluster sorting protein 2	*PACS2*	XM_024982233.1	1441-1540
3-phosphoinositide dependent protein kinase 1	*PDPK1*	XM_024985289.1	3235-3334
Phosphofructokinase, muscle	*PFKM*	NM_001075268.1	2058-2157
Phosphorylase kinase regulatory subunit alpha 2	*PHKA2*	NM_001191545.1	1344-1443
Pyruvate kinase M1/2	*PKM*	NM_001075268.1	902-1001

Peroxisome proliferator activated receptor gamma	*PPARG*	NM_181024.2	1562-1661
Protein kinase AMP-activated non-catalytic subunit gamma 2	*PRKAG2*	XM_024991251.1	1528-1627
Glycogen phosphorylase, muscle associated	*PYGM*	NM_175786.2	1585-1684
Ras protein-specific guanine nucleotide releasing factor 1	*RASGRF1*	NM_001191457.1	1946-2045
Stearoyl-CoA desaturase 5	*SCD5*	NM_001076945.1	1059-1158
Thyroid hormone receptor beta	*THRB*	XM_005226186.4	2641-2740

[1]GenBank sequence identifier for each mRNA accession
[2]Target position within each sequence

2.2.6. Statistical Analysis

2.2.6.1. DNA methylation data processing

Raw methylation data was filtered and processed within Excel (v2012) to exclude unreliable CpG units. Firstly, CpG units with no data values and more than one silent peak were deleted. CpG units that could not be distinguished from one another (contained the same methylation level values for each individual sample), were combined into one and relabelled into a single CpG (for example CpG 3-5). Then, samples with more than 10% missing data values were deleted. For analysis within treatments, standard deviation (SD) was calculated for each CpG within individual tissues, and CpGs with SD less than 0.02 were removed from further analysis. Finally, CpG units with more than two missing values were also removed from further statistical analysis. For principal component analysis (PCA) between tissue, SD was calculated including all tissues, and CpG units with SD value less than 0.02 were deleted. CpG units with more than 10% missing data values were excluded from the analysis. For comparison of EpiTYPER and WGBS methylation results, average methylation of each fragment was calculated using the average function within Excel (v2012).

2.2.6.2. Gene expression data processing and normalization

Raw gene expression counts were pre-processed for quality control (QC) and normalized using nSolver® Analysis Software v4.0 (NanoString Technologies, Inc). Specifically, the data were quality controlled based on the manufacturer's default recommendations for QC parameters set for each sample; imaging QC (Field of view (FOV) counted/FOV counts; cut-off 75% FOV), binding density (measure of reporter probe density on the cartridge surface within each sample; cut-off range = 0.05–2.25), positive control linearity (linear regression R^2 = $\log_2$ known (positive controls)/$\log_2$ measured (positive controls), cut-off = R^2 value ≥ 0.95) and positive control limit of detection (indicates whether the counts for the positive control probe and target sequence are

significantly above the counts of the negative probes; cut-off set at 2 SD above means of negative control). Samples that passed the QC parameters were subsequently normalized following the manufacturer's instructions. The normalization procedure was based on geometric means of manufacturer's induced positive controls, and housekeeping genes. The housekeeping transcripts consisting of five genes; *EEFIA2, GAPDH, HMBS, PPIA*, and *YWHAZ* were used to normalize the analysis of LD and SM muscle samples while *GAPDH, HMBS*, *PPIA*, and *YWHAZ* were used to normalize the analysis of LV samples. The housekeeping genes were selected based on average expression count (cut-off = 100), and percentage co-efficient of variation following manufacturer's recommendations. Positive control normalization accounted for technical variation between samples, cartridges, and hybridization using a normalization factor range of 0.3 to 3. The housekeeping gene normalization calculates the geomean of five housekeeping genes to adjust for differences in any possible technical mRNA quality variation across the samples. The normalization factor for the housekeeping gene normalization ranges from 0.1 to 10. Normalized data were exported into Excel (v2012) for further sorting before further analysis was carried out. Genes with expression count of ≥ 50 were considered robust enough to analyze although genes with less than 75% of their sample counts below 50 were excluded from further statistical analysis. Principal component analysis (PCA) was conducted using the normalized gene expression data after data processing for each fragment.

2.2.6.3. Differential methylation and gene expression analysis

Statistical analysis was performed using SAS (v9.4, SAS Institute Inc., Cary, NC, USA). Data were first tested for normality using the PROC Univariate procedure. Non-normally distributed data were transformed using either the box-cox transformation via PROC TRANSREG procedure in SAS, square-root, cube-root, or log-transformation in Excel (v2012). Transformed data were further tested for normality and only normally distributed data were further used for subsequent

analysis. Differential analysis was initially conducted using PROC MIXED to test for the random effect of sire nested within RFI. The model consisted of Diet, RFI, their interaction, and slaughter day as fixed factors, age set as a co-variate, and sire nested within RFI as a random factor. Since the effect of sire was not found to be significant in any of the models, data was then analyzed using PROC GLM with the same fixed factors and co-variate as listed above. If slaughter day and age of the animal was not significant, it was removed from the model. The least-square means were determined using the PDIFF function in SAS. Residuals from the model were screened for normality using PROC Univariate procedure within SAS (v9.4). If residuals were not normally distributed, original data were then transformed and re-analyzed. Significant p-values were reported from type III sums of squares. The significance threshold of a difference between prenatal diet and genetic potential for RFI or their interaction was set at $P \leq 0.05$. Means are reported as LSmeans ± standard error (SE) and means of transformed data are reported as back-transformed means with a 95% confidence interval (CI). Pairwise correlation between individual CpGs showed a high percentage of significant correlation (59.63%). As a result, multiple testing correction was not performed to avoid false negative results.

To compare EpiTYPER and WGBS semen methylation results, average methylation for each of the putative DMRs from the semen EpiTYPER methylation analysis was calculated using Excel (v2012). Data was then tested for homogeneity using PROC UNIVARIATE procedure in SAS. The mean methylation of each treatment group within each DMR was assessed using PROC TTEST procedure within SAS (v9.4, SAS Institute Inc., Cary, NC, USA). For WGBS, all statistical analysis was conducted and detailed by (Foroutan, 2020)

2.2.6.4. Principal component analysis

PCA was performed to profile the gene expression differences within LD, SM, LV, and TS using the prcomp package (default stats package v3.5.1) in R (version 4.1.1; R Core Team, 2021).

Dimensions were reduced and variance maximized by extracting principal components (PC) from the analysis based on the cumulative variance explained. A threshold of ≤ 80% explained variance was used to select the number of PCs for further steps in the analysis. Two PCs were selected and correlated with the original data. The ggbiplot R package (https://github.com/vqv/ggbiplot) and stat ellipse function, at 95% confidence interval in R (version 4.1.1; R Core Team, 2021) was used to plot a biplot between PC1 and PC2, representing the component with most variation in the dataset to visualize the pattern of gene distribution between tissues and the contribution of each gene to the respective PCs.

2.3. Results

2.3.1. Comparison of methylation associated with prenatal diet between EpiTYPER and WGBS methylation

Prenatal diet had no significant association with average semen methylation of the analyzed target (Table 2.6) in the current study. From the WGBS study, Ldiet bull progeny had significantly higher methylation levels compared to their Mdiet counterparts (negative mean methylation values). For EpiTYPER, Mdiet bull progeny displayed higher methylation levels *COLQ* and *INSL3* while Ldiet had higher methylation levels compared to Mdiet group for *INSL3*.

Table 2.6 Comparison between mean methylation difference (WGBS) and average CpG methylation (EpiTYPER)

WGBS				EpiTYPER			
					Average methylation level		
DMR	Chromosomal Location	Mean methylation difference[1]	q-value	CpG Analyzed[2]	Mdiet	Ldiet	P-value
ALDH3B1	chr29:45537743-45538325	-0.30	2.00E-10	5;7-8;9; 10;14-15; 18,21;22;23-24;26;28	0.89	0.89	0.51
COLQ	chr1:152538021-152538485	-0.27	6.90E-14	2-3;4;5-6;7;8;9;11;14;16-17;23;24-25;26;27;29;30	0.83	0.80	0.56
DPP6	chr4:116706545-116706690	-0.25	0.04	2;5-6;7-8;9-12;13;14-15;16-17;18	0.87	0.87	0.98
GRB10	chr4:5274461-5274881	-0.23	0.01	3;4;16-17;18;19	0.88	0.88	0.71
HDAC4	chr3:118258328-118258946	-0.20	0.0003	1;4;6;16;23;27;31-32	0.87	0.87	0.94
IGF2R-WGBS	chr9:96278763-96278943	-0.30	0.01	7;17;24-25;26	0.89	0.89	0.67
IGFN1	chr16:79717301-79717467	0.36	0.0002	2;6;7	0.93	0.93	0.76
INSL3	chr7:5326381-5326499	-0.21	0.03	4;5;6;7;8;9-10;11;14;16	0.62	0.57	0.19
RASGRF1	chr21:25311278-25311694	-0.27	0.002	1;2;9;13-15;17	0.86	0.87	0.29
SCD5	chr6:97507091-97507186	-0.31	0.01	4-5;6;8-9;10-12;13-14	0.83	0.80	0.38

[1]Mean methylation difference between Mdiet and Ldiet bull progeny from WGBS data analysis. Negative value denotes higher methylation in Ldiet progeny. [2]CpG units used in calculating average methylation for fragments analyzed.

2.3.2. Principal component analysis of gene expression

In the PCA analysis, two PCs (PC1 and PC2) explained most of the variation (85.9%) (Figure 2.1) in the data set with 56.1% explained by PC1 and 29.8% explained by PC2. Therefore, the two PCs were used in profiling the pattern of gene expression between LD, SM, LV, and TS tissues represented by colored ellipses. From the PCA ggbiplot (Figure 2.1), expression patterns of LD and SM overlap with each other and are positively correlated to both PC1 and PC2. LV expression patterns are slightly positively correlated with PC1 but negatively correlated to PC2. TS negatively correlates with PC1 and has a weak positive correlation with PC2. Additionally, correlation between the genes and their contribution to the PCs is denoted by the direction and length of the arrows within the ggbiplot. PC1 positively correlates with the expression of *COLQ, GRB10, IGFN1, INSR, MSTN, MYF5, PFKM, PKM*, *PYGM*, and *THRB* while *ACOT7*, *IGF1R*, *IGF2R, PHKA2, SCD5, ALDH3B1, PRKAG2, RASGRF1, PPARG, INPP5A, HDAC4, DPP6, PDPK1, KCNIP1, PACS2, MEF2A* and *INS3* negatively correlates with PC1. *ACADVL, IGF1*, and *IGF2* had no correlation with PC1. For PC2, There is a positive correlation with the expression of *ACADVL, COLQ, HDAC4, IGF1, IGF2, IGF1R, INSR, MSTN, MYF5, PACS2,* and *PHKA2*. PC2 negatively correlates with *PRKAG2, DPP6, IGF2R, RASGRF1, PPARG, INPP5A, PDPK1, KCNIP1, MEF2A, PKM, IGFN1, PFKM, PYGM*, and *GRB10* while *ALDH3B1,* ACOT7, *INSL3, SCD5,* and *THRB* had no correlation with PC2. Genes positively correlated with the PCs implies the genes had higher contribution to the variation captured by the respective PCs (Figure 2.1)

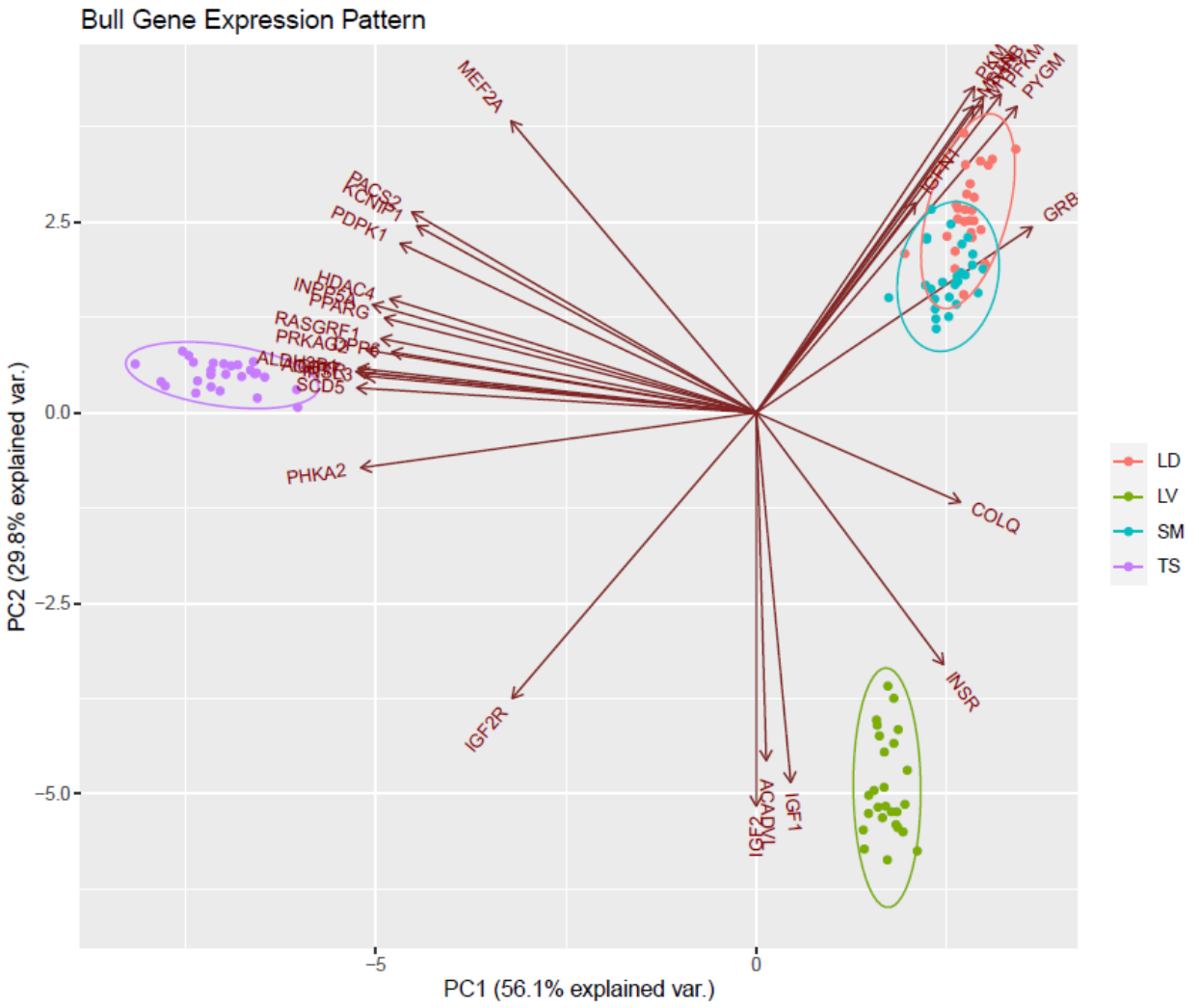

Figure 2.1 A ggbiplot of PCA profiling the gene expression pattern between LD, SM, LV, and TS. Variables are indicated as arrows showing the direction and relative contributions to the PCs. Ellipses grouping the samples according to expression patterns between tissues; *Longissimus* dorsi LD, *Semimembranosus* muscle (SM), liver (LV) and testis (TS).

2.3.3. Semen DNA methylation in bull progeny

Prenatal dietary treatment significantly influenced the methylation of the DNA fragments associated with *ALDH3B1* (CpG 18), *IGF2R*-DMR2 (CpG 3-4), *GRB10* (CpG 18) and *INSL3* (CpG 5) (Table 2.7). Mdiet bull progeny had greater methylation levels compared to the Ldiet groups for all significant CpGs (Table 2.7). Genetic potential for RFI affected the methylation levels in *ALDH3B1* (CpG 22), where LRFI group had higher methylation levels compared to HRFI group [(LRFI vs HRFI; 0.88 ± 0.01 vs 0.90 ± 0.01, respectively)]. Prenatal diet and RFI interaction influenced methylation in *DPP6* (CpG 18), and *IGF2R*-WGBS [(CpG 24-25); (CpG 26)]. In *IGF2R*-WGBS, Ldiet-LRFI group had significantly higher methylation levels than Ldiet-HRFI but were similar compared to other diet-RFI groups in both CpGs (Figure. 2). For *DPP6,* Mdiet-HRFI bull progeny had a higher methylation level than the Ldiet-LRFI group, but similar to other counterparts [(Mdiet-HRFI (85, [86.2, 82.8]); Mdiet-LRFI (87.7, [89.1, 86.2]); Ldiet-HRFI (86.2, [88.4, 83.9]); Ldiet-LRFI (83.6, [88.6, 77.9)].

Table 2.7 Impact of prenatal feed restriction during early to mid-gestation and RFI on DNA methylation in semen of bulls as assessed by EpiTYPER analysis

DMR	CpG	Diet		P-value
		Mdiet[1]	Ldiet[1]	
ALDH3B1	18	0.95 ± 0.01	0.93 ± 0.01	0.05
IGF2R-DMR2	3-4	0.22 ± 0.02	0.15 ± 0.01	0.01
GRB10	18	0.90 ± 0.01	0.87 ± 0.01	0.001
INSL3	5	0.51 ± 0.04	0.41 ± 0.03	0.05

[1]Data represent methylation percentage for CpG in semen and expressed as LSmeans ± SE. Significance set at $P \leq 0.05$.

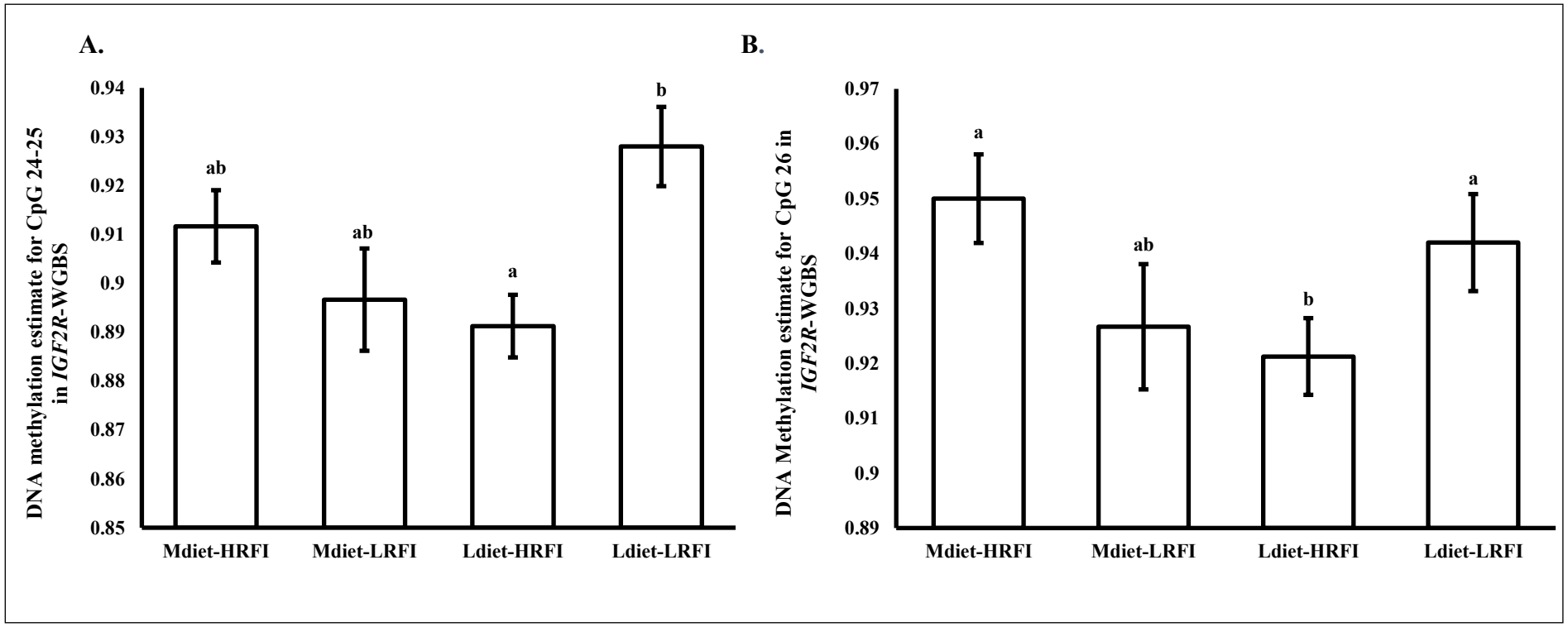

Figure 2.2 Bar graphs indicating differential methylation between prenatal diet and RFI interaction groups for A) CpG 24-25 B) CpG 26 in *IGF2R*-WGBS fragment. Bars represent LSmeans estimates, and error bars represent SE. [a,b] indicates significant differences between the diet-RFI groups.

2.3.4. Effect of prenatal diet and genetic potential for RFI on gene expression in *longissimus* dorsi

In LD, prenatal diet had a significant effect on the expression of *PDPK1*, with Ldiet having higher mRNA abundance compared to Mdiet bulls (Table 2.8). RFI was associated with the expression of *IGFN1*, with LRFI bulls having higher mRNA relative abundance than HRFI group (Table 2.8). The interaction of prenatal diet and RFI was significantly associated with the expression of *PFKM*, where the Ldiet-HRFI steer group had a significantly higher expression level than Mdiet-HRFI but did not differ compared to other diet and RFI combinations [Mdiet-HRFI (11816 [405.70, 408.80]); Mdiet-LRFI (12981.16 [405.30, 417.40]); Ldiet-HRFI(13988.33 [411.00, 418.20]); Ldiet-LRFI(12562.94 [403.80, 416.00])] groups. All other genes analyzed did not have any significant differences in the mRNA abundance between treatment groups.

Table 2.8 Effect of prenatal diet and genetic potential for RFI on gene expression in Longissimus dorsi muscle of bull offspring at slaughter as assessed by NanoString.

Gene	Diet		P-value
	Mdiet[1]	**Ldiet**[1]	
PDPK1	372.99 ± 14.59	429.58 ± 11.72	0.01
	RFI		
	HRFI[1]	**LRFI**[1]	
IGFN1	1255695.1, [1080077.8, 1468501.5]	1758876.2, [1451681, 2151802.4]	0.02

[1]Data represent the gene expression estimates within LD muscle and are expressed as least square means ± SE. If the data is non-normal, data are the back-transformed mean and 95% CI in []. Significance set at $P \leq 0.05$.

2.3.5. Effect of prenatal diet and genetic potential for RFI on gene expression in semimembranosus muscle in bull progeny

The effect of genetic potential for RFI, and prenatal feed restriction during gestation, on gene expression in SM of bull offspring is shown (Table 2.9). Prenatal diet was associated with the expression of *PDPK1* and *THRB*. Ldiet progeny had higher expression of both *PDPK1* and *THRB* compared to their Mdiet counterparts. Genetic potential for RFI was associated with the expression of *PACS2*, with LRFI progeny having higher mRNA abundance than HRFI progeny (Table 2.9).

Table 2.9 Effect of prenatal diet and genetic potential for RFI on gene expression in Semimembranosus muscle of bull offspring at slaughter as assessed by NanoString.

Gene	Diet		P-value
	Mdiet[1]	Ldiet[1]	
PDPK1	333.07 ± 11.42	374.11 ± 9.20	0.01
THRB	588.73 ± 30.07	671.66 ± 24. 33	0.01
	RFI		
	HRFI[1]	LRFI[1]	
PACS2	529.36 ± 13.12	584.3 ± 20.20	0.04

[1]Data represent the gene expression estimates within SM muscle and are expressed as least square means ± SE. If the data is non-normal, data are the back-transformed mean and 95% CI in []. Significance set at $P \leq 0.05$.

2.3.6. Effect of prenatal diet and genetic potential for RFI on gene expression in testis

Prenatal diet was not significantly associated with the expression of the genes analyzed in testis tissue. Genetic potential for RFI was associated with expression levels of *HDAC4*, and *IGF1R* (Table 2.10). LRFI bull progeny had a higher mRNA abundance of both *HDAC4* and *IGF1R* as compared to HRFI progeny.

Table 2.10 Effect of genetic potential for RFI on gene expression in testis tissue of bull offspring at slaughter as assessed by NanoString.

Gene	HRFI[1]	LRFI[1]	P-value
HDAC4	20470.7, [19228.0, 21578.5]	22155.7, [20891.3, 23290.2]	0.01
IGF1R	3410.49 ± 127.43	2842.6 ± 196.19	0.02

[1]Data represent the gene expression estimates within TS tissue and are expressed as least square means ± SE. If the data is non-normal, data are the back-transformed mean and 95% CI in []. Significance set at $P \leq 0.05$.

2.4. Discussion

In-utero feed restriction alters fetal organ and tissue development leading to metabolic and endocrine programming that may persist in the postnatal period (Bispham et al., 2003; Chadio et al., 2007). Even in the absence of any phenotypic consequences, prenatal nutrition has been shown to alter the methylation and expression of genes associated with metabolism and growth (Lan et al., 2013). A previous study that utilized the same group of bull progeny showed that the Ldiet bull group recorded a higher growth rate between 10 to 16 months of age (Johnson et al., 2019). Therefore, in this study, we investigated the molecular mechanisms regulating the phenotypic changes in beef cattle progeny due to feed restriction during early to mid-gestation.

2.4.1. Comparative semen methylation analysis (WGBS vs EpiTYPER)

Genome-wide methylation techniques including WGBS have been used as discovery approaches in identifying differentially methylated regions (DMRs) (Chatterjee et al., 2017). Due to a potential number of factors such as overestimation of statistical significance, inadequate sequencing depth, and use of inappropriate statistical tests, biases could be introduced in WGBS findings (Chatterjee et al., 2017). Therefore, these results are sometimes subject to validation analysis to determine the accuracy of WGBS results (Chatterjee et al., 2017). The EpiTYPER assay has been used in validating DMRs identified by WGBS methylation analysis (Chatterjee et al., 2017; Jin et al., 2013). EpiTYPER enables a relatively high throughput quantitative measurement of DNA methylation at targeted CpG sites, and we have utilized this technology to attempt to verify WGBS results obtained by Foroutan et al. (2020) when they identified DMRs associated with prenatal diet in semen DNA of bulls used in the current study.

In the WGBS study, the influence of prenatal dietary treatment identified 652 DMRs with 352 unique DMR-associated genes (Foroutan, 2020). Comparing the prenatal diet groups, 77.8% of the identified DMRs had higher methylation levels in Ldiet progeny (Foroutan, 2020). Using

the WGBS results to choose DNA fragments associated with genes that might influence biological functions related to growth and metabolism, we analyzed 10 normally distributed methylated DNA fragments from DMR-associated genes using EpiTYPER. We compared the influence of prenatal diet on average CpG methylation levels from each fragment to the mean methylation differences from the WGBS analysis. From the results (Table 2.7), the prenatal diet did not significantly influence methylation levels of any of the genes analyzed with EpiTYPER. We did observe similar methylation levels in DMR *RASGRF1*. Methylation levels of this DMR in the Ldiet group were higher than in the Mdiet group, which agrees with the results from the WGBS analysis. Our approach to comparing the average methylation levels from the EpiTYPER and WGBS was similar to Chatterjee et al. (2017) who reported that the accuracy of validating whole genome methylation data significantly improved when multiple CpG sites rather than single sites were used. Hence, in general, we speculate that the EpiTYPER methylation in this study could not validate the association of prenatal diet to methylation as observed in the WGBS study.

The variation we observed in the results could be attributed to several factors. Firstly, the general principle of quantifying methylation counts varies between the two techniques i.e., sequence-based vs mass spectrometry-based which could introduce some level of technical variation in the data output. Also, WGBS data underwent bioinformatic analysis including data quality control, identification, and quantification of methyl-cytosine to determine the DMRs. Bioinformatic analysis also could introduce some level of bias such as overestimation of statistical significance when analyzing methylation data (Chatterjee et al., 2017). Furthermore, in the EpiTYPER methylation analysis, the data underwent several data processing procedures that resulted in the deletion of CpG sites and individual samples that were deemed unreliable. Thus, potential data contributing to a complete overview of a DMR could have been omitted from the

final statistical analysis. Additionally, bisulfite sequencing approach is subject to biases that could lead to false-positive due to incomplete conversion cytosine or overestimation of the methyl-cytosine (Olova et al., 2018; Owa et al., 2018). We conclude that to design a true test of the WGBS analysis we would want to limit our choice of potential DMRs to those with the lowest q-values, as opposed to also considering DMR-associated genes that have higher, yet still significant q-values. We chose a variety of genes that could have potential effects on growth and development, which were interesting to relate back to our observed difference in growth of the bull progeny.

2.4.2. Effect of prenatal feed restriction and genetic potential for RFI on semen CpG methylation

The formation of functional spermatozoa involves a series of epigenetic reprogramming of male germ cells during spermatogenesis (Kiefer & Perrier, 2020). These modifications are essential in protecting the paternal genome during the fertilization process and ensuring the stabilization of information crucial to genomic imprinting, post-fertilization reprogramming, and embryonic development (Carrell, 2012; Champroux et al., 2018). The reprogramming process is sensitive to external factors resulting in epigenetic marks that could persist and be inherited by subsequent generations (Wu et al., 2015). Both epidemiological and animal studies have shown that aberrant semen DNA methylation is associated with infertility (Poplinski et al., 2010; Tang et al., 2018; Verma et al., 2014). As a result, the current study analyzed methylation in association with relation to prenatal diet, genetic potential for RFI, and/or their interaction in bull semen.

In the current study, Mdiet bull progeny had greater methylation levels in DNA associated with genes involved in testicular development and spermatogenesis, *ALDH3B1* (CpG 18) and *INSL3* (CpG 5). *ALDH3B1* is a member of the aldehyde dehydrogenase family that plays a role in oxidative and aldehyde stress defense and is highly expressed in lungs, kidneys, and testis (Marchitti et al., 2007; 2010). The expression of *INSL3* is implicated in testicular descent and

maintenance of semen production, and it's reported to be highly expressed in Leydig cells of testis in livestock including bulls (Pitia et al., 2017; Siqin et al., 2013). Recently, Liu et al. (2019) reported that *INSL3* could be a candidate gene for epigenetic regulation for bulls. The authors showed that differential methylation of genes including *INSL3* in bull semen resulted in altered expression of the gene, sub-optimum semen quality and fertility, as well as performance of their progeny. In sheep, *ALDH3B1* expression has been reported to be downregulated in testes of nutrient restricted rams and its expression could indicate the capacity of sperm production (Fan et al., 2018; Marchitti et al., 2010). Hence, transcriptional activities of *ALDH3B1* could potentially be sensitive to nutritional status. The current study did not conduct gene expression analysis in semen, however, in the studies by Johnson et al. (2019, 2020), where the study was performed on the same bull progeny, the prenatal diet had no significant influence on the expression of genes in the testes of bull progeny, and semen quality parameters. Therefore, Mdiet bull progeny displaying higher methylation as compared to Ldiet group may signify a lack of biological consequence of the methylation of these DMRs. However, further research on the semen transcriptome and subsequent quality of progeny produced would be required to ascertain the effect of *ALDH3B1* and *INSL3* methylation levels due to gestational maternal feed restriction on fertility.

Genomic imprinting of a gene is a genetic mechanism where a single allele of specific parental origin is expressed (Lim & Maher, 2010). Imprinting of genes are essential in establishing and maintaining the epigenetic landscape of subsequent generations via methylation (Bajrami & Spiroski, 2016). Aberrant genomic imprinting therefore could result in several undesirable phenotypic consequences (Gosden et al., 2003; Marques et al., 2004). We observed higher methylation in two maternally expressed imprinted genes *IGF2R*-DMR2 (CpG 2-3) and *GRB10* (CpG 18). *IGF2R* is involved in the regulation of spermatogenesis and fetal growth (Carvalho et

al., 2012). *GRB10* is a constituent of the growth factor receptor bound (GRB) protein family that inhibits the activation of the PI3K/Akt pathways restricting insulin receptor signaling when phosphorylated by mTORC1 (Yao et al., 2012). Hence, expression of *GRB10* negatively regulates insulin sensitivity and growth (Holt & Siddle, 2005; Yu et al., 2011). To the best of our knowledge, both *IGF2R* and *GRB10* have not been reported to potentially impact reproductive development nor sperm parameters. Therefore, investigating the potential impact of these methylation patterns in subsequent generations could highlight a potential transgenerational effect of the current results.

2.4.3. Effect of prenatal feed restriction and genetic potential for RFI on gene expression in *Longissimus dorsi*, *Semimembranosus* muscle, and liver tissues

Prenatal feed restriction has been associated with offspring postnatal metabolic adaptations including altered feed intake and metabolism (Coupé et al., 2012). Subsequently, functions associated with metabolic pathways such as PI3K/Akt pathways are also affected (Tingey, 2005; Yoshida et al., 2014). *PDPK1* is a signaling mediator gene involved in several physiological pathways including the PI3K/mTOR signaling pathways (Kuramoto et al., 2021; Loor et al., 2005; Wullschleger et al., 2006). *PDPK1* within the PI3K pathway phosphorylates and activates Akt, which in turn promotes protein synthesis and growth (Schiaffino & Mammucari, 2011). Both LD and SM muscle expressions of *PDPK1* were significantly associated with gestational prenatal dietary treatment, and the Ldiet group had greater expression levels compared to the Mdiet group in both muscle tissues. Studies of IUGR in humans and rats indicate a reduced expression of *PDPK1* and other effectors of PI3K pathway as a result (Ozanne et al., 2005b, 2022). In Ozanne et al. (2022), the activities of PI3K pathway was elevated in adipocytes of growth retarded rats with an elevated uptake of glucose. Therefore, higher expression of PDPK1 in our study could mean that Ldiet bulls could potentially be undergoing an adaptation to an increase energy uptake. Furthermore, according to Johnson et al. (2019), Ldiet bull progeny showed a tendency for a faster

growth rate than the Mdiet group between 10 to 16 months of age. Hence, the greater expression level of *PDPK1* in the Ldiet group at slaughter in the current study possibly suggests a long-term adaptive response of the PI3K pathway due to prenatal programming to potentially promote growth during postnatal stages.

The *PFKM* gene is a muscle distinct subunit of a tetrameric enzyme, phosphofructokinase (PFK), and a glycolytic regulatory enzyme involved in skeletal muscle metabolism and glucose storage (Fleming-Waddell et al., 2007). *PFKM* plays a role in the glycolytic pathway by catalyzing the rate-limiting transfer of phosphate from ATP to fructose-6-phosphate (Cole & Eastoe, 1988; Fleming-Waddell et al., 2007). Hence, the expression of *PFKM* in skeletal muscle correlates with the glycolytic potential of muscle (Rani et al., 2020). In the current study, prenatal diet and RFI interaction influenced the expression of the *PFKM* gene in LD muscle, where the Ldiet-HRFI group had significantly higher expression compared to Mdiet-HRFI but was similar to other diet-RFI groups. In a study by Costa et al. (2022), restricting the diet of pregnant does by 50% during early to mid-gestation impacted the energy metabolism of restricted offspring at birth by altering the energy investment phase of their glycolytic process and resulted in a greater *PFKM* abundance in LD of the restricted offspring. The result from the current study and that of Costa et al. (2022) suggest that limiting nutrient intake during critical gestation stage may have postnatal impact on glycolytic activities of LD muscle. Additionally, it is possible that in the postnatal environment in this study, genetic potential for RFI amplified the expression of *PFKM*. According to Xu et al. (2020), feed-efficient pigs showed a decreased expression of genes involved in glycolysis. Hence, in the current study, the genetic potential for lower feed efficiency may have had the opposite effect and resulted in increased glycolytic process in the Ldiet-HRFI group. Therefore, increased

expression of *PFKM* in Ldiet-HRFI bulls in our study indicates a possible adaptation of LD to increasing glycolytic potential during postnatal stages.

IGFN1 comprises a transcriptionally complex genomic locus that encodes multiple protein variants; mainly in skeletal muscle and heart via alternative splicing (Baker et al., 2010; Li et al., 2017). In skeletal muscle, *IGFN1* plays a role in myoblast fusion and differentiation via its interaction with actin nucleating protein COBL (Cracknell et al., 2021). In the current study, LRFI bull progeny had significantly higher *IGFN1* expression than the HRFI group in LD muscle. Association of the impact of genetic potential for residual feed intake on either *IGFN1* or myoblast fusion remains elusive. Hence, future studies on the effects of level of dietary intake or metabolic efficiency on *IGFN1* expression are required to properly explain its role in postnatal skeletal muscle development.

PACS2 is a multifunctional sorting protein located at, and regulates the functions of, mitochondrial-associated membranes (MAMs). The MAMs are structures consisting of mitochondria and endoplasmic reticulum (ER) contact sites involved in ER-mitochondria Ca^{2+} transport, lipid metabolism, and stress response (Li et al., 2020; Thomas et al., 2017; Zhang et al., 2021). Additionally, MAMs have been implicated in metabolic functions via nutrient and hormonal signaling (Su & Wang, 2019), and the expression of *PACS2* has been positively correlated with the functions of MAMs (Arruda et al., 2014). LRFI bull progeny had greater *PACS2* expression compared to the HRFI group in SM muscle in the current study. From our results, we assume that the higher expression of *PACS2* may indicate that the genetic potential for improved feed efficiency might increase mitochondrial sensitivity and promote nutrient uptake in SM muscle of LRFI bull progeny.

The liver is an essential metabolically active tissue regulating growth and metabolism and could potentially be affected by early gestational feed restriction. Studies have shown that maternal feed restriction during early and mid-gestation affects fetal liver development (Crouse et al., 2019; Smith et al., 2021; Vonnahme et al., 2003; Xue et al., 2019). This alteration potentially influences postnatal metabolism and growth. Thus, we sought to investigate the long-term impact of gestational feed restriction on the liver as information on that effect in beef cattle is lacking. Prenatal feed restriction and/or RFI had no significant influence on gene expression in LV in this study. In bovines, early prenatal feed restriction resulted in decreased fetal weight and size. However, following realimentation, the fetal organ weight and size were similar to those of the control group during the last trimester (Long et al., 2009). Similarly, reduced fetal liver weight due to early to mid-gestation was restored to normal after feed realimentation (Meyer et al., 2010). Therefore, lack of significance in LV in our study could potentially be due to timing of feed restriction and realimentation during gestation, which might have reversed hepatic response to nutrient restriction.

2.4.4. Effect of prenatal diet and RFI on gene expression in testis of bull progeny

Bull reproductive capabilities are essential to sustaining beef cattle production. The reproductive development of bulls could potentially be altered by restricted nutrient supply to pregnant dams during gestation (Silva et al., 2001; Yilmaz et al., 2006). In the present study, early to mid-gestation feed restriction had no significant effect on testis gene expression. However, genetic potential for RFI influenced the expression of *HDAC4* ($P = 0.01$) and *IGF1R* ($P = 0.02$).

Histone modification including acetylation and deacetylation could occur in tissues due to prenatal programming as well as nutritional cues from the postnatal environment (Lillycrop et al., 2005). *HDAC4* belongs to the class II HDACs whose mRNA is found to be highly expressed in tissues including the testis and skeletal muscle (Liu et al., 2006), and is involved in transcriptional

repression of genes (Wang et al., 2014). The role of *HDAC4* in testicular growth and reproductive development remains to be uncovered. In the current study, LRFI bull progeny had greater expression levels of *HDAC4* compared to their HRFI counterparts. At the time of conducting this study, little information is known about the impact of selection for feed efficiency (RFI) on *HDAC4* expression in testis. However, *HDAC4* has been implicated in influencing male fertility by regulating the physiological function of osteocalcin, a hormone that promotes testosterone production in the testis (Makinistoglu & Karsenty, 2015; Oury et al., 2011). It could be that selection for high feed efficiency potentially results in a reproductive adaptation in the LRFI group. While further investigation would be required to determine these assumptions, genetic potential for RFI might play a role in testicular functions in bull progeny.

Sertoli cells (SCs) are an essential component of testicular development and spermatogenesis (Griswold, 1998). The number of SCs has been correlated to testicular size, germ cell number, and sperm output (Orth et al., 1988). *IGF1R* mainly mediates physiological activities of *IGF1* by activating PI3K/Akt pathway (Baker et al., 1993), which is important in SC development and proliferation (Pitetti et al., 2013). Additionally, plane of nutrition is a major factor in the postnatal sexual development of bulls where high feed intake increased *IGF1* concentration, testicular size, and sperm production (Brito et al., 2007). In addition, an increased plane of nutrition in bull calves improved their metabolic status and resulted in heavier testicular weight and higher expression of genes in testicular function and SC development (Coen et al., 2021). In our study, *IGF1R* expression was significantly associated with genetic potential for RFI, and its expression was greater in the HRFI group compared to the LRFI group. These results agree with those of Johnson et al. (2020), who also reported an upregulation of *IGF1R* in the testis of HRFI bull progeny. Although the current study was carried out in the same animals, we used

NanoString gene expression technology as compared to RNA seq analysis by Johnson et al. (2020), and hence, validated the results from the RNASeq analysis for *IGF1R* gene. Additionally, the HRFI bull progeny had larger scrotal circumferences, reached puberty faster, and tended to have superior average sperm motility, which positively correlated with *IGF1R* expression (Johnson et al., 2019). Therefore, we speculate that higher *IGF1R* expression promoted PI3K/Akt pathway activities in the testis of HRFI bull progeny leading to improved testicular development.

2.5. Conclusion

Limited nutrient supply during gestation could be detrimental to the development of offspring, potentially influencing the expression of genes via altered DNA methylation and consequently affect their postnatal productive performance. In the current study, restricting prenatal diet during early to mid-gestation resulted in differential CpG methylation of DMRs (*ALDH3B1, INSL3, IGF2R*-WGBS, *GRB10*) in semen of bull progeny. These DMRs are involved in essential physiological and biological processes including testicular development, spermatogenesis, and genomic imprinting. Interestingly, the methylation levels were higher in the non-restricted bull group. Although, no expression analysis was carried out on the semen to assess the potential transcriptional consequences, these DMRs could be reproductive markers related to maternal diet and potential DMRs for future research investigations. The use of EpiTYPER methylation analysis to validate the DMRs identified from WGBS study showed no significant association of prenatal diet to average methylation in the current study. This is possibly due to potential technical variation between analyses, and further studies would be required to ascertain these variations. In muscle tissues a higher expression of gene (*PDPK1*) involved in PI3K-pathway in restricted progeny suggests a possible metabolic adaptation to prenatal diet in support for growth during postnatal stages. Genetic potential for RFI was associated with higher *IGF1R,* which stimulates growth and

development in TS tissue. Neither prenatal diet nor genetic potential for RFI showed any association with gene expression in LV tissue perhaps due to timing of feed restriction allowing for prioritization of nutrient supply to essential organs including LV. These results elucidate some molecular mechanisms underlying prenatal diet and postnatal factors on progeny performance and contributes to growing knowledge nutritional epigenetic effects in cattle.

Chapter 3. Impact of prenatal feed restriction during early to mid-gestation and genetic potential for RFI on DNA methylation and gene expression in *Longissimus dorsi*, *Semimembranosus* muscle, and liver in steer progeny

3.1. Introduction

Human epidemiological studies associate intrauterine growth retardation with various physiological disorders including insulin resistance, obesity, and cardiovascular diseases (Barker, 1995, 2004). In recent times, the use of animal models such as rodents to replicate these findings provided additional evidence of fetal developmental programming occurring due to maternal gestational experiences (Greenwood & Bell, 2003). Ruminant fetal programming studies suggest that important economic traits could be programmed with a persistent long-term effect on livestock productivity (Funston & Summers, 2013; Thomas, 2011; Wu et al., 2006). In beef cattle production, extreme climatic variation and seasons result in suboptimal quality and shortages of feed. Low-quality feed or limited availability of feed might coincide with cow gestational periods affecting fetal development (Allison, 1985; Duarte et al., 2013; Enk et al., 2001).

Skeletal muscle constitutes about 40% of body weight and is susceptible to maternal gestational experiences such as stress and nutrient restriction (Beauchamp & Harper, 2016; Costa et al., 2021; Zhu et al., 2006; Zurlo et al., 1990). In cattle, myogenesis initiates during the embryonic into the fetal period in two phases that overlap i.e., primary and secondary myogenesis, which initiate primarily in the first and second trimesters, respectively (Du et al., 2010). The success of both myogenic processes is crucial to postnatal growth performance as the total number of myofibers that forms the muscle is determined at birth and postnatal muscle development is hypertrophy of pre-formed myofibres (Davis & Fiorotto, 2009; Du & Zhu, 2009). Hence, a limited supply of nutrients during early to mid-gestation could impair fetal skeletal muscle development. According to Quigley et al. (2005), the number of myofibers formed in ovine fetuses during mid-

gestation was 20% lower when feed was restricted during early pregnancy. Muroya et al. (2021) reported that a 40% decrease in total feed requirement during gestation led to a marked reduction in fetal muscle weight of calves during the last trimester. Similarly, in sheep, restricting prenatal diet to 50% between days 28 to 78 of gestation resulted in decreased secondary myofibers (Zhu et al., 2004). Furthermore, prenatal feed restriction altered the expression of genes regulating metabolism and myogenesis including the Insulin-Like Growth Factor (*IGF*) family of genes and myogenic regulatory factors *MYOD1* and *MYOG* (Paradis et al., 2017). Impairment of fetal skeletal muscle development could potentially alter postnatal metabolic functions and growth. Zhu et al. (2006) reported that postnatal muscle characteristics, including myofiber number and composition were altered due to intrauterine growth restriction (IUGR) in early gestation and consequently impacted muscle growth in sheep. Currently, there are limited studies on the long-term consequences of fetal programming in beef cattle.

DNA methylation is an epigenetic mechanism that involves the addition of a methyl group to the 5'-carbon position of cytosine in a cytosine-guanine dinucleotide (CpG) residue (Wang & Wu, 2018). Methylation at promoter regions results in the silencing of genes by inhibiting the binding of transcription factors (Busslinger et al., 1983; Huang et al., 2014). The mechanism is an integral part of developmental processes such as X-chromosome inactivation and genomic imprinting (Allen et al., 1992). Hence, aberrant methylation of genes could alter phenotypes, and in livestock, traits can be affected (Andrés et al., 2021). Prenatal nutrition levels i.e., under- or over-nutrition, has been implicated in modifying fetal DNA methylation patterns and consequently affecting the offspring's productive performance (Thompson et al., 2020). Several studies show that prenatal nutrition alters DNA methylation in goats (Li et al., 2018), cattle (Paradis et al., 2017), and sheep (Lan et al., 2013).

Studies on postnatal consequences of prenatal feed restriction, especially in beef cattle, are limited and the involvement of dietary restriction in shaping DNA methylation and expression of genes regulating metabolism and postnatal growth and development needs to be further elucidated. Previously, our research group identified differentially methylated regions (DMRs) associated with prenatal diet in semen of bull offspring using whole-genome bisulfite sequencing (WGBS) (Foroutan et al., 2021). In the current study, we employed the use of quantitative individual fragment bisulfite sequencing analysis (EpiTYPER) to determine whether similar DMRs were present in skeletal muscle of steer offspring from the same experiment. In particular, we examined *Longissimus dorsi* (LD) and *Semimembranosus* (SM) muscles, as well as liver (LV) tissue. Therefore, the objective of this study is to determine the effect of early to mid-gestational feed restriction in steer progeny on 1) DNA methylation in LD and SM muscles, and LV, and 2) expression of genes regulating metabolism and postnatal muscle development in the same tissues.

3.2. Materials and methods

3.2.1. Animals and experimental design

The experimental protocol for this study was approved by the Animal Care and Use Committee at the University of Alberta (Animal Use Protocols 135, 457, 483, and 877), under the CCAC guidelines on the care and use of farm animals in research, teaching, and testing (Canadian Council on Animal Care, 2009). Eighty-four purebred Angus heifers were examined for residual feed intake (RFI) using the GrowSafe System® (GS) automated feed recording system (Vytelle® Ltd., Calgary, Alberta, Canada), as described below, were used for the experiment. The housing of heifers and dietary tests were carried out at the University of Alberta Roy Berg Kinsella Research Ranch (Kinsella, Alberta, Canada). The population from which these heifers originated was

described previously (Mao et al., 2013). The animals and experimental design of this study were detailed by Devos et al. (2021) and Meale et al. (2021).

Calculation of residual feed intake

Heifers whose ages were between ~9 and 12 months were housed in dry lot pens which contained feed bunks monitored by GS for their initial RFI test using protocols described by Basarab et al. (2003) and (Mao et al., 2013). The description for the RFI test procedure, categorization of heifers' into dietary groups and sire selection for breeding is detailed in the animal and experimental design section of chapter 2 in this thesis.

Estrus synchronization, artificial insemination, and pregnancy detection of heifers

The estrus synchronization, artificial insemination, and pregnancy detection of heifers protocol was similar to those employed for the bull progeny as described in chapter 2 of this thesis.

Segregation into diet treatment groups

Pregnant heifers (n = 69, out of the starting 84 heifers) were assigned to either one of two dietary treatments through stratified randomization (considering RFI, current body weight, backfat depth, sire, and conception date), and remained in these groups from day 30 to day 150 of gestation. One of the diets (moderate diet or Mdiet) was formulated to provide growth of 0.73 kg/day ADG (roughly 100% of NRC recommendations for growing pregnant heifers) and consisted of brome grass hay supplemented with oat grain (n = 36). The second diet (Ldiet) which consisted of mainly brome grass hay (n = 33) was formulated for 0.54 kg/day ADG and represented approximately 74% of Mdiet (Meale et al., 2021). To account for heifer growth and the increasing weight of the conceptus, the ration was adjusted approximately once per month after the heifers were weighed (Table 3.1). Individual intakes of brome grass-mixed hay were recorded by the GrowSafe System® to monitor consumption of diets within groups and measure differences between groups. This

enabled the use of the individual animal as an experimental unit, as group feeding of H- and L-RFI cattle was required to test the expression of the RFI phenotype since RFI is measured in a group setting. Heifers were fed the entire hay allocation once a day in the GrowSafe System®. Oats were provided in separate bunks before the hay was fed to ensure even access and consumption. Feed samples of hay and oat grain were collected weekly and pooled monthly before being sent for nutrient analysis (Parkland Laboratories, Red Deer, AB, Canada). A portion of each pooled feed sample was dried in an oven at 80 °C for a minimum of 72 h to calculate dry matter. Wet chemistry methods were employed by Parkland Laboratories to determine crude protein (CP; method 981.10, Helrich, 1990); acid detergent fibre (ADF; ANKOM Technology method 8, based on method 973.18, Helrich, 1990); neutral detergent fibre (NDF; ANKOM Technology method 9, based on Van Soest et al., 1991) ; Ca, Na, K and Mg (atomic absorption using a Perkin Elmer 5000 atomic adsorption spectrophotometer following sulphuric acid/hydrogen peroxide digestion; Ca and Mg by absorption, and Na and K by emission); P (ammonium molybdate/ ammonium metavanadate colorimetric method, manual adaptation of AAFRD SCDC automated method F004.A); total digestible nutrients (TDN) (grasses %TDN = 4.898 + (89.796 × (1.0876 - (0.0127 × ADF))); oat grain %TDN = 4.898 + (89.796 × (0.9265 - (0.00793 × ADF)))) (Pennsylvania State, 1981). Cobalt iodized salt (Windsor Salt Ltd., Pointe-Claire, QC, Canada) was provided free choice with a vitamin premix included (Vitamin A, D, E-10 M Vitamin Premix for Livestock Feeds, Hi-Pro Feeds LP, Sherwood Park, Alberta) at a rate of 66 mL per liter of salt. Upon a subsequent pregnancy check mid-way through the diet treatment, six open heifers were removed from the feeding trial, three from each from Ldiet and Mdiet. After 150 days of gestation, all heifers were housed together and offered free choice mixed grass hay until approximately 2 months after birth. Nutrient analysis of all feedstuffs fed to heifers from the start of the feeding trial (30

days of gestation), until when they were grazing pasture with their calves, can be found in Table 12.

Calves were born between March 18th and April 23rd, 2013 (total n = 56, male n = 24; of the 63 heifers in the experiment at calving time, six calves died at birth from unrelated causes (crushing, size of the calf, calf positioning at birth), and one heifer was found to no longer be pregnant). At birth, male calves were castrated using an elastic band. Calves remained with their dams and grazed mixed tame and native grass pastures until weaning in November 2013. At weaning, heifers and steers were separated from each other and subsequently, all steers were fed and managed according to industry standards for feedlot production of finished cattle in Alberta. One steer from the LRFI-Mdiet group got his head caught in a feeder and died in late December 2013. Two weeks before the proposed first slaughter date all steers were measured for backfat thickness at the 12-13th rib using an Aloka-500 V scanner equipped with a 17 cm, 3.5 MHz linear array transducer (Overseas Monitor Corporation Ltd., Richmond, BC, Canada). This backfat measurement was used to group the animals into three slaughter dates such that steers with > 6 mm were slaughtered first, and all four treatment groups were represented at each slaughter date (Meale et al., 2021)

Table 3.1 Dietary adjustments of M- and L-diets fed to pregnant heifers from 30 to 150 days of gestation. Diets consisted of different proportions of brome grass hay and oat grain to facilitate either 0.54 kg/d or 0.73 kg/d ADG.

Period	1[a]		2[a]		3[c]		4[d]		5[e]	
Diet amount (kg Dry Matter/heifer/d)	Mdiet	Ldiet	Mdiet	Ldiet	Mdiet	Ldiet	Mdiet	Ldiet	Mdiet	Ldiet
Crude Protein	0.93	0.77	0.97	0.83	1.06	0.91	1.13	0.98	1.18	0.99
Acid Detergent Fibre	3.26	3.70	3.31	3.97	3.59	4.33	3.82	4.69	4.00	4.13
Neural Detergent Fibre	4.84	5.34	4.92	5.74	5.35	6.26	5.69	6.78	5.96	6.03
Calcium	0.04	0.05	0.05	0.06	0.05	0.06	0.05	0.07	0.05	0.06
Phosphorus	0.03	0.02	0.03	0.02	0.03	0.02	0.03	0.02	0.03	0.03
Potassium	0.16	0.18	0.16	0.20	0.18	0.21	0.19	0.23	0.20	0.20
Magnesium	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Total Digestible Nutrients	5.32	4.01	5.63	4.31	6.16	4.70	6.58	5.09	6.89	5.44

[a] Start date 1st AI group = July 20, 2012; 2nd AI group = Aug. 13, 2012.
[b] First adjustment: Sept. 4, 2012.
[c] Second Adjustment: Oct. 15, 2012.
[d] Third adjustment: Nov. 6, 2012.
[e] Fourth adjustment: Nov. 22, 2012.

Table 3.2 Nutrient analysis of feeds fed to pregnant heifers during the feeding trials (brome grass-mixed hay and oat grain)[a], and after the feeding trial (free choice mixed grass hay)[b]

Feed Type	Brome grass hay (n = 6)	Oat grain (n= 4)	Free-choice hay (n=3)
Dry Matter (%)	86.25 ± 1.11	87.31 ± 1.29	89.58 ± 1.02
Composition (%), Dry Matter Basis			
Crude Protein	9.66 ± 0.72	11.44 ± 0.43	13.54 ± 3.25
Acid Detergent Fibre	46.08 ± 1.26	12.91 ± 1.88	41. 78 ± 2.57
Neutral Detergent Fibre	66.59 ± 2.01	23.17 ± 2.53	59.02 ± 11.83
Calcium	0.66 ± 0.12	0.12 ± 0.001	0.93 ± 0.54
Phosphorus	0.22 ± 0.23	0.43 ± 0.04	0.27 ± 0.01
Potassium	2.27 ± 0.23	0.60 ± 0.03	1.87 ± 0.58
Magnesium	0.21 ± 0.04	0.13 ± 0.01	0.19 ± 0.04
Sodium	0.01 ± 0.0001	0.01 ± 0.005	0.01 ± 0.0001
Total Digestible Nutrient	50.00 ± 1.44	78.90 ± 1.34	54.91 ± 2.93

[a] Feed sample was collected weekly and pooled monthly. Averaged values and standard deviations for the monthly analyses are shown.

[b] Samples of hay were collected from representative bales in each source (two sources). Average values and standard deviations for the two sources are shown.

3.2.2. Sample collection

Slaughter and tissue collection was conducted as previously described by Devos et al. (2021). When the steers reached 6–8 mm backfat, they were transported to the abattoir at the Agriculture and Agri-Food Canada Lacombe Research and Development Centre (Lacombe, AB, Canada) (mean age of 512.1 ± 10.1 (SD) d) and slaughtered by captive-bolt stunning and exsanguination. One approximately 10 g sample each *Longissimus dorsi*, *semimembranosus* muscles, and liver biopsies were aseptically collected from steer calves at slaughter (n = 23; Mdiet-HRFI = 4, Ldiet-HRFI = 6, Mdiet-LRFI = 5, Ldiet-LRFI = 8) within 30–45 min post-mortem. All tissue samples were snap-frozen in liquid nitrogen and stored at -80°C.

3.2.3. Selection of DMR-associated genes

Selection of DMR-associated genes is described in chapter 2 of this thesis.

3.2.4. DNA extraction and methylation analysis

Muscle and liver tissues were grounded into powder with a mortar and pestle under liquid nitrogen and then stored in a -80℃ freezer. Approximately 100 mg of each tissue were aliquoted into 2 ml microcentrifuge tubes to be used for DNA extraction. The DNA extraction was performed in duplicate for each LD and SM muscle, respectively, then combined. For the LV, only a single extraction per individual was performed. The extraction was performed using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), following the Spin-Column protocol with a few modifications. An extra 620 µl of DNA lysis buffer (prepared in-house; 25 ml of 0.5 M NaCl, 12.5 ml of 50 mM Tris-HCl pH 8.0, 25 ml of 50 mM EDTA, 50 ml of 4% SDS, and 137.5 ml MilliQ water (the total volume is 250 ml) was added during the tissue lysis step. The tissue samples were homogenized using Mini-Beadbeater-24 (BioSpec, Bartlesville, OK, USA). As well, an extra 20 µl proteinase K was added during the protein digestion stage, and an extra 200 µl AL buffer and

an extra 200 µl 100% ethanol were used for de-salting the DNA. DNA quantification was performed using the NanoDrop 2000C (Thermo Fisher Scientific, Waltham, MA, USA) for nucleic acid concentration, while quantification of double-strand DNA in the samples was determined using the Invitrogen Qubit™ 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

The DNA methylation analysis was performed by Genome Quebec using the EpiTYPER MassARRAY technology (Agena Bioscience, San Diego, CA, USA). A total of 15 target amplicons (Table 3.3) were analyzed. All methylation procedures were similar to those described in chapter 2 of this thesis

Table 3.3 Genes and chromosomal location of the 15 CpG that were selected for the methylation analysis.

Gene name	Gene symbol	Chromosomal location[1]	CpG Analyzed[2]
Acyl-CoA dehydrogenase very long chain	*ACADVL*	19:26938525 -26939292	9/10
Aldehyde dehydrogenase 3 family member B1	*ALDH3B1*	29:45537643 -45538425	24/28
Collagen-like tail subunit of asymmetric acetylcholinesterase	*COLQ*	1:152537871-152538635	29/32
Dipeptidyl peptidase like 6	*DPP6*	4:116520561-116521160	17/18
Growth factor receptor-bound protein 10	*GRB10*	4:5274311-5275031	20/20
Histone deacetylase 4	*HDAC4*	3:118258228-118259046	30/33
Insulin-like growth factor 2 receptor-DMR2	*IGF2R-DMR2*	9:96220873-96221574	24/25
Insulin-like growth factor 2 receptor-WGBS	*IGF2R-WGBS*	9:96278463-96279243	26/35
Immunoglobulin-like and fibronectin type III domain containing 1	*IGFN1*	16:79717001 -79717767	8/10
Insulin like 3	*INSL3*	7:5326081-5326799	15/16
3-phosphoinositide-dependent protein kinase 1	*PDPK1*	25:2062070-2062783	25/26
Phosphorylase kinase regulatory subunit alpha 2	*PHKA2*	X:124497025-124497732	14/16
Protein kinase AMP-activated non-catalytic subunit gamma 2	*PRKAG2*	4:114315277-114316030	17/19

Ras protein-specific guanine nucleotide releasing factor 1	*RASGRF1*	21:25311128-25311844	16/17
Stearoyl-CoA desaturase 5	*SCD5*	6:97506791-97507486	15/15

[1] Chromosomal location is within genome assembly ARS-UCD1.2/bosTau9
[2] CpG Analyze: Analyzable CpG sites/Total CpG sites in each amplicon

3.2.5. RNA extraction and NanoString gene expression analysis

Frozen LD, SM muscle, and LV samples were ground in liquid nitrogen using a mortar and pestle. Approximately 100 mg of each sample (n=23 for each tissue type) were aliquoted into 2 mL micro-centrifuge tubes. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). The details of the RNA extraction are detailed in chapter 2.

Gene expression analysis was performed on 100 ng of total RNA (~ 44 ng/μL) extracted from the LD and SM muscle, and LV, using the nCounter RNA gene expression assay (NanoString Technologies, Seattle, Washington, USA) and their Elements TagSets technology. Probe A and B for each transcript associated with putative DMRs from the methylation analysis, or for transcripts chosen based on their biological and functional role in metabolism and muscle development, were synthesized by Integrated DNA Technologies (IDT) using publicly available bovine sequences listed in Table 3.4. All hybridization, purification, and data quantification steps were similar to those described in chapter 2.

Table 3.4 List of genes analyzed by NanoString nCounter genes expression analysis in LD, SM, and LV.

Gene Name	Symbol	Accession No.[1]	Position[2]
Acyl-CoA dehydrogenase very long chain	*ACADVL*	NM_174494.2	656-755
Acyl-CoA thioesterase 7	*ACOT7*	NM_001075682.2	769-868
Aldehyde dehydrogenase 3 family member B1	*ALDH3B1*	NM_001075518.1	1621-1720
Collagen-like tail subunit of asymmetric acetylcholinesterase	*COLQ*	NM_001035297.4	1009-1108
Dipeptidyl peptidase like 6	*DPP6*	NM_174040.2	1873-1972
Growth factor receptor-bound protein 10	*GRB10*	NM_001192586.1	1651-1750
Histone deacetylase 4	*HDAC4*	XM_024990281.1	4436-4535
Insulin-like growth factor 1	*IGF1*	NM_001077828.1	346-445
Insulin-like growth factor 1 receptor	*IGF1R*	NM_001244612.1	2767-2866
Insulin-like growth factor 2	*IGF2*	NM_174087.3	401-500
Insulin-like growth factor 2 receptor	*IGF2R*	NM_174352.2	8126-8225
Immunoglobulin-like and fibronectin type III domain containing 1	*IGFN1*	XM_024976573.1	801-900
Inositol polyphosphate-5-phosphatase A	*INPP5A*	XM_024986036.1	758-857
Insulin like 3	*INSL3*	NM_174365.2	109-208
Insulin receptor	*INSR*	XM_002688832.2	1818-1917
Potassium voltage-gated channel interacting protein 1	*KCNIP1*	NM_001013604.1	276-375
Myocyte enhancer factor 2A	*MEF2A*	NM_001083638.2	1751-1850
Myostatin	*MSTN*	NM_001001525.3	703-802
Myogenic factor 5	*MYF5*	NM_174116.1	661-760
Phosphofurin acidic cluster sorting protein 2	*PACS2*	XM_024982233.1	1441-1540
3-phosphoinositide-dependent protein kinase 1	*PDPK1*	XM_024985289.1	3235-3334
Phosphofructokinase, muscle	*PFKM*	NM_001075268.1	2058-2157
Phosphorylase kinase regulatory subunit alpha 2	*PHKA2*	NM_001191545.1	1344-1443
Pyruvate kinase M1/2	*PKM*	NM_001075268.1	902-1001
Peroxisome proliferator-activated receptor gamma	*PPARG*	NM_181024.2	1562-1661
Protein kinase AMP-activated non-catalytic subunit gamma 2	*PRKAG2*	XM_024991251.1	1528-1627
Glycogen phosphorylase, muscle associated	*PYGM*	NM_175786.2	1585-1684
Ras protein-specific guanine nucleotide releasing factor 1	*RASGRF1*	NM_001191457.1	1946-2045
Stearoyl-CoA desaturase 5	*SCD5*	NM_001076945.1	1059-1158
Thyroid hormone receptor beta	*THRB*	XM_005226186.4	2641-2740

[1]GenBank sequence identifier for each mRNA accession
[2]Target position within each sequence

3.2.6. Statistical analysis

3.2.6.1. DNA methylation data processing

The processing of DNA methylation data is the same as described in chapter 2

3.2.6.2. Gene expression data processing and normalization

All gene expression data processing and normalization steps followed similar steps as those described in chapter 2.

3.2.6.3. Differential methylation and gene expression analysis

The test for normality and differential methylation and gene expression analysis followed the same step as described in chapter 2 of this thesis. A spearman correlation analysis was conducted between CpG methylation of a DMR and its corresponding gene expression within each tissue to evaluate the association between DNA methylation and gene expression using the PROC CORR function within SAS (v9.4). Significant association was set at $P \leq 0.05$. Pairwise correlation within tissues and between individual CpGs showed a high percentage of significant correlation with each tissue (LD = 34%, SM = 35%, and LV = 27%). As a result, multiple testing correction was not performed to avoid false negative results.

3.2.7. Principal component analysis

PCA was performed to profile patterns within DNA methylation and gene expression with respect to LD, SM and LV using the prcomp package (default stats package v3.5.1) in R (version 4.1.1; R Core Team, 2021). Dimensions were reduced and variance maximized by extracting principal components (PC) from the analysis based on the cumulative variance explained. A threshold of $\leq$ 80% explained variance was used to select the number of PCs for further steps in the analysis. Two PCs were selected and correlated with the original data. The ggbiplot package (https://github.com/vqv/ggbiplot) and stat ellipse function, at 95% confidence interval in R

(version 4.1.1; R Core Team, 2021), was used to plot a PCA plot between PC1 and PC2, representing the components with most variation in the dataset to visualize expressed gene distribution between tissues.

3.3. Results

3.3.1. Principal component analysis results

PCA displays the distribution and clustering of DNA methylation (Figure 3.1) and gene expression (Figure 3.2) between the tissues (LD vs SM vs LV). The arrows represent each gene, its direction represents its correlation between the genes as well to the PCs and the length represent the level of contribution to the PC. Genes which form angles ≥ 30° from the midpoint of the plot to either of the respective to the PCs were considered to have a relatively high correlation to the PCs and those that form a 90° are considered to have no correlation to the PCs. Figure. 3.1 shows the PCs (DNA methylation) score plot for PC1 against PC2, which explains 47.9% and 11.4% of the variance within the methylation data analyzed respectively. Also, from Figure 3.1, methylation of DNA fragments *ACADVL*, *DPP6, HDAC4, INSL3, IGF2R*-WGBS, *PDPK1* and *RASGRF1* were positively correlated to PC1 while *COLQ*, *IGF2R*-DMR2 and *GRB10* genes were negatively correlated with PC1. *PHKA2* displayed no correlation with PC1. PC2 was positively correlated with *INSL3, GRB10, PHKA2* and *PDPK1*, while *COLQ*, *DPP6, ACADVL,* and *RASGRF1* were negatively correlated with the PC. *IGF2R*-DMR2, *IGF2R*-WGBS, and *HDAC4* had almost no correlation with PC2. The PCA for gene expression (Figure 3.2) shows PC1 and PC2 explaining 64.5% and 10.5% variation within the gene expression data respectively. DNA fragments *ACADVL, IGF1, IGF2*, *IGF2R, INSR* and *PHKA2* positively correlates with PC1 while *ACOT7, ALDH3B1, COLQ, DPP6, GRB10, HDAC4, IGF1R, IGFN1, INPP5A, INSL3*, *KCNIP1, MEF2A, MSTN, PPARG, PRKAG2, PYGM*, and *THRB* negatively correlates with PC1. *SCD5* shows no

correlation with PC1. PC2 positively correlates with *DPP6, KCNIP1, PFKM*, and *PRKAG2* and negatively correlates with *ACADVL, ACOT7, ALDH3B1, COLQ, HDAC4, IGF1, IGF2, IGF2R, INPP5A, INSL3, INSR, MEF2A, MSTN, PHKA2, PPARG*, and *THRB*. *PKM, IGF1R* and *PACS2* have no correlation with PC2. Genes positively correlated with the PCs implies the genes had higher contribution to the respective PCs vice versa. From the PCA plots, we see that both the DNA methylation (Figure 3.1) and expression (Figure 3.2) patterns for LV are clustered to the right of PC1 and distinctly different from the patterns observed in the muscle tissues. Between LD and SM, we see that the methylation patterns in the two muscles overlap, while there is much less overlap in gene expression patterns. This implies that the methylation pattern between the two muscle types is relatively similar compared their patterns in gene expression.

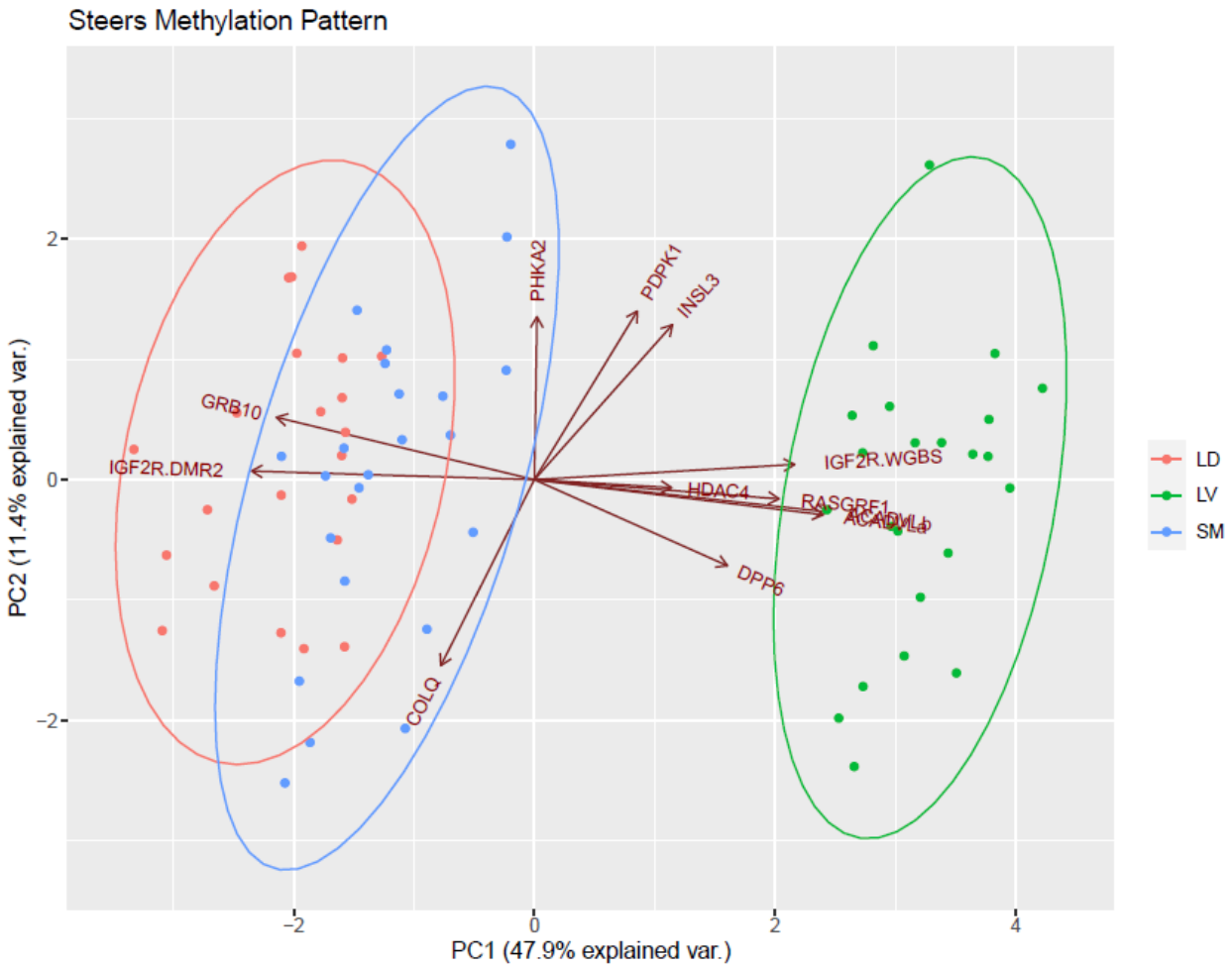

Figure 3.1 Principal component analysis for DNA methylation between tissues with red, blue, and green ellipses representing *Longissimus dorsi* (LD), *Semimembranosus* muscle (SM), and liver (LV) tissues respectively. The sample individuals are represented by dots and clustered according to tissue (coloured). The variables are represented by the arrows within the figures, and their directions represent correlation between them and other variables, and the PCs.

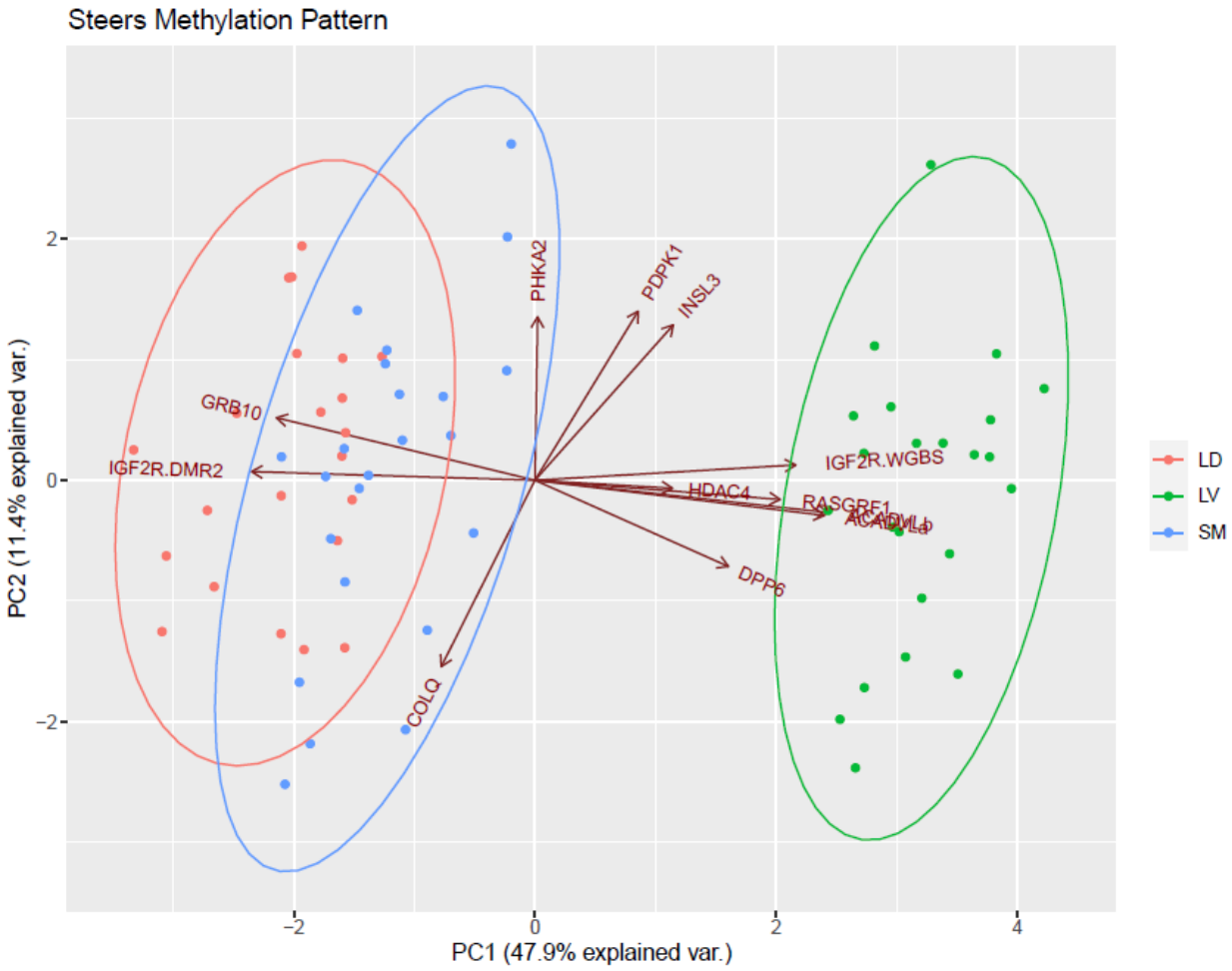

Figure 3.2 Principal component analysis for gene expression distribution between tissues with red, blue, and green ellipses representing *Longissimus dorsi* (LD), *Semimembranosus* muscle (SM), and liver (LV) tissues respectively. The sample individuals are represented by dots and clustered according to tissue (coloured). The variables are represented by the arrows within the figures, and their directions represent correlation between them and other variables, and the PCs.

3.3.2. Effect of prenatal diet and RFI on DNA methylation in LD muscle

In LD muscle, genetic selection for RFI influenced the methylation of the DNA fragments associated *ACADVL* (CpG 11), *ALDH3B1* (CpG 5), *INSL3* (CpG 6 and 16), and *RASGRF1* (CpG 2) (Table 3.5). LRFI steer progeny displayed higher methylation for *ACADVL*, *INSL3* and *RASGRF1* while for *ALDH3B1*, the HRFI group had higher methylation levels (Table 3.5). The methylation of *COLQ* (CpG 7) and *PDPK1* (CpG 16) were affected by the interaction between diet and RFI (Figure 3.2). The Mdiet-HRFI group showed significantly higher methylation levels than the Ldiet-HRFI group for *COLQ*, and for *PDPK1* the Mdiet-LRFI group displayed higher methylation levels compared to Ldiet-LRFI group. An interaction effect was also detected for *HDAC4* (CpG 6), but there were no significant differences between treatments in the post-hoc test.

Table 3.5 Effect of prenatal diet and genetic potential for RFI on DNA methylation in longissimus dorsi muscle of steer offspring at slaughter as assessed by EpiTYPER.

DMR	CpG	RFI		P-value
		HRFI [1]	LRFI[1]	
ACADVL	11	0.40 ± 0.01	0.44± 0.009	0.03
ALDH3B1	5	0.77 ± 0.02	0.70 ± 0.02	0.02
INSL3	6	0.37 ± 0.02	0.44 ± 0.02	0.03
	16	0.60 ± 0.01	0.65 ± 0.01	0.01
RASGRF1	2	0.83 ± 0.007	0.85 ± 0.007	0.04

[1]Data represents methylation percentage for CpG within LD muscle and are expressed as least square means ± SE/ If the data is non-normal, data are the back-transformed mean and 95% CI in []. Significance set at $P \leq 0.05$.

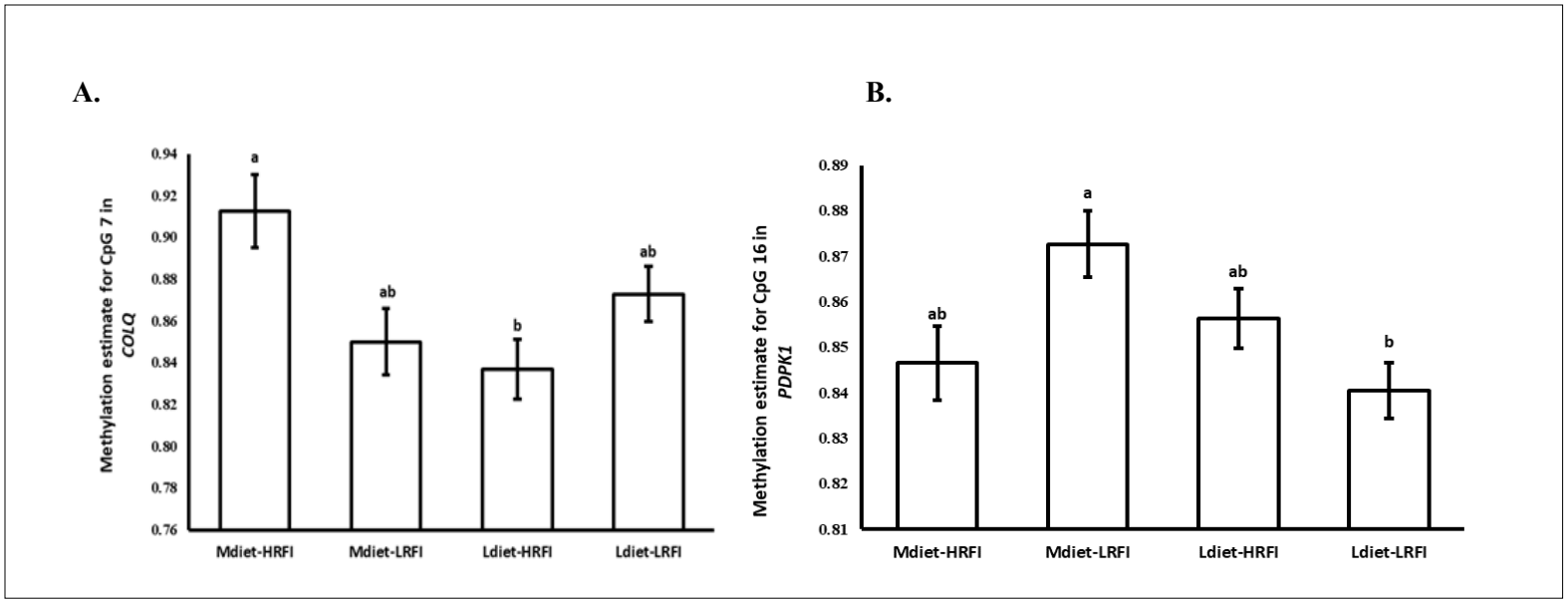

Figure 3.3 Bar graph displaying differential methylation between prenatal diet and RFI interaction steer sub-groups for A) *COLQ* (CpG 7) and B) *PDPK1* (CpG 16). Bars represent LSmeans estimates, and error bars represent SE. a,b indicates significant differences between the treatment groups.

3.3.3. Effect of prenatal diet and RFI on DNA methylation in *semimembranosus* muscle (SM)

In SM muscle, prenatal diet impacted methylation of the DNA fragments associated with *IGF2R*-WGBS (CpG 17), *INSL3* (CpG 5), and *RASGRF1* (CpG 5-6) (Table 3.6). Ldiet progeny displayed higher methylation levels for *RASGRF1* while Mdiet steer progeny displayed higher methylation levels for significant CpGs in *IGF2R*-WGBS, and *INSL3*. Genetic potential for RFI influenced the methylation of *ACADVL* (CpG 7-8), *GRB10* (CpG 15 and 16-17), and *IGFN1* (CpG 6) (Table 3.6). HRFI progeny displayed higher methylation levels for significant CpGs in *ACADVL*, and *IGFN1* while methylation levels for significant CpGs in *GRB10* were higher in LRFI steer progeny. Prenatal diet and RFI interaction influenced methylation for *ALDH3B1* (CpG 18), *COLQ* (CpG 11), *IGF2R*-DMR2 (CpG 3-4), and *SCD5* (CpG 8-9) (Figure 3.3). In three DMRs (*ALDH3B1, COLQ,* and *SCD5*), Mdiet-HRFI had significantly lower methylation compared to at least two or more other diet-RFI groups. In *IGF2R*-DMR2, Mdiet-LRFI steer group had significantly lower methylation compared to their counterparts (Figure 3.3). An interaction effect was also detected for *DPP6* (CpG 2), but there were no significant differences between treatments in the post-hoc test.

Table 3.6 Effect of prenatal diet and genetic potential for RFI on DNA methylation in *Semimembranosus* muscle of steer offspring at slaughter as assessed by EpiTYPER.

		Diet		
DMR	**CpG**	**Mdiet[1]**	**Ldiet[1]**	**P-value**
IGF2R-WGBS	17	0.87 ± 0.03	0.80 ± 0.02	0.05
INSL3	5	0.27 ± 0.01	0.25 ± 0.01	0.04
RASGRF1	5-6	84.0, [82.1,85.8]	86.0, [85.1, 86.8]	0.008
		RFI		
		HRFI[1]	**LRFI[1]**	
ACADVL	7-8	0.79 ± 0.005	0.77 ± 0.005	0.01
GRB10	15	0.63 ± 0.01	0.68 ± 0.01	0.02
	16-17	50, [47.3,52.5]	53.7, [50.7, 56.4]	0.03
IGFN1	6	0.45 ± 0.01	0.41 ± 0.01	0.002

[1]Data represents methylation percentage for CpG within SM muscle and are expressed as least square means ± SE/ If the data is non-normal, data are the back-transformed mean and 95% CI in []. Significance set at $P \leq 0.05$.

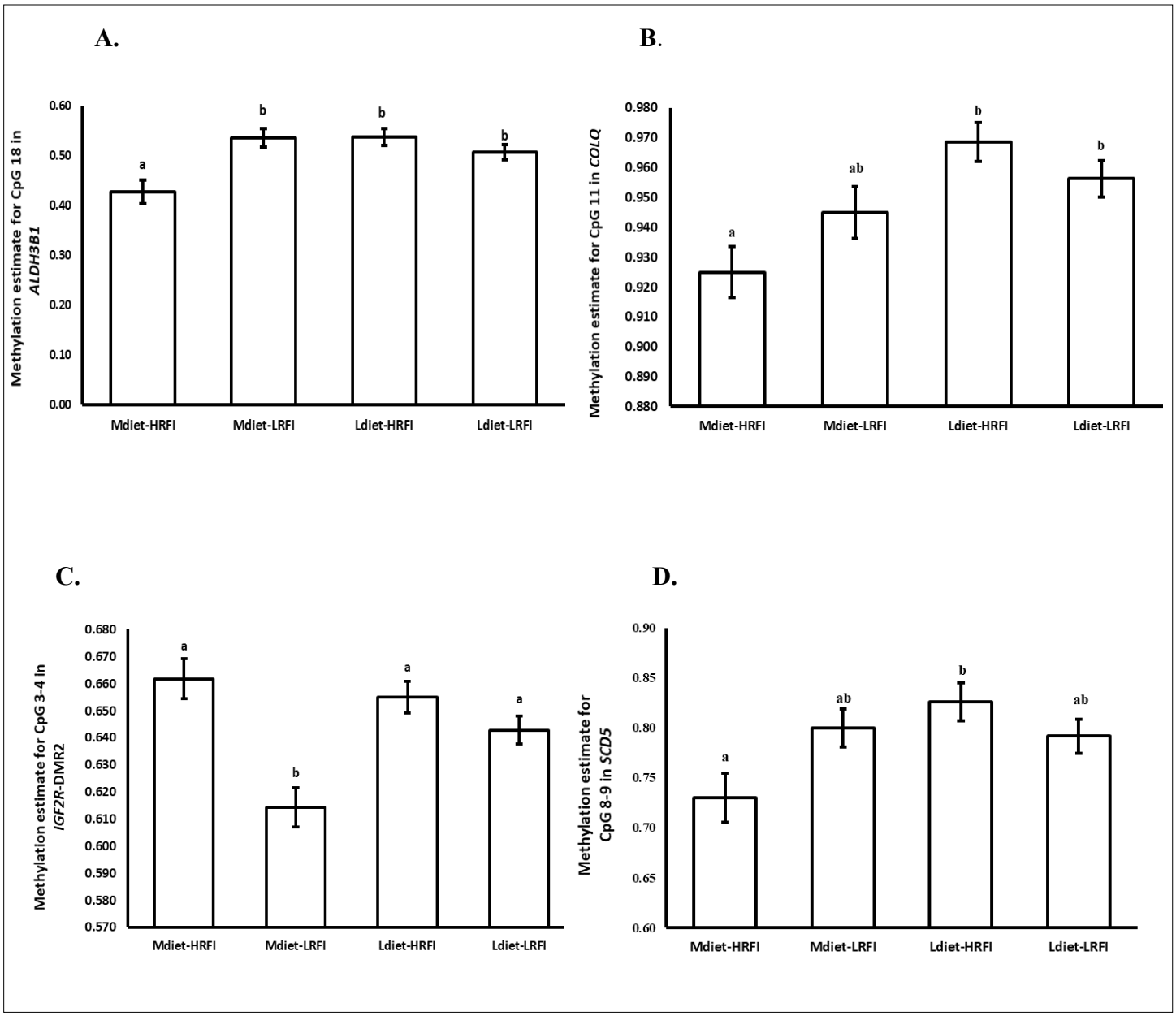

Figure 3.4 Bar graphs displaying differential methylation associated with the interaction of prenatal diet and RFI upon steer treatment groups in SM muscle for A) *ALDH3B1* (CpG 18), B) *COLQ* (CpG 11), C) *IGF2R*-DMR2 (CpG 3-4) and D) *SCD5* (CpG 8-9). Bars represent LSmeans estimates, and error bars represent SE. [a,b] indicates significant differences between the diet-RFI groups

3.3.4. Effect of prenatal diet and RFI on DNA methylation in liver (LV)

In LV tissue, prenatal dietary treatment affected the methylation of DNA fragments associated with *ALDH3B1* (CpG 18) and *COLQ* (CpG 16-17 and 24-25) (Table 3.7). The Mdiet steers displayed higher methylation levels for significant CpGs in both *ALDH3B1* and *COLQ*. Genetic selection for RFI influenced the methylation of *COLQ* (CpG 24-25), *IGF2R*-DMR2 (CpG 10), *GRB10* (CpG 5), and *PDPK1* (CpG 11) (Table 3.7). The HRFI steers displayed higher methylation levels in significant CpGs for *COLQ* and *PDPK1*, while LRFI steers displayed higher methylation levels for significant CpGs in *IGF2R*-DMR2 and *GRB10*. The interaction of prenatal diet and genetic potential for RFI was associated with methylation levels of CpGs within *HDAC4* (CpG 6 and 33), *INSL3* (CpG 14), *RASGRF1* (CpG 5-6), and *SCD5* (CpG 6). In *HDAC4* (CpG 6), there was no differences between individual treatment groups while for *HDAC4* (CpG 33), the Mdiet-HRFI group displayed lower methylation levels than Mdiet-LRFI group [(Mdiet-HRFI (144.9, [119.5, 163.6 CI]), Mdiet-LRFI (157.0, [154, 160.7 CI]), Ldiet-HRFI (152.5, [146.6, 157.9 CI]), Ldiet-LRFI (112.0, [100.5, 121.5 CI), expressed as back-transformed mean and 95% CI]. In *INSL3*, Mdiet-LRFI group had higher methylation levels than Mdiet-HRFI group but levels similar to other steer groups (Figure 3.4A). In *RASGRF1*, Ldiet-HRFI methylation levels were similar to Mdiet-LRFI but lower compared to other steer groups Mdiet-HRFI and Ldiet-LRFI (Figure. 3.4B). For *SCD5*, the Mdiet-LRFI group was more highly methylated compared to all other diet-RFI steer groups (Figure 3.4C).

Table 3.7 Effect of prenatal diet and genetic potential for RFI on DNA methylation in liver of steer offspring at slaughter as assessed by EpiTYPER.

DMR	CpG	Diet		P-value
		Mdiet[1]	**Ldiet**[1]	
ALDH3B1	18	64.7, [61.1, 68.0]	55.4, [47.8, 61.3]	0.03
COLQ	16-17	0.94 ± 0.02	0.89 ± 0.01	0.04
	24-25	0.73 ± 0.006	0.71 ± 0.005	0.03
		RFI		
		HRFI[1]	**LRFI**[1]	
COLQ	24-25	0.73 ± 0.006	0.71 ± 0.005	0.001
IGF2R-DMR2	10	0.38 ± 0.008	0.40 ± 0.007	0.04
GRB10	5	0.36 ± 0.01	0.39 ± 0.009	0.03
PDPK1	11	0.92 ± 0.02	0.85 ± 0.02	0.02

[1]Data represents methylation percentage for CpG within LD muscle and are expressed as least square means ± SE/ If the data is non-normal, data are the back-transformed mean and 95% CI in []. Significance set at $P \leq 0.05$.

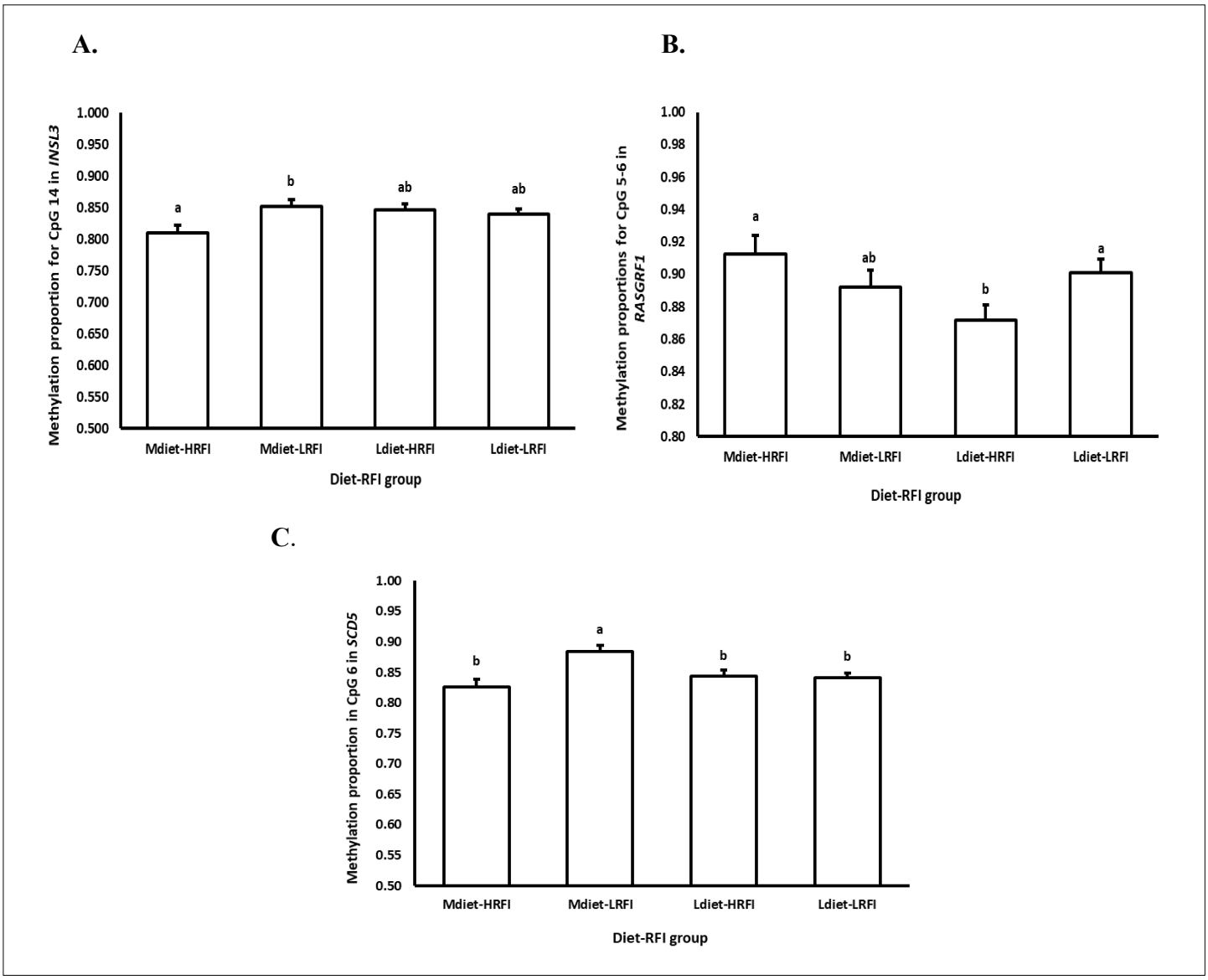

Figure 3.5 Bar graphs displaying differential methylation associated with the interaction of prenatal diet and genetic potential for RFI in steer LV tissue for A) *INSL3* (CpG 14), B) *RASGRF1* (CpG 5-6), and C) *SCD5* (CpG 6). Bars represent LSmeans estimates, and error bars represent SE. [a,b] indicates significant differences between the diet-RFI groups.

3.3.5. Effect of prenatal diet and genetic potential for RFI on gene expression in LD muscle

Prenatal diet was associated with the expression of *HDAC4*, where steers whose dams received Mdiet displayed higher expression levels than steers from the Ldiet treatment (Table 3.8). *IGFN1* and *PPARG* were differentially expressed when comparing steer groups that differed in the genetic potential for RFI (Table 3.8). LRFI steer progeny had higher expression of *IGFN1* while HRFI steers had higher *PPARG* expression (Table 3.8). Expression of all other genes analyzed within LD muscle were not influenced by prenatal diet, the genetic potential for RFI, nor their interaction.

Table 3.8 Effect of prenatal diet and genetic potential for RFI on gene expression in *Longissimus* dorsi muscle of steer offspring at slaughter as assessed by NanoString.

Gene	Diet		P-value
	Mdiet[1]	Ldiet[1]	
HDAC4	277.2 [240.1, 317]	225.8 [203.4, 249.3]	0.02
	RFI		
	HRFI[1]	LRFI[1]	
IGFN1	22555 ± 2237.82	30858 ± 1976.39	0.01
PPARG	159.2 [115.4, 219.5]	105.8 [90.2, 124.1]	0.02

[1]Data represents the gene expression estimates in LD muscle and are expressed as least square means ± SE/ If the data is non-normal, data are the back-transformed mean and 95% CI in []. Significance set at $P \leq 0.05$.

3.3.6. Effect of prenatal diet and genetic potential for RFI on gene expression in SM muscle

The expression of *HDAC4* was influenced by both prenatal diet and RFI treatments (Table 3.9). For prenatal diet, *HDAC4* was upregulated in Mdiet steers compared to their Ldiet counterparts, and for RFI, LRFI steers displayed higher expression levels. Prenatal diet was associated with the expression of *MYF5* (P = 0.05) and was upregulated in Mdiet steers compared to Ldiet steers (Table 3.9). *IGFN1* expression was also associated with the genetic potential for RFI (P = 0.02) with LRFI steers again displaying a higher expression. (Table 3.9). The interaction between prenatal diet and RFI was significant for the expression of *PACS2* (P = 0.01), Ldiet-LRFI expression levels were similar compared to Mdiet-HRFI but significantly lower than Mdiet-LRFI and Ldiet-HRFI (Figure 3.5).

Table 3.9 Effect of prenatal diet and genetic potential for RFI on gene expression in *Semimembranosus* muscle of steer offspring at slaughter as assessed by NanoString.

Gene	Diet		P-value
	Mdiet[1]	**Ldiet[1]**	
HDAC4	187.92 ± 8.11	154.81 ± 6.53	0.005
MYF5	75.72 ± 4.81	62.62 ± 3.87	0.05
	RFI		
	HRFI[1]	**LRFI[1]**	
HDAC4	157.20 ± 7.80	185.53 ± 6.89	0.01
IGFN1	22464.9 [17942.1, 27495.5]	31020 [36016.9, 26396.1]	0.02

[1]Data represents the gene expression estimate in SM muscle and are expressed as least square means ± SE/ If the data is non-normal, data are the back-transformed mean and 95% CI in []. Significance set at $P \leq 0.05$.

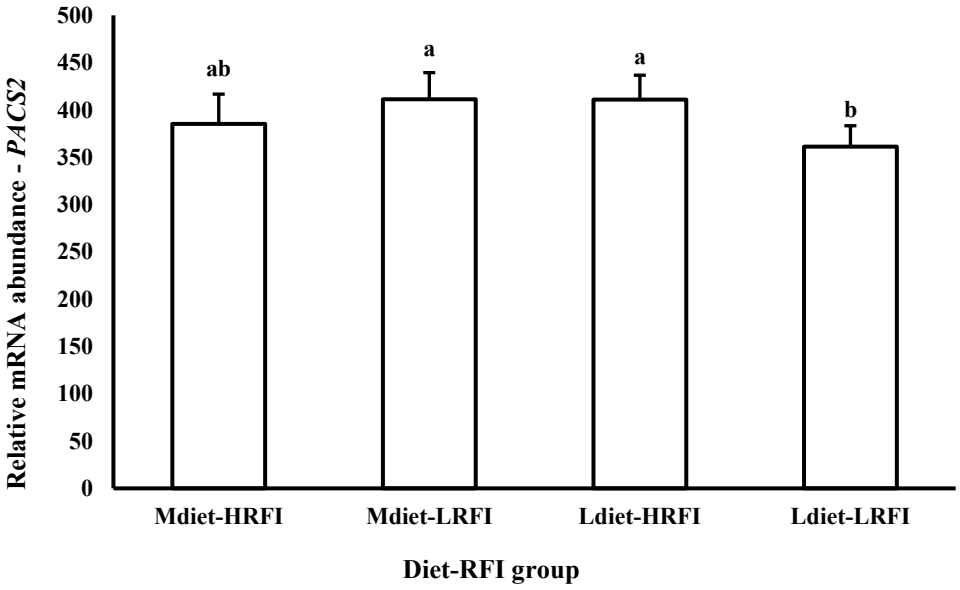

Figure 3.6 Bar graph indicating differential gene expression between prenatal diet and RFI interaction in steer groups for *PACS2*. Bars represent LSmeans estimates, and error bars represent SE. a,b indicates significant differences between the diet-RFI groups.

3.3.7. Effect of prenatal diet and genetic potential for RFI on gene expression in LV tissue

The genetic potential for RFI was associated with the expression of *IGF2* and *PDPK1* (Table 3.10). The expression of both *IGF2* and *PDPK1* were elevated in HRFI steers compared to their LRFI counterparts (Table 3.10). The interaction of prenatal diet and genetic potential for RFI affected the expression of *MEF2A*, where the Mdiet-HRFI group had higher expression than the Mdiet-LRFI and Ldiet-LRFI steer groups [Mdiet-HRFI (101.5 [70.8, 145.7 CI]), Mdiet-LRFI (75.3 [68.5, 82.8 CI]), and Ldiet-LRFI(84.0 [79.5, 88.8 CI]), expressed as back-transformed mean and 95% CI].

Table 3.10 Effect of genetic potential for RFI on gene expression in liver tissue of steer offspring at slaughter.

Gene	RFI		P-value
	HRFI[1]	LRFI[1]	
IGF2	9020.9 [8057.5, 10099.4]	8210 [772.4, 8728.3]	0.04
PDPK1	342.39 ± 14.44	299.14 ± 12.76	0.04

[1]Data represent the gene expression estimates in LV tissue and are expressed as least square means ± SE/ If the data is non-normal, data are the back-transformed mean and 95% CI in []. Significance set at $P \leq 0.05$.

3.3.8. Correlation between DNA methylation and gene expression

Methylation of CpG 11 in DMR ACADVL was positively correlated with its gene expression (r = +0.45, P= 0.04) in LD muscle. All other correlation analysis between the CpGs and gene expression showed no significant correlation.

3.4. Discussion

Developmental processes (prenatal and postnatal) have been reported to be programmed by maternal nutrition during gestation and/or progeny postnatal environment. Evidence from fetal programming studies has revealed significant associations between fetal development, the molecular mechanism (methylation), and maternal nutrition in ruminant livestock (Lan et al., 2013; Paradis et al., 2017; Wang et al., 2013). However, postnatal studies into these phenomena are still under investigation, especially in cattle. The current study investigated the molecular mechanisms regulating progeny performance at the postnatal level as a result of prenatal feed restriction, as well as selection for the genetic potential for RFI. Our aim was also to determine potential epigenetic markers associated with prenatal diet in muscle and liver tissues. Hence, we selected DMRs identified by WGBS in semen obtained from a subset of the offspring in our main experiment. The DMRs were based on methylation differences between M- and L-diet offspring and were chosen with reference to genes in close proximity that had biological functions related to growth and metabolism. The extra genes that we could include in the gene expression analysis were based on biological and functional roles related to growth and metabolic processes.

3.4.1. Differential methylation and gene expression patterns between tissues

We conducted PCA to observe patterns of DNA methylation and gene expression between LD, SM, and LV to investigate if the methods we were employing to measure DNA methylation and gene expression (EpiTYPER and NanoString, respectively), were yielding results reflective

of biology within the tissues. The methylation PCA shows that the ellipses of LD and SM overlap (Figure 4A) indicating a similar methylation pattern within the muscle tissues. In contrast, the PCA for gene expression displays a similar pattern between the two muscle tissues with respect to PCI, but almost no overlap between the muscle tissues for PC2. In both figures 4A and B the methylation and gene expression patterns for LV tissue display a distinct pattern from either LD or SM. The clustering of the muscle tissues away from the liver gives us evidence that the DNA methylation and gene expression values we are measuring are reflective of true biological variation at the tissue level. It might be expected that for LD and SM the DNA methylation levels are more closely related than expression levels as DNA methylation is more stable and related to genetic potential, and gene expression is more reflective of the environment and has more inherent variation, when examining tissue-level differences (Jaenisch & Bird, 2003).

We identified the methylation of 10 CpG sites within potential DMRs and their associated genes (*ACADVL, ALDH3B1, DPP6*, *GRB10*, *IGF2R*-DMR2, *IGF2R*-WGBS, *IGFN1*, *PDPK1, RASGRF1*, and *SCD5*), involved in metabolism and/or skeletal growth function, that were influenced by either prenatal diet, RFI and/or their interaction in LD, SM, and LV tissues. Comparatively, prenatal feed restriction had a higher influence on the methylation at slaughter in SM muscle than in LD and LV. Prenatal feed restriction resulted in differential methylation of 10 CpG sites in eight DMRs in SM compared to one CpG site in LD muscle and four CpG sites in three DMRs in LV. This differential response to either prenatal diet or RFI could be attributed to biological or functional differences between the tissues analyzed. Both LD and SM muscles are identified as glycolytic muscles with slight differences in their myofiber and metabolic properties involved in varied myogenic processes (De Las Heras-Saldana et al., 2019; Herault et al., 2014). Additionally, transcriptome and functional analysis by Herault et al. (2014) showed that mRNA

abundance and composition varied between LD and SM muscle and further revealed that postnatally, both muscle tissues are involved in varied biological functions. Therefore, it is possible that in the current study, methylation in the SM muscle was influenced to a greater extent by prenatal diet at slaughter due to potential functional and biological differences between the tissues analyzed.

In beef cattle studies, Paradis et al. (2017) and Devos et al. (2021) reported differential methylation in response to gestational feed restriction in LD and SM muscle at the fetal stage, as well as at weaning and slaughter. Similar to the results in the current study, Devos et al. (2021) reported that at slaughter, differential methylation was influenced by maternal diet to a greater extent in SM muscle were compared to LD at slaughter. Since, both studies were carried out in the same steer groups and with the same tissue samples, the similarity in our results validates the accuracy of procedures and protocols for methodology employed by both studies. Conversely, Paradis et al. (2017) reported that due to prenatal feed restriction, there was a higher response of methylation in fetal LD muscle relative to semitendinosus (ST) muscle. The discrepancies between our results to those of Paradis et al. (2017) could be due to factors including the timing of maternal feed restriction. In our study, the feed was restricted during early to mid-gestation compared to mid to late-gestation in Paradis et al. (2017). Additionally, methylation was analyzed in fetal tissues relative to adult tissues in the current study. The direct effect of maternal feed restriction may be still present in the LD of Paradis et al. (2017), while both the direct and metabolic consequences are manifested in the SM of the current study. This is particularly possible as Devos et al. (2021) also observed that prenatal feed restriction resulted in higher methylation levels in LD at birth but the effects were undetectable at slaughter. A study in sheep showed that prenatal feed restriction during early gestation negatively impacted myogenic processes in fetal LD muscle

compared to *Semitendinosus* muscle (Gauvin et al., 2020). Hence, the LD muscle may be relatively more susceptible to the influences of prenatal diet during gestation during the fetal stage as compared to ST or SM muscle. Therefore, the result from the current study suggests a potential differential requirement of nutrients by either muscle type during pre- and/or post-natal muscle development. Consequently, both muscle types could respond differently to prenatal diets at different stages of fetal and post-natal development.

3.4.2. Repeatability of EpiTYPER quantification of DNA methylation

We analyzed methylation of *IGF2R*-DMR2, previously analyzed by Devos et al. (2021) from the same tissue (LD, SM, and LV) at slaughter and animal group as in the current study to assess the repeatability and validate the methylation measured by EpiTYPER. The raw methylation data for from the study by Devos et al. (2021) and the current study as well as the results of the statistical analysis were compared to each other. Although DNA extraction for both studies was conducted at different times, all other methodologies were similar. After data processing and filtration of unreliable CpGs from the raw methylation data, both studies ended up with the same CpGs after processing in SM and LV tissue while for LD, the current study analyzed 7 CpGs vs 11 CpGs in Devos et al. (2021). Although at first glance the methylation results appear in the same range of values, we have observed some variation in the methylation counts for CpGs between both studies particularly for LV tissue. Therefore, this raises concern about the repeatability of the EpiTYPER technology in measuring slight differences in methylation, and that methylation results from the current study with no potential secondary evidence of other biological differences such as differences in gene expression, methylation of other CpGs within the same fragment, and correlation to gene expression and/or similar methylation trend between treatments group or tissues, will be discussed with some caution. As a result, we have focused our discussion on those

methylation results that display either similar significant differential methylation for more than one CpG within a DNA fragment, and/or significant gene expression result, or a correlation between gene expression and significantly differentially methylated CpGs.

3.4.3. Impact of prenatal diet on methylation and gene expression

Evidence from fetal programming studies in cattle have highlighted the potential long-term impact on progeny metabolic and growth performance induced by maternal dietary regime (Barcelos et al., 2022; Hoffman et al., 2017; Zago et al., 2020). This information allowed us to hypothesize that perhaps prenatal diet might have a long-term influence the underlying regulatory mechanisms i.e., DNA methylation as well as gene expression within the same tissue**.** Maternally expressed *IGF2R* gene is known to regulate embryonic and fetal development with peak expression detected during gestation (Bebbere et al., 2013). The *IGF2R* gene also controls *IGF2* signaling which also is essential for fetal growth resulting in the degradation of its availability (Bebbere et al., 2013). Hence, the improper establishment of *IGF2R* imprint could result in the dysregulation of the expression of these growth-regulatory genes and potentially impair development (Ghanipoor-Samami et al., 2018). Methylation in SM was influenced by prenatal diet for the *IGF2R*-WGBS DMR, and the Mdiet progeny displayed higher methylation levels. Alternatively, LD and LV tissues showed no difference in methylation of *IGF2R*-WGBS in response to prenatal diet, and in original semen WGBS (Foroutan, 2020), the Ldiet progeny displayed higher methylation for the potential DMR. Therefore, it appears there is a link between methylation of DNA in the vicinity of *IGF2R* that is associated with prenatal nutrition, but its exact location or trend is not consistent. Further examples of this are methylation of *IGF2R*-DMR2. The methylation pattern of *IGF2R*-WGBS in SM muscle of this study is similar to semen *IGF2R*-DMR2 methylation previously described in chapter 2 of this thesis, which could imply that the *IGF2R* could potentially be a

methylation marker due to prenatal diet. Studies have shown that tissues including the liver are given priority to nutrient supply in cases of sub-optimal maternal nutrient supply (Du et al., 2010b) and also LD muscle differentiates at a faster rate during myogenesis compared to SM. Both LD and LV may have been prioritized nutrient-wise during the feed restriction stage in the current study hence, the less impact on methylation. We did not observe any association of the expression of *IGF2R* with prenatal dietary treatment. According to Zhan et al. (2019), the expression of *IGF2R* tends to be inconsistent in tissues including *Longissimus dorsi* and liver in ruminant. Hence, it could be that the lack of significant association of *IGF2R* expression with prenatal diet might be due to the fluctuation of its expression. Therefore, we speculate that *IGF2R* methylation could potentially be a gestational marker for maternal diet, but more intense studies concentrating on *IGF2R* methylation and gene expression at different stages of growth and development in cattle, with respect to differential prenatal diets, are required.

3.4.4. Impact of selection for RFI on methylation and gene expression

Selection for RFI had a greater influence on methylation in LD and LV tissues relative to SM in the current study. In comparison to prenatal diet, we observed a greater effect on methylation associated with RFI, particularly for LD (Diet vs RFI; one CpG vs five CpGs) and LV (Diet vs RFI; four CpGs vs seven CpGs). In SM, methylation impact was slightly higher in response to prenatal diet (10 CpGs vs eight CpGs). For methylation impacted due to selection for RFI, LRFI group displayed higher methylation levels across the tissues compared to HRFI (LRFI vs HRFI; 13 CpGs vs six CpGs). Although with respect to gene expression, an equal number of significantly differentially expressed genes (two) were observed in each of the tissues. Perhaps, selection for RFI has a relatively stronger influence on DNA methylation relative to gene expression. A study conducted by Rocha et al. (2019) identified some DMRs in liver associated with RFI in cattle,

however this is the only study beside the current study investigating RFI and methylation association in cattle. Therefore, further research is needed to determine the molecular mechanism regulating the genetic potential for RFI in cattle.

Our results for the differences between tissues echo those from Devos et al. (2021) who also reported a greater impact of RFI on methylation in LV at slaughter, with the LRFI group displaying higher methylation levels. Additionally, the authors revealed that at birth CpG methylation of potential DMRs examined in LD muscle was more often associated with the genetic potential for RFI rather than prenatal diet, which could indicate that genetic potential for RFI could be greater effector of methylation in LD muscle at slaughter.

The methylation pattern in the *ACADVL* DMR was significantly associated with genetic potential for RFI in LD (CpG 11) and SM (CpG 7-8). Methylation for LRFI steers was higher in LD while HRFI steers displayed higher methylation in SM. *ACADVL* has been implicated in initiating mitochondrial β-oxidation of fatty acids and stimulating glucose synthesis (Antunes et al., 2013; Aoyama et al., 1995). It is expressed in tissues such as the liver, heart, and skeletal muscle with a high metabolic rate. A study by (Herault et al. (2014) showed that differential metabolism between SM and LD muscle resulted in differential *ACADVL* expression in adult swine and LRFI animals have been reported to possess a lower metabolic rate in previous studies (Zhang et al., 2017). In our correlation analysis, methylation in CpG 11 in LD muscle, which was significantly associated with RFI showed a positive correlation ($r = 0.45$, $p = 0.04$) with *ACADVL* expression and although expression analysis was not significant for the effect of RFI, the HRFI expression estimates were higher compared to LRFI group in LD muscle. There was neither a correlation between the significant CpG 7-8 and gene expression in SM, nor a significant association of RFI with gene expression in this tissue. Therefore, selection for RFI could result in

a greater impact on muscle glucose homeostasis in LD compared to SM and potentially emphasize tissue differential long-term response to energy consumption.

The *GRB10* gene interacts and binds with *IGF1R* and insulin receptor (*IR*) to regulate nutrient demand and glucose homeostasis postnatally, and influence the deposition of muscle fat (Cowley et al., 2014; Smith et al., 2007). Genetic potential for RFI was associated with *GRB10* methylation in SM and LV where LRFI steer progeny had higher methylation compared to HRFI counterparts in both tissues. The higher methylation response in LRFI groups in SM and LV could be a result of potential lower energy intake by the group during postnatal periods. Both SM and LV tissues are chiefly involved in metabolic activities during postnatal life. Therefore, *GRB10* could potential be a regulator of both muscle and liver metabolism due to RFI.

IGFN1 is a z-band-associated sarcomeric protein that interacts with kyphoscoliosis peptidase (KY) and eukaryotic translation elongation factor 1A (eEF1A) protein (Blanco et al., 2001; Mansilla et al., 2008). KY and eEF1A are known to play a role in muscle maintenance and muscle atrophic conditions respectively (Blanco et al., 2001; Mansilla et al., 2008). Methylation CpG 6 in HRFI steers progeny was significantly higher in SM muscles in this study. Additionally, our expression analysis showed a greater mRNA expression in LRFI groups in both LD and SM muscles. Currently, the direct function of *IGFN1* in the muscle remains to be elucidated with available literature associating its expression with muscle-associated disease conditions (Cracknell, 2019; Li et al., 2017). Higher methylation in the HRFI group in SM, accompanied with lower expression, may indicate that methylation is a negative inhibitor of expression in this tissue, and might be consequential in terms of metabolic functions with respect to RFI. As well, higher mRNA expression in the LRFI group in both muscles could be an indication of a difference in

muscle maintenance between the two RFI groups in this study. Further interrogation would be required to properly associate these findings to postnatal performance traits.

An essential metabolic pathway involved in muscle development is the phosphoinositide-3 kinase (PI3K) signaling pathway via its regulation of the metabolic roles of insulin (Petersen & Shulman, 2018). The *PDPK1* gene activates downstream kinases involved in the PI3K pathway promoting protein accretion and consequently muscle growth (Barile et al., 2012). HRFI progeny showed significantly higher *PDPK1* methylation in LV tissues. The selection for feed efficiency has revealed that there is a possible impact on visceral organs in ruminants including liver (Zhang et al., 2017). Basarab et al. (2003) reported that LRFI cattle have relatively lower liver weights compared to HRFI cattle. Additionally, in the study by Devos et al. (2021), the authors revealed that at slaughter, methylation in liver was mainly associated with RFI in the DNA fragments they investigated. Expression analysis showed no significant influence of RFI on *PDPK1* expression in the current study. Therefore, it could be that the methylation we have measured doesn't influence expression of *PDPK1* with respect to RFI classification, or that the physiological conditions we measured expression of *PDPK1* under were not conducive to show this relationship. *PDPK1* might be a potential postnatal methylation marker related to RFI in cattle, but more specific research needs to be conducted.

3.4.5. Influence of diet and RFI interaction on gene expression

Selection for RFI in cattle influences traits such as feeding behaviour and nutrient utilization even during gestation (Hafla et al., 2013). As a result, RFI potential could interact with diet availability and consequently influence DNA methylation and/or gene expression. Mitochondria-associated membranes (MAMs) are membranes that represent contact sites that the endoplasmic reticulum forms with mitochondria within individual cells (Rieusset, 2018). The activities of MAMs have

been shown to regulate metabolic processes such as energy homeostasis (Liesa & Shirihai, 2013). The *PASC2* gene encodes a protein that regulates the activities of MAM (Myhill et al., 2008). The expression of *PACS2* is significantly influenced by prenatal diet and RFI interaction in SM muscle in this study. The current study did not conduct methylation analysis of *PACS2* and hence, we could not determine whether methylation may, or may not influenced its transcriptional activities. The Ldiet-LRFI steer groups had significantly lower expression levels of *PACS2* in SM than the other steer groups. Decreased expression of *PACS2* impairs the MAMs, induces endoplasmic reticulum (ER) stress, and accelerates cellular catabolic activities (Hamasaki et al., 2013). Studies in other animal species reported that maternal nutrition during gestation could affect fetal MAM formation as well as a down-regulation of *PACS2* and potentially affect fetal metabolism (Zhao et al., 2017). It could be that prenatal feed restriction and the potential for less energy consumption during postnatal stages might have impacted SM metabolism hence the lower *PACS2* expression in the Ldiet-LRFI sub-group. Therefore, we speculate that lower expression in Ldiet-LRFI associated with prenatal feed restriction and genetic potential for RFI might be a sign of potential metabolic and cellular stress to the progeny.

MEF2A is a member of the MEF2 family of transcription factors that interact with myogenic regulatory factors to influence skeletal muscle development by activating muscle-specific genes (Molkentin & Olson, 1996). *MEF2A* positively regulates skeletal muscle differentiation and its polymorphism in cattle has been associated with body growth (Chen et al., 2010; Juszczuk-Kubiak et al., 2012; Wang et al., 2018). In the current study, methylation analysis of *MEF2A* could not be conducted, hence, the discussion of its significance is centered on the gene expression analysis. The expression of *MEF2A* in LV was influenced by the interaction of prenatal diet and genetic potential for RFI, and expression was upregulated in the Mdiet-HRFI animals

compared to other steer groups. The role of *MEF2A* in regulating glucose metabolism has mainly been elucidated in liver (Knight et al., 2003; Maruta & Yamashita, 2020; Wang et al., 2004). Although *MEF2A* expression is predominantly involved in skeletal muscle development, there was no influence of prenatal diet, RFI, and/or their interaction in either LD or SM muscle in this study. A study by Foroutan et al. (2021) reported contradictory results to our study, the authors found significant associations of *MEF2A* expression to the genetic potential for RFI in SM and LV, and also observed an influence of prenatal diet and RFI interaction on *MEF2A* expression in *Longissimus thoracis* (LT). The expression was higher in LV tissue of bulls with the genetic potential for LRFI as compared to HRFI in their study, opposite to the results of our current observations. The contrasting results in our study to those of Foroutan et al. (2021) could be due to differences in cattle-type (bulls vs steers) used in both studies.

3.4.6. Methylation differences between bulls and steers due to prenatal diet

Comparing the semen methylation results (chapter 2) to those from muscle and liver in the current study, we observed similar associations of prenatal diet with differences in methylation for potential DMRs associated with genes *ALDH3B1*, *IGF2R*-WGBS and *INSL3*. SM muscle displayed significant differences for Mdiet verses Ldiet steers for at least one CpG in all three DMRs. In LV, CpG (18) within *ALDH3B1* was also significantly different between prenatal diet treatments, where Mdiet progeny displayed higher methylation levels. In the bull study, Mdiet groups were highly methylated in all the DMRs compared to the Ldiet group. In this study, *IGF2R*-WGBS and *INSL3* methylation were in a similar direction to that of the bull study (higher methylation in the Mdiet group) in SM. However, *ALDH3B1* methylation levels were higher in Ldiet steer progenies in SM muscle. *ALDH3B1* and *INSL3* are both functionally related to testicular development and spermatogenesis, and their roles in muscle development and

metabolism remain unclear. Hence, the significant association of their methylation to gestational maternal diet in the current study potentially indicates that these DMRs might be prenatal epigenetic markers and could be subject to further investigations.

3.5. Conclusion

In conclusion, results from this study revealed some variations in the raw data counts from EpiTYPER methylation analysis within the same tissues. These findings indicate a potential technical source of variation in the technology. However, the differential methylation and gene expression patterns in response to prenatal feed restriction between the tissues suggests that the results from the current study do reflect the biological and functional differences that exist between these tissues and that gives confidence in the results as well as the methodology employed for this study. Prenatal feed restriction during early to mid-gestation had a greater influence than genetic selection for RFI on the methylation of potential DMRs associated with metabolic regulatory genes in SM muscle at slaughter, perhaps because of its greater involvement in postnatal metabolism and growth processes. Additionally, the results also indicate a tissue-specific response to either maternal diet and/or genetic potential for RFI. The selection for higher feed efficiency in LRFI cattle resulted in a higher methylation level of several DNA fragments analyzed compared to HRFI, particularly in LD and LV tissues. Hence, at slaughter, RFI could be a greater epigenetic effector relative to gestational feeding regime. Furthermore, we observed some similar methylation patterns as a result of prenatal diet in DMRs in our semen methylation study (chapter 2) and tissues in the current study. Therefore, these DMRs could potentially be candidate markers for prenatal diet and possible future epigenetic studies. Our findings will contribute to the growing knowledge on the epigenetic modifications associated with gestational management practices (prenatal diet

and selection for feed efficiency) and possibly set the foundation for further studies on fetal programming to improve the genetic potential in beef cattle.

Chapter 4. General Conclusion

Studies have elucidated the impact of feed restriction at critical stages of gestation on several economically important traits in livestock including beef cattle. Emerging evidence into the molecular mechanism of this phenomenon suggests the possible involvement of epigenetic modification. The majority of this research evaluates this mechanistic involvement at the fetal stage while studies into long-term postnatal consequences are lacking. Therefore, the purpose of this thesis is to evaluate and further increase our understanding of the epigenetic mechanism (DNA methylation) and expression of genes regulating the influence of prenatal feed restriction and the genetic potential for RFI in cattle progeny at slaughter.

The first study was conducted in bull progeny to validate the DNA methylation associated with prenatal diet from a prior WGBS study using EpiTYPER technology and investigate the effect of prenatal feed restriction and/or genetic potential for RFI on DNA methylation in semen and gene expression in muscle, liver, and testis tissue. We first hypothesized that DMRs associated with prenatal diet in semen using EpiTYPER would be similar to that of the WGBS and prenatal feed restriction and RFI would result in differential semen methylation as well as gene expression at slaughter in muscle, liver, and testis. In this study, our comparative methylation analysis showed that prenatal diet had no significant influence on average methylation between dietary treatment (Mdiet v Ldiet) groups when analyzed with EpiTYPER. Although two out of the 10 analyzed DMRs showed a similar trend in methylation pattern i.e., higher methylation in the Ldiet bull group compared to the Mdiet group. While the EpiTYPER technology has been utilized in validating whole-genome methylation studies, there are potential factors including the principle of methylation quantification between the two technologies, and bioinformatic analysis that could result in variations in the results. Also, out of over 300 identified DMR-associated genes, we only

selected and analyzed 10 DMR-associated genes for the current study. Therefore, an extensive and comprehensive validation study would be required to further validate the WGBS methylation results. For the analysis of methylation of individual CpG sites and prenatal diet, we found a significant association between prenatal diet and methylation in DMRs-associated genes involved in testicular development and genomic imprinting. In cattle, semen methylation is essential in spermatogenesis and has well been related to male infertility (Carrell, 2012; Takeda et al., 2017). Verma et al. (2014) showed those bulls with varying semen quality and fertility had differential methylation patterns of genes involved in essential biological processes. Although we did not investigate the expression of these significant genes in semen that could further reveal possible transcriptional and biological consequences, these findings point to perhaps a potential reproductive methylation marker associated with maternal nutrition during gestation which could be inherited transgenerationally by subsequent progenies.

Gene expression analysis for LD and SM tissues showed that Ldiet bull progeny had higher expressions of *PDPK1* gene. *PDPK1* is an activator gene involved in the PI3K pathway, which promotes protein synthesis and growth. In a study involving the same bull groups, at 10 to 16 months of age, the restricted bulls were reported to exhibit a faster growth rate compared to their unrestricted counterparts (Johnson et al., 2019). Furthermore, the Ldiet groups had higher expression levels of the *THRB* in SM muscle, a gene whose expression is reported to increase metabolic processes in muscle (Bloise et al., 2018; Liu et al., 2012). The result from the current study, therefore, indicates that restricting maternal diet during periods of skeletal muscle formation might result in an adaptation in support of increasing metabolism and possibly in support of growth.

The second study evaluated the impact of prenatal diet and genetic potential for RFI on DNA methylation and gene expression in LD, SM, and LV tissues in steer progeny. We hypothesized that restricting maternal feed during early to mid-gestation and/or RFI would result in differential methylation and expression of genes regulating muscle development at slaughter. The results from the methylation analysis revealed a differential response to prenatal diet between the tissues. SM muscle methylation had a higher response to dietary restriction at slaughter suggesting a potentially greater long-term response to influences of gestational feeding. Due to the prioritization of nutrients for essential tissues such as the liver, heart, and brain in situations of maternal malnutrition during gestation, nutrient supply to tissues including skeletal muscle is minimized, possibly increasing its susceptibility to prenatal diet (Bauman et al., 1982). Additionally, myofiber and functional differences between muscle types might have also influenced the variation in response to gestational diet. Relative to prenatal diet, we observed that genetic potential for RFI had a greater influence on both methylation and expression of genes involved in metabolism and growth-related functions. The relatively little influence of prenatal diet in all tissues compared to RFI may be attributed to the timing of maternal feed restriction during gestation in our study. Studies have shown that there is the possibility of either compensatory growth or metabolic adaptation in support of growth in some cases of prenatal malnutrition, especially when it occurs during early gestation (Hornick et al., 2000). The majority of livestock research on fetal programming is conducted during late gestational periods and hence most effects reported are when feed is restricted during late gestation, a period of extensive fetal growth. Therefore, at slaughter, the impact of prenatal diet on DNA methylation and/or gene expression may have been compensated for or minimized while RFI had a greater influence on steers at slaughter. Finally, we observed a similar methylation trend in *IGF2R* and *INSL3* DMRs

associated with prenatal diet in both semen and muscle in this study. In both cases, Mdiet groups displayed higher methylation levels. This implies that DMR-associated genes might be epigenetic markers regulated by maternal nutrition during gestation. The findings from this research provide insight into the potential molecular mechanisms underlying fetal programming and contribute to growing knowledge on the possible epigenetic regulation in beef cattle, and potentially set the basis for further research studies.

Studies have shown altered methylation pattern in fetuses and neonatal progeny due to maternal nutrition (Downing et al., 2011; Lan et al., 2013; Lillycrop et al., 2008). Also, DNA methylation has been to be a stable epigenetic modification and regulates transcriptional activities of gene (Hermann et al., 2004). Hence in this research we hypothesized that prenatal feed restriction during gestation would result in a long-term modification of DNA methylation in cattle progeny. Our results however show an inconsistent influence of prenatal diet on DNA methylation in muscle and liver tissues at slaughter. Furthermore, RFI had greater influence on methylation compared to prenatal diet. To the best of our knowledge, no other study evaluated the effect of prenatal diet on DNA methylation and gene expression at slaughter. Therefore, we conclude that prenatal diet during early to mid gestation may have little or no impact on methylation in muscle and liver of progeny in beef cattle in postnatal periods of life. The greater impact of RFI also suggests that other traits or biological processes may have greater influence on molecular modification compared to gestational maternal diet. Therefore, further investigation on the long-term impact of prenatal diet during postnatal periods on DNA methylation and gene expression on a larger sample of individuals, tissues and DMRs to establish the association between gestational maternal diet and epigenetic modifications.

4.1. Limitations of study

The current study had some limitations that might have influenced the results observed. Firstly, the smaller sample size for each treatment used for the study may have impeded our ability to detect consistent significant and/or non-significant results. Additionally, the extensive processing of both methylation and gene expression data resulted in the deletion of multiple methylation data for some individual samples and/or deletion of the entire individual from statistical analysis. In the process, we may have excluded crucial data points, which could have affected the results observed. Some other limitations of the research were that for study 1, only a few DMR-associated genes were selected for validation of the WGBS study (352 vs 15 DMR-associated genes). This is a relatively small sample size employed to the validation study and might not be the true representation of the methylation trend that were observed in the WGBS semen methylation study. Additionally, bull muscle, liver and testis DNA methylation were not analyzed as there was no longer tissue samples left for these animals. Hence, in bull progeny a simultaneous investigation of DNA methylation and gene expression could not be performed. Testing both DNA methylation and gene expression at the same time in our bull progeny would give us more confidence that we could connect prenatal nutrition to DNA methylation, then to changes in biological function.

4.2. Future directions

The current study observed some significant potential molecular modification associated with either prenatal diet and/or genetic potential for RFI. However, some knowledge gaps that arose from the study that would require further interrogation include:

- Assess the repeatability of EpiTYPER technology identification and quantification of DNA methylation within the same tissue. In the current study, there were some variations in the methylation data of *IGF2R*-DMR2 between the current study and

those from Devos et al. (2021). Conducting a repeatability study would determine whether the results from the technology are indeed reliable for DNA methylation identification and/or validating whole genome methylation studies.

- Evaluate the influence of prenatal diet on DNA methylation of *PDPK1* gene, whose expressions were significantly associated with prenatal diet. The gene is a growth-promoting gene, and it was highly expressed in Ldiet bulls in the current study. Therefore, assessing the epigenetic modification possibly regulating its expression would assist in determining whether *PDPK1* is an epigenetic marker regulated by maternal nutrition.

- Determine the possible transgenerational passage of DMR-associated genes involved in male reproductive development (*ALDH3B1* and *INSL3*) and genomic imprinting (*IGF2R* and *GRB10*) that were significantly associated with prenatal diet in offspring of the bull used in this study. This would assist in determining the possibilities of transgenerational epigenetic influence on fertility and/or progeny development during gestation.

- Evaluate the influence of prenatal diet on DNA methylation in *INSL3* and *IGF2R*-WGBS in other tissues. The methylation of these DMRs was significant with prenatal diet and displayed similar patterns in both semen and SM muscle. Therefore, if similar results are found in other tissues regulating metabolism, it could establish the DMRs are stable nutritional epigenetic markers.

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