

ABSTRACT

Title of Dissertation: INTEGRATIVE GENOMIC, EPIGENETIC AND METABOLOMIC CHARACTERIZATION OF BEEF FROM GRASS-FED ANGUS STEERS

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Beef constitutes a main component of the American diet and still represent the principal source of protein in many parts of the world. Currently, the meat market is experiencing an important transformation; consumers are increasingly switching from consuming traditional beef to grass-fed beef. People recognized products obtained from grass-fed animals as more natural and healthy. However, the true variations between these two production systems regarding various aspects remain unclear. This dissertation provides information from closely genetically related animals, in order to decrease confounding factors, to explain several confused divergences between grain-fed and grass-fed beef. First, we examined the growth curve, important economic

traits and quality carcass characteristics over four consecutive years in grain-fed and grass-fed animals, generating valuable information for management decisions and economic evaluation for grass-fed cattle operations. Second, we performed the first integrated transcriptomic and metabolomic analysis in grass-fed beef, detecting alterations in glucose metabolism, divergences in free fatty acids and carnitine conjugated lipid levels, and altered β -oxidation. Results suggest that grass finished beef could possibly benefit consumer health from having lower total fat content and better lipid profile than grain-fed beef. Regarding animal welfare, grass-fed animals may experience less stress than grain-fed individuals as well. Finally, we contrasted the genome-wide DNA methylation of grass-fed beef against grain-fed beef using the methyl-CpG binding domain sequencing (MBD-Seq) method, identifying 60 differentially methylated regions (DMRs). Most of DMRs were located inside or upstream of genes and displayed increased levels of methylation in grass-fed individuals, implying a global DNA methylation increment in this group. Interestingly, chromosome 14, which has been associated with large effects on ADG, marbling, back fat, ribeye area and hot carcass weight in beef cattle, allocated the largest number of DMRs (12/60). The pathway analysis identified skeletal and muscular system as the preeminent physiological system and function, and recognized carbohydrates metabolism, lipid metabolism and tissue morphology among the highest ranked networks. Therefore, although we recognize some limitations and assume that additional examination is still required, this project provides the first integrative genomic, epigenetic and metabolomics characterization of beef produced under grass-fed regimen.

INTEGRATIVE GENOMIC, EPIGENETIC AND METABOLOMIC
CHARACTERIZATION OF BEEF FROM GRASS-FED ANGUS STEERS

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Preface

The chapters of this manuscript have appeared in peer-reviewed journals or are presently in preparation for submission. I highly appreciate the collaboration of each of my coauthors in these studies; it greatly improved the quality of research and writing of manuscripts. At the time of writing, Chapter 3 has been published in a recognized peer-reviewed journal, while Chapters 2 and 4 are still in process.

Chapter 2:

Carrillo JA, He Y, Li Y, Liu J, Song J: **Growth curve, important economic traits and beef quality characteristics of Grass-fed Angus steers. In preparation.**

We would like to acknowledge the Wye Angus Farm staff, especially Mr. Edward C. Draper and Ms. Lisa M. Yoash for their collaboration through all these years.

Chapter 3:

Carrillo JA, He Y, Li Y, Liu J, Erdman RA, Sonstegard TS, Song J: **Integrated metabolomic and transcriptome analyses reveal finishing forage affects metabolic pathways related to beef quality and animal welfare.** *Sci Rep* 2016, **6**:25948.

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Chapter 4:

Carrillo JA, He Y, Li Y, Liu J, Song J: **Methylome analysis of beef from grass-fed Angus steers reveals enrichment of differentially methylated regions in chromosome 14. In preparation.**

Dedication

To my beloved wife “Maripaz” and my two adorable daughters “Montserrat” and
“Macarena” whose unconditional encouragement and support made this
accomplishment possible. You have been my inspiration.

To my parents, the reason of what I become today. Thanks for your unconditional
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List of Abbreviations

ADG: Average daily gain

BCTRC: Boneless, closely trimmed retails cuts

BF: Back fat

BSE: Bovine Spongiform Encephalopathy

BSTFA: Bistrimethyl-silyl-trifluoroacetamide

CLA: Conjugated linoleic acid

DHA: Docosaheptaenoic acid

DMR: Differentially methylated region

Dress: Dressing percentage

EDTA: Ethylenediamine tetraacetic acid

EPA: Eicosapentaenoic acid

FAO: Food and Agriculture Organization of the United Nations

Grade: Quality grade

HCW: Hot carcass weight

HPA: Hypothalamic-pituitary-adrenal

IMF: Intramuscular fat

IPA: Ingenuity Pathway Analysis

LW: Live weight

MACS: Model Based Analysis of ChIP-Seq

MBD-Seq: Methyl-CpG binding domain sequencing

MBD: Methyl binding domain

MBM: Meat and Bone Meal

REA: Ribeye area

REA/CWT: Ribeye area/carcass weight ratio

SAM: Sympathetic-adrenal-medullary

SIRA: Stable isotope ratio analysis

TF: Transcription factor

TMAO: Trimethylamine-N-oxide

U.S.: United States of America

USDA: United States Department of Agriculture

USHHS: United States Human and Health Service

WBSF: Warner–Bratzler shear force

Yield: Yield grade

1. Literature Review

Introduction

Beef represents a main diet component in the USA and constitutes the major source of protein in many other countries [1]. It is well known that beyond amino acids, beef provides other nutrients such as lipids, vitamins and minerals, which are essential for normal growth and development. These factors participate in maintaining the homeostasis of an organism; hence their disequilibrium causes not exclusively nutritional diseases; genomic instability has been linked to mineral deficiency as well [2]. Food delivers nutrients and energy, however its acquisition also demands energy expenditure. In the modern world the relationships among food, energy and health are very complex and sensitive to exogenous factors, thus the raising of serious concerns about high-level production systems. Climate change, undernourishment and over-nutrition are factors associated with serious health conditions. Occasionally, the symptoms appear later and aggravate even further in individuals that suffered restrictions or excess of nutrients during early development or childhood [3].

Production of meat is a biological process that requires the interaction of the animal with the immediate environment; normally individuals obtain most of the necessary nutrients from the food. In conventional production models, nutrients are carefully considered and the ration is adjusted according to the requirements, providing a roughly perfect balanced diet. Contrarily, grass-fed animals extract almost all nutrients from the grass, which usually provides insufficient amount of necessary elements for an efficient production. The quality of grass depends on many factors,

such as soil, plant developmental stage, climate and region that would influence the development and growth of muscle as well.

Herbivorous face several nutritional challenges; plant matter is rich in fiber with relatively low protein and fat, and the majority of nutrients are contained within hard cell walls. Mammals lack the enzymes required for the breakdown of plant cell walls, therefore they only can access part of the nutrients contained in plants. Intriguingly, many of the microorganisms that inhabit the rumen can synthesize cellulytic enzymes, providing the ruminants the capability to digest plant-based material through microbial fermentation of the ingesta that occurs in the rumen. Consequently, a symbiotic relationship exists between microbes and ruminants; the animal provides nutriment and living space for the microbes and receives absorbable fermentation by-products, including fatty acids and sugars.

The ruminant micro-flora includes billions of protozoa, bacteria and fungi, which act anaerobically and many of them are difficult or impossible to culture experimentally. The rumen community dynamically adjusts to the diet therefore the type and proportion of microorganisms will reflect the substrate that should be degraded. After the microbial breakdown occurs, the animal continues the digestion through a series of enzymatic reactions. Many of these biochemical processes are highly but not exclusively genetically regulated. Epigenetic modifications, and especially DNA methylation, play major roles in many conditions and diseases, such as cancer [4]. For instance, mice that have been exposed to a lifelong Western-style diet, exhibited altered DNA methylation in colon [5]. Studies suggest that certain diets may influence genomic and gene-specific DNA methylation in systemic and target tissues,

altering genomic stability and transcription of different genes [6]. Additionally, another report suggests a relationship between DNA methylation and physical activity and energy balance [7], which totally differ in grass and grain fed styles. Thus, gene expression and DNA methylation profiles could be indicators of diet styles in cattle and may be used as biomarkers to trace the origin of beef as well.

Current Meat Consumption

According to a recent study that considers agricultural supply and dietary intake data from the USDA, the USHHS and FAO for estimation of meat and protein consumption, the US citizens consume 125.9 to 166.5 g of meat and 175.2 to 209.4 g-eq from the USDA protein foods group daily [8]. Similar results are obtained from employing the USDA database alone, domestic red meat consumption was 62.8 kg per person for the year of 2015; of those 35 kg were beef.

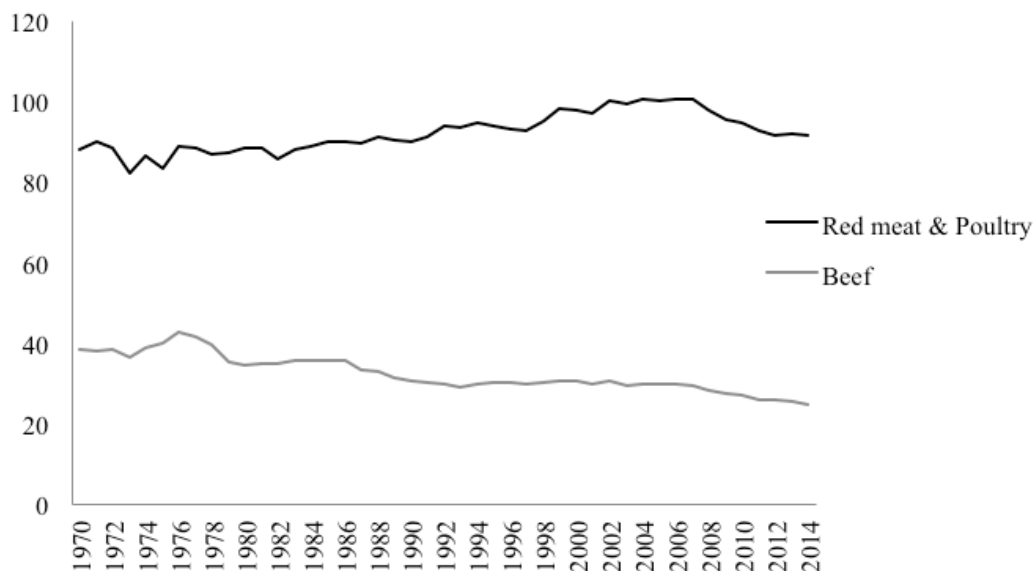


Figure 1.1 Total meat per capita disappearance, retail weight (kg) from 1970 to 2014.

Despite the rise of the global demand of meat, the US consumption of red meat has decreased in recent years [9]. Figure 1.1 shows that the US total meat consumption has been steadily declining since 2006. Meat constitutes the main source of protein, however red and processed meats comprise the majority of total meat intake. Estimation from nutrient supply data, indicates that total meat represents 43.1 % of the whole protein available in the US food supply [8]. More economically developed countries normally consume high level of meat, however consumption below 10 kg per capita, is considered deficient and still common in many regions, particularly developing countries [10].

Beef and other animal products

The US is the largest producer of beef worldwide and correspondingly constitutes the leading fed-cattle industry in the world, with the 4th biggest cattle inventory behind India, Brazil and China, and followed by the European Union, Argentina and Australia. The US herd comprises 89,800,000 heads, constituting 9.31% of the total bovine world population [11]. The domestic industry produces predominantly high quality grain-fed beef for internal and export use. In 2014, the US produced in total 11,016.7 million kg of beef and exported 11% the production, generating \$6.52 billion in revenue [12]. Interestingly, the US also imports lower-value beef intended for processing to cover the high local demand of processed meat products.

Historical records obtained from the USDA demonstrates that imported beef and initial stock for each year remain moderately stable, however the domestic beef production peaked in 2002 reaching 27,192 million pounds for a total beef production of 31,016 lb. in the same year (Figure 1.2).

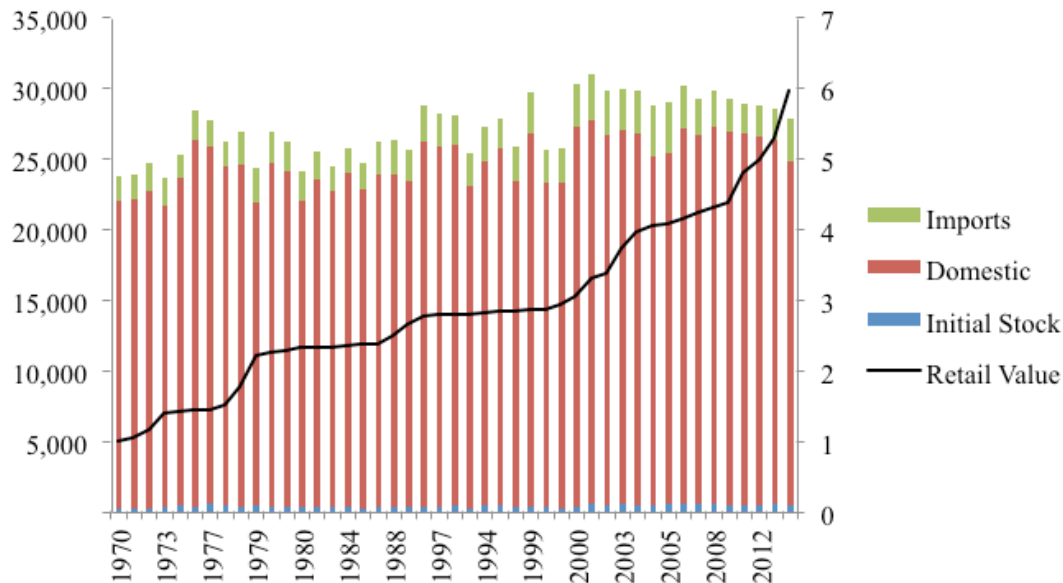


Figure 1.2 Historical Total US Beef Production and Retail Value from 1970 to 2014
Data includes Initial Stock, Imports and Domestic production (million pounds); Retail Value (\$ per pound)

Although the US is the world's largest producer of beef, it also imports more beef than any other country. The imports mainly comprise lower-value grass-fed lean product that is destined for processing, mainly into ground beef. In 2014 the imports surpassed slightly 10% of the total beef supply. Figure 1.3 shows historical data from 1989; revealing that imported beef reached the maximum level of 3,679 million pounds in 2004. Interestingly, from 2010 to 2013 the US was a net exporter of beef. In 2014 imports exceeded exports as a result of the domestic production decline, which was triggered by severe drought in the South and high price of grains that caused producers to diminish their herds between 2009 and 2014 [11]. Although the total beef production has decreased in the last years, the beef retail price increased vertiginously, demonstrating the preference of the market; people consume less but high-quality beef.

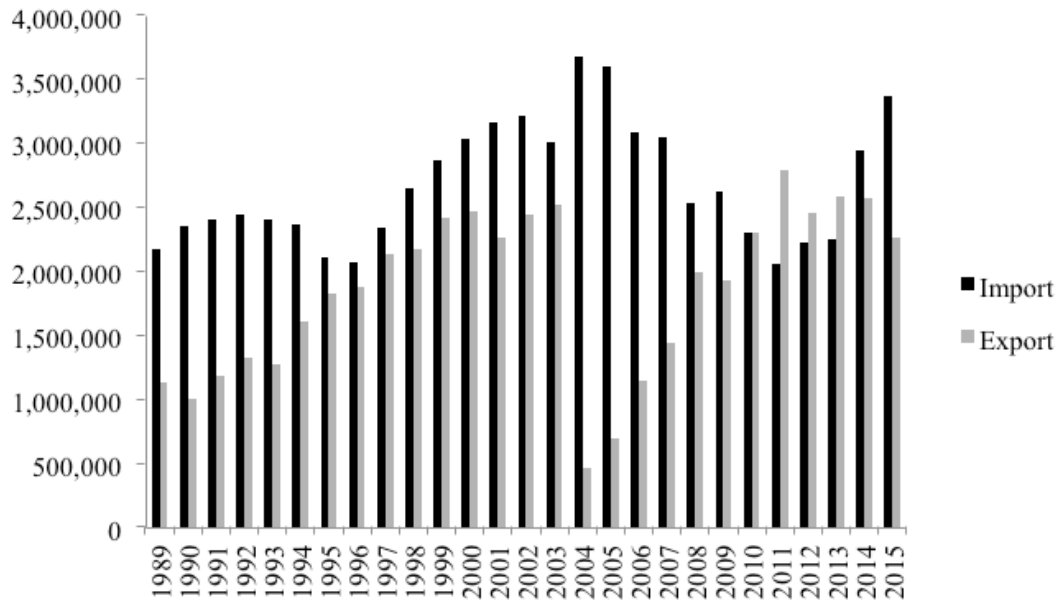
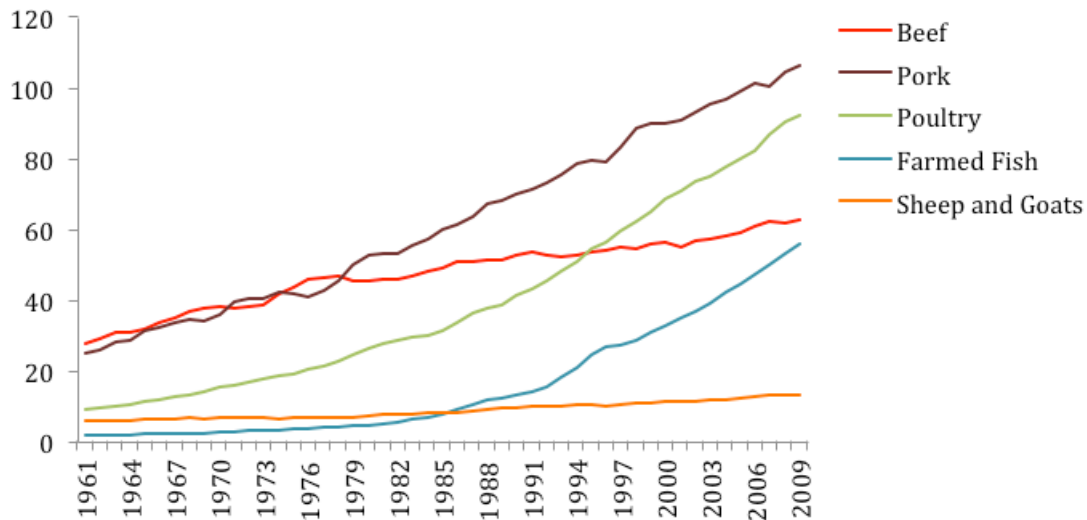


Figure 1.3 US import and export historical data.
 *y axis: Carcass weight x 1000 pounds

Global meat consumption is continuously increasing congruently with the world population growth. Current projections estimates that in 2050 the world population of humans will be 9.6 billion, thus based on current growth trend 9 billion could be reached by the year of 2040 [13]. Figure 1.4 illustrates the global animal protein production considering five types of meat; poultry and farmed fish are the two groups that have the highest increment, followed by pork and then beef. These trends coincide with the preference for meat in the highest population growth rate regions of the world, like Asia and part of Africa. Interestingly, the global beef production increased 2.27 fold in 48 years, from 27.7 million tons in 1961 to 62.8 million tons in 2009. Relative similar, 2.19 fold change increment has been observed for the sheep and goats group, which raised from 6 to 13.2 million tons in the same period of time. Annual growth of meat production in developing countries to 2030 is projected at 2.4 percent [14].



Source: Worldwatch, FAO

Figure 1.4 World animal protein production from 1961 to 2009

*The y-axis expresses million tons

Trend of grass-fed beef consumption

In the last 15 years, the US grass-fed industry has undergone an exponential expansion and current projections predict that this trend will continue, offering a good opportunity for producers. Retail sales of nationally produced grass-fed beef exceeded \$400 million in 2013, compared to less than \$5 million in 1998, when only around 100 producers were really involved in grass-fed beef production [15]. Another source, mentions that despite the generally decreasing consumption of red meat, beef from alternative production systems (natural, organic and grass/forage-fed) comprises 3% of the US market and has expanded around 20% yearly in recent years [12].

In 2012, a research that evaluates consumer purchase habits reported that 43% of all surveyed customers had purchased “Grass-fed” or “Locally raised” beef within the past year [16]. Interestingly, the demand for grass-fed beef has increased 25-30% annually in the last decade. Certainly this progression will not persist forever,

however recent market research suggests that this rate will not decelerate considerably in the near future, and predicts that grass-fed beef will become more than a niche product, comprising 30 to 40% of the total beef market in 10 years [16].

Causes of current trend

The grass-fed movement began in the mid-90s, as a consumer-driven market, without any promotion to publicly support or expand the idea and having limited demand in only small parts of the country. The true consumer concern started in 2003 when the Bovine Spongiform Encephalopathy (BSE) or commonly known as “Mad Cow Disease” hit hard the US beef industry. The BSE comprises a progressive neurological disease of cattle, which symptoms are similar to a disorder observed in sheep, known as scrapie. BSE results from infection of an extremely unusual infection agent. Currently, the most acceptable theory establishes that the agent constitutes an altered form of a normal cellular protein, called prion [17]. The origin of the disease remains uncertain, however it may result from feeding cattle with scrapie containing meat and bone meal (MBM) or from consuming MBM derived from a cow or other animal that developed the disease.

Therefore, people became concerned about the diet that these beef cattle received during their lives. Grass-fed animals obtain their nutrient directly from pastures thus it is unlikely for the animals to acquire BSE in this type of feeding system. Then, several customers turned to grass-fed beef based on the perception that it was safer than grain-fed beef. Consequently, distributors and retailers that have ignored grass-fed beef for a long time were suddenly interested, facilitating the expansion of this tendency.

A research focused in food attributes found that consumers rated “taste”: 8.7, “hormone-antibiotic free”: 7.9, “environmental impact”: 7.5 and “grass-fed”: 7.2, on a scale from 1 to 10 [16]. For instance, grass-fed beef normally complies with all of the previous concerns, having a positive environmental impact and avoiding the use of hormones and antibiotics, while providing flavorful and juicy beef. Lately, the word “natural” has been heavily used in the culinary art and approximately one third of customers mention that they are eating “natural” food more frequently [15]. Again, grass-fed beef advocates say that beef from animals on a regimen free of hormones, steroids and antibiotics is fully “natural” and provides a rich and robust taste.

People concerns about beef

Worldwide and particularly in developed countries, the market preference is changing from consuming grain-fed to grass-fed produced beef [18]. Public perception is that grass-fed animals are more natural, healthy and environmental friendly. Although intuitively it is easy to agree with this idea, some product attributes, which have been defined by the market for years, could be difficult to achieve in grass-fed beef [19]. Consequently, some consumers are willing to switch to grass-fed beef but still they are seduced by the flavor of the grain-fed beef. Beside the inherent quality aspects of the final product (tenderness, juiciness, fat content, flavor) and price, main consumer concerns can be classified in three different groups: Human health, Animal welfare, and Environmental impact and Bioenergy.

Human Health

Many of red meat components, such as cholesterol and triglycerides could have negative health impact, particularly when they are excessively ingested [20, 21]. The intestinal microbiota converts carnitine (a compound of red meat) to a heart-damaging compound trimethylamine-N-oxide (TMAO), a metabolite linked to atherosclerosis and whose levels strongly suggest high cardiovascular disease risks [22]. Recently, World Health Organization (WHO) evaluated the carcinogenicity of the consumption of red meat and processed meat, and concluded that based on limited evidence red meat consumption causes cancer (mainly colorectal) in humans. However based on sufficient evidence processed meat was classified as carcinogenic to humans, and experts also concluded that each 50-gram portion of processed meat eaten daily increases the probability of intestinal neoplasia by 18 % [23].

Previous studies, based on meta-analysis of epidemiological observations also confirmed the association between processed meat intake and gastric cancer risk, and type-2 diabetes as well [24, 25]. The neoplasia cause is still unclear but the increase in nitrosamines has been pointed as possibly responsible. Greater red meat intake was strongly related to elevated risk of breast cancers that were estrogen and progesterone positive (ER+/PR+) but not for those receptors negative cases (ER-/PR-) [26]. Gene variants involved in iron metabolism [hemochromatosis (HFE) H63D and transferrin C2 (TfC2)] have been associated with the establishment of Alzheimer's disease, suggesting that brain accumulation of iron could correlate with development of neurodegenerative diseases [27]. Meat contains a lot of iron, which if consumed in large amounts, could raise the iron level in the brain and predispose for Alzheimer's

disease. The exact mechanism remains unknown but while iron accumulates the nerves myelin gets damaged, disrupting the normal neurological communication.

Industrial practices that are employed to improve revenue, such as the use of transglutaminase—an enzyme that catalyzes the formation of isopeptide bonds between proteins—to maintain the meat cut integrity, could also have an impact in human health [28]. Other researchers have criticized the methodology and the lack of consideration of factors employed in those studies, fueling the ongoing controversy regarding health implications of beef consumption.

A simplistic consideration is that humans and pre-hominids have been eating meat for millions of years and that human physiology has been adapted ever since [29]. The high incidence of diabetes 2 and obesity is relatively new thus is hard to associate meat ingestion—especially as a single factor—with these disorders. Blaming an ancient food for relatively new diseases makes absolutely no sense. Processed meats have been associated with higher incidence in coronary heart disease and diabetes, but not red meats [30].

Despite the possible consequences of red meat consumption cited previously and the ongoing controversy, beef still constitutes a main component in the American diet; a proof of this is that steaks remain as a highly desirable option in upscale menus and restaurants. It is well known that meat consumption correlates with macro-economic indicators [31]. Per capita consumption in some industrialized countries is high while in developing countries it remains below 10 kg/year and must be considered deficient, leading to under-nourishment and malnutrition. FAO estimates that more than 2 billion people suffer deficiencies in key vitamins and minerals, especially vitamin A,

iodine, zinc and iron [10]. These deficiencies appear in people that have restricted access to micronutrient-rich food, such as meat, fish, fruits and vegetables. Most people with deficiencies live in low-income countries and often present more than one insufficiency, worsening the problem and consequently the quality of life. For these individuals, especially women and children, highly nutritious foods such as beef are particularly valuable to fulfill their daily nutritional requirement.

In emerging countries, cattle are most of times raised naturally, consuming principally any type of grass. This situation presents several difficulties, such as parasites control, seasonal availability of forage, long period of termination, old animals and lack of uniformity of the final products, among others. However it also presents an opportunity to use natural pastures in a more effective manner to produce better and more amount of food in order to alleviate the shortage of nutrients in those populations.

Animal Welfare

Contemporary society considers animal welfare an ethical issue and assumes its responsibility for improving it, seriously. Logic dictates that animals raised in their natural habitats should be healthier and lively than individuals nurtured in artificial environments. This is particularly true for some of the traditional systems, which have been developed based on production efficiency and ignoring main principles of animal welfare. Nevertheless, world population is growing faster than ever before and to supply the food demand, new strategies should be created and implemented urgently. The controversy that involves animal welfare, environment conservation and sustainability is presently one of the greatest public interest that should be

resolved by employing technology and combined effort among different international organizations, government institutions and social groups.

Environmental Impact and Bioenergy

Regarding the environmental impact of animal agriculture, ruminants lately have been accused of being a major player in the green house effect, as a consequence of their methane emission and nitrous oxide production from manure applied to soils. Agricultural activity and particularly livestock production accounts for approximately a fifth of the total impact of the global greenhouse-gas emissions, hence contributing to climate change and its negative health implications, comprising the risk for food production in several regions of the planet [32]. Besides the methane production, grass-feeding systems require more extensive areas than the conventional methods to provide the necessary nutrients for beef production, promoting the deforestation of rainforests. The alternative use of land represents another problem for the grass-fed systems; the continuous increment of the cost of land limits the use of it as pastures. High quality soils generally are more productive as crops than as pastures.

Controversially, ruminants still represent the main source of nutrients for humans in many areas of the world and offer an alternative to obtain renewable energy from biomass instead of conventional fossil fuels [33]. Interestingly, although many microbial enzymes exist, only a few of them are currently used as biocatalyst. The discovery and characterization of novel enzymes should be facilitated by the availability of innovative metagenomic approaches and the large number of annotated metagenome sequence databases [34]. The biochemical decomposition of the lignocellulose biomass constitutes the key point in the biofuel research area.

Agriculturalists should produce the best possible products, using the resources efficiently with a minimal ecosystem effect. Thus, identification of microorganisms, genes and biological pathways related to the biochemical degradation of the cellulose is necessary for better understanding of this complex process that has diverse practical applications.

Problems and challenges of grass-beef production

Although most cattle consume forages through their lives, a distinction between grass-fed and grain-fed animals must be considered. Grass-finished animals have grazed only on pastures or other forages, and most importantly have been fattened only on grass or forages to achieve an adequate commercial final weight. Grass-fed operations require large amounts of forages, which normally implicate the use of extensive areas, demanding special expertise and solid management-operator skills.

The animal weight and carcass characteristics depend heavily on the type and quality of forage administered to the cattle. Thus, achievement of an efficient growth curve demands animals to have a year-round access to good-quality forage, something not easy to attain in many regions or under specific circumstances. The seasonal growth pattern and nutrient content variation of plants make the constant provision of high-quality forage a difficult and costly endeavor.

The infrastructure of grass-fed systems differs from the already established conventional operations, which involve high-density housing with elevated provision of grain in the diet. Temperature represents an important factor in beef production; during cold weather animals require more energy to maintain body functions first and then for growth and fat deposition. Contrarily, in warmer conditions, reduced feed

intake presents a problem in providing sufficient nutrients for efficient production of high quality beef.

Uniformity of the product comprises another challenge for grass-fed models; variation could be caused by difference in genetics, forage characteristics and management practices. Consequently, producers have decreased variation by using breeds with special characteristics and determined type of forage as well as paying careful attention to grazing and management practices. A study that compares carcass characteristics and palatability of beef in grass-fed versus grain fed Limousin-cross steers, concluded that ribeye roasts and ground beef from forage feeding cattle had slightly less beef flavor and more off-flavor than grain-fed beef [35]. Another report suggested that beef taste scores and flavor intensity were superior in loins from grain steers compared to rye grass fed steers [36]. Monoculture finishing has been linked to off-taste, thus diverse pastures should be employed for grass-fed systems. Accordingly, taste difference between grass-fed and grain-fed beef exists and is well documented.

Results from studies addressing tenderness of beef between grass- and grain-fed beef can be controversial however; results from comparing appearance are consistent. Most studies suggest no difference in tenderness of beef comparing both types of feeding systems [36, 37]. Regarding appearance, one study reported that grass-fed carcasses were lighter, coarser in lean texture and had more yellow fat than carcasses from grain finished individuals. Although yellow fat tastes better and is considered healthier, steaks from grass-fed animals were darker during retail display and were unacceptably dark after 5 days of display [37]. Another report mentioned that

yellowness of subcutaneous fat was lower in carcasses from steers feeding on grain, as well [36]. A review of nine papers published in 2003 concluded that grass-fed beef is lower in tenderness, flavor and acceptability ratings than grain-fed beef [38]. Considering retail price, high quality grass-fed beef is generally more expensive than grain-fed beef; grass-fed cattle need longer time, additional land and superior pastures to reach the market.

Status of beef study

Meat constitutes a main source of protein and energy for many species, and has contributed to the welfare of man for millions of years. Before, humans have relied on hunting of wild animals to obtain their meat provision. In the US, buffalo, deer, and elk herds constituted the principal meat supply for natives and early settlers. Later the declining number of wild animals and the introduction of different species (cattle, sheep and pigs) from Europe generated the development and expansion of domesticated herds for food supply. Since then, the efforts to improve animal production have been continuous and include different disciplines such as animal husbandry, agronomy, management and genetics. Around 1800, 90% of Americans were on farms producing food and clothes for their own use or for selling, compared to the current 2% of the population constituted by farmers.

Significant studies

In live animals, muscle performs different biological functions; from generating movement to regulating body temperature therefore, the study of muscle includes several scientific fields like physiology, anatomy, biochemistry, physics and genetics

among others. However this project focuses mainly in the composition and quality of beef, and how these traits relate to genetics and epigenetics. Particularly we are interested in the overall quality of beef obtained from grass-fed animals and the “real” differences between grass and grain fed beef.

Many studies conducted in different countries and environments have mentioned the composition of beef regarding the type of feeding systems [38-40]. Most of the conclusions refer to fat and protein content of beef. Grass-fed steers were lower for monounsaturated fat and have greater n-3 fat and less n-6 fat than conventional steers, however the Warner-Bratzler shear force was not different from organic beef but tends to be greater than conventional beef [41]. The same study performed in 2014, suggests the existence of a potential market for grass-fed beef in the US but also points out that quality and consistency of grass-fed beef should be improved [41]. A Japanese study that evaluates feeding systems regarding cost, palatability and environmental effects concluded that high hay and grass-only-fed systems could reduce feed cost by 60 and 78% respectively. In the palatability test, 62.5% considered grass-only-fed acceptable delicious and 15% extremely delicious. Grass-only fed showed the lowest environmental effect, producing 2.04 tons of eq compared to 9.32 for the high concentrated group [42].

People consider grass-fed beef an alternative for eating more naturally, however the need for general improvement motivates research in different beef aspects. Epidemiological studies, looking for *E. coli* shedding and contamination rates in grass-fed beef, have been performed [43, 44]. Grass-fed cows were concurrently shedding *E. coli* O157; occurrence of rainfall, feeding silage and lactating were found

to be predictors of shedding. The study also demonstrated moving animals to a new paddock had a negative effect on shedding and that high shedding events are rare in grass-fed beef herds [43]. Regarding contamination rates, coliform concentration did not vary between grass-fed and conventional beef. Furthermore, the data indicated that there are no clear food safety differences between these two types of production systems [44]. Another important area of research comprises the identification of biomarkers for grass-fed beef. For consumers, it is critical to determine accurately the quality and origin of the beef. Regarding meat tenderness, DJ-1 and Peroxiredoxin 6 were consistently correlated with Hsp20 and μ -calpain respectively; in *longissimus thoracis* and *semitendinosus* muscle in three European breeds (Aberdeen Angus, Limousin, Blond d'Aquitaine). Additionally, Hsp70-Grp75 appears to be relevant for several pathways involved in meat tenderness [45]. Another report suggests Hsp70-1B to be a potential biomarker of low tenderness in beef [46]. It is well known that IMF is associated with meat quality; consequently pathway and network analyses pointed three transcription factors (TF) (*PPARGC1A*, *HNF4G*, and *FOXP3*) as key regulators of carcass IMF.

PPARGC1A is a transcription factor that activates distinct hormone receptors and other TFs implicated in the regulation of white adipocyte differentiation. Additionally, *PPARGC1A*, a main regulator of muscle fiber composition, mitochondrial content and energy homeostasis has demonstrated co-association with 108 genes; many of them have been linked to cellular assembly and organization processes, and tissue morphology and skeletal muscle development as well [47]. *HNF4G*, a nuclear hormone receptor expressed in various tissues (kidney, pancreas,

small and large intestines, testis) in mice has been predicted as critical for IMF regulation in bovines[48]. *FOXP3* plays a main role in the regulation of the immune system [49] however, it also has been reported as a regulator of genes that were activated during adipogenesis in humans [50]. These 3 TF act cooperatively with other interacting genes (*CAPN6*, *STC2*, *MAP2K4*, *EYAI*, *COPS5*, *XKR4*, *NR2E1*, *TOX*, *ATF1*, *ASPH*, *TGSI*, and *TTPA*) as determiners of carcass and beef quality traits [51].

For feeding type confirmation, several approaches have been proposed. The analysis of perirenal and subcutaneous fat was considered for volatile compounds identification, resulting more consistent the first type of fat tissue [52]. Higher levels of 2,3-octanedione were identified in carcasses from pasture animals, confirming the ability of this molecule to trace green herbage feeding systems. Animals receiving high concentrate level presented lower skatole and high terpenoids values than animals consuming lower concentrate levels. Therefore, terpenes, 2-3 octanedione and skatole can trace grass feeding system in beef [52]. Another study reports the use of stable isotope ratio analysis (SIRA) from muscle and tail hair to confirm accurately the diet of cattle. Additionally, it indicates that analysis of tail hair sections provides information about the diet changes that have occurred in the last 12 months [53]. Carotenoid, color and reflectance levels of subcutaneous fat have also been used for distinguishing pasture fed animals from individuals receiving barley-based concentrated diet [54].

Regarding animal welfare, a recent study considering total protein, lysozyme, cortisol, serum and fecal corticosterone, and GR- α gene expression suggests that open

systems are less stressful than tie-stall housing, consequently the previous indicators considered together could be effective biomarkers of stress and disease susceptibility [55]. Pasture-feeding influences metabolism of skeletal muscle and gene expression; from the 212 differentially expressed genes 149 correspond to previously identified genes, which have been classified mainly in “protein metabolism and modification”, “signal transduction”, “cell cycle”, “developmental processes” and “muscle contraction” biological processes according to the function gene annotation [56]. Selenoprotein W was under-expressed in pasture-fed animals and could be considered a genetic marker for grass-based systems [56].

More recently, diverse epigenomic studies have been performed in bovine muscle. DNA methylation of regulatory regions of the PPARG1 and FABP4, which have been differentially expressed between IMF and lean part of *longissimus dorsi*, were lower in the IMF than in lean portion of the muscle, suggesting that DNA methylation is involved in the regulation of tissue-specific expression on adipogenic and lipogenic genes [56]. Another study reported the DNA methylation profiles and their relationships with gene expression and miRNA in beef, providing basis information for understanding the epigenetic mechanism of muscle development and growth [57]. Metabolomics approaches also have been employed in the analysis of beef; metabolite profiling could differentiate beef adulterated with pork meat [58], classify meat samples according to their storage time and predict the storage time [59], and estimate the microbial counts of different microorganisms and the sensory scores of beef considering storage conditions as well [60].

Structure, physiology and composition of muscle

Muscle constitutes a soft tissue that possesses ability to contract, providing movement to different parts of the body. Muscular tissue is formed during embryonic development through a process called myogenesis, in which myoblasts fused together to form multi-nucleated fibers named myotubes. The myoblast differentiation occurs in different stages; the first one comprises cell cycle exit and the initiation of expression of certain genes. The second step encompasses the alignment of myoblasts among them. The third and final stage consists in the actual cell fusion and the presence of calcium ions is crucial for it. During this, actin is recruited to the membrane plasma, followed by adjacent apposition and the formation of a pore that becomes rapidly wide. Distinct genes and protein products—currently under investigation—express and appear throughout myogenesis, correspondingly.

Commonly, muscle tissue is classified according to the function and body location into three types: striated or skeletal, non-striated or smooth, and cardiac muscle. The last two types contracts involuntarily but can be activated through the interaction of the central nervous system, the peripheral plexus and endocrine stimulation as well. Contrarily, skeletal muscle contracts voluntarily upon influence of the central nervous system. Distinctive types of muscle respond differently to neurotransmitters and endocrine substances such as noradrenalin, acetylcholine, adrenalin and nitric oxide among others.

Myocytes constitutes the unit of muscle tissue; they are elongated cells with length and width ranging from millimeters to approximately 10 centimeters and from 10 to 100 micrometers, respectively. These cells can be organized in tissues that may be

smooth or striated, depending on the presence or not of repeated arrangements of contractile proteins called myofilaments. The skeletal and cardiac muscles contain sarcomeres, which are the basic unit of striated muscle. Sarcomere comprises two structural proteins called myosin (thick filament) and actin, which forms the thin filament (Figure 1.5). The muscle contraction is produced by the interaction between these two microfilaments. The molecular basis for this interaction is the binding of myosin to actin filaments, allowing myosin to act as a motor that drives filament sliding. Myosin can be considered a molecular motor that converts energy in the form of ATP to mechanical energy, generating force and movement [61].

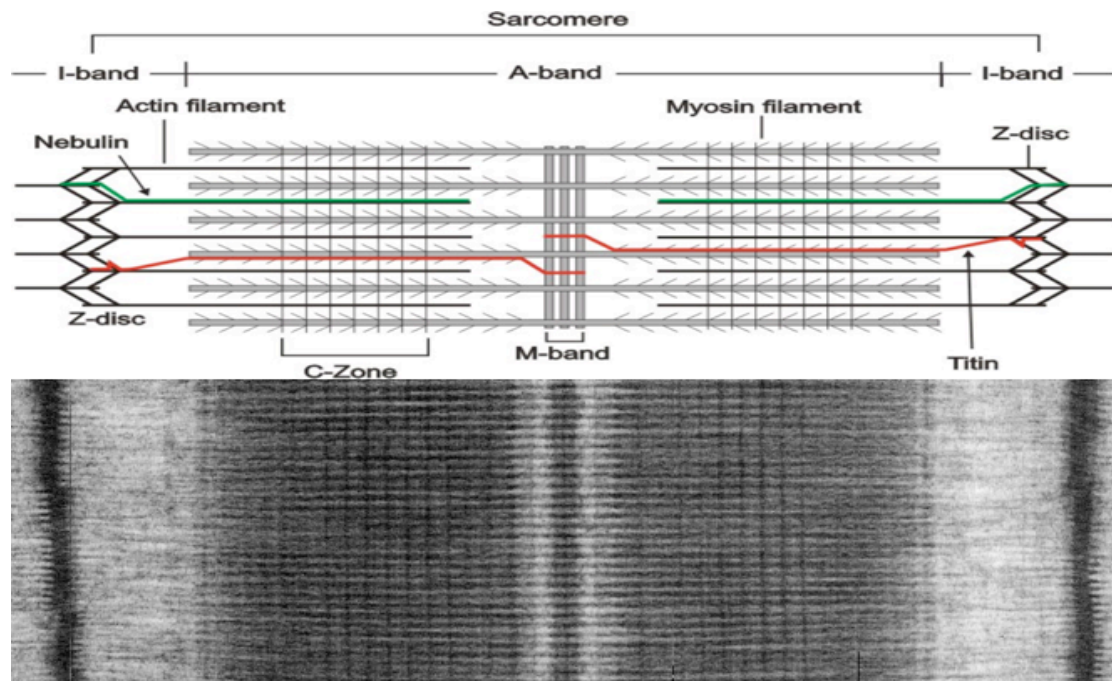
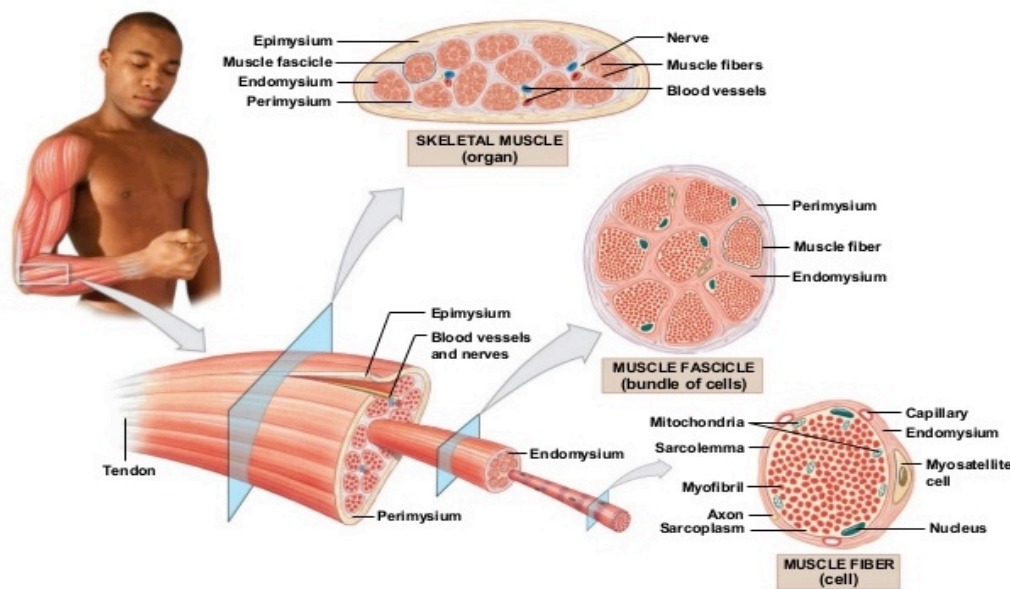


Figure 1.5 Sarcomere, electron micrograph with a schematic representation of the structural components

Actin and myosin filaments within a myofibril are contained in the sarcomeres. All myofibrils are settled parallel to the cell long axis with their sarcomeres lying side by

side. A layer of areolar connective tissue, called endomysium, which represent the deepest and smallest component of connective tissue, surrounds each individual myocyte (Figure 1.6). Single muscle fibers group together to form bundles known as fascicle. Each of these fascicles is covered by another fibrous sheath called perimysium. This structure contains mainly collagen, blood vessels, nerves and intra-muscular fat as well. Finally, the most outer layer of connective tissue that surrounds the entire skeletal muscle constitutes the epimysium. This layer protects the muscle against friction with other muscles and adjacent structures such as bones and cartilages. It is continues with fascia and forms the tendons, where it becomes thicker and stronger for providing insertion to the bones.



Source: <http://image.slidesharecdn.com>

Figure 1.6 Structural organization of skeletal muscle

Muscle is composed of water, protein and amino acids, minerals, fats and fatty acids, vitamins and other bioactive components, and small amount of carbohydrates. Table 1.1 summarizes the beef composition from a nutritional prospective. Meat contains all essential amino acids and constitutes a good source of highly bio available minerals and vitamins, particularly Vitamin B12 and iron; which are absent in vegetarian diets.

Table 1.1 Nutritional composition of meats per 100g

Product	Water	Protein	Fat	Ash	Kilojoules
Beef (lean)	75.0	22.3	1.8	1.2	485
Beef carcass	54.7	16.5	28.0	0.8	1351
Veal (lean)	76.4	21.3	0.8	1.2	410

Conventional evaluation of beef

Beef grading system in the United States

Following the guidelines of the USDA, beef is graded as a whole carcass in two ways: a) Quality Grade and b) Yield Grade. For a consumer prospective, Quality Grade should be considered more relevant since they are easy to understand and use as a selection criterion at the moment of purchase. Contrarily, producers depends heavily on both grading systems; varying according to the method employed by the corresponding market.

Quality Grade

A quality grade is obtained from an evaluation of combined factors that influence the palatability of the cooked meat. Palatability is based mainly on tenderness, juiciness and flavor of the meat. USDA quality grades serve to reflect dissimilarities in expected eating quality among slaughter cattle and their carcasses. The considered

variables comprise carcass maturity, texture, firmness, color of the lean part of the meat, and the amount and spreading of intramuscular fat. Whole carcass is graded based on two components: level of intramuscular fat (marbling) and the physiological age of the animal (maturity). The grade of a beef cut could be a relevant decision factor for many consumers at the retail store, however for producers it is critical because the compensation depend directly upon the obtained grade. The grading includes eight levels, from best to worst: 1) Prime, 2) Choice, 3) Select, 4) Standard, 5) Commercial, 6) Utility, 7) Cutter, 8) Canner. Figure 1.7 depicts the relationship between marbling, maturity and carcass quality grades. Generally, the Prime, Choice Select and Standard grades are restricted for young animals (A and B). The Commercial, Utility, Cutter and Canner groups comprise of mature animals, normally C, D and E maturity levels.

Degrees of Marbling	Maturity ²				
	A ³	B	C	D	E
Slightly Abundant	PRIME				
Moderate			COMMERCIAL	COMMERCIAL	
Modest	CHOICE				
Small					
Slight	SELECT		UTILITY	UTILITY	
Traces					
Practically Devoid	STANDARD			CUTTER	

¹Assumes that firmness of lean is comparably developed with the degrees of marbling and that the carcass is not a "dark cutter."

²Maturity increases from left to right (A through E).

³The A maturity portion of the Figure is the only portion applicable to bullock carcasses.

Figure 1.7 Quality grades table. Relationship between marbling, maturity and quality grade

Marbling

The intermingling and distribution of fat within the lean part of the meat is called marbling. It refers to the similarity of a piece of marble, which shows different patterns of grain colors in its surface. For marbling grading, a ribeye is obtained between the 12th and the 13th ribs, then inspectors visually evaluate the amount and dispersion of marbling at the cut surface. Beef cuts displaying high level of marbling are prone to be tender, juicy and flavorful, thus carcasses with greater amount of marbling receive higher quality grades Degree of marbling is the main determiner of quality grade. The marbling scores are nine, as they can see in Figure 1.8.

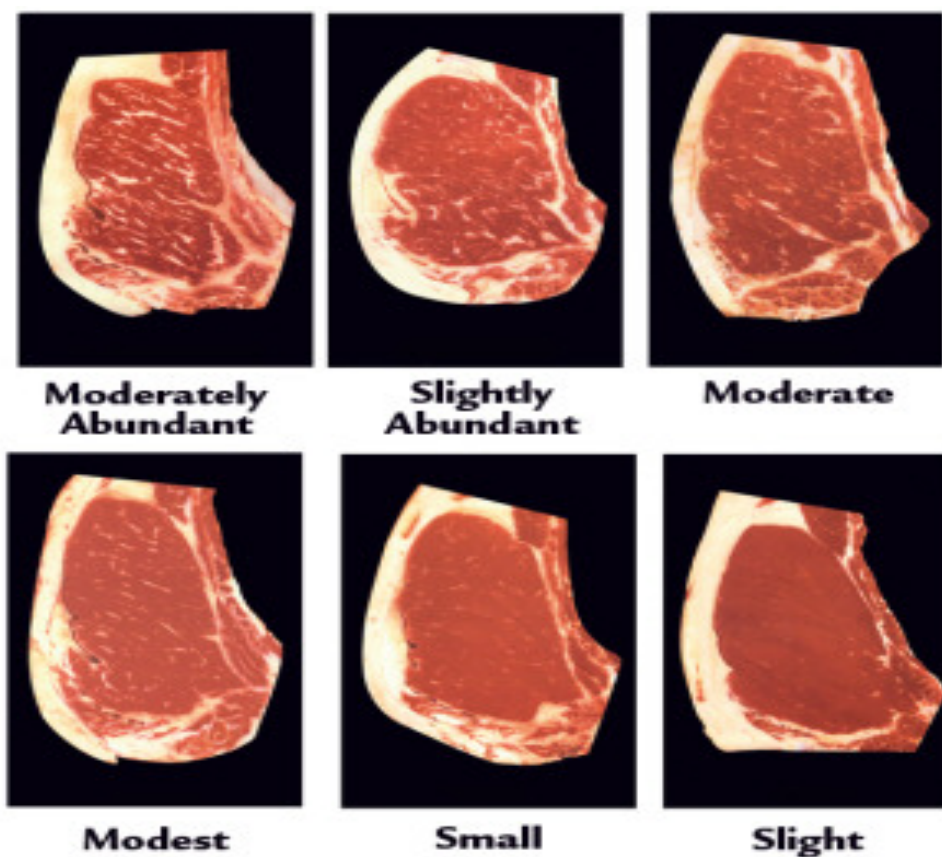


Figure 1.8 Marbling grades. Ribeyes displaying the nine USDA categories of marbling

Maturity

Animals develop at different rates due to different factors (e.g., breed, environment, forage). Maturity refers to the physiological stage of the animal rather than the chronological age; it includes the skeletal characteristic, and the color and texture of the lean part of the carcass. When cattle get old, the cartilage and bone structures change; cartilages ossify and bones become progressively tougher. This observation allows graders to determine the bone maturity grade. Mature animals display a dark red-purple color with a coarser texture, which is distinct than the clean bright pink observed in young individuals. There are five categories in maturity. Table 1.2 shows the approximate ages for the corresponding maturity grades.

Table 1.2 Levels of carcass maturity with the corresponding age approximation

Carcass Maturity	Approximate age
A	9-30 months
B	30-42 months
C	42-72 months
D	72-96 months
E	> 96 months

Yield Grade

Yield grade estimates the carcass cutability; it represents the combined yield of closely trimmed, boneless retail cuts from the round, loin and chuck, expressed as percentage (% CTBRC). It is a prediction of the amount of usable lean, edible beef from a carcass. Yield Grade is calculated considering the next factors: 1) External fat thickness over the ribeye, 2) Hot carcass weight, 3) Ribeye area, 4) Percentage of internal fat (KPH–kidney, pelvic and heart–). There are five Yield Grades, ranging from 1 to 5. Lowest numbers characterize more yield of the carcass, denoting greater

lean to fat ratio. Table 1.3 shows the Yield grades and the corresponding yield of closely trimmed, boneless retail cuts.

Table 1.3 Yield Grades and its corresponding expected proportion of Closely Trimmed Boneless Retail Cuts (CTBRC)

Yield Grade	%CTBRC
1	>52.3
2	50 - 52.3
3	47.7 - 50
4	45.4 - 47.7
5	<45.4

Hot Carcass Weight

It is the weight of the carcass immediately after slaughter and removal of the hide, head, gastrointestinal tract and internal organs; at that moment the carcass is still hot or unrefrigerated. Hot carcass weight represents normally around 60-64% of live animal weight, however it depends on other factors such as fasting period before slaughter, transportation distance and amount of muscling and finishing. Hot carcass weight is critical for yield grade determination.

Fat thickness

It is measured at a three-four point of the length of the ribeye from its chine bone side (Figure 1.9). This measurement estimates reasonably the overall carcass amount of fat. Nevertheless, this measure is adjusted according to visual observation performed by the inspector in order to account for evident differences in the distribution of fat in other areas of the carcass.

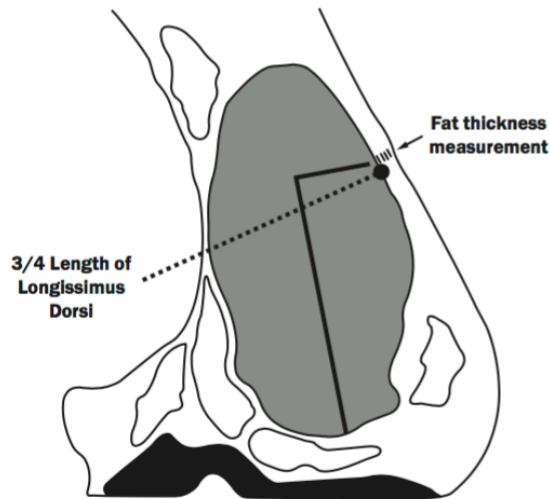


Figure 1.9 Fat Thickness measurement location

Kidney, Pelvic and Heart Fat Percentage

Fat normally accumulates around the kidneys, heart and inside the pelvic cavity. A measurement is obtained based on visual observation. Carcass Kidney, Pelvic and Heart Fat normally ranges from 1 to 5% of the carcass weight.

Ribeye Area

Ribeye area is measured at the 12th rib using a particular plastic grid. The grid is placed on the surface of the ribeye, then all squares in which the meat surrounds a dot is counted and then divided by 10; the resulting number represent the area of the ribeye in square inches (Figure 1.10). It normally ranges from 9 to 17 square inches in regular carcasses.

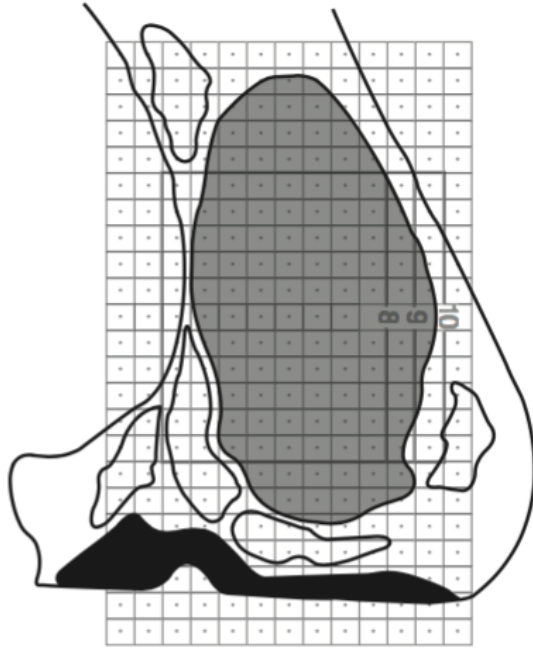


Figure 1.10 Method of measurement for Ribeye Area.
Squares in which the meat surrounds a dot is counted and then divided by 10; the resulting number represent the area of the ribeye in square inches

Yield Grade Calculation

To obtain the final Yield Grade based on the USDA standards, the following formula is used: $YG = 2.5 + (2.5 \times \text{adjusted fat thickness, inches}) + (.20 \times \text{KPH}\%) - (.32 \times \text{ribeye area, sq. inches}) + (.0038 \times \text{hot carcass weight, lbs.})$

Most of the times, inspectors avoid the use of the formula and grading is performed based on their professional training and experience. However the use of the formula depends on the situation and should be employed for more accurate evaluations.

Warner-Bratzler Shear Force

The Warner-Bratzler shear force (WBSF) constitutes the standard and most popular method for measuring the tenderness of meat. The basis of the method consists in cutting a piece of meat with a blade attached to a machine that registers the force

employed during the procedure [62, 63]. In the past, this method has been recognized as the most accurate for assessing the tenderness of meat. Nevertheless, other authors have challenged the precision of the method [64-66]. In 1994, the National Beef Tenderness Plan Conference recognized the necessity of standardization of the Warner-Bratzler procedure based on studies that demonstrated the great amount of variation among institutions [67]. For measuring tenderness in this study, the WBSF according to the standardized protocol method has been utilized [68].

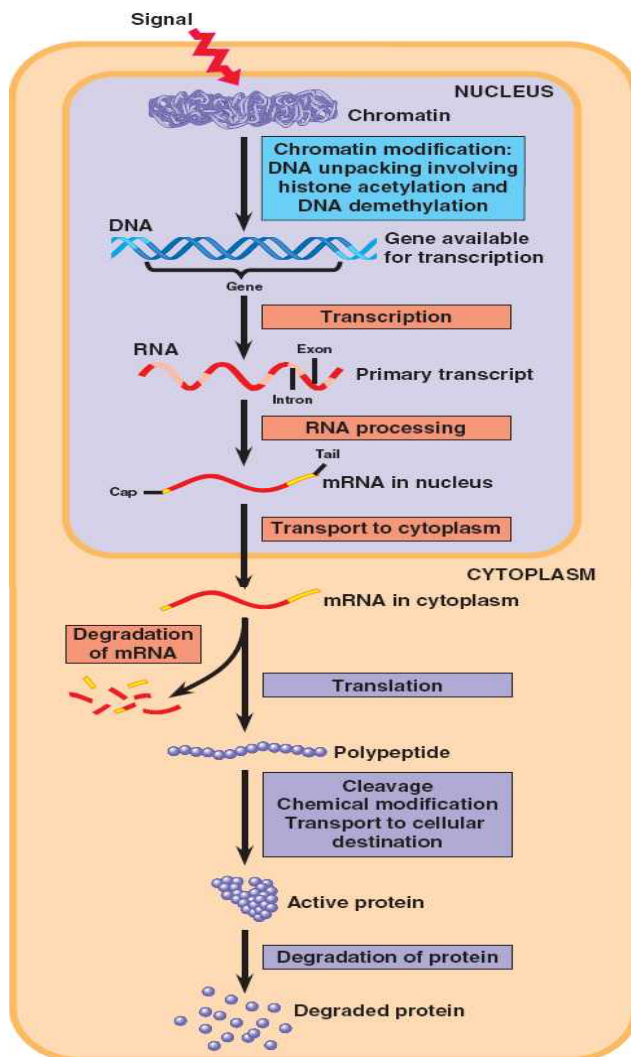
Genomic and epigenomic evaluation

Genomics constitutes a branch of biotechnology that includes DNA sequencing, recombinant DNA, computational biology and bioinformatics to sequence, assemble and examine the organization and function of genomes. It also comprises the study of relationships among genes and the interaction of the genome (complete set of DNA within a cell) with the corresponding environment, allowing the study of complex diseases and traits—regulated by a combination of genetic and environmental factors—such as cancer, diabetes and human height. Contrarily, genetics focuses in the examination of structure and role of single genes. Therefore genomics opens new opportunities for diagnosis, prognosis, prevention and treatment of many complex disorders, and study of many complex traits as well.

Gene Expression

Gene expression comprises the process by which information contained in a gene is employed for synthesis of functional molecules that most of the times are proteins, however in non-protein coding genes (transfer RNA or small RNA) the final product

is a functional RNA. Gene expression is a mechanism that all living organisms employed to create the biochemical machinery necessary for life. Gene expression includes various stages: transcription, RNA splicing, translation and post-translational modification of the protein. All these steps need meticulous regulation to provide the cell control over its structure and function. Hence, the cell gene expression profile determines the function and capability of that specific cell.



Source: <http://www.slideshare.net>

Figure 1.11 Gene expression and its regulatory mechanisms

Each stage in the central dogma of genetics (DNA to RNA to protein) offers the cell a prospective switch for functional self-regulation through fine-tuning of the type and amount of produced proteins (Figure 1.11). Therefore, gene expression constitutes the foundation for cellular differentiation, development, adaptability and evolution of any living organism; becoming the fundamental level of phenotype definition.

Regulation of Gene Expression

Regulation of Gene Expression refers to the process that modulates the rate and manner of gene expression, thus it controls the amount and timing of synthesis of a gene functional product. Regulation of gene expression involves a vast number of complex interactions among genes, RNA molecules, transcription factors, and other biochemicals; fine-tuning of this system is critical to maintain cell function and homeostasis within an organism. Basically, regulation of gene expression occurs at two levels: a) Transcriptional level and b) Post-transcriptional level.

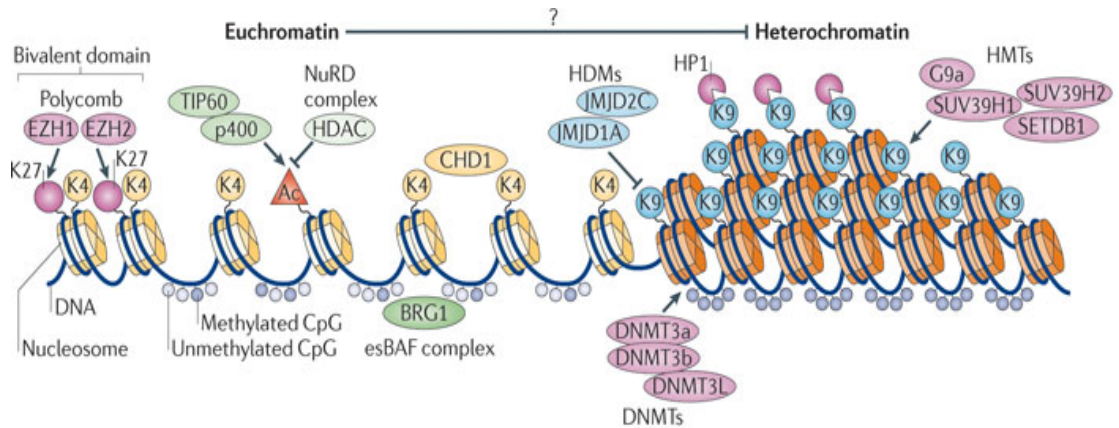
Transcriptional regulation

This mechanism determines the amount and timing of messenger RNA (mRNA) that a gene produces to serve as a template for its correspondent final product; this way the cell can adjust to various internal and external signals. Transcription regulation involves transcriptional factor proteins and other molecules that precisely control the amount of RNA produced at a determined moment through different mechanism of actions. Although the strategy for transcription regulation varies between prokaryotes and eukaryotes, some features remain the same for both groups.

Transcriptional regulation in eukaryotes depends on the physical properties of the DNA and the interplay of protein known as transcription factors [69]. The most relevant structural difference in the DNA of eukaryotes against prokaryotes is the condensation of DNA in a structure called chromatin. Regulation of transcription constitutes the most common form of gene control.

Chromatin State

Nuclear DNA does not exist in linear strands in eukaryotic cells; it appears highly packaged and wrapped around protein octamers (histones) in order to fit into the nucleus. This arrangement of the DNA influences the physical accessibility of parts of the DNA molecule at determined moments [70]. Chromatin exists in two forms called euchromatin and heterochromatin (Figure 1.12). The euchromatin displays an uncondensed, loose DNA configuration; which makes it more accessible to polymerases or cofactors necessary for transcription. Contrarily, heterochromatin shows a highly compacted chromatin that is normally not accessible, inhibiting the transcription [71]. The arrangement of DNA is a dynamic process that sequesters or exposes genes for transcription; this mechanism becomes an effective way of regulation that many times affect large regions of chromosome such as it occurs in genomic imprinting [72].



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Figure 1.12 Euchromatin and Heterochromatin states in DNA of embryonic stem cell. Significant epigenetic marks comprise histone modifications and inclusion of various core histone variants (orange and yellow cylinders), which affect DNA accessibility and modulate the efficiency of transcription. The relevant histone modifications, H3K4me3 (active), H3K9me3 and H3K27me3 (repressive) are positively controlled by certain histone methyltransferases (HMTs), G9a—known as EHMT2—, SUV39H1, SUV39H2 and SETDB1; and negatively regulated by the corresponding histone demethylases (HDMs) JMJD2C—known as KDM4C—and JMJD1A—also known as KDM3A—. Active (K4) and repressive (K27) marks can be located in promoters of developmental genes to inhibit their expression while permitting fast activation through transcription factors Enhancer of zeste homologue 1 (EZH1) and Enhancer of zeste homologue 2 (EZH2). Histone acetylation constitutes an active mark, the acetyl group (orange triangle) could be added by the TAT-interacting protein of 60 kDa (TIP60; also known as KAT5)—p400 and removed by histone deacetylases (HDACs) present in repressive complexes such as nucleosome-remodelling (NuRD) complex. DNA methylation typically occurs on CpG islands in promoter regions and heterochromatin. DNA methyltransferases (DNMTs) such as DNMT3a-DNMT3b or DNMT3L promote DNA hypermethylation, however DNA is generally unmethylated in euchromatic areas [73].

Many post-transcriptional modifications that arise in the tail of core histones facilitate and influence histone rearrangement (Figure 1.13). These modifications can be established by different enzymes (e.g. acetyltransferases, methyltransferases, deacetylases), which add or remove the correspondent covalent modification, such as acetyl groups, methyl groups, ubiquitin, and phosphates [74].



Figure 1.13 Histones and its main post-transcriptional modifications. Acetylation (blue), methylation (red), phosphorylation (yellow) and ubiquitination (green). The number in gray under each amino acid symbolizes its position in the sequence

Transcription Factors and Enhancers

Transcription factors comprise several proteins that initiate and regulate the expression of genes by binding to particular DNA sequences. Transcription factors have DNA-binding domains (normally up to 10^6 -fold higher affinity for their target sequences than for the rest of the DNA), which allow the binding to specific DNA sequences known as promoters sequences or enhancers [75]. These highly conserved sequences have been used to classify the transcription factors in several families, such as the MADS box-containing proteins, SOX proteins, and POU factors [76]. Transcription factors can also be catalogued by their 3 dimensional protein structure, involving basic helix-turn-helix, helix-loop-helix, and zinc finger proteins. Many of

these transcription factors bind the DNA in the promoter region close to the transcription start site (TSS) and assist to form the transcription initiation complex [77, 78]. Other transcription factors bind to regulatory regions that can promote or inhibit transcription of the corresponding gene; which could be located thousands of base pairs in any direction from the regulatory sequence. Sequence-specific transcription factors are considered the main mechanisms of gene regulation in prokaryotic and eukaryotic organisms.

In eukaryotes, regulation of gene expression by transcription factors is considered to be combinatorial—requires the coordinated interactions of multiple proteins—, while in prokaryotes a single protein is usually all that is required. In prokaryotes, RNA polymerase can access almost any promoter region in the DNA without the presence of any activator or repressor. Hence, the “ground state” is considered to be “on” or non-restrictive. Contrarily, in eukaryotes the “ground state” of gene expression is determined as restrictive because although some promoters might be present, they are inactive if some sort of recruitment to the promoter is not performed by the transcription factors [69].

Post-transcriptional regulation

After the transcription of DNA into mRNA, diverse mechanisms determine how much mRNA will be translated into proteins, thus post-transcriptional regulation constitutes the control of gene activity at the RNA level, before the translation of the gene. Most of genes that encode different RNAs are transcribed in the nucleus by RNA polymerase I, II and III, however the first transcript almost never constitutes the mature, functional product. These precursor RNAs must experience post-

transcriptional processing, which includes 5' capping, 3' polyadenylation and splicing of pre-mRNA, and in several occasions RNA editing as well. The cytoplasmic level of mature RNA defines the rate of translation that depends on mRNA transport, storage and degradation processes.

DNA methylation

DNA methylation comprises the addition of a methyl (CH₃) group to a DNA molecule. DNA methylation occurs in both prokaryotes and eukaryotes, and is conserved in most main eukaryotic groups, such as plants, several fungi and animals. Interestingly, the yeast *Saccharomyces cerevisiae* and the nematode worm *Caenorhabditis elegans* have lost DNA methylation mechanism [79]. In plants, DNA methylation exists in CG, CHG, and CHH (H=A, C, T) contexts, however in mammals DNA methylation mostly occurs in both strands of the symmetric CG sites, while non-CG methylation predominates in embryonic stem (ES) cells [80]. One of the most widely studied DNA methylation process constitutes the covalent addition of a methyl group to the 5-carbon of the cytosine ring, resulting in 5-methylcytosine (5-mC). DNA methylation regulates the function of the DNA, methyl radicals project into the DNA major groove preventing transcription to occur.

The human genome contains approximately 1.5% of 5-methylcytosine [81]. Globally, genomic DNA shows heavily methylated CpG sites whereas CpG islands located near promoters of normal somatic cell remain unmethylated in a tissue specific context, allowing transcription. Generally, a methylated CpG island in the promoter region of a gene represses gene expression, turning the gene “off”. DNA methylation is critical for normal development and has been associated with several key biological

processes such as gene silencing, X-chromosome inactivation, genomic imprinting, repression of repetitive elements, ageing and oncogenesis [82-85]. Furthermore, DNA methylation has also been related with initiation of sexual dimorphism, DNA repair and progression through cell division checkpoints and repression of transposable and retroviral elements in mammals [86-89]. DNA methylation can alter gene transcription in two ways: a) Physically preventing the binding of transcriptional proteins to a gene and b) Methylated DNA may be bound by methyl-CpG-binding proteins (MBDs), which can recruit additional molecules to the site, such as histone deacetylases and other chromatin remodeling proteins that alter the histones to form a more compact and inactive state of chromatin, called heterochromatin. The association between DNA methylation and chromatin structure is critical for normal function. Patients with Rett syndrome show loss of methyl-CpG-binding protein 2 (MeCP2) and in cancer, the dynamics of methyl-CpG-binding domain protein 2 (MBD2) within DNA methylated regions has been associated with the oncogenic transformation of cells [90, 91].

DNA methylation mechanism

In mammals DNA methylation mainly occurs through the action of two classes of enzymatic activities, *de novo* methylation and maintenance methylation. *De novo* methylation establishes DNA methylation patterns early in development while maintenance methylation preserves DNA methylation after every cellular DNA replication cycle. Mammalian DNA methyltransferases (Dnmts) include three members, divided in two clusters, which are structurally and functionally different. The *Dnmt3a* and *Dnmt3b* establish the *de novo* methylation pattern whereas *Dnmt1*

maintains this arrangement throughout chromosome replication and repair [92]. *Dnmt1* shows a higher specificity (30 to 40-fold change) for hemymethylated sites [93]. Nevertheless, the functional division is not strict, since *Dnmt1* is needed for de novo methylation at non-CpG cytosines and possibly in CpG islands as well [94].

Regulatory factor for de novo DNA methylation: Dnmt3L

The *Dnmt3* group comprises two active *de novo* members (*Dnmt3a* and *Dnmt3b*) and one regulatory factor, the Dnmt3-Like protein (*Dnmt3L*). The active members have analogous similar catalytic domains, contrary to the *Dnmt3L* that lacks the essential residues in the C-terminal for DNA MTase activity. *Dnmt3a* and *Dnmt3L* are essential for methylation in most imprinted regions in germ cells [95]. *Dnmt3L* co-localizes and co-immunoprecipitates with *Dnmt3a* and *Dnmt3b*, and enhances *de novo* methylation by these two MTases [96, 97]. The interaction between Dnmt3L and Dnmt3a depends on the minimal regions localized in the C-terminal of both proteins, which have the typical fold of Class I Adomet-dependent MTases [98]. Nevertheless, the S-adenosyl-L-homocysteine product of the methylation reaction was only observed in *Dnmt3a* and not in *Dnmt3L*, demonstrating that *Dnmt3a* constitutes the catalytic element of the complex whereas Dnmt3L is incapable to bind to Adomet, being inactive [99].

CpG spacing and de novo DNA methylation pattern

The superimposing of Dnmt3a structure with a M. HhaI-short oligonucleotide complex suggests that the two active sites remain in the DNA major groove and that Dnmt3a could methylate two CpGs separated by one helix turn in a single binding

event. The cycle of activity of Dnmt3a on long DNA strands showed an association of methylated CpG sites at distances of 8 to 10 base pairs, and the structural model of oligomeric Dnmt3a bound to DNA could explain this arrangement. Analogous pattern was detected in the frequency of CpG sites in differentially methylated regions of a dozen of maternally imprinted genes in mouse [100]. Those results indicate a basis for the analysis of differentially methylated regions in imprinted genes, including CpG spacing evaluation and nucleosome modification. Additionally, an 8 to 10 base pair periodicity has been observed in non-CpG methylation in embryonic stem cells [81].

DNA methylation and histone modifications

The relationship between DNA methylation and histone modification is well known [101]. DNA methylation profiles better correlates with histone modification patterns than with genomic sequence background. Generally, DNA methylation associates with the presence of H3K9 methylation and the absence of H3K4 methylation, respectively. In somatic cells, H3K4 methylation may prevent gene promoter from *de novo* DNA methylation [102]. Negative correlation between H3K4 methylation and allele-specific DNA methylation at differentially methylated regions has also been reported [103]. Another study suggests that demethylation of H3K4 is necessary for establishing DNA methylation of imprinted genes during oogenesis [104].

Protein interaction analyzes demonstrated a particular interaction between Dnmt3L and the extreme amino terminus of histone H3; this interaction was intensely repressed in presence of H3K4 methylation nevertheless it was unresponsive to alterations at other positions [105]. This suggests that *Dnmt3L* constitutes a probe of

H3K4 methylation and in case of lack of methylation *Dnmt3L* promotes *de novo* DNA methylation by coupling activated Dnmt3a to the corresponding nucleosome. Many studies also suggest the existent of a correlation between H3K9 methylation and DNA methylation, though the mechanism of how H3K9 contributes to DNA methylation still remains uncertain, specifically in mammals [106, 107].

Methyl-CpG Binding Domain Sequencing (MBD-seq)

Many approaches have been developed to study DNA methylation profiles. However the most comprehensive laboratory method for determining DNA methylation at high resolution is bisulfite sequencing, which is recognized as the “gold standard” method for DNA methylation detection. Bisulfite sequencing provides a single base resolution status but the cost of sequencing whole-genomes and the necessity of large number of sample to achieve sufficient statistical power limit its utility in many ways. An alternative method is reduced representation bisulfite sequencing that employs bisulfite sequencing to a reduced part of the genome but only capturing around 12% of all CpGs [108].

MBD-Seq uses the methyl binding domain of methyl binding proteins MBD2 or MBDL3L1 to capture methylated DNA fragments with a posterior sequencing of those fragments employing NGS approach [109]. Although affinity-based capture methods lack the single nucleotide resolution of bisulfite sequencing, they still provide a good representation of DNA methylation. MBD-Seq and MeDIP sequencing demonstrated efficiency to detect differentially methylated regions (DMRs) and both methods detect similar methylome proportions. Interestingly, MBD-Seq captures specifically methylated CpGs, contrary to MeDIP that binds to

DNA fragments containing any methylated cytosine. In conclusion, MBD-Seq is highly specific, sensitive and practical to ascertaining DMRs, and laboratory automation simplify its use on a large scale [110].

Micro RNA

Micro RNAs comprise small non-coding RNA molecules of approximate 18 to 22 nucleotides that play key roles in post-transcriptional gene regulation. In animals, miRNAs regulate their target by translational repression, and messenger RNA (mRNA) destabilization and degradation. Since the discovery of the first microRNA, other several hundreds have been identified in animals, plants and viruses [111-113]. Micro RNA downregulates gene expression through base-pairing with the 3' untranslated region (3' UTR) of the target mRNA [114], constituting another level of regulation of gene activity in several organisms.

miRNA gene families and nomenclature

Micro RNA genes comprise one of the most abundant gene families, and are broadly dispersed in plants, animals and viruses. Currently, there are 1881 and 808 catalogued miRNAs for human and cow respectively. Interestingly, many of the functions of these miRNAs still remain undetermined [115].

Generally, there are multiple miRNA loci with similar sequences that result from gene duplication [116]. This event makes the classification process a difficult task that should be standardized. Although, the rules have not yet been well defined, it is usually assumed that miRNAs with perfect-matching sequences at position 2-8 of the mature miRNA belong to the same family. As an example, the human genome

contains the let-7 family, which includes 14 paralogous loci. There are 196 miRNA families conserved among mammals and 34 miRNA families are phylogenetically conserved from *C. elegans* to humans [115, 117]. Several miRNAs evolve from the same origin but diverge in the miRNA seed region; miR-200c and miR-141 belong to the well-conserved miR-200 superfamily but their seeds differ only by one nucleotide. Experimental deletion of each locus demonstrated that the targets of miR-200c and miR-141 scarcely overlap, showing the relevance of the seed sequence in the evolution and function of the miRNAs [118].

The nomenclature of miRNA genes is still moderately inconsistent. The first discovered miRNA genes received their names according to their phenotypes (*lsey-6* and *lin-4*) while the others miRNAs identified by cloning or sequencing were named numerically (e.g., *lin-4* homologues in other species are named *mir-125*). Genes that encodes miRNA sisters—nearly identical sequences except for one or two nucleotides—are labeled with lettered suffixes (*miR-124a* and *miR-124b*). When the identical mature miRNA is generated from various distinct loci in the genome, numeric suffix are included in the name of the corresponding miRNA loci (e.g., *mir-194-1* and *mir-194-2* produce the same mature *mirR-194*). Another more unusual case is when each locus generates two mature miRNAs but one derives from the 5' strand and the other from the 3' strand of the precursor (*miR-125a-5p* and *miR-125a-3p*). Normally one arm is more prevalent and functional (the “guide” strand) than the other arm (miRNA*), which typically degrades.

Biogenesis and silencing mechanism of miRNA

The biogenesis of miRNAs is under fine-tune temporal and spatial regulation; and their alteration is related with many disorders, such as cancer and genetic disorders [119]. Most of the identified miRNA genes are located in intergenic regions or oriented inversely to neighboring genes, thus it is suspected that they are transcribed independently [120, 121]. Interestingly, some miRNA genes are transcribed simultaneously with their corresponding host gene, suggesting a coupled regulation of miRNA and protein-coding genes [122].

Micro RNA genes are generally transcribed by RNA polymerase II (Pol II) to produce a stemloop including primary miRNA (pri-miRNA), which can range from hundreds of nucleotides to tens thousands bases. Micro RNAs contained in Alu-repetitive elements, constitute an exception that can be translated by RNA Pol III [123]. Similarly to mRNAs, Pol II transcribed-pri-miRNAs have 5' capping structures and poly-A tail and might be spliced [124]. Interestingly, most mammalian miRNAs are contained in introns and the corresponding miRNA processing occurs before the splicing [125].

In the nucleus, a complex of proteins called the processor, processes the pri-miRNA; the main components of this complex constitute the RNase III enzyme Drosha and the double-strand RNA-binding domain (dsRBD) protein DGCR8/Pasha. The microprocessor complex cleaves the pri-miRNA stem by assessing the distance from the single-stranded/ double-stranded RNA junction, generating an approximately 70-nucleotide hairpin precursor miRNA, called pre-miRNA [126]. Following Drosha processing, pre-miRNA is exported into the cytoplasm. The 2-nt 3' overhang, typical

characteristic of cleavage mediated by RNase III, is identified by Exportin-5, which transfers the pre-miRNA into the cytoplasm through a Ran-GTP-dependent mechanism [127]. Subsequently, another RNase III enzyme, Dicer, cleaves the pre-miRNA to generate the mature approximately 22 nucleotide miRNA:miRNA* duplex. The Dicer enzyme interacts with the dsRBD proteins TRBP/Loquacious, and in human PACT [128].

TRBP recruits the Argonaute protein Ago2 and combined with Dicer produce a trimeric complex that initiates the formation of the RNA-induced silencing complex (RISC) [129]. The miRNA strand with lesser stability of base-pairing at its 5' end is integrated into RISC, while the miRNA* strand is normally degraded [130]. Typically, the ratio of miRNA to miRNA* is approximately 100:1 however, when the two RNA strands are functional, the proportion can be much lower. After integrated into RISC, the miRNA guides the complex to the target by base-pairing complementation. When perfect or almost-perfect complementarity occurs, the target mRNA can be cleaved and destroyed, repressing their translation [131].

For cleavage to occur, RISC must contain an Argonaute protein capable of endonucleolytic slicing. In mammals, the only enzyme having this property is Ago2 [132]. Generally, animal miRNAs base-pair imperfectly with their target mRNA and endorse repression of translation rather than slicing and degradation. In this type of inhibition, target mRNAs are not vigorously destroyed but can be destabilized owing to deadenylation and posterior decapping. The mechanism of translation repression by miRNAs still remains uncertain; nevertheless some evidences suggest that miRNAs interfere translation initiation while others indicate block of elongation

[133]. The new complex, formed by Argonaute proteins, miRNAs and their target mRNAs accumulates in the processing bodies (P-bodies), which are cytoplasmic centers of mRNA degradation [134].

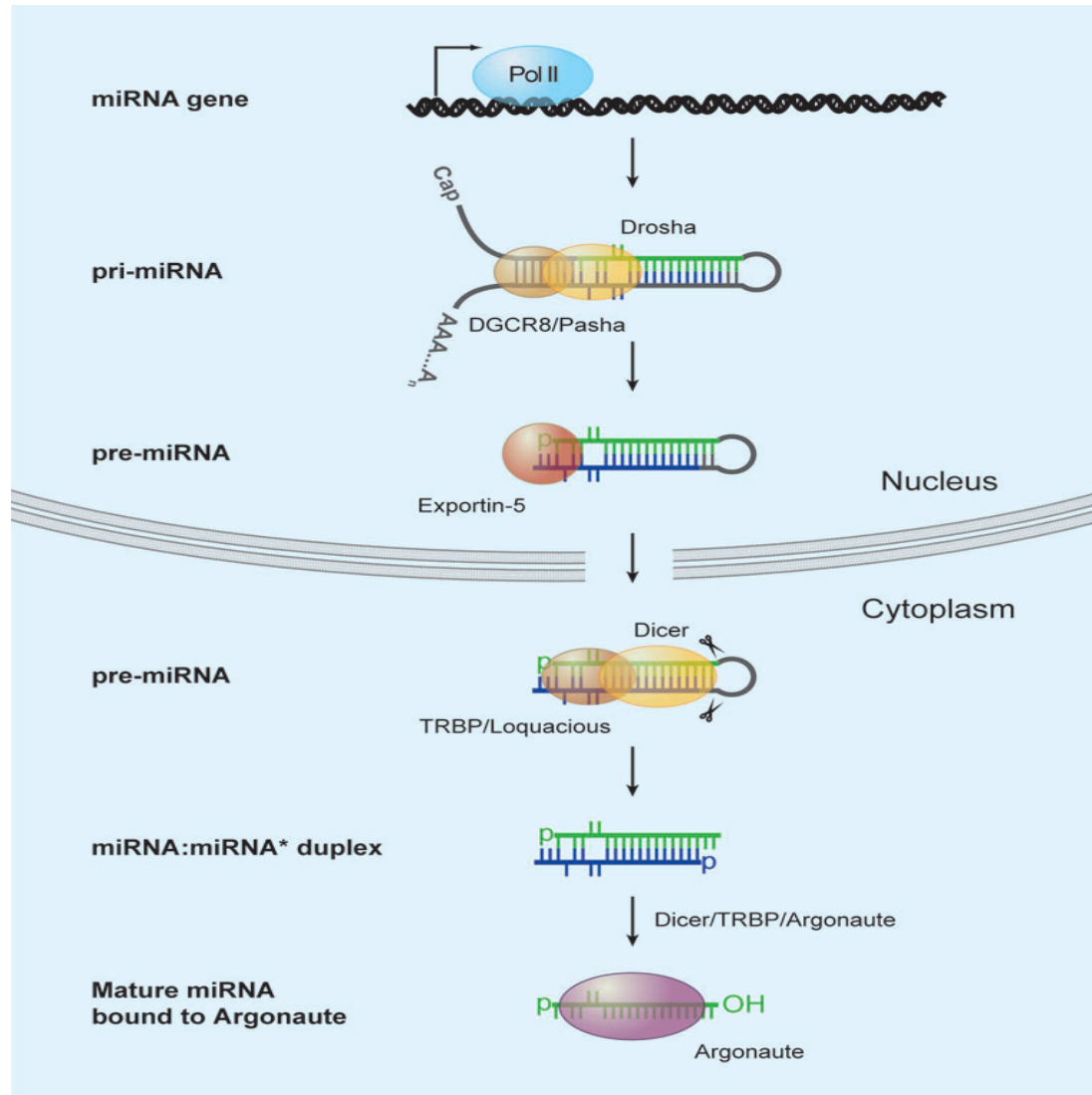


Figure 1.14 miRNA biogenesis

miRNA genes are normally transcribed by RNA polymerase II (Pol II), producing primary miRNAs (pri-miRNAs). Next, the RNase III endonuclease Drosha and the double-stranded RNA-binding domain (dsRBD) protein DGCR8/Pasha cleave the pri-miRNA to generate the precursor miRNA (pre-miRNA) in the nucleus. The pre-miRNA is transported into the cytoplasm by the Exportin-5, where it is cleaved by another RNase III endonuclease called Dicer, in combination with the dsRBD protein TRBP/Loquacious, releasing the 2-nt 3' overhang which includes a ~21-nt miRNA:miRNA* duplex. The miRNA strand is incorporated into an Argonaute-containing RNA-induced silencing complex (RISC), while the miRNA* strand is typically destroyed.

Metabolomic analysis

Metabolomics constitutes the study of biochemical processes involving metabolites, which are the intermediates and final products of metabolism. Metabolomics integrates different strategies to identify and quantify metabolites by using sophisticated analytical tools with statistical and multi-variant methods for data analysis and inference. Novel high-throughput analysis methods allow the study of the whole collection of metabolites in a biological cell, tissue or organism called metabolome. Metabolomic studies provide information about the physiological state of the respective organism at one specific moment based on its metabolite profiles. The characterization of the metabolome has multiple applications in several biological disciplines, including the developing of novel diagnostic tools and treatments in medicine [135, 136]. Commonly, metabolomics is employed in conjunction with other –omics techniques such as transcriptomics, proteomics and epigenomics to understand the biology, genetic perturbation and interaction of an organism with its environment [137].

Diverse analytical technologies have been used to examine metabolites in different tissues and organisms [138]. In order to analyze many metabolites simultaneously (nontargeting profiling), mass spectrometry combined with different chromatographic separation techniques such as gas or liquid chromatography or nuclear magnetic resonance (NMR) has been employed in many studies [139, 140]. The vast diversity of biochemicals and the large divergences in their abundances make metabolomic analysis very challenging, thus still there is no single technology available to explore the whole metabolome. Additionally, development of more accurate and faster

bioinformatics and statistical tools for dealing with complex and massive data generated in metabolomics studies is necessary.

Contrarily to target profiling that uses internal standards for comparison and quantification of determined compounds in molar units, non-target profiling compares two biological states and reports analytes that based on statistical analysis, qualitatively differ in both conditions. NMR spectroscopy theoretically constitutes an excellent tool for non-target metabolic profiling in a small number of metabolites, because this method identifies spectral features originating from any compound that contains hydrogen or carbon [141]. With this approach, analyses can be performed straightforwardly in body fluids, cell or intact tissues without the need for chemical extraction or derivatization of the compounds, making the process more convenient and practical.

Wye Angus Herd

The Wye Angus herd was established in 1937 with the purchase of 18 yearling heifers and 2 bulls [142]. From these heifers, 10 were paternal half-sisters, the rest conforms two clusters of 4 and 2 half-sisters respectively, and two unrelated heifers. There is no known genetic relationship between the 3 groups and the remaining two heifers [143]. In 1939, 8 additional heifers from an external source were incorporated into the herd (a group of 4 and another group of 2 were paternal half-sisters). In the following two decades, Wye imported 19 bulls from the British Islands. Seven pairs of those bulls were paternal half-sibs and most of the other bulls had less than 6% of genetic relationship among them [143]. Approximately, 75% of the current Wye germplasm derived from those bulls. The herd was closed for external breeding since 1958 and

has remained closed ever since. Therefore, the genetic similarity of the herd offers an invaluable opportunity for performing basic and applied research, particularly genomic and epigenomic research in cattle.



Figure 1.15 Wye Angus farm, different feeding systems

Rationale and Significance

In this project we would like to unfold some of the facts behind the established controversy between grass- and grain fed beef. Although some researchers have reported no significant differences between these types of meat, consumers recognize grass-fed beef as a more natural and healthy product. Additionally, they understand that grass-fed beef production is more humane compared to other traditional systems. These confused points somewhat reflect the current consumption trend of grass-fed beef, which further limits producers to make wise management decisions. We think the main reasons of those unclear aspects are attributed to the difficulty of organizing

a cohort study of grass-fed project, resulting in the heterogeneity of sampled beef cattle, besides the longer time and cost of this kind of research. In response to the market needs, we want to provide a scientific evidence to explain why grass-fed beef could be better. Here we hypothesize that different diet styles, especially grass- and grain-fed, could result in physiological, nutritional and quality variation of beef. To test the hypothesis, we used a herd that has been closed for almost 70 years; the use of closely genetically related animals constitutes a good model for epigenetic studies and enhances the detection of the effect of diet as well. Information of the growth curve and evaluation of important economics traits in Angus steers that have been raised under the grass-fed regimen provide an initial reference point for the multi-omics analyses projected in this study. From a practical point of view, it is critical to recognize the factors that would impact this model of production to make a sound decision in adopting or rejecting it. In the first part of the study, we focus in the differences of those qualitative and quantitative economic traits between grass and grain fed animals, nevertheless a conclusive evaluation should include an economical assessment based on these scientific results and the market conditions at the moment of analysis.

The final goal of beef cattle breeders is to produce meat of the best quality in the shortest period of time in order to maximize the economic revenue. However, pursuing of this objective demands understanding of the biological processes that individuals suffer through their lives. Most of these processes comprise genetic and environmental components that affect the development of the animal and the final product as well. In this trial, we employ genetically similar animals to diminish the

genetic influence for a more efficient analysis of the diet as an environmental factor. Consequently, we explore the transcriptome and metabolome of animals from both groups to obtain gene activity profiles for each feeding style, and then link those profiles to the metabolites detected in the same group. Taking together both profiles (transcriptomic and metabolomic) for a joint pathway analysis provides valuable information that individually should be very difficult to obtain.

The last part of this project investigates the epigenetics, mainly DNA methylation, related to both types of feeding systems. Conceptually, epigenomic modifications can influence gene expression without altering the sequence of the corresponding genome. Therefore, it is critical to analyze the methylome in conjunction with the transcriptome to make comprehensive biological inferences about their relationship, which can help to comprehend better the concepts of gene expression and regulation, especially via DNA methylation.

Thus this project will contribute to further our understanding of the development of animals—principally information regarding important economic traits—. Additionally, it will allow the characterization of gene expression and metabolites profiles, and the recognition of relevant pathways for grass-fed animals. Finally, this study will identify the epigenetic markers—principally DNA methylation— associated to this type of feeding style.

All these results will provide valuable information for the beef industry and specially cattle producers. Furthermore, it will significantly benefit the scientific community and be useful in a wide range of forthcoming applications. Therefore, this project consist of:

1. Analysis of the growth curve and economic important traits in grass-fed Angus steers.
2. Transcriptomic and metabolomics examination, and significant joint pathway identification of animals raised on grass.
3. Identification of methylome profile associated with grass-feeding style.

2. Growth curve, important economic traits and beef quality characteristics of Grass-fed Angus steers

Abstract

The increasing demand for natural products is currently transforming the meat industry, making grass-fed beef a valuable option for improving profits. However, the transformation of conventional operations to grass-fed systems comprises many modifications, such as logistic, technological and financial, that could be very complex and expensive, involving economic risk. Therefore, in this study we analyzed the growth curve, important economic traits and quality carcass characteristics over four consecutive years in closely related grass-fed Angus steers, to reduce the genetic effect on the results. We found that grass-fed animals require around 188 additional days to reach the market weight and average daily gain (ADG) in grain-fed steers (1.075 ± 0.12 kg) was approximately 70% higher than grass-fed individuals. A regression analysis demonstrates interaction ($p < 2^{-16}$) between feed and age, thus individual regressions were fitted for each regimen style, obtaining almost perfect linear curves for both treatments, which could be easily applied in practical situations due to its simplicity. Six of eight carcass traits were different between grain and grass fed steers. Hot-carcass weight, dressing, back-fat and quality grade were superior in grain-fed individuals, contrarily to yield grade and ribeye area/carcass ratio which were better in grass-fed steers ($p < 0.05$).

Interestingly, the meat tenderness was certainly low and similar in both treatments ($p = 0.25$), indicating the feasibility to produce really tender meat with animals under grass-fed diet. Nevertheless, according to the quality grade analysis grain-fed

carcasses were higher ranked compared to grass-fed carcasses ($p < 1.185^{-05}$), regardless of similar tenderness. The results will provide valuable information for better understanding of the growth evolution of animals in grass-feeding systems, especially from weaning to termination. Additionally, the study will expand the knowledge about the quality of meat obtained from animals that received exclusively grass, becoming relevant information for economic evaluation and management decision for grass-fed cattle operations.

Introduction

Beef represents a main source of nutrients in many parts of the world and remains as a highly desirable meal especially in the US and other western countries. The muscle contains not only protein; it provides energy, minerals and vitamins as well. Although the importance of beef in the regular diet is undeniable, its impact in human health is still controversial. Red meat consumption has been associated with several pathologies such as cancer, coronary heart disease and diabetes [144-147]. According to some studies, the lipid profile and antioxidant contents of the meat are correlated with several observed outcomes [148, 149]. Additionally, a recent publication raised the concern about the content of toxic metals (zinc, chromium and nickel) in cow meat as a cause of the anthropogenic contamination [150]. Contrarily, other scientists mentioned that red meat consumption could be beneficial, providing high quality protein that increases satiety and boosts stamina [151].

Currently, the beef market is increasingly changing towards grass-fed animals. This adjustment has been generated by consumer's perception that animals raised on pastures and maintained in more natural environments have less detrimental impact

on the environment and yield healthier products. Rationally, it is totally coherent; grazing animals stay on pastures, consuming their food *ad libitum* and moving freely, exercising their muscles. Differently, feedlot animals received high-energy diet while they are confined to restricted spaces, preventing them to move easily for a more efficient feed conversion. The latest condition has been considered the traditional way to produce beef in the US since 1950's [152]. This system allows producing large number of animals in small areas, making the process more efficient by reducing costs such as housing and transportation [152]. Thus, customers have habituated to consume feedlot beef, which has specific characteristics that are difficult to achieve under other circumstances. Nevertheless, public consciousness about health, environment and animal welfare are inducing agriculturalists to adjust to more organic systems. Accordingly, the meat industry is progressively experiencing an expansion of the grass-fed beef to meet the growing demand [12].

The aim of this study was to determine the real divergences between grain-fed and grass-fed animals. First we ascertained the growth curve and compared several physiological traits on both groups. Secondly, we explored the beef quality traits to demonstrate whether a real difference exists or not between these two types of meat. Ultimately, our result provides scientific evidence for economic assessments and management decisions for beef cattle production systems transformation or modification.

Material and Methods

The animals were originated from the WYE Angus farm. This herd has been closed to any animal importation for almost 70 years hence it produces very homogeneous

progeny [142]. The use of a single breed and genetically similar individuals allows noise decreasing and enhancing the detection of diet effect in the study. The population comprised 92 Angus steers that have been treated and raised equally until weaning. At that moment, animals were randomly assigned to either grass or grain-fed style. From then on, the animals received exclusively one type of ration until the day of termination. The grain group consumed a conventional diet consisting of corn silage, shelled corn, soy bean and trace minerals. The grass-fed steers received grazed alfalfa when available and bailage during wintertime. The alfalfa has been harvested from land without any fertilizers, pesticides or other chemicals used. The individuals in this group consumed no animal, agricultural or industrial byproducts and never receive corn or any type of grain. The grain-fed and grass-fed clusters consisted of 52 and 40 individuals respectively. The animals were commercially harvested at George G. Ruppertsberger and Sons, Inc (Baltimore, MD) when they reached the market weight (approx. 470 kg).

Nutritional Analysis

An external laboratory analyzed the forage and hay used in both types of feeding regimen to determine their nutritional characteristics (Dairy One - Forage Laboratory, Ithaca, NY). The samples were obtained following the company recommendations and immediately shipped for analysis.

Genetic background

Genomic DNA has been extracted from blood using the Wizard[®] Genomic DNA Purification Kit (Promega, Cat # A1120). After extraction and quality assessment, 65

DNA samples have been properly shipped to GeneSeek Company, which achieved the genotyping using the GGP-HD BeadChip (80k SNPs). Subsequently, the data have been analyzed with PLINK, which comprises a whole genome association analysis toolset [153].

Body weight and carcass traits measurement

Body weights were recorded at birth, weaning and then monthly until slaughter. At termination, live weights (LW) and hot carcass weights (HCW) have been measured and dressing percentage (Dress) calculated. Concurrently, back fat (BF), ribeye area (REA), ribeye area/carcass weight ratio (REA/CWT), yield grade (Yield) and quality grade (Grade) measurements were obtained.

Warner-Bratzler Shear Force

After carcasses were maintained for 14 days in 4°C chambers for maturation, steaks from the *longissimus dorsi* muscle were cut at the level of the 12th intercostal space, vacuum packaged and immediately frozen at -20°C. Later, to assess the Warner-Bratzler shear force (WBSF), the steaks were thaw at 4°C and then cooked to an internal temperature of 70°C employing a George Foreman grill. An Oakton digital thermometer (Temp JKT Acorn series) was used to measure the core temperature of the steaks. After the samples cooled down to room temperature, six cores of tissue (1.27 cm in diameter and parallel to the fibers direction) were cut employing a hand-held coring device. The Warner-Bratzler shear forces were measured individually in each core utilizing an Instron #5442 Test Machine (Norwood, MA). The representative WBSF value for each sample was obtained averaging the

measurements of six cores from the same steak. The sample size for WBS measurement constitutes 57 animals from three consecutive years.

Blood Analysis

Five 14-month-old animals from each group were selected at random to undergo blood test analysis. Three 10 ml tubes of blood were collected through jugular puncture; the first and second tubes were coated with ethylenediamine tetraacetic acid (EDTA) and heparin respectively, the last one contained no additive. All samples were immediately storage in ice and shipped to the lab. The samples were analyzed in a commercial lab (BioReliance) following the standard procedures for typical hematological parameters, plus carbon dioxide and glucose content.

Statistical Analysis

The statistical analysis was performed in R (free software environment for statistical computing and graphics) employing the “nlme” package, which can fit and compare Gaussian linear and nonlinear mixed-effects models [154, 155]. Treatment results are reported as least squared means with a significance threshold of $P < 0.05$. For quality grade analysis, a Fisher test was employed to compare number of individuals assigned to different categorical groups.

Results

Nutritional Analysis

To check the nutrients in the diet, an external laboratory (Dairy One, Ithaca, NY) performed the analysis of the diet administered to the animals. As expected, the

content of non-fiber carbohydrates (NFC) and starch is much higher in the grain diet, providing more total digestible nutrients (TDN) and available energy as well. Contrarily, proteins, lignin, fiber matter and ash were more abundant in the grass than in the grain diet. All these differences were anticipated, however the magnitude of those differences should be known and considered in this study. Thus, Table 2.1 shows the specific values reported in the laboratorial analysis.

Table 2.1 Feed Components Analysis

Components	Dry matter basis (%)	
	Grain Diet	Grass Diet
Crude protein	13.9	17.5
Adjusted crude protein	13.9	17.5
Soluble Protein	47	28
Acid Detergent Fiber	18.4	42.4
Neutral Detergent Fiber	29.7	56.6
Lignin	2.6	7.1
NFC	47.1	18.4
Starch	35.6	0.2
Crude fat	2.9	2.5
Ash	6.47	10.12
TDN	73	60
NEL, Mcal/Lb	0.77	0.56
NEM, Mcal/Lb	0.79	0.56
NEG, Mcal/Lb	0.51	0.3

Genetic Background

To minimize the influence from genetic background, we completed genetic background cluster analysis between Wye Angus and external Angus animals based on SNP genotyping. The genetic analysis performed in 65 animals suggested no genetic differences between the two groups (grain-fed and grass-fed). Figure 2.1a shows the PCA result from the 65 examined animals. There is clearly no grouping of animals regarding diet style, suggesting that the randomization process immediately

after weaning was successful in order to cancel out the possible genetic difference between groups.

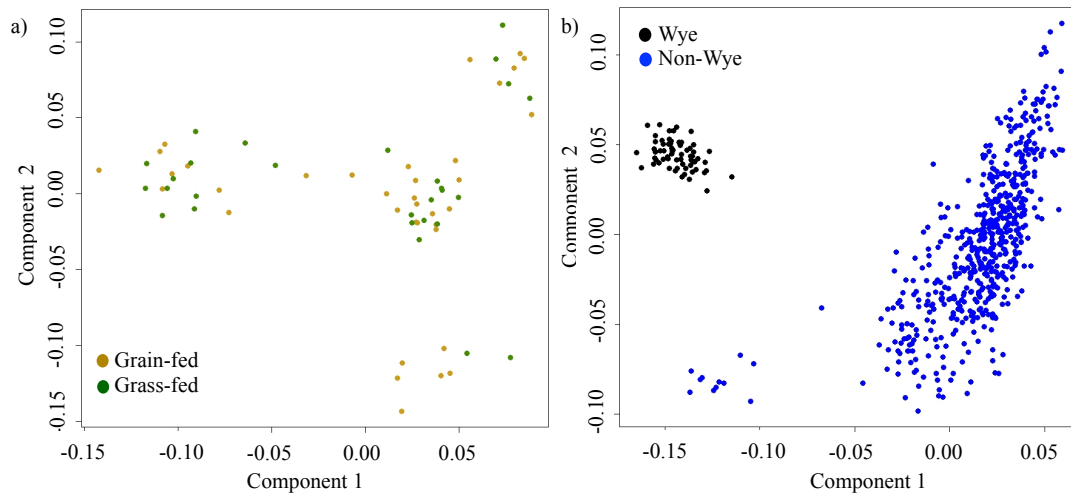


Figure 2.1 Principal component analysis grass-fed against grain-fed beef.
a) PCA in grass and grain-fed Wye Angus steers employing 58K SNPs b) PCA obtained after adding 487 closed Angus individuals selected for minimized influence of MHC, using approximately 20K shared SNPs.

Figure 2.1b, represents the result of the same PCA analysis but including 487 closed Angus animals selected for minimized influence of MHC over 16 years [156]. It demonstrates that Wye Angus animals are genetically very homogeneous, clustering tightly and separately from the new added individuals. This detail remains critical for performing epigenetic studies, which also is in our scope of interest.

Growth Curve

At weaning, the animals showed a random difference of 13 kg between groups with 174.5 and 187.5 kg for grain-fed and grass-fed steers respectively. After correction, the weaning weight adjusted at 205 days (WW205) was also significantly higher for grass-fed animals, averaging 218.1 kg against the 206.7 kg observed in grain-fed

individuals (p-value 0.009). The final weights for both groups were similar (459.6 kg for grass-fed and 472.4 kg for grain-fed steers, $P>0.05$). Nevertheless, the variation of weight at termination within grain-fed animals and grain-fed individuals was similar, both groups showed a standard deviation (SD) of approximately 35 kg. The maximum and minimum final weights with 564.7 kg at 286 days post-weaning and 392.8 kg at 448 days post-weaning correspond to a grain-fed and a grass-fed steer respectively. The animals that received grass were in average 644.1 days old at the moment of slaughter. Regarding the finishing period after weaning and considering four consecutive years, grass-fed animals needed 188 additional days to achieve the market requirement. Grain fed animals demonstrated around 68% higher average daily gain than the grass-fed counterpart, ranging from 0.889 to 1.406 kg. The behavior of the three most significant traits in Table 2.2 was expected, however similar final weights and distinct weaning weights adjusted at 205 days between animals from different feeding styles were unanticipated.

Table 2.2 Statistical descriptors of economic traits in grass and grain-fed Angus steers

Traits	Feed-Style	Min.	Median	Mean	Max.	p-value
Weaning Weight	Grain-Fed	135.6	173	174.6±21.28	224.1	0.0054
	Grass-Fed	137.4	186	187.5±21.83	244.9	
Weaning Weight 205	Grain-Fed	144.7	208.2	206.7±20.65	250.8	0.0095
	Grass-Fed	185.5	217	218.1±19.71	269.4	
Final Weight	Grain-Fed	402.8	467.2	471.1±36.49	564.7	0.1353
	Grass-Fed	392.8	452.7	459.6±35.87	544.3	
Age at termination (days)	Grain-Fed	394	459	456.5±24.31	499	3.54 ⁻¹⁶
	Grass-Fed	619	644	644.1±20.47	713	
Post-weaning days at termination	Grain-Fed	254	282	273.9±12.79	286	<2.2 ⁻¹⁶
	Grass-Fed	448	454	454.2±5.13	461	
Average daily gain	Grain-Fed	0.888	1.056	1.075±0.12	1.406	1.38 ⁻¹⁴
	Grass-Fed	0.453	0.635	0.639±0.09	0.861	

The growth curve for each feeding style in four consecutive years can be observed in Figure 2.2. The year effect is clearly observed; animals from the year 2011 and 2014 are at the top and bottom of both clusters correspondingly. However, the slopes of the curves within each feeding system are very similar. The shortest period of termination corresponds to the grain-fed steers in 2013 with only 254 days after weaning. Interestingly, the grass-fed group from the same year shows the longest finishing period with 448 days post-weaning.

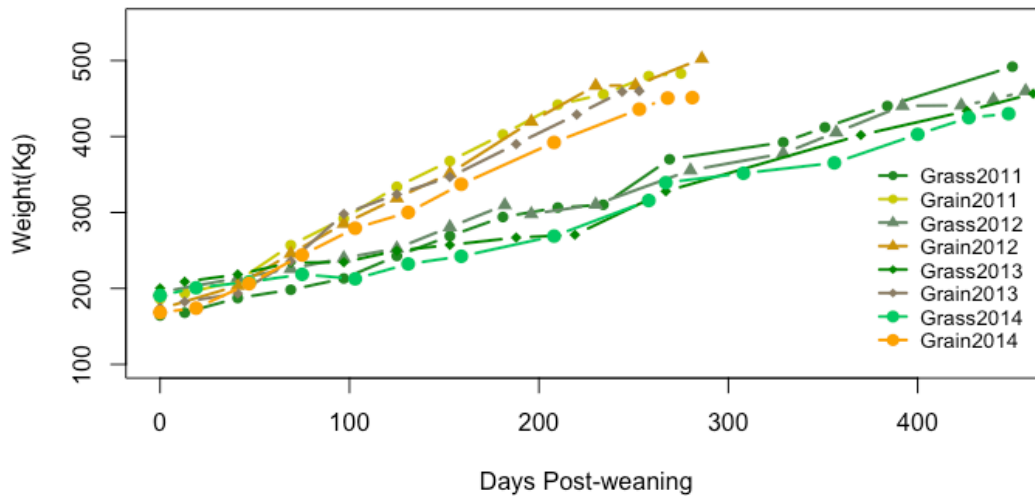


Figure 2.2 Growth curves of grass and grain fed Angus steers in four consecutive years

The result of a linear model fitting $Y = \beta_0 + \beta_1(X) + \beta_2(Z) + \beta_3(XZ) + e$; indicates an interaction (p-value $< 2^{e-16}$) between feeding style (X) and age of animals (Z), thus both factors should be considered in the model for maximizing the explanation of variation in the population ($R^2 = 0.92$). Figure 2.3 shows the individual measurements of body weight from weaning to termination and the curves obtained from an individual simple linear regression fitting for each feed type. The intercept and coefficient were 171.32 and 1.140 for the grain-fed group, and 174.78 and 0.614 for

the grass-fed group respectively. The fitted simple linear regressions extend from 0 to 286 and 461 days post-weaning for the grain and grass-fed clusters correspondingly.

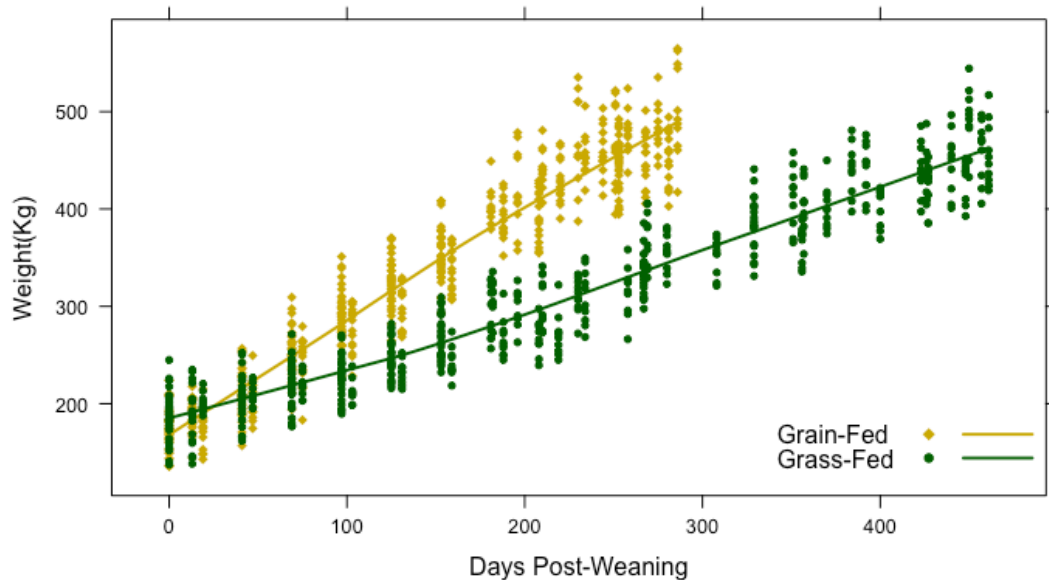


Figure 2.3 Individual body weights measured at different time points and the corresponding regression lines for grass and grain fed Angus steers

Carcass traits

We found the carcass weight in grain-fed animals was 290.3 kg, which was 35.6 kg heavier than the carcasses from grass-fed individuals. Interestingly, the weight of the carcasses was fairly homogeneous within both groups, showing a similar standard deviation of approximately 23 kg. The dressing of steers ranged from 51.1 to 66.5 and 58.8 to 65.8 percent in grass and grain finished animals correspondingly. Overall, the dressing of animals that received grain was more homogeneous and 6.4% higher than the grass-fed individuals. Regarding back fat, grain-fed carcasses presented 0.282 inches (0.71 cm) more sub-cutaneous fat than carcasses from grass-fed animals. The maximum back fat measured in grass-fed individuals corresponded to 0.5 inch (1.27 cm), contrasting with the maximum of one inch (2.54 cm) observed in a 444

days-old grain-fed steer. The results from the carcass traits evaluation are summarized in Figure 2.4.

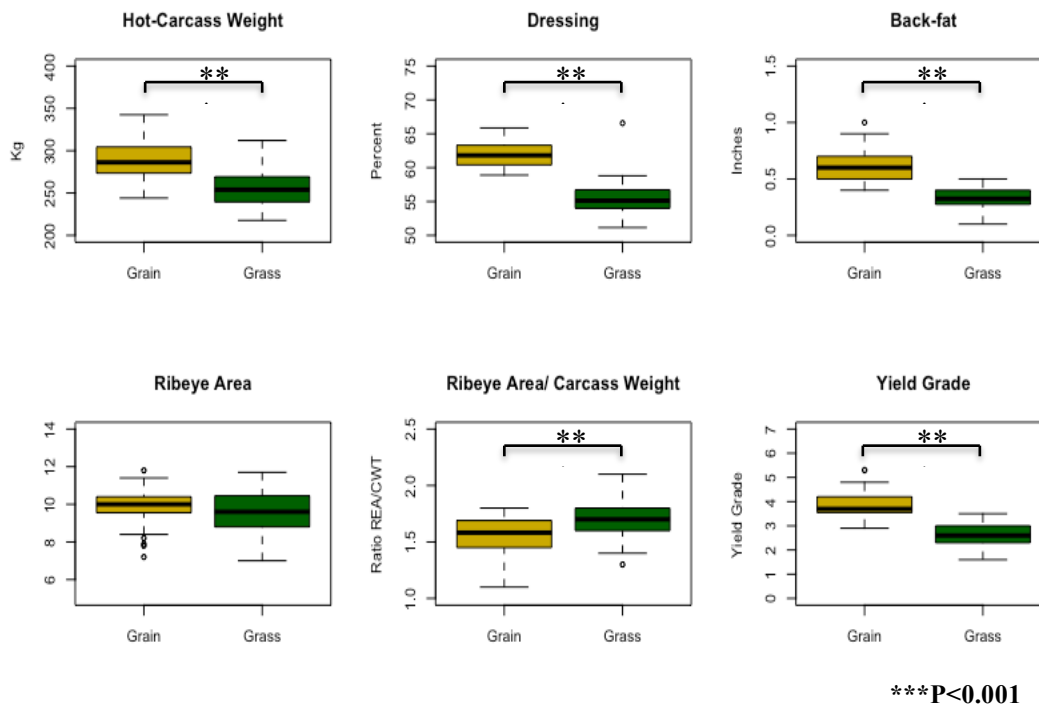


Figure 2.4 Carcass traits evaluation in Angus steers under different feeding systems

Interestingly, the ribeye area was similar for both groups (p-value 0.41); the minimum value was 7 in² for a grass-fed steer and the maximum score corresponded to a grain-fed animal with 11.8 in². However, giving heavier grain-fed carcasses and similar ribeye area in both groups, the ribeye area/carcass weight ratio was higher for grass-fed carcasses, with a mean of 1.7 ± 0.20 against the 1.54 ± 0.16 average ratio observed in the grain-fed cluster.

Carcasses from grass-fed animals showed lower yield grades, which generally were 1.22 points lower than the grain-fed carcasses that averaged 3.86 ± 0.58 . As a reminder, USDA yield grades are ranked numerically from 1 to 5. Yield grade 1

represents the highest yielding carcass and Yield grade 5, the lowermost. Considering quality grades (Figure 2.5), a larger proportion of grain fed carcasses (30 of 40) were classified within Choice, comparing to the fraction of grass fed carcasses (29 of 40) that were assigned to inferior Select categories ($p < 1.185^{-05}$).

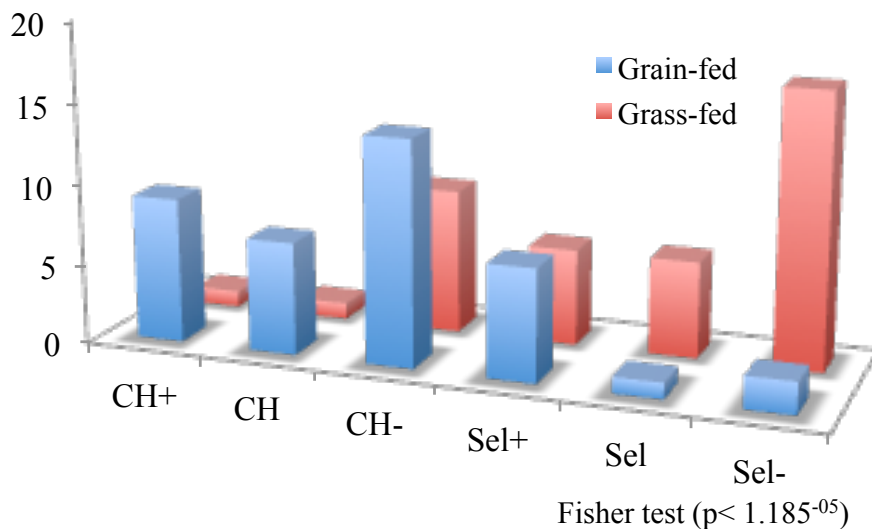


Figure 2.5 Quality Grade of carcasses regarding feeding regimen

Warner-Bratzler Shear Force

The analysis of measurements acquired from the Warner-Bratzler shear force test, indicates no difference in tenderness of beef from animals receiving grass or grain diet from weaning to termination ($p > 0.25$). However, longissimus dorsi muscles from grass-fed animals were slightly tender than the grain-fed individuals, requiring 0.16 less kg force to cut the samples (Figure 2.6). The necessary force to cut the grain-fed strips was 2.27 kgf in average. Generally, the steaks from both groups were fairly tender, with a WBS maximum value of 3.39 and 3.44 for the grain and grass-fed group respectively

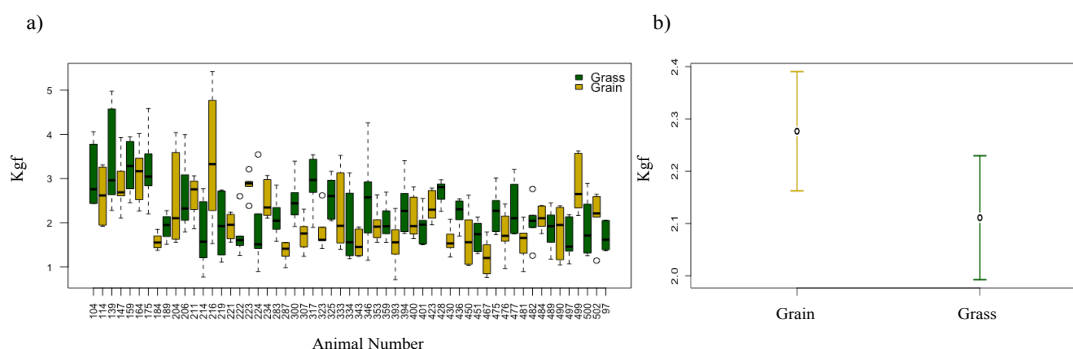


Figure 2.6 WBSF measurements in grass-fed and grain-fed beef
a) WBSF by animal and feeding style; b) Means of WBS in grain and grass-fed *longissimus dorsi* muscle

Blood analysis

The routine laboratory blood analysis yielded normal values for all parameters and showed no difference between animals under grass or grain diet, except for carbon dioxide (CO₂) and glucose with a corresponding p-value of 0.04 for both components (Table 2.3)

Table 2.3 Blood parameters in grain-fed and grass-fed animals

Test	Grain	Grass	Unit	p-value
White Blood Cells	11.13±2.13	8.35±4.58	x10 ³ cells/uL	0.25
Neutrophils	3.23±1.59	2.48±0.50	x10 ³ cells/uL	0.34
Lymphocytes	6.68±1.52	6.61±1.41	x10 ³ cells/uL	0.94
Monocytes	0.54±0.18	0.49±0.09	x10 ³ cells/uL	0.63
Eosinophils	0.47±0.49	0.45±0.20	x10 ³ cells/uL	0.92
Basophils	0.11±0.02	0.08±0.02	x10 ³ cells/uL	0.06
Large Unstained Cells	0.08±0.06	0.03±0.01	x10 ³ cells/uL	0.11
% Neutrophils	28.60±9.67	24.60±3.95	%	0.41
% Lymphocytes	60.36±10.16	65.00±5.15	%	0.38
% Monocytes	4.84±1.12	4.90±0.91	%	0.92
% Eosinophils	4.42±4.50	4.34±1.83	%	0.97
% Basophils	1.06±0.42	0.67±0.37	%	0.16
% Large Unstained Cells	0.72±0.39	0.36±0.16	%	0.09
Red Blood Cells	8.19±0.49	8.29±0.36	x10 ⁶ cells/uL	0.72
Hemoglobin	12.34±1.71	12.58±1.00	g/dL	0.79
Hematocrit	34.38±4.71	34.84±2.62	%	0.85
Mean Corpuscular Volume	41.88±4.26	42.02±2.74	fL	0.95
Mean Corpuscular Hemoglobin	15.04±1.54	15.20±1.06	pg	0.85
Mean Corpuscular Hemoglobin Concentration	35.92±0.31	36.14±0.23	g/dL	0.24
Red Cell Distribution Width	18.62±0.67	19.44±0.88	%	0.13
Platelets	510.60±134.34	384.20±187.40	x10 ³ cells/uL	0.25
Mean Platelet Volume	5.94±0.27	6.98±2.36	fL	0.35
CO ₂	21.94±2.78	25.38±1.69	mmol/L	0.04
Glucose	101.60±15.20	85.00±3.87	mg/dL	0.04

Discussion

In this study, we evaluated the grass-fed beef production in steers from a closed herd to minimize the genetic effect while maximizing the diet effect in the results. The nutritional analysis (Table 2.1) confirms our expectation regarding nutrients disparities and quantifies the correspondent differences between diets. It is well known that nutritional requirements change with age and physiological stage of the animal, however the periodical adjustments made in highly efficient production systems for increasing gain is difficult to achieve in grass-fed regimens. The nutrient composition of grass depends on many factors such as pasture species and habits, developmental stage of grass, season, region and climate conditions, among others. Therefore, our study comprises four cycles of production in consecutive years, following the same treatment from birth to termination to assess some of these possible sources of variation.

As expected, the big differences between diets were crude protein, fiber and energy content. High dietary protein level is critical for development and early growth, however high energy level is particularly important during finishing period. Those differences are reflected in longer termination periods for grass-fed individuals, ranging from 448 to 461 days in comparison with the 254 to 286 days post-weaning required by the grain-fed steers to achieve similar final weights). The average daily gain for grass-fed steers was 0.639 kg, a reasonable value comparing to the 0.490 kg found in a study using several breeds but much lower than the 1.04 kg reported in Bonsmara steers [157, 158]. Animals that received grain gained 0.440 kg more weight daily than its grass-fed counterpart, a value comparable to a previous found

difference of 0.480 kg [157]. The reason for the differentially ADG between grass and grain-fed systems could be mainly related to the level of energy supplied in the feed and the energy expenditure that animals experienced in both conditions [159]. Normally, grass-fed animals are more exposed to adverse climate conditions, which could interfere the period of grazing and increase the energy demand for thermo regulation, limiting the amount of nutrients for body growth.

Interestingly, despite total randomization of animals, the adjusted weaning weight at 205 days differed between feeding styles; grass-fed group was in average 11 kg heavier than grain-fed individuals. This involuntary aspect should be considered during inference. However, the results of this study are still robust and provide valuable information for the beef industry and especially for beef producers.

Generally the market defines the final weight of animals; therefore we adjusted our experiment to the reality of beef production. Animals were sent for termination when they reached a satisfactory weight and upon slaughterhouse requirement and schedule. Although the final weight was similar for both groups (p-value 0.13), a slight difference of 12 kg was favorable for the grain-fed steers. At the moment of termination, grass and grain-fed animals averaged 644 and 456 days old respectively, meaning that grass-fed animals generally need extra 188 days for finishing.

The effect of year is notorious in Figure 2.2; 2014's animals were the inferiors within their correspondent groups, suggesting that conditions during this period of time affected negatively the animals' performance. Nevertheless it is also clear that year effect was similar in both groups, implying no interaction between feeding style and year effect. However, the linear regression model indicated an interaction between

feed and days post weaning thus individual regression lines were fitted for both groups. For the two clusters, the growth was linear and constant from weaning to termination; similar trend was previously observed in Bonsmara grass-fed steers in a 2-year period, in grass-fed tropical-breed steers and in grass-fed buffalo as well [158, 160, 161]. Displaying similar deviations from the fitted line, growth curves from both conditions were fairly homogeneous. Alternatively, other more complex models could increase the accuracy of prediction but the almost perfect linearity provides a simple method to estimate growth for practical uses and particularly for management decisions.

Regarding carcass traits, six of the eight explored characteristics were different between animals that received grass or grain. Grain-fed animals produced heavy carcasses (290.25 kg) with 36 kg more than grass-fed steers. Accordingly, a review of nine publications including several cattle breeds concluded that grass-fed animals produce lighter carcasses than grain-fed individuals [38]. Dressing reflects the amount of carcass regarding the animal's live weight thus grain-fed dressing was 6.45% higher than grass-fed, constituting a relevant economic difference for beef production. Accordingly, a similar value of 54.2 %, compared to the 55.4% observed in this study, was previously reported in grass-fed tropical breeds [161]. Since the slaughter process constitutes a constant cost regardless the weight of animals, high dressing score is highly appreciated in the beef industry.

Grain-fed carcasses with 0.626 in (1.59 cm) had 1.8 fold more back fat than grass-fed carcasses with 0.343 in (0.87 cm). This variation remains close to the 2-fold change reported earlier in a study that mentioned the positive association of subcutaneous fat

with sarcomere length and negative correlation with shear force. Additionally, the same authors suggest that fatter carcasses endure less shortening of muscle fibers and promote enzymatic proteolysis during the progression of rigor mortis [162]. However the increasing of subcutaneous fat beyond 10.2 mm (0.40 in) did not affect sarcomere length, consequently would not contribute for enhancing beef tenderness [162]. Another study suggested that thinner layer of subcutaneous fat in steaks would allow easier access of heat during cooking, reducing cooking time and incrementing cooking loss, affecting beef tenderness [163].

Interestingly, the ribeye area in carcasses from different diet regimens showed no difference with values of 9.85 (63.54 cm²) and 9.60 sq inch (61.93 cm²) for grain and grass-fed respectively, contrasting with another result that reported a similar value of 61 sq in (61 cm²) for grass-fed carcasses but different area of 71 sq in (11 cm²) for grain-fed carcasses [162]. A speculation for the reported differences is that animals receiving grain use the abundant energy to put on lean tissue to the limit of their genetic potential, with the excess going to fat tissue formation. Therefore, the similarity of ribeye area observed in our study could be explained by the genetic resemblance of animals and that both groups received satisfactorily the least energy required to achieve an optimum lean tissue deposition.

The ribeye area/carcass weight ratio diverged between both feed regimens. After knowing the divergence of carcass weight and the similarity of ribeye area, this result is evident. Nonetheless, it is important to consider that lighter grass-fed carcasses produce similar amount (measured in area) of highly valuable cuts (steaks) compared

to carcasses from grain-fed steers. This is particularly relevant for high quality grass-fed beef production, which most of its revenue depends on steaks trading.

The average Yield Grade for grain-fed carcasses was 3.86 compared to the 2.64 value assigned to grass-fed carcasses. Interestingly, a previous study reported no significant difference between grain and grass finished carcasses with 2.4 and 2.1 yield grades correspondingly [162]. Another study found a better yield grade of 1.4 in Angus x Hereford grass-fed steers that were employed as control and never received any supplement [164]. Yield grades assess the total amount of boneless, closely trimmed retail cuts from the carcass, however the evaluation mainly considers the high-valuable parts of the carcass, as the round, loin, rib and chuck. A low Yield Grade is preferred because it indicates higher cutability than larger numbers, therefore grass-fed steers produce carcasses with better percentage of BCTRC than grain-fed individuals, demonstrating that grass-fed beef production could increase revenue, and particularly the benefits generated through the commercialization of high-valuable cuts.

However, a recent study using molecular breeding values suggests that increasing genetic potential for yield grade was unlikely to improve general carcass quality given its large detrimental effect on quality grade. For instance, concurrent genetic improvements for yield grade and marbling constitute a more appropriate strategy to achieve better overall carcass quality [165]. We also observed that although grass-fed carcasses showed better yield grades, their quality grades were generally inferior to its grain-fed counterpart; 75% of grain-fed carcasses were classified as Choice while only 27.5% of grass-fed carcasses received the same grade (Figure 2.5). Furthermore,

only 2.5% of grass-fed carcasses reached the maximum score observed in this study (CH+) compared to the 22.5% of grain-fed carcasses that were classified as CH+. Contrarily, 42.5% of grass-fed carcasses received the worst grade attained (Sel-), against only 5% of grain-fed carcasses with the equivalent score. These observations concerning quality grade suggest that high quality grass-fed beef production is feasible but not easy to succeed. Therefore beef producers should evaluate the economic consequences of transforming conventional to grass-fed systems, especially considering whether the extra compensation for “green” products counterbalances the loss of “standard” quality with the correspondent economic punishment.

Regardless consumers’ tradition or habits, tenderness always has been central for beef quality judgment and became a common desirable characteristic across consumers from different cultures. We found no difference in WBS between different diet types, however the grass-fed shear force was slightly lower than the grain-fed value, with their correspondent score of 2.11 and 2.27 kgf. This finding antagonizes previous studies that reported lower WBS values for grain-fed compared to forage-fed carcasses, however the shear forces obtained in our study for both groups were low compared to others [41, 162]. As mentioned before, subcutaneous fat could enhance less shortening of muscle fibers and increase post-mortem enzymatic proteolysis, determining beef tenderness, nevertheless we found different back-fat values but similar tenderness measures in both groups, suggesting than other factors such as genetics or epigenetics could be critical for tenderness of beef. Genetically similar animals present in both feed styles were still very young at the moment of slaughter;

therefore we speculate that the homogeny of tenderness was sustained by the genetic makeup and the lack of enough time for the age factor to manifest.

Conclusion

Grass-fed steers showed a distinct growth curve compared to its grain-fed counterpart, requiring around 180 extra days to reach similar terminal weight. The average daily gain of grain-fed animals (1.075 kg) was approximately 70% higher than grass-fed individuals. Grass-fed steers produce lighter carcasses, with reduced subcutaneous fat and less dressing than grain-fed individuals. Interestingly, while the tenderness of beef was comparable in both groups, the quality grade was better for grain-fed animals, indicating risk to produce really tender grass-fed beef that will not be recognized as high-value meat under the traditional evaluation system.

3. Integrated metabolomic and transcriptome analyses reveal finishing forage affects metabolic pathways related to beef quality and animal welfare

Abstract

Beef represents a major dietary component and source of protein in many countries. With an increasing demand for beef, the industry is currently undergoing changes towards naturally produced beef. However, the true differences between the feeding systems, especially the biochemical and nutritional aspects, are still unclear. Using transcriptome and metabolome profiles, we identified biological pathways related to the differences between grass- and grain-fed Angus steers. In the *latissimus dorsi* muscle, we have recognized 241 differentially expressed genes (FDR<0.1). The metabolome examinations of muscle and blood revealed 163 and 179 altered compounds in each tissue (P<0.05), respectively. Accordingly, alterations in glucose metabolism, divergences in free fatty acids and carnitine conjugated lipid levels, and altered β -oxidation have been observed. The anti-inflammatory n3 polyunsaturated fatty acids are enriched in grass-finished beef, while higher levels of n6 PUFAs in grain finished animals may promote inflammation and oxidative stress. Furthermore, grass-fed animals produce tender beef with lower total fat and a higher omega3/omega6 ratio than grain-fed ones, which could potentially benefit consumer health. Most importantly, blood cortisol levels strongly indicate that grass-fed animals may experience less stress than the grain-fed individuals. These results will provide deeper insights into the merits and mechanisms of muscle development.

Introduction

In many parts of the world, beef constitutes a main dietary component and a major source of protein. Beyond amino acids, beef also provides abundant energy and essential vitamins and minerals such as iron, which is frequently deficient in the human diet [2, 166]. The beef industry in developed countries is currently undergoing changes and is facing increased demands especially for natural meat [18]. This transformation is partly driven by the public concern regarding the environmental impacts of intensive livestock production, animal welfare, meat quality, and the flavor of beef [167, 168]. Recently, consumers have preferred naturally-raised organic beef because they consider it to be safer and higher quality as this type of beef is obtained without using antibiotics or extrinsic hormones. Compared to its grain-fed counterpart, grass-fed beef is lower in total fat content and richer in omega-3 fatty acids, characteristics which could be more beneficial for human health [39, 167]. As more humane husbandry becomes an integral part of quality assurance programs for consumer-accepted sustainable animal production [169], such changes will also affect other production components like animal health, breeding, production management, downstream processing, meat quality, nutrient value and environmental impact.

As we know that animal growth, health, and meat quality are controlled by genetic and environmental factors. Considering diet regimen as a nutritional welfare variable, it influences the animal as a whole entity, affecting nutrient assimilation, disease resistance and behavior. Consumers often postulate that raising animals entirely on an all forage diet might be healthier for the animals as it is closer to the environment in which they have evolved. The general public understands that grain finished systems

seem unnatural [166]. Although many reports claim that grass finishing is healthier and better for the environment, there is conflicting information regarding the benefits of grass-fed beef [170-172]. Unfortunately, we found very little research quantifying the effects of natural production on fatty acid profiles, meat quality and animal welfare. On animal production, there is a severe lack of solid evidence that beef from grass-fed animals have these advantages, which further prevents animal producers interested in meeting the demand for grass finished beef from making fully informed management decisions.

Beef quality and behavior traits also share the property of being concurrently regulated by gene sets. The identification of genes that control critical beef quality traits, such as tenderness, fatty acid content, and marbling will help to unveil the regulatory networks and physiological mechanisms behind these clusters. Several reports suggest that gene expression would be mediated by environmental factors at the transcriptional and translational levels in different species [173, 174]. For example, dietary polyunsaturated fatty acids (PUFA's) dramatically affect the expression of genes, regulating four families of transcription factors: sterol regulatory element binding proteins (SREBPs) 1 and 2, liver X receptors (LXRs), hepatic nuclear factor-4 (HNF-4) alpha and peroxisome proliferator activated receptor (PPAR) alpha, beta and gamma [175]. These transcription factors play major roles in fatty acid, cholesterol, triglyceride, hepatic carbohydrate, and bile acid metabolism, which demonstrate a complex interaction between diet, genotype and gene activity, and the diverse mechanisms of action of dietary small bioactive components [176-178]. The major approaches in metabolomic studies include mass spectrometry–

(MS) and nuclear magnetic resonance (NMR)–based techniques. Detection of metabolites using MS-based techniques comprises several methods including gas chromatography (GC), high performance liquid chromatography (HPLC), and capillary electrophoresis (CE). The high throughput metabolomics technologies allow for the assessment of global low-molecular-weight metabolites and show great potential in biomarker discovery [179, 180]. Such studies will help to identify biologically meaningful metabolic networks that control cellular responses to environmental factors, including diet [181, 182]. To date, few studies have investigated the nutritional regulation of the whole metabolome; therefore, the characterization of metabolite levels in beef should provide a unique opportunity to ascertain ‘metabolic signatures’ or ‘molecular hubs’ as biomarkers related to beef quality and different metabolomics platforms.

Metabolomic studies systematically assess the unique chemical elements produced during specific cellular processes by describing the metabolites profiles of individuals that undergo similar biological processes and transformations [183]. The fingerprint of a product could be characterized by its metabolites content [184]. The quality or origin of a food is basically determined by its biochemical components, which also will define the metabolites profile. RNA-Sequencing (RNA-Seq) analysis interrogates the level of mRNA from an organism to estimate its gene expression profile at a given moment. Identification of active and repressed genes in different regimens would lead to the discovery of the biological pathways affected by these functional differences. Accordingly, the aim of this study is to characterize molecular signatures from cDNA expression and metabolomics in blood and muscle originated from grass-

fed and grain-fed steers to ascertain pathways and functional differences and to identify the differences in meat quality with implications for public health and animal welfare. Additionally, the discovery of involved pathways would benefit the meat industry in producing healthier and higher quality products.

Material and Methods

Animals

The steers came from a closed Wye Angus herd with very similar genetics. The grass-fed group was comprised of steers that received alfalfa and orchard grass hay, clover and orchard grass pasture, or orchard grass and alfalfa pasture. The grass-fed individuals consumed grazed alfalfa upon availability and bales during winter, so they were not exposed to any corn, any form of grain, or any form of feed by-products. The alfalfa and grass hay were harvested from land that has had minimal fertilizer and no application of pesticides or inorganic chemicals. The control group was fed a conventional diet consisting of corn silage, soybean, shelled corn and minerals. The pastures were managed without fertilizers, pesticides, or any chemical additives. In order to demonstrate the nutritional differences between diets, a sample from each regimen was collected in two consecutive years. The analytical results summarized in columns for both diets (Table 2.1) reflect the averaged value from those two years estimated for each individual parameter. When the animals reached the required market weight, they have been accordingly prepared, weighted and shipped for termination. To avoid any extrinsic variation during all these processes—from the previous day at the farm to the moment of sacrifice—, both groups were treated

similarly. Animals were shipped late afternoon (around 5:00 pm) the date before and were fasted but with free access to water until the moment of termination. We considered that season of slaughter could influence some results—especially the functional—, but we reproduced the time frame that grass-fed animals need to achieve the weight determined by the market. At the slaughter plant, 10 ml whole blood sample from the jugular vein was collected in EDTA tubes and directly stored at -80°C. Blood collection for both groups were performed immediately before slaughtering; the environmental condition and time of collection were similar for the two groups (approximately between 6:30-9:30 am). Immediately after termination, a small piece of *longissimus dorsi* muscle was obtained from each hot carcass at the level of the 12th intercostal space and immediately frozen in dry ice for posterior analysis. Commercially, the *longissimus dorsi* muscle is highly valuable and constitutes a reference for beef quality studies, allowing reasonably comparison of different results among them. All animal experiments were conducted following NIH guidelines for housing and care of laboratory animals and in accordance with The University of Maryland at College Park (UMCP) regulations after review and approval by the UMCP Institutional Animal Care and Use Committee (permit number R-08-62).

Warner-Bratzler Shear Force

Immediately after slaughter, carcasses were deposited and maintained in 4°C chambers for maturation during 14 days. Then, steaks of one inch (2.54 cm) from the *longissimus dorsi* muscle were cut at the 12th intercostal space, vacuum packaged and immediately frozen at -20°C. For the Warner-Bratzler Shear Force assessment,

the steaks were firstly thaw at 4°C and then cooked using a George Foreman grill to a core temperature of 70°C. To measure the internal temperature of the steaks, an Oakton digital thermometer (Temp JKT Acorn series) was employed. After the samples cooled down to room temperature, six cores of tissue of 1.27 cm in diameter and parallel to the fibers were obtained employing a hand-held coring device. The Warner–Bratzler shear force (WBSF) was determined for each core utilizing an Instron #5442 Test Machine (Norwood, MA). The final WBSF value was calculated averaging the measurements of six cores from the same steak.

Metabolomic and Metabolite analyses

Samples description and preparation

Global biochemical profiles were determined from whole blood and muscle tissue in eight individuals from each group: the grain-fed group displayed a younger age (range 443-490 days) than the grass-fed group (range 619-667 days). Metabolomic profiling analysis was performed by Metabolon as previously described [185]. Each sample received was accessioned into the Metabolon LIMS system and was assigned a unique identifier by the LIMS that was associated with the original source identifier only. All samples were maintained at -80 °C until processed. Samples were prepared using the automated MicroLab STAR[®] system from Hamilton Company. A recovery standard was added prior to the first step in the extraction process for QC purposes. Sample preparation was conducted using aqueous methanol extraction process to remove the protein fraction while allowing for maximum recovery of small molecules. The resulting extract was divided into four fractions: one for analysis by

UPLC/MS/MS (positive mode), one for UPLC/MS/MS (negative mode), one for GC/MS, and one for backup. Samples were placed briefly on a TurboVap[®] (Zymark) to remove the organic solvent. Each sample was then frozen and dried under vacuum. Samples were then prepared for the appropriate instrument, either UPLC/MS/MS or GC/MS.

Ultra-high Performance Liquid Chromatography / Mass Spectroscopy (UPLC/MS/MS)

The LC/MS portion of the platform was based on a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo-Finnigan linear trap quadrupole (LTQ) mass spectrometer, which consisted of an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. The sample extract was dried and then reconstituted in acidic or basic LC-compatible solvents, each of which contained 8 or more injection standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic positive ion optimized conditions and the other using basic negative ion optimized conditions in two independent injections using separate dedicated columns. Extracts reconstituted in acidic conditions were gradient eluted using water and methanol containing 0.1% formic acid, while the basic extracts, which also used water/methanol, contained 6.5mM ammonium bicarbonate. The MS analysis alternated between MS and data-dependent MS² scans using dynamic exclusion. Raw data files were archived and extracted as described below.

Gas Chromatography / Mass Spectroscopy (GC/MS)

The samples destined for GC/MS analysis were re-dried under vacuum desiccation for a minimum of 24 hours prior to being derivatized under dried nitrogen using bistrimethyl-silyl-trifluoroacetamide (BSTFA). The GC column was 5% phenyl and the temperature ramped from 40° to 300° C in a 16 minute period. Samples were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization. The instrument was tuned and calibrated for mass resolution and mass accuracy on a daily basis. The information output from the raw data files was automatically extracted as discussed below.

Quality Assurance / Quality Control

For QA/QC purposes, additional samples were included with each day's analysis. These samples included extracts of a pool of well-characterized human plasma, extracts of a pool created from a small aliquot of the experimental samples, and process blanks. QC samples were spaced evenly among the injections and all experimental samples were randomly distributed throughout the run. A selection of QC compounds was added to every sample for chromatographic alignment, including those under test. These compounds were carefully chosen so as not to interfere with the measurement of the endogenous compounds.

Data extraction and compound identification

Raw data was extracted, peak-identified and QC processed using Metabolon's hardware and software. Compounds were identified by comparisons to library entries of purified standards or recurrent unknown entities. Metabolon maintains a library

based on authenticated standards that contain the retention time/index (RI), mass to charge ratio (m/z), and chromatographic data (including MS/MS spectral data) on all molecules present in the library. Furthermore, biochemical identifications are based on three criteria: retention index within a narrow RI window of the proposed identification, nominal mass match to the library ± 0.2 amu, and the MS/MS forward and reverse scores between the experimental data and authentic standards. The MS/MS scores are based on a comparison of the ions present in the experimental spectrum to the ions present in the library spectrum. While there may be similarities between these molecules based on one of these factors, the use of all three data points can distinguish and differentiate biochemical. More than 3,500 commercially available purified standard compounds have been acquired and registered into LIMS for distribution to both, the LC and GC platforms for determination of their analytical characteristics. Poly unsaturated fatty acids mentioned in Table 3.1 were identified from beef samples following this approach

Table 3.1 Polyunsaturated Fatty acids in beef

Biochemical	Mean Grain*	Mean Grass*	Fold Change	p-value	q-value
eicosapentaenoate (EPA; 20:5n3)	0.6103	1.9478	0.31	0.0000	0.0001
docosapentaenoate (n3 DPA; 22:5n3)	0.7342	1.3533	0.54	0.0034	0.0043
docosahexaenoate (DHA; 22:6n3)	0.7733	1.3363	0.58	0.0013	0.0023
linoleate (18:2n6)	1.0001	0.8906	1.12	0.5070	0.1831
linolenate [alpha or gamma; (18:3n3 or 6)]	0.4920	2.0852	0.24	0.0000	0.0000
dihomo-linolenate (20:3n3 or n6)	1.2911	0.7161	1.80	0.0050	0.0055
arachidonate (20:4n6)	1.5782	0.7931	1.99	0.0095	0.0092
adrenate (22:4n6)	1.0393	0.9055	1.15	0.3768	0.1484
docosapentaenoate (n6 DPA; 22:5n6)	2.2328	0.5677	3.93	0.0001	0.0004
dihomo-linoleate (20:2n6)	1.2098	0.9403	1.29	0.6033	0.2092
mead acid (20:3n9)	1.7035	0.8769	1.94	0.0541	0.0343

***Units of measure correspond to relative mass spectrometry intensities measured in equivalent amounts of muscle tissue.**

Statistical Analysis

Missing values (if any) were assumed to be below the level of detection. However, biochemicals that were detected in all samples from one or more groups but not in samples from other groups were assumed to be near the lower limit of detection in the groups in which they were not detected. In this case, the lowest detected level of these biochemicals was imputed for samples in which that biochemical was not found. Following log transformation and imputation with minimum observed values for each compound, Welch's two-sample t-test was used to identify biochemicals that differed significantly between experimental groups. Pathways were assigned for each metabolite, allowing examination of overrepresented pathways.

Gene Expression Analysis

mRNA Extraction and RNA-Seq Library Preparation

The mRNA was extracted directly from muscle samples employing the Oligotex Direct mRNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. For synthesis of the first cDNA, SuperScript II Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA) with random hexamers (Invitrogen, Carlsbad, CA) was applied in a final volume of 40 μ l. A combination of Polymerase I (NEB, Ipswich, MA), 2 U of RNase H (Invitrogen) and 30 nmol of deoxyribonucleotides, followed by a 2.5 hour of incubation at 25°C, was used to generate the second cDNA strand. For an optimal quality, QIAquick PCR purification kit (Qiagen) was employed to enrich the PCR products. Index libraries were assembled utilizing Multiplex Sample preparation Oligonucleotide Kit (Illumina, San Diego, CA) complying with the

provided instructions. After, 50 µl of the purified samples was placed in a Bioruptor sonicator (Diagenode, Denville, NJ) for 40 min, obtaining double cDNA fragments of approximately 200 to 500 bp. The yielding fragments were repaired employing the end repair module (NEB) while the subsequent 3'A addition was achieved by Klenow Fragment (NEB). T4 DNA ligase (NEB) generated the ligation of the Solexa adaptors (Illumina) to the double-stranded cDNA fragments. The amplification step comprised the use of index and adaptor primers during 18 PCR cycles. The resulted PCR products with a length of 200 to 500 bp were filtrated by QIAquick Gel purification kit (Qiagen). Finally, clustering and sequencing was performed on the Hi-Seq 2000 (Illumina) platform.

RNA-Seq Data Analysis

An ultrafast, memory efficient short read aligner called Bowtie was used to align the sequenced reads to the BosTau6 (Bos_Taurus_UMD_3.1) reference genome, which has been downloaded from the UCSC Genome Bioinformatics site (<http://genome.ucsc.edu>)[186]. Data quality assurance, data manipulation and file format conversion have been achieved through the implementation of different programs: SAMtools, bedtools, HTSeq and the R based packages, ShortRead, Rsamtools and Biostrings [187-189]. The GenomicRanges and edgeR programs were employed for the statistical analysis (Bioconductor, <http://www.bioconductor.org>) [190, 191]. GenomicRanges reported the reads counts and edgeR performed the gene expression analysis, fitting a negative binomial distribution model and then applying a generalized linear model (GLM) likelihood ratio test. In this approach, the common, trended and tagwise variations were estimated, increasing the sensitivity of the

method to detect genuine and minimal differences, this facilitates posterior functional and pathway analyses. Additionally, 13 mitochondrial protein-coding genes were extracted from the RNA-Seq result to undergo a clustering analysis. This step was supported by previous outcomes from functional examinations that pointed out the importance of mitochondria activity in muscle [192, 193].

RNA-seq Validation

qPCR was employed for result verification (iCycler iQ PCR system, Bio-Rad, Hercules, CA). The qPCR reactions were performed in a final volume of 20 ul, employing the QuantiTect SYBR Green PCR Kit (Qiagen) following the manufacturer's instructions. For primer design, the NCBI tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi>) was used considering that all amplicons would embody at least one intron. Appendix *III* shows the primers sequences.

Pathway Analysis

Differentially expressed gene functions were annotated using the Ingenuity Pathway Analysis (IPA) tool (<http://www.ingenuity.com/products/ipa>). The program inquires the IPA Knowledge database for information and direct relationships of genes and endogenous chemicals. This creates algorithmically generated networks and clustering of the data into biological functions and diseases that are overrepresented in the scrutinized data. Resulting networks are created de novo based upon input genes, proteins or chemicals. The program also determines the high representation of signaling and biological pathways. The method employs the Fisher's exact test,

determining the proportion of genes mapped to a function or pathway in the sample and then compares it to the ratio in the reference set. Statistical similarity means no biological effect. The result consisted of functional networks, disease and disorders, molecular and cellular functions, physiological system development and function, top canonical pathways, upstream regulators, and top toxicity lists.

Integrated pathway-level analysis of transcriptomic and metabolomics data

The web-based Integrated Molecular Pathway-Level Analysis (IMPaLA) (<http://impala.molgen.mpg.de>) was employed for the joint pathway analysis of gene expression and metabolite concentration; it performed an over-representation analysis with the provided lists of genes and metabolites employing 11 databases, which included more than 3000 pre-annotated pathways [194]. The results list the pathways that may be altered at the transcriptional level, the metabolomic level, or both. This approach provides combined evidence of pathway deregulation and permits to identify biological pathways that would not have been distinguished by independent single functional level analyses. For this case, all entities present in the pathways were used as background lists. Then, a hypergeometric test determined the significance of each pathway based on its overlap with the uploaded lists. The result reports pathways that include at least one gene or metabolite from the input arrays. The table comprises of pathway name, source, size, and overlap with the provided entities, and p-values obtained with the appropriate statistical test for each pathway. The joint p-values have been calculated following a previously described method [195]. To control for multiple comparisons, the false discovery rate approach was

applied, assigning a q-value for every listed pathway [196]. By clicking on the pathway name, the user is directed to the source database and a summary for the correspondent pathway is displayed.

Results

Growth curve and beef tenderness with diet styles

The growth curve of grass- and grain-fed Angus steers is shown in Figure 3.1a. As expected from the difference in nutrients (mainly energy) provided by the feeding regimens, affected rate of gain between groups, the average daily gain (ADG) from weaning to termination was linear in both groups, but certainly displayed different slopes as grass-fed animals required approximately 200 additional days to reach minimum acceptable slaughter weight. The average final weights were 479.04 and 492.40 kg for the grain-fed and grass-fed categories ($p > 0.05$). According to the Warner-Bratzler Shear Force (WBS), diet type had no significant impact on beef tenderness ($p > 0.05$) (Figure 3.1b).

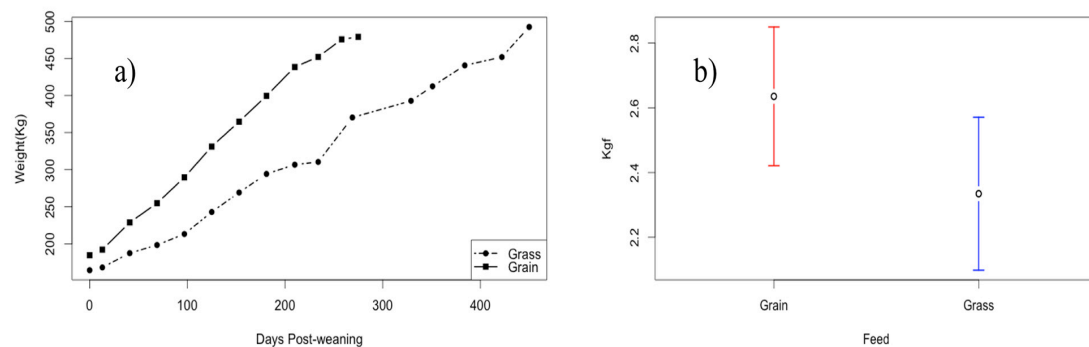


Figure 3.1 Growth curve and beef tenderness in different diets.

a) Body weights trajectories in grass-fed and grain-fed steers from weaning until termination (Grass, Grain: n=30 and 36, respectively) b) Warner-Bratzler Shear Force expressed in kilogram-force (kgf) in muscle obtained from grain-fed and grass-fed animals.

Animal welfare and feed regimen

Initially, blood samples from animals receiving grass or grain diet were analyzed using standard hematological parameters, plus carbon dioxide and glucose content (Table 2.3). We found that most parameters were similar in both groups except for carbon dioxide and glucose (Figure 3.2). However, the metabolome examination revealed that glucose and cortisol levels in blood differed significantly between animals from the two feeding systems (Figure 3.3).

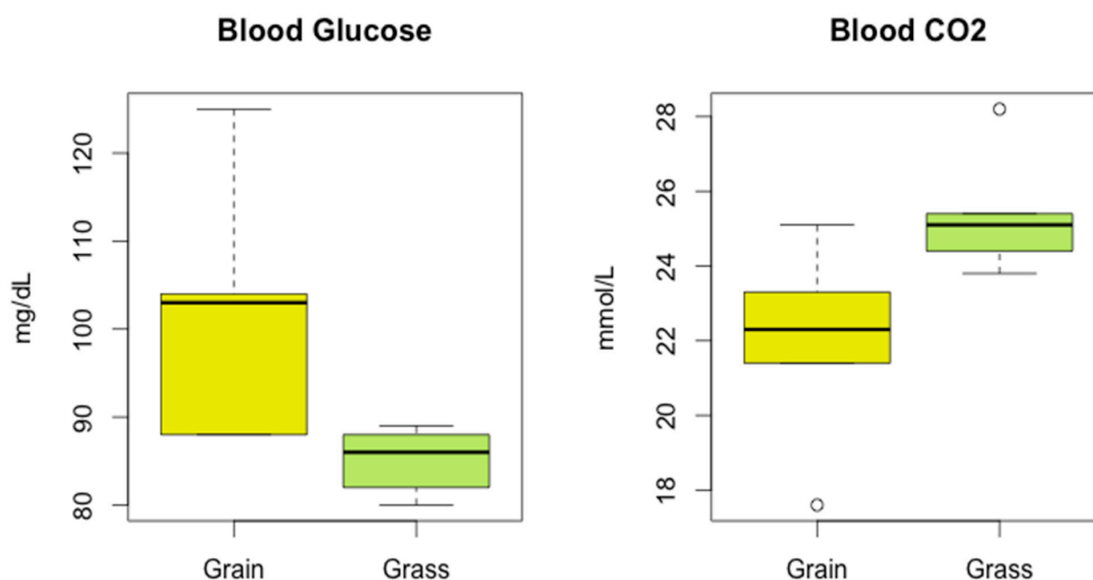


Figure 3.2 Glucose and CO₂ blood levels in grain- and grass-fed steers.

a) Blood glucose level obtained through conventional clinical analysis, represented in milligram per deciliter (mg/dL) (n=10 for each group); b) Carbon dioxide measured in blood and expressed as milimol per liter (mmol/L) (n=10 for each group)

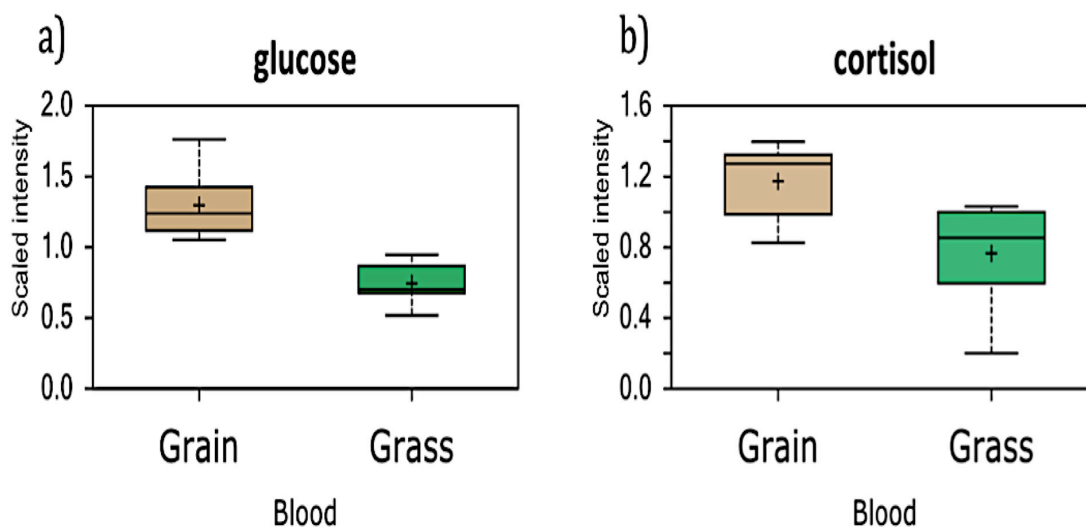


Figure 3.3 Glucose and cortisol metabolomics blood levels in grain and grass fed animals.

a) Glucose level measured in blood employing metabolomic approach (y axis represents mass spectrometry scaled intensity, n=8 for each group) **b)** Relative mass spectrometry scaled intensities of cortisol determined in blood of grass and grain fed steers (n=8 for each group)

Fatty acid content of beef and diet style

A variety of fatty acids are found in muscle but for its relevance in human health, we examined the polyunsaturated fatty acids (PUFA) concentration of beef (Table 3.1). Eleven members of this group were recognized and showed significant variation between groups of animals that received either grass or grain, suggesting that type of feed might influence the final concentration of PUFAs in beef. Fold change was calculated as grain/grass mean, implying that values < 1 represent more abundance of a biochemical in grass; contrarily, values > 1 mean higher compound concentration in grain samples. Interestingly, all omega 3, eicosapentaenoate, docosapentaenoate and docosahexaenoate, were significantly higher in grass-fed beef. The opposite was observed for linoleate, dihomolinenate, arachidonate, adrenate, docosapentaenoate, and dihomolinoleate, which happen to be omega 6 fatty acids.

The metabolomic analysis detected 131 lipid molecules that have been summarized in 20 sub pathways according to their properties and functions. Figure 3.4 shows that most groups—excepting fatty acid metabolism (acyl carnitine) and fatty acid, amide—averaged higher lipid intensity in muscle from grain fed individuals, suggesting greater total fat concentration in this type of beef based on the subset of total lipid population measured with this metabolomic approach.

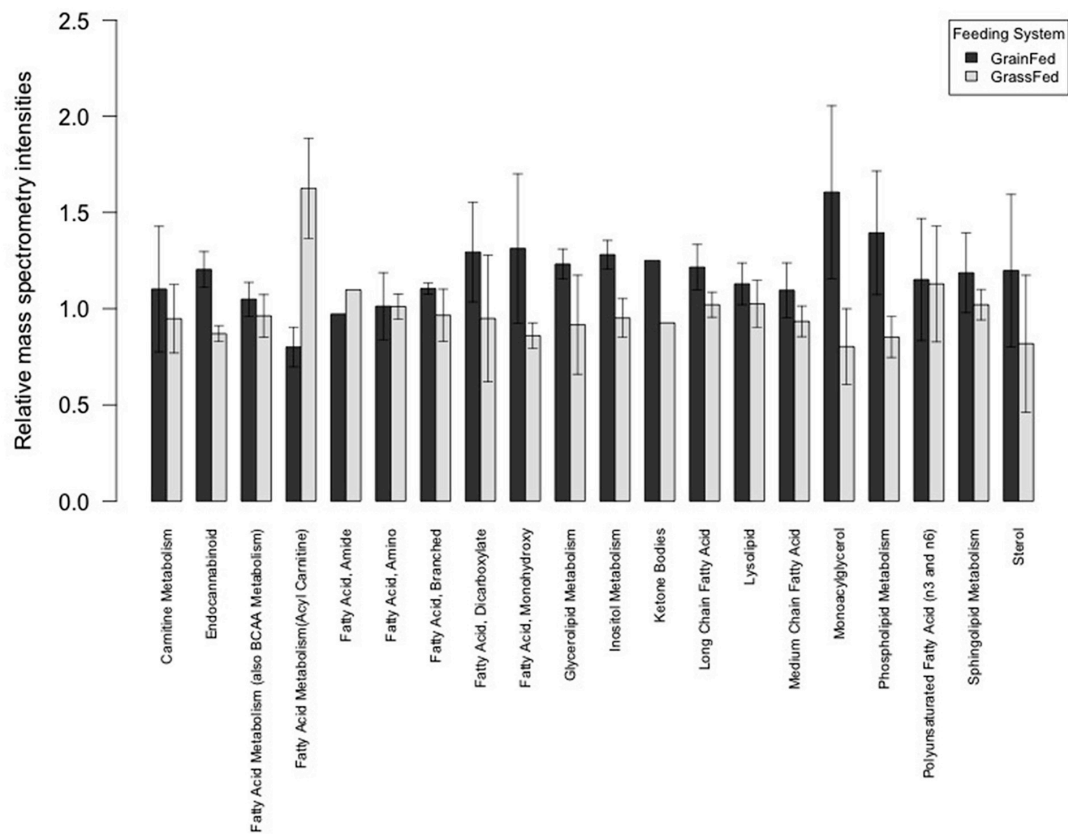


Figure 3.4 Averaged relative mass spectrometry intensities of 131 lipid molecules detected in muscle of grain-fed and grass-fed animals.
There are 20 sub-pathways according to its physical and/or functional properties. Sub-pathways Fatty Acid, Amide and Ketone Bodies only have one member thus error bars are absent in these two groups

Metabolomics Analysis

We examined a total of 326 and 353 known compounds in blood and muscle, respectively. A summary of the numbers of biochemicals that achieved statistical significance ($p \leq 0.05$), as well as those approaching significance ($0.05 \leq p \leq 0.1$) is shown in Table 3.2. Principal component analysis and hierarchical clustering revealed a perfect separation between grass and grain finished muscle tissue and blood that may be indicative of the divergences in global metabolism (Figure 3.5 and Figure 3.6). These figures demonstrate that both tissues—blood and muscle—are valid to classify the origin of the animal regarding their level of metabolites, considering the 326 and 353 correspondingly.

Table 3.2 Summary of identified biochemicals according to the Welch's test.

Significantly Altered Biochemicals	<u>Grain Fed</u> <u>Grass Fed</u>	
	Whole Blood	Muscle Tissue
Total biochemical $p \leq 0.05$	179	163
Biochemicals	↑92 87↓	↑117 46↓
Total biochemical $0.05 < p < 0.10$	25	33
Biochemicals	↑14 11↓	↑26 7↓

*Arrows indicate up- and down-regulation of the compounds in grass-fed individuals

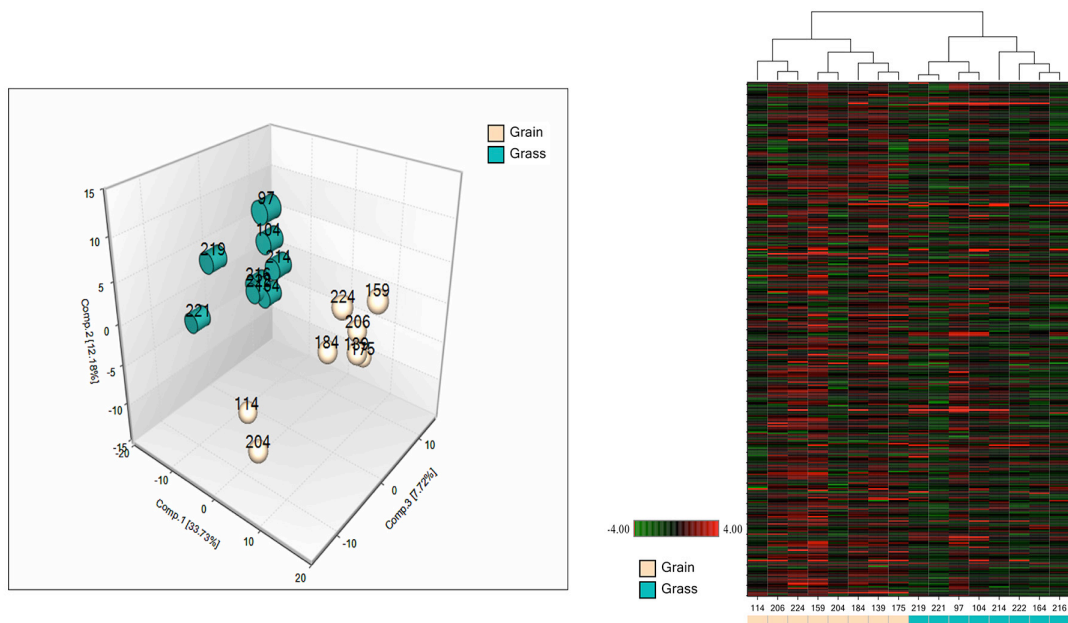


Figure 3.5 Principal Component Analysis (PCA) and hierarchical clustering obtained from employing metabolic concentration of all metabolites detected in muscle tissue. Turquoise and light-brown colors represent grass and grain-fed groups, respectively. In the heatmap red color denotes enrichment of the corresponding molecules, contrary to green color that represents depletion (n=8 for each group)

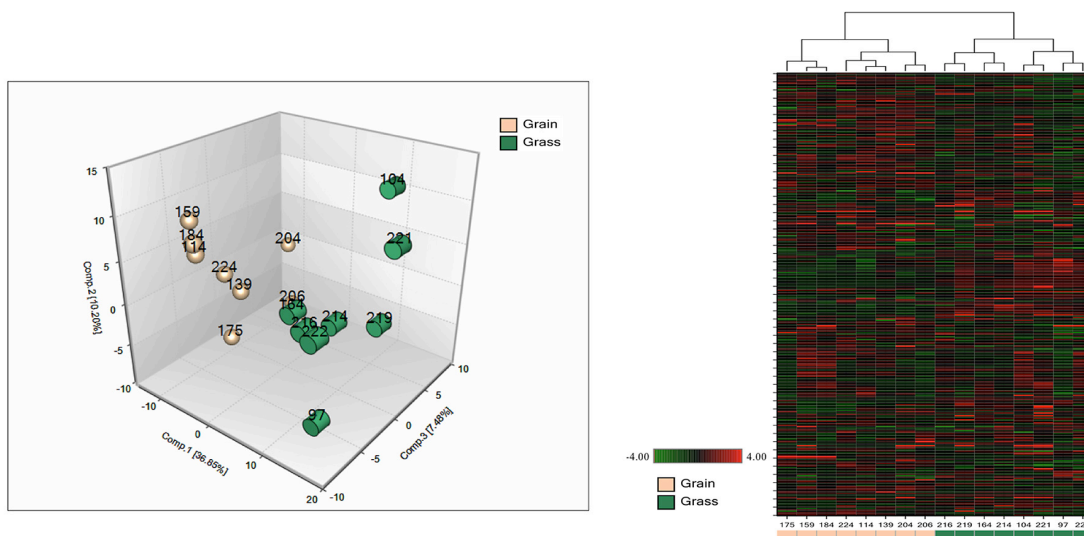


Figure 3.6 Principal Component Analysis (PCA) and heatmap acquired from metabolites relative expression calculated in blood of grain- and grass-fed steers. In the hierarchical clustering red color shows higher level of the corresponding biochemical, opposite to green that means reduction. Light brown and green colors represent grain and grass-fed individuals in both graphs (n=8 for each group).

We know that metabolites are product of biochemical reactions thus differences in those components are manifestations of distinct physiological states. Both groups clustered tightly in muscle, demonstrating a smaller variation of metabolites levels than in blood. Additionally, random forest analysis based on the biochemicals detected in this dataset resulted in a predictive accuracy of 100% between dietary conditions. The results show that the calculated values are greater than random chance, suggesting that those biochemicals can be of interest as biomarkers for the specific types of diet. Figure 3.7 indicates the top 30 metabolites and their classifications, summarized by the legend color code.

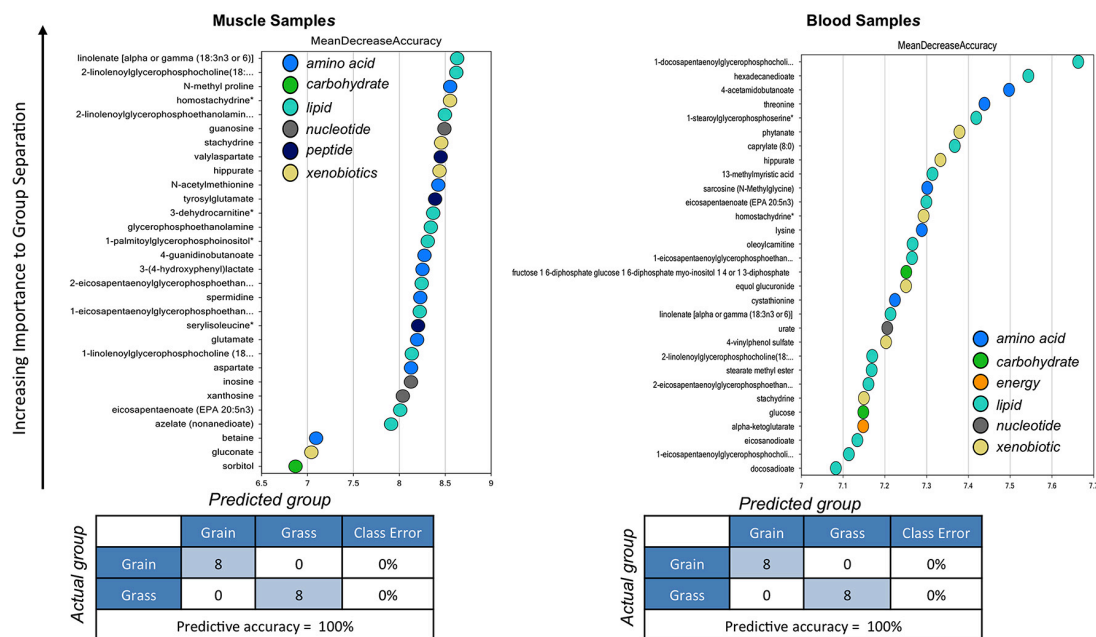


Figure 3.7 Random forest analyses obtained from muscle and blood. Random forest analysis consists in a supervised classification technique based on an ensemble of decision trees that has proven to be a valuable statistical tool for identifying biomarkers of interest. Random forest analysis based on the biochemicals detected in this dataset resulted in a predictive accuracy of 100% between dietary conditions. This value is greater than random chance (50%), suggesting these metabolites may be of interest as biomarkers. The y-axis represents the molecules in order of importance for group classification, from top to bottom. The legend represents the type of biochemical and the table below each chart shows the prediction accuracy based on the random forest result (actual against predicted group)

In both tissues the top two biochemicals are lipids, followed by an amino acid. It is well known that both elements are critical to define beef quality, a trait that heavily depends on marbling. Surprisingly and despite the different level of energy in the different diets, carbohydrates compounds barely appear once in muscle and twice in blood, respectively.

Glucose and Cortisol level

The level of cortisol found in the blood metabolomic analysis coincided with the trend observed in the regular blood test (Figure 3.3b). Our study showed that grass-fed animals had lower cortisol levels, suggesting that animals maintained on pastures, following their natural habits, displayed less stress than animals maintained in restricted spaces with limited walking distances and high energy diets. The observed differences in blood glucose, CO₂, and cortisol concentrations, reflect broad phenotypic traits that may be altered through many different mechanisms and these traits themselves could also affect other metabolic pathways. Thus, the result shows that grass-fed and grain-fed treatments generate broad metabolic divergences, providing an excellent opportunity to study interactions between nutrients and the genome.

Gene Expression Analysis

To study the gene activity in both types of diet, mRNA from muscle was extracted to construct libraries for RNA-Seq analysis on Illumina Hi-Seq 2000. Each regimen was represented by two randomly chosen biological replicates, with a total number of 32,401,755 and 30,428,052 reads for grass-fed and grain-fed samples, respectively.

The raw data has been deposited in NCBI database and can be access through this link: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE70248>. Mapping levels of the samples were adequate and similar, averaging 73 % in the grain-fed group and 76 % in the grass-fed cluster. Table 3.3 shows the mapping details of the samples.

Table 3.3 Sample descriptors for RNA-Seq analysis

Sample	Reads Processed	Aligned Reads	Aligned (%)
Grain_184	15,260,337	11,288,315	73.97
Grain_224	17,141,418	12,349,227	72.04
Grass_104	15,158,827	11,532,709	76.08
Grass_222	15,269,225	11,634,528	76.20

After analysis, we found 241 genes displaying differential expression ($FDR < 0.1$). From these genes, 59 and 182 demonstrated down- and up-regulation in grass-fed animals. The MA plot ($M = \log \text{ratio}$ and $A = \text{mean average}$) was used to illustrate the magnitude distribution of altered gene expression based on diet (Figure 3.8a). The red dots represent the significant differentially expressed genes; the positive values on the y-axis are higher expressed in the grass-fed group however the negative ones showed a higher expression in grain-fed animals. Figure 3.8b shows the clustering of samples according to the expression level of up-regulated genes in the grass-fed group.

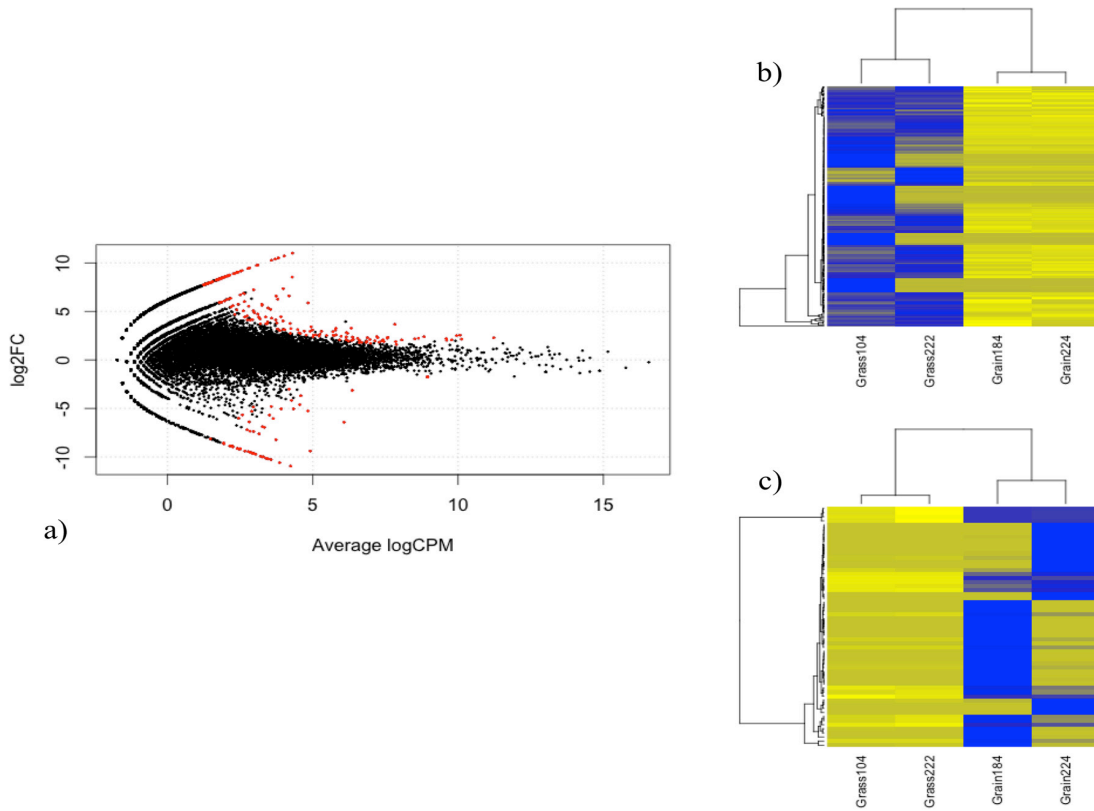


Figure 3.8 Differentially expressed genes

a) MA plot shows the significant differentially expressed genes in red, (y-axis depicts the log2 fold change of gene expression between grass and grain fed groups; x-axis represents the average log reads count per million for each gene) **b)** Hierarchical cluster of samples according to up-regulated genes in the grass-fed group **c)** Heatmap obtained using down-regulated genes in grass-fed individuals

The dendrogram showed that the expression of these 182 genes were more consistent within the grain-fed group. In the other hand, Figure 3.8c illustrates the clustering of samples based on genes that were up regulated in grain-fed animals; in this case the variation of gene expression between replicates was larger for the grain-fed group. The lists of genes based on expression directionality are provided in the Appendix IV and Appendix V. The log2fold-change levels ranged from -10.94 to 10.99, being almost symmetrical for both orientations. According to the FDR value, the next well-recognized genes *KRT5*, *GJAI*, *MDHI*, *CA12*, *CHST13*, *AMBP*, *GSTT3*, *NDUFA4* were identified among the top 10 entities.

Pathway Analysis

The complete set of differentially expressed genes was submitted for the IPA analysis. However, from the total of 241 genes, only 193 were considered for further analysis, as the rest represented unmapped or duplicates genes. For the analysis, the log2 ratio cutoff was set to 1. Only genes and direct relationships were included. The preeminent canonical pathways comprised of oxidative phosphorylation, mitochondrial dysfunction, acute phase response signaling, farnesoid X receptor/retinoid X receptor (FXR/RXR) activation, and protein ubiquitination pathways. Its corresponding p-values and ratios could be seen in Table 4. The top diseases and disorder cluster notably included skeletal and muscular disorders and metabolic diseases; despite those pathways ranked third and fourth, we consider them relevant to this study, such as molecular and cellular functions; nucleic acid metabolism; small molecule biochemistry; DNA replication, recombination and repair; energy production; and cell-to-cell signaling and interaction, integrated the classification. Additional information about the top networks, top lists and top molecules were also obtained from the IPA analysis. Detailed information could be seen in Appendix I.

Table 3.4 Top canonical pathways from IPA analysis

Name	p-value	Ratio
Oxidative Phosphorylation	2.14e-11	13/109 (0.119)
Mitochondrial Dysfunction	6.03e-09	13/171 (0.076)
Acute Phase Response Signaling	1.65e-04	8/169 (0.047)
FXR/RXR Activation	1.67e-04	7/127 (0.055)
Protein Ubiquitination Pathway	2.43e-03	8/255 (0.031)

For a more specific inquiry, we performed individual runs employing subsets of genes according to its expression levels. The array of analyzed under-expressed genes comprises 42 entities and the correspondent top canonical pathways were represented by FXR/RXR activation, acute phase response signaling, LXR/RXR activation, complement system and arginine degradation I. Contrarily, the over-expressed genes list included 151 elements, showing oxidative phosphorylation, mitochondrial dysfunction, protein ubiquitination, telomere extension by telomerase, and phospholipase C signaling as the highest ranked biopathways (Appendix II).

Mitochondrial protein-coding genes

The bovine genome includes 13 mitochondrial protein-coding genes. Considering previous reports that mentioned the essential role of mitochondria in muscle cells and our result from the enrichment analysis, we decided to evaluate the gene expression levels for all of them from the RNA-Seq data and then perform a cluster analysis. Figure 9a depicts distribution of reads from RNA-Seq analysis across the mitochondrial chromosome; it clearly shows the different gene expression profiles between grass and grain-fed individuals. Additionally, Figure 3.9a allows visualizing grossly the location and magnitude of peaks, suggesting which genes can be more relevant for each condition as well. We found that most genes increased their expression levels in grain-fed animals compared to grass-fed animals. Although the dendrogram in Figure 3.9b, which shows the expression level of the 13-mitochondrial genes, indicates more similarity between grass-fed samples, the resulting heatmap demonstrates a perfect association of the individuals regarding their type of diet.

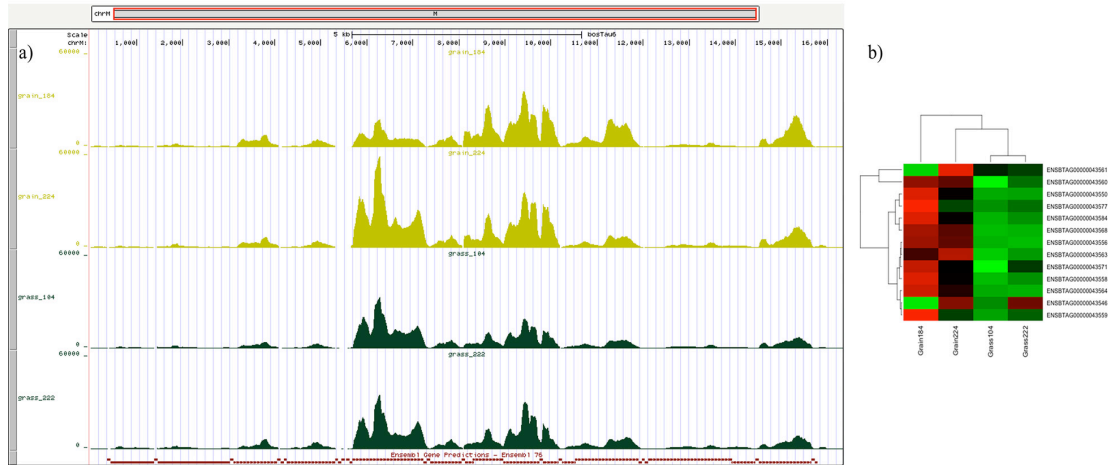


Figure 3.9 Bovine mitochondrial coding genes

a) Peaks obtained from the alignment of reads to the mitochondrial genomic reference during RNA-Seq analysis; yellow and green peaks correspond to grain and grass-fed samples respectively b) Heatmap of the 13 mitochondrial protein-coding genes according to their expression levels. The dendrogram demonstrates that grass-fed animals have a more consistent mitochondrial gene expression profile than grain-fed individuals

Analysis for verification of the RNA-Seq results

q-PCR analysis has been performed in 10 significant DE genes. Figure 3.10 demonstrates the certainty of our method, which detect the right direction of the gene expression change in 100% of the genes. The gray bars represent the log2 Fold change values from qPCR and the black bars the corresponding value detected within the RNA-Seq analysis. It is also noticeable the unsteady values reported by the q-PCR and RNA-Seq approaches. However, the q-PCR approach always yielded lower expression and the difference from RNA-Seq approach was quite stable across samples. Genes *ENSBTAG00000031295*, *ENSBTAG00000016688* and *ENSBTAG00000047914* presented the largest discrepancy between methods; meanwhile gene *ENSBTAG00000022120* exhibited parallel result in both techniques.

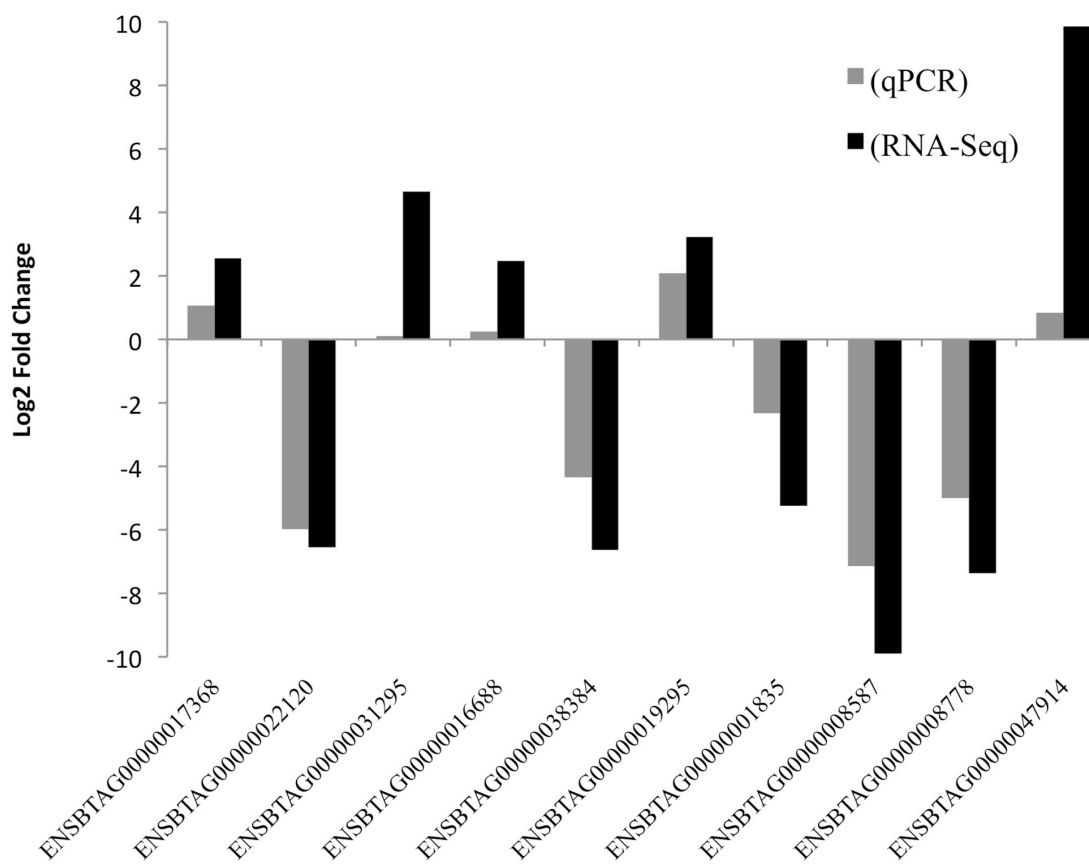


Figure 3.10 Validation of the RNA-Seq result by qPCR
The y-axis represents the log 2 fold change of gene expression; x-axis shows the Ensembl names of genes employed for validation. Black and gray bars depict the RNA-Seq and qPCR results, respectively

Integrated pathway-level analysis

The result comprises 2,196 canonical pathways from 11 databases (Dataset can be accessed: <http://www.nature.com/article-assets/npg/srep/2016/160517/srep25948/extra/srep25948-s5.xls>). Each row represents a pathway that not only has independent p and q values for genes and metabolites; but also joint p and q values, which makes the result more comprehensible. A systematic way to link analyses from different functional levels always has been problematic. The applied method is just one of the proposed approaches that fit well for our purposes. Unsurprisingly, metabolism was

the number one pathway in the list with a joint q-value of 3.81^{-30} . The citric acid cycle (TCA), and respiratory electron transport also had significant q-value of 3.95^{-15} . According to the KEGG database, oxidative phosphorylation appears first, including 16 genes and 3 metabolites with a joint q-value of 5.11^{-11} . The same bio-pathway also arises first in the IPA analysis. Protein digestion and absorption, purine metabolism, amino acyl-tRNA biosynthesis, vitamin digestion and absorption, biosynthesis of unsaturated fatty acids, fat digestion and absorption, pentose phosphate pathway, arginine and proline metabolism, mineral absorption, citrate cycle (TCA cycle), and taste transduction are the significant KEGG pathways ($q < 0.05$) that included both, genes and metabolites respectively. We chose the KEGG database for our results because it describes pathways more specifically and more detailed than other databases, which frequently define pathways with too broad barriers that are complex to interpret biologically.

Discussion

Our studies demonstrate that growth rate, gene expression, and metabolite levels in LD muscle are different in response to the grass-fed and grain-fed regimens, indicating that the diet-type influences gene expression and other physiological pathways, thus modulating the host metabolism to regulate its own meat composition and quality. Additionally, previous studies in our laboratory have identified a large series of candidate genes that showed such differences in expression related to beef tenderness [197, 198]. Taking all of the results together, we can explore the metabolomic profiles in grass-fed and grain-fed beef. The major objective of this

project is to determine if these differences are real, and to discover more related effects.

Polyunsaturated fatty acids

It is well known that muscle from grain-fed animals contains a higher percentage of total fat than that of grass-fed animals, and that the location (inter- or intramuscular) and composition of this fraction could influence both meat tenderness and quality [199, 200]. Our results summarized in Figure 3.4 also confirm the lower total lipid concentration in muscle obtained from grass fed individuals. However, muscle from grass-fed cattle possessed elevated levels of the n3 lipids eicosapentaenoate (EPA), docosapentaenoate (DPA), and docosahexaenoate (DHA). Conversely, n6 polyunsaturated fatty acids (PUFAs) such as dihomo-linolenate, arachidonate, and docosapentaenoate (DPA n6) were diminished in these animals. Our results are in agreement with published studies demonstrating muscle from grass-fed animals has an enrichment of n3 to n6 PUFAs [201, 202]. The omega3/omega6 ratio has been linked to metabolic perturbations like type 2 diabetes, adipose and liver inflammation. Omega-6 and omega-3 promote and reduce inflammation respectively [203, 204]. Omega-3 EPA, DPA, and DHA were also 5 to 10 fold higher in the blood of grass-fed steers compared to grain-fed animals which may reflect dietary intake. Although a modest elevation in a portion of n6 fatty acids was observed in the blood of grass-fed cattle, these observations could reflect recent feeding compared to grain counterparts prior to sacrifice as suggested by 6 to 22 fold elevations in multiple food components including homostachydrine and stachydrine found in plants, such as alfalfa, and higher levels of bile acids, such as glycocholate and glycochenodeoxycholate in the

muscle and blood. Notably, lower levels of n6 PUFAs such as arachidonate in the muscle tissue of grass-fed animals may suggest a decreased capacity for the generation of inflammatory lipid mediators. The arachidonic acid, precursor of the pro-inflammatory eicosanoids, is released from membrane phospholipids during inflammatory activation to produce prostaglandins and leukotrienes [205]. Dietary supplementation with long chain omega-3 fatty acids could exert protective effects in various inflammatory disorders. During inflammation, the secreted EPA competes with arachidonic acid for enzymatic metabolism, resulting in less synthesis of inflammatory and chemotactic derivatives. Furthermore, the endocannabinoids oleic ethanolamide and palmitoyl ethanolamide were reduced in muscle from grass-fed animals. Oleic and palmitoyl ethanolamide cannot stimulate cannabinoid receptors, but can stimulate PPAR-alpha activity and have been shown to be elevated in diabetic obese patients [206], suggesting a potentially similar metabolic disruption in grain finished cattle. PPAR-alpha is a ligand-activated transcription factor with a main regulation effect of lipids metabolism in the liver. PPAR activation produces up-regulation of genes implicated in transport of fatty acids, fatty binding and activation, and peroxisomal and mitochondrial fatty acid beta-oxidation [207].

Lipid oxidation

Interestingly, medium and long chain fatty acids including pelargonate, palmitate, and stearate were elevated in grass-fed blood samples, but modestly reduced in related muscle tissue. Differences in fatty acid levels between these matrices may reflect alterations in lipid metabolism or recent feeding as described earlier that may impact the lipid signature. Diminished fatty acid abundance in muscle in grass-fed cattle was

in agreement with evidence in the literature demonstrating that this meat exhibits less marbling compared to grain finished counterparts [208]. Similarly, lower levels of myristate and palmitate were reported in grass- versus grain-fed meat [39, 209]. Differences in fatty acid levels may indicate a change in complex lipid hydrolysis considering lower levels of glycerol and several monoacylglycerols including 2-oleoylglycerol in the blood and muscle of grass-fed cattle suggest decreased triglyceride availability and/or degradation compared to grain counterparts. Additionally, carnitine conjugated fatty acids such as stearyl carnitine and oleoyl carnitine were reduced in muscle tissue, but accumulated in the blood of grain-fed animals and may be indicative of a change in fatty acid β -oxidation. Specifically, higher levels of carnitine conjugated fatty acids in the blood of grain-fed cattle may suggest inefficient fatty acid oxidation as suggested by the depletion of free carnitine. Furthermore, modestly higher levels of the dicarboxylic fatty acids azelate and undecanedioate in muscle from grain-fed individuals may reflect altered β -oxidation. Dicarboxylic fatty acids are generated when the terminal methyl group of a fatty acid is converted into a carboxyl group and are often indicative of ω -oxidation. Although the catabolism of fatty acids typically occurs via β -oxidation in the peroxisomes and/or mitochondria, fatty acid ω -oxidation occurs in the smooth endoplasmic reticulum and often serves as a rescue pathway for conditions where β -oxidation is disrupted or overwhelmed. Interestingly, inefficient lipid oxidation in grain-fed animals may still be higher than grass-fed counterparts as suggested by modestly elevated levels of the ketone body 3-hydroxybutyrate (often generated in the presence of excess acetyl-CoA and historically a marker of lipid oxidation in datasets) in the

blood and muscle. Furthermore, the grain diet may be ketogenic. Together, these observations suggest complex lipid hydrolysis and lipid availability may be greater in muscle from grain-fed diet and result in elevated lipid oxidation that may be partially disrupted. In contrast, lipid accumulation in the blood of grass-fed animals may suggest dietary feeding decreased incorporation into triglycerides and decreased absorption by tissues.

Glucose metabolism

Compared to grass-fed animals, animals finished on a grain diet exhibited considerable higher levels of blood glucose. This finding may reflect a difference in dietary intake or decreased uptake of glucose by tissues. Similarly, significantly higher levels of sorbitol pathway metabolites fructose and sorbitol, often generated by the reduction of glucose excess in muscle from grain-fed diet, may be indicative of increased glucose availability to the tissue. In humans, it has been reported that the increased activity of the sorbitol pathway of glucose metabolism is one of the leading factors in the pathogenesis of diabetic complications [210]. The negative effects of increasing the sorbitol pathway activity under hyperglycemic conditions comprise intracellular sorbitol accumulation with an increment in osmotic stress and production of fructose, which is more potent than glucose to generate non-enzymatic glycosylation [211]. Controversially, other groups have reported that increased activity of the sorbitol pathway could be beneficial rather than detrimental because the pathway detoxifies toxic lipid peroxidation products. In contrast, elevated levels of maltopentaose, maltotetraose, maltotriose and maltose in muscle of grass-fed individuals may indicate glycogen degradation in order to maintain glucose

homeostasis. Differences in glycogen availability may be of interest in future studies considering that glycogen in post-mortem muscle can be converted to lactate, causing the meat to become more acidic and less predisposed to browning. A study in lambs demonstrated that lairage time exerts a slight effect on carcass quality traits, affecting most of the meat quality parameters at 24h post-mortem. Additionally, the glycogen content in liver and longissimus dorsi muscle decreased as lairaged time increased (0 to 12 hours). However, the muscle from lambs that were sacrificed after 3 hours of lairage had the highest pH and shear force measures [212]. Grain-fed cattle may also exhibit enhanced glycolysis as evidenced by the accumulation of pyruvate and lactate in the blood as well as increased glucose utilization for anabolic growth as suggested by the accumulation of the pentose phosphate pathway (PPP) metabolites ribose 5-phosphate and the isobar for pentulose 5-phosphate in the muscle. The pentose phosphate pathway is a biochemical process paralleling to glycolysis that produces NADPH and 5-carbon sugars; its main role is anabolic rather than catabolic, thus its metabolites of higher concentration observed in grain fed animals coincided with their metabolic stage. Furthermore, PPP facilitates NADPH regeneration necessary for glutathione reduction, nucleotide synthesis and anabolic growth. Indeed, reduced levels of NADPH in the muscle of grain-fed animals may suggest utilization for biogenesis and therefore may require recycling via glucose shuttling.

Cortisol level and animal well-being

Theoretically, changes in glucose homeostasis may be mediated by cortisol, which is a naturally occurring glucocorticoid produced by the adrenal glands [107, 213, 214]. Related to metabolism, cortisol assists in the maintenance of blood glucose level

through the stimulation of gluconeogenesis in the liver, from non-carbohydrate substrates (amino acids, glycerol, lactate and propionate)[215]. Additionally, cortisol inhibits the glucose uptake in muscle and adipose tissue, and stimulates the fat breakdown in the last type of tissue to released glycerol, one of the substrates used for gluconeogenesis. Thus, higher cortisol levels in grain-fed blood suggest that grain feeding might increase glucose availability for uptake by muscle tissue and subsequently promote utilization for glycolytic metabolism and anabolic growth [216-218]. Intriguingly, cortisol has been recognized as the “stress hormone” for its higher levels during the “fight or flight” response to stress [219-221]. Thus, plasma cortisol level has been used to assess the stress in different species [222-225]. In humans, stress-induced hyperglycemia is commonly observed in patients admitted to the intensive care unit (ICU), even when the blood glucose level has been previously normal [226]. The elevation of cortisol could result from the activation of the hypothalamic-pituitary-adrenal axis. Cortisol increased 4 fold immediately after extenuating exercise in humans, returning to the baseline within 17 hours [200, 227]. Housing system constitutes an important factor in animal welfare; measurement of total protein, lysozyme, cortisol, serum and feces corticosterone concentration and GR- α gene expression demonstrated that tie-stall causes more stress than loose system in cows [55]. Plasmocortisol levels in dogs was used to determine the level of stress during confinement in public shelters; the results suggest that confinement induces a prolonged activation of the hypothalamic-pituitary-adrenal axis with possible implications for welfare of confined dogs [222]. From an economic prospective, stress can negatively influence meat quality [228]. However, no

difference in plasma cortisol concentration associated with feeding system was observed after transportation and immediately before slaughter in lambs [228]. Recently, it has been reported that hair cortisol could be useful for measuring chronic stress in dairy cattle [229]. As we have known, stress is associated with increased levels of circulating corticosteroids, mainly cortisol (corticosterone) [230, 231]. Theoretically, there are two biopathways associated with initial stress response to environmental stimuli, the sympathetic-adrenal-medullary (SAM) axis and the hypothalamic-pituitary-adrenal (HPA) axis [232], respectively. Activation of the HPA axis results in a significant release of the steroid hormone from the cortex of the adrenal glands. Systemic cortisol concentrations increase immediately after threat and last for a long time and also recur if the threat is still present [233]. Physiologically, cortisol has multiple effects on organ systems involved in glucose homeostasis, blood pressure regulation, behavior modification, inflammation, and immune function. Corticosteroids can bind to the glucocorticoid receptors in the cytoplasm, and then the complex is transferred to the nucleus, acting as transcription factors to modulate gene expression down-stream [234-236]. Since the binding sites are ubiquitous throughout the genome, corticosteroid activation during stress could cause wide modifications in gene expression [237, 238]. Considering that both groups of animals have been treated under similar conditions during loading, transport and termination, the observed difference in cortisol level could be generated by a combination of factors present in each type of feeding system. Grain-fed animals received high-energy diet in confinement, predisposing them to suffer more metabolic disorders or infectious diseases than their grass-fed counterpart. Additionally, grain-fed animals were

maintained in groups and fed *ad libitum*, therefore the competition among individuals could become another source of stress. Consequently, long-term exposure to higher cortisol level may cause immune depression, suggesting that confined animals fed with grain diets are more prone to infections and diseases, a relevant matter for animal welfare and public health as well [239, 240]. Interestingly, Japanese Wagyu cattle breeders feed their animals with enormous amount of grain twice daily from 11 months of age until slaughter (28 to 30 months of age). From 11 to 18 months, the concentrate content of diet increases from approximately 37 to 87 % with a concomitant decrease of roughage [241]; nevertheless the animals appear to be perfectly healthy and calm. The cattle are only eating, seating or sleeping, and they look fine. However, the natural behavior of ruminants is to graze, consume grass freely and ruminate on the pasture, following its essential instinct. Thus, instead of considering cortisol as a marker of “pure stress” we conceive that cortisol level could be indicative for abnormal feeding or adverse environmental circumstances for cattle against instinctive feeding. Therefore, the higher cortisol level in the blood of the grain-fed group observed in our study strongly suggests that grass-fed animals generally suffered less adverse circumstances. We think grass-fed animals mainly benefit from moving freely, exercising their muscles and expressing innate behavior in a more natural habitat.

Gene expression analysis and functional annotation

RNA-Seq and posterior functional analyses broadly concurred with the findings revealed by the metabolomics data. IPA reported oxidative phosphorylation as the top pathway. Oxidative phosphorylation occurs in the mitochondria of cells, which use

their structure and enzymes to produce ATP. During this process electrons from donors are transferred to acceptors generating energy, which is used to form ATP. Mitochondrial dysfunction was the next pathway according to IPA; it arises when the reactive oxygen species (ROS)-mediated oxidative stress overcomes the antioxidant defense system. Metabolic fluctuations can trigger oxidative stress and cause mitochondrial dysfunction. Unexpectedly, the pathways oxidative phosphorylation, mitochondrial dysfunction, and protein ubiquitination, included among the top five significant, only comprised up-regulated genes in the grass-fed group, suggesting that these biological processes are sped up in the grass-fed animals.

The most represented cellular compartment was certainly the mitochondria. The 13 genes of the mitochondria genome are potential biological markers for diet type in beef. We found nucleic acid metabolism; small molecule biochemistry; DNA replication, recombination and repair, energy production; and cell-to cell signaling interaction as top molecular and cellular functions, which are primarily associated with the metabolites divergences observed in the two groups. The metabolomic and transcriptomic analyses lead to similar results making our findings very reliable and suggesting that both approaches could be combined for more solid conclusions. Thus, we implemented the integrated pathway-level analysis, which resulted in oxidative phosphorylation as the most significance pathway. Certainly, 16 genes and 3 metabolites assigned to this pathway demonstrated notable divergences between diet regimens. However, to identify which are more critical for altering the content and quality of the beef needs more advanced genome editing methods in the future.

Conclusion

Our results provide evidence that grass-fed animals produce tender beef with lower total fat, higher omega3/omega6 ratio and superior protein content than grain-fed animals, which are beneficial for the health of consumers. Finally, the higher blood cortisol level in the grain-fed group strongly suggests that grass-fed animals experience less stress; mainly from moving freely, exercising their muscles and expressing usual species behavior in a more natural environment.

4. Methylome analysis of beef from grass-fed Angus steers reveals enrichment of differentially methylated regions in chromosome 14

Abstract

The meat market is under current transformation. Consumers increasingly prefer grass-fed beef instead of the conventionally produced beef. Traditionally, the physiological age of the animal and intramuscular fat constitute the main determinants for beef quality. These two traits differ comparing grass-fed to grain-fed beef, presenting a difficulty for grass-fed beef to achieve high-quality standards under the existing grading system. It is well known that meat quality is closely controlled by genetic and environmental factors, such as nutrients. Beef quality traits, such as tenderness, marbling and fatty acids content, share the property of being concurrently regulated by gene sets. Therefore, the identification of possible epigenetic factors that would influence those genes is critical for improving meat quality. In the current study, we compared the whole-genome DNA methylation of grass-fed against grain-fed beef employing MBD-Seq method, identifying 60 differentially methylated regions (DMRs) (FDR <0.1). Most DMRs (50 of 60) demonstrated higher methylation level in grass-fed muscle, suggesting an overall increase of methylation in muscle genomic DNA from grass-fed individuals. Remarkably, chromosome 14 allocated the largest number of DMRs (12 of 60); this chromosome has been associated with large effect on milk fat percentage and composition, marbling, back-fat and carcass weight, traits that also have diverged comparing both types of diet. Nearly half of DMRs contained CpG islands and around 30 % of DMRs were located either upstream or downstream of CpG islands. The pathway analysis from the

annotated DMRs revealed skeletal and muscular system as the top physiological system and functions, while carbohydrates metabolism, lipid metabolism and tissue morphology have been identified among the top networks. G-protein-coupled receptor (GPCR) and amyloid precursor protein (APP) were recognized as “hub” molecules in those networks, indicating their relevance for the corresponding physiological function. Finally, the motifs TACAAA and TATGTA were recognized in the DMRs, indicating that these sequences could play a main role in establishing DNA methylation.

Introduction

Humans have been consumed wild animals, including different types of bovines, since prehistoric times. Modern cattle originated from a small number of ancestors domesticated around 10,500 years ago, revealing the long history of human’s beef consumption [242]. Currently, beef represents a main component of the American diet and constitutes the major source of protein in many regions [1]. Beef provides diverse nutrients, such as amino acids, energy, vitamins and minerals that play essential roles in several physiological functions. Therefore, beyond nutritional diseases its deficiencies also cause other disorders; folate and mineral deficiencies have been linked to colon cancer and genomic instability respectively [2, 243]. For instance, some components of red meat, such as cholesterol, triglycerides and heme (the iron porphyrin component of hemoproteins; which gives meat its red color) could adversely affect human health, especially when meat intake is excessive [20, 21, 244]. Controversially, other studies reported that red meat consumption could be beneficial,

providing high quality protein that increases satiety and boosts stamina [151], helping to manage muscle tissue loss especially in advance-age individuals [245].

In the last years, the market preference has been increasingly changing from consuming grain-fed to grass-fed animals [18]. People recognize products obtained from grass-fed animals as more natural, healthy and humanely produced than conventionally obtained products. However, the true differences between these two types of beef production regarding various aspects remain unclear. Concerning human health, grass-fed beef generally has higher concentration of several nutrients such as conjugated linoleic acid (CLA), omega-3 fatty acids, antioxidants and some vitamins, than grain-fed beef [41, 246, 247]. Contrarily, total fat content of grass-fed beef is lower than grain-fed beef [247, 248]. Besides the advantage on consuming less fat, nutritionists emphasize on the omega-3 fatty acid content, nevertheless the amount of omega-3 fats provided by grass-fed beef, compared to other sources such as salmon, becomes irrelevant considering the recommended daily intake [249]. Definitely, grass-fed beef has a better fat profile than standard beef, nevertheless it cannot be considered a good source of omega-3 fats and its overconsumption, which can be stimulated by its reputation for healthfulness, should be avoided. Regarding animal welfare, bovines seem to enjoy eating grains, and the use of grains as a source of energy is fine while plenty of roughage is available. The problems appear when the proportion of grain becomes excessively high, altering the homeostasis and forcing the organism to its physiological limits. Additionally, animals in high input systems are normally maintained in crowded and less favorable conditions, according to their natural habits, than grass-fed animals. The ruminant digestive system has evolved to

efficiently breakdown plant matter therefore consuming only grass, according to their innate instinct, should be certainly more natural than others conventional systems.

Despite its benefits, grass-fed beef presents some drawbacks regarding meat attributes, which have been defined by the market for years and could be challenging to achieve under the grass-fed regimen [19]. As an example, animals produced only with grass are older than the traditional raised animals at the moment of termination. Therefore, the effect of age of animal could compromise different characteristics of the product, such as tenderness, juiciness and fat contents, affecting the overall quality and grading of beef. In addition, grass-fed systems depend on various hard-to-control variables (climate, regional conditions, forage types and management practices), producing more heterogeneous beef than grain-fed systems. Most importantly, the physiological and biochemical mechanisms of why the grass-fed beef is better than grain-fed beef remain unclear.

It is well known that genes and environmental factors, such as nutrients, closely control growth and meat quality. For instance, tenderness, fatty acids content and marbling, also are concurrently regulated by gene sets. Numerous studies indicate that environmental elements could influence gene expression by regulating the transcription and translation processes in several species [174, 250]. Dietary polyunsaturated fatty acids (PUFA's) considerably alter gene expression through transcription factor modulation, involving four clusters of transcription factors; liver X receptors (LXRs), hepatic nuclear factor-4 (HNF-4) alpha, sterol regulatory element binding proteins (SREBPs) 1 and 2, and peroxisome proliferator activated receptor (PPAR) alpha, beta and gamma [175]. These transcription factors intervene

directly in the metabolism of lipids, carbohydrates and bile acids. For the post-transcriptional regulation, consumption of isothiocyanate sulforaphane modulates gene expression and alternative gene splicing in a phosphatase and tensin homolog (PTEN) null preclinical murine model of prostate cancer, suggesting that diet may impact the synthesis of different isoforms. The report also demonstrates the complexity of the interaction between diet, genotype and gene activity, and the diverse mechanisms of action of dietary small bioactive components [178]. All of those involve very sophisticated epigenetic process.

However, the ideal model for epigenetic studies is the one that includes organisms with identical genomes. In mammals, this characteristic is observed in monozygotic twins or clones. The limited number of natural occurrences and the cost of producing clones restrict the use of such models experimentally. The next preferred model for epigenomics comprises individuals from highly inbred lines, thus in the present study we used steers from the Wye Angus herd, which produces animals with very homogeneous genetics, as a result of being closed for breeders importation for almost 50 years [143].

DNA methylation is the most studied and well-characterized epigenetic mark. In mammals, the addition of a methyl group occurs generally at the 5' position of the cytosine ring within CpG dinucleotides, which are underrepresented in the genome as a result of an enzymatic reaction that deaminates the 5mC to thymine [251, 252]. DNA methylation has been associated to transcription silencing, having a main role in gene regulation, development and carcinogenesis [253]. Alteration of DNA methylation occurs typically in human tumors, displaying overall genomic

hypomethylation and site-specific CpG island hypermethylation [254]. Diverse biological processes such as genetic imprinting, X chromosome inactivation, repressing of transposon and maintenance of repressed centromeric satellite repeats, have been linked to DNA methylation [251, 255, 256]. Moreover, DNA methylation has been associated with different stages of genes expression regarding its localization; methylation of promoter is linked with gene repression, gene body methylation has different effects on gene activity and intergenic methylation may influence gene expression through enhancer regulation [102, 257, 258].

The enzymes DNMT1, DNMT3a and DNMT3b catalyze the addition of the methyl group to the correspondent cytosine. The last two target the unmethylated CpGs to trigger the conversion and are recognized as the *de novo* methyltransferases [259]. Differently, DNMT1 maintains the methylation pattern of the cells, identifying the hemimethylated CpGs in the parental strand and reestablishing methylation in the daughter strand during DNA replication [260]. DNMTs knock out mice died during embryo development, recounting the relevance of methylation at this stage [261]. A study in Korean cattle, found that methylation level in promoter region of genes PPARG1 and FABP4 were inversely correlated with their corresponding mRNA levels in *longissimus dorsi* muscle, suggesting that DNA methylation control the expression of adipogenic and lipogenic genes in IMF and muscle [262]. Another group reported, that some SNPs of the bovine methyltransferases 3b gene have been associated with beef quality traits in Snow Dragon cattle indicating that DNMT3b highly influence the quality of meat [263]. Differentially methylated regions (DMRs) have been identified in beef with divergent tenderness in Angus steers [198].

Genome-wide DNA methylation affects muscle fiber density and drip loss in chickens [264]. Environmental factors that affect T2D, including obesity, exercise and diet, have also been found to alter the epigenome [265]. An epigenome wide association study (EWAS), performed using blood of 479 Caucasian individuals, found that DNA methylation levels at three CpG sites (cg27146050, cg16672562 and cg22891070) within intron 1 of *HIF3A* were associated with adult body mass index (BMI) [245]. Other researchers reported a significant association between DNA methylation at CpG loci and skeletal muscle mass (SMM) in 1,550 middle-age UK twins, suggesting that methylome of whole blood could be informative of SMM variation [266]. Thus, we hypothesize that different diet regimens could be associated with particular genome-wide DNA methylation profiles, which also would influence meat quality in cattle. Although many studies have been performed addressing DNA methylation, a detailed characterization of this phenomenon is still necessary to understand its implications in gene expression and determination of specific phenotypes, acting alone or in combination with other epigenetic marks. Additionally, there is no study examining global DNA methylation in grass-fed beef. Therefore, the goal of this study is to characterize the DNA methylation profile associated to each feeding regimen and provide novel information to understand the influence of diet, modulated by DNA methylation, in the phenotypic determination.

Material and Methods

Verification of the genetic background of animals

The Wizard® Genomic DNA Purification Kit (Promega, Cat # A1120) has been employed for extraction of genomic DNA from blood obtained from jugular puncture. After passing a rigorous quality control and proper identification, 65 DNA samples have been shipped for external processing and genotyping based on the GGP-HD BeadChip (80k SNPs) from GeneSeek. Lastly, the data analysis step has been performed in our laboratory applying the PLINK platform that embraces a set of tools for whole genome association analysis [153].

DNA extraction and MBD-Seq library preparation

Samples from two previously randomly selected steers, for RNA-seq analysis, have been used to extract muscle genomic DNA employing the Wizard Genomic DNA purification kit (Promega, A1120). DNA concentration was checked by the Qubit dsDNA Broad-Range Assay (Invitrogen, Q32850). MBD-seq method, utilizing the MethylCap kit (Diagenode, C02020010) identified methylated DNA regions. Firstly, DNA has been extracted and dissolved to reach 0.1 µg/µl for a final volume of 40 µl in a 1.5 ml tube. Then using the Bioruptor® Sonicator, DNA was cut into 300-500 bp fragments and filtered on agarose gel to visualize the size of the resultant segments. Secondly, approximately 142 µl of capture reaction mix containing 12 µl of DNA but without MethylCap protein, have been prepared. From this mixture, 119 µl of capture reaction mix were incubated with 1 µl of diluted MethylCap protein at 40 rpm on a rotating wheel for 2 hours and at 4°C to allow the binding occurs. The rest of the mix

was employed later as input sample. Magnetic beads captured the methylated DNA fragments. Subsequently, free DNA has been washed off and elutes obtained. All input and fractions have been refined using the MiniElute PCR Purification Kit (QIAGEN, 28006). Lastly, qPCR (iCycler iQ PCR system, Bio-Rad) has been performed for each individual in duplicates to check the enrichment efficiency. To estimate relative fold enrichments the method $2^{-\Delta\Delta C_t}$ was used, comparing values of a positive *TGFB3* to a negative primer pair *MON2*, between treated (methyl DNA) and control (input DNA) samples.

The library for sequencing has been prepared following the next protocol. For the end repair of the fragmented methylated DNA, NEBNext[®] End Repair Module (NEB, E6050S) has been employed. Later, using DNA Polymerase I, Large (Klenow) Fragment (NEB, M0210L) a 3' A was ligated. Additionally, T4 ligase (Promega, M1801) was used to add a pair of Solexa adaptors (Illumina) to the repaired ends. For fragment selection, filtration in a 2% agarose gel has been performed. Phusion[®] Hot Start High-Fidelity DNA Polymerase (NEB, M0530S) purified the PCR DNA templates. After filtration, DNA quality was tested. The DNA library was diluted and the concentration verified using the Qubit assay (Life Technology, Q32850). Lastly, the sequencing was performed in the Solexa 1G Genome Analyzer platform (Illumina) according to manufacturer protocols.

MBD-Seq data analysis

All sequence files have been tested and passed the quality control implemented using FastQC software. Subsequently, files have been aligned to the BosTau6 (Bos_Taurus_UMD_3.1) reference genome. The same parameters used for the

previous RNA-seq analysis have been applied for the alignment process [247]. While originally each fragment consisted of 50 nucleotides, they have been trimmed 10bp at 5' and 5bp at 3' to maintain high quality sequences. The bRemoveDuplicates option included in the DiffBind package was used to remove duplicated reads, a relevant step for the analysis method that we implemented. Model Based Analysis of ChIP-Seq (MACS) software performed the peak-calling step for each sample individually [197]. The program models empirically the shift size of the reads and uses a dynamic Poisson distribution to calculate the local bias, making the estimates more accurate. DiffBind package implemented in R identified the Differentially Methylated Regions (DMRs) computing differentially bound sites from affinity data [267]. The input for DiffBind comprises the binary bam files containing aligned reads and the set of peaks identified by MACS. The program builds a matrix from the consensus peaks, considering a "minimum overlap" of 2 for this corresponding case. After a contrast between conditions has been established, DiffBind executes an edgeR analysis, which constitutes an empirical Bayes method [191]. The pre-defined method TMM (Trimmed Mean of M-values) that subtracts the controls tags and account for the effective library size of samples has been used for normalization. The significance cut-off value for DMR identification was set to < 0.1 false discovery rate (FDR).

DNA methylation profile

To visualize the genome-wide DNA methylation profile regarding different type of diets, the reads were mapped to different genomic features employing the ngs.plot software [268]. This bio-informatic tool program has been specifically designed for visualizing next-generation sequencing (NGS) samples at functional genomic regions.

The reads resulting from the previous precipitation step in the corresponding diet regimen, were piled up and plotted against “genebody”, “CGI” and ‘tss’. Firstly, the enquired regions were normalized to be of equal length following one of the two available algorithms; this step is particularly useful for “genebody”, “CGI” and custom regions. Then, the vectors have been normalized according to the corresponding library size to generate values in Reads Per Million of mapped reads, making different NGS samples comparable regardless of variation in sequencing depth.

Genomic and functional annotation

After identification of DMRs, the software ChIPpeakAnno was used for the genomic annotation of the corresponding regions[269]. ChIPpeakAnno generates information about the overlaps, distances and relative position for the requested features. For the enrichment analysis, genes previously annotated based on the nearest TSS or overlaps with the DMRs have been considered for an Ingenuity Pathway Analysis (IPA). The analysis provides a long and detailed report, however the most relevant parts are: molecular and cellular functions, networks, canonical pathways, diseases and disorders, and physiological system development and function. For motifs identification, sequences within and around the resulting DMRs have been extracted and then analyzed by the motifStack program.

MBD-seq validation through bisulfite sequencing

For treatment of 500 ng of DNA, sodium bisulfite conversion reagents were employed following the regular manufacturer protocol (EZ DNA Methylation Golden

Kit, ZYMO Research, D5005). Primers for PCR have been designed using MethPrimer (<http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi>). Firstly, equal amounts of DNA from samples from each group (grain-fed and grass-fed) were pooled together, serving as a template for the bisulfite conversion and the bisulfite PCR. Next, the purification of the PCR resultants has been achieved using QIAquick Gel Extraction Kit (QIAGEN, 28704). Vectors (pGEM-T Vector System I, Promega, A1360) have been added to the refined PCR products, then transformed to DH5 α cells (Z-Competent™ E. Coli Cells - Strain Zymo 5 α , ZYMO Research, T3007), and checked for positive insertions after incubation at 37°C overnight. Subsequently, ten white colonies from each cluster were cultured overnight in a shaker maintained at 37°C. For plasmid DNA isolation, Zyppy™ Plasmid Miniprep Kit has been used (ZYMO Research, D4036). For sequencing, the ABI 3730 machine employed M13 reverse primer and BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, 4337456). Finally, QUMA software (<http://quma.cdb.riken.jp>) analyzed the DNA methylation level for the corresponding group in each examined DMR.

Results

Genetic Background

The result from the SNP analysis demonstrated similar genetics among the 65 enquired Wye Angus steers. The chart obtained from the PCA analysis revealed no clustering between grain-fed and grass-fed animals (Figure 4.1a). Additionally, it confirmed the effect of randomization during animal assignment to either grass or grain feeding regimen. In epigenetic studies, it is critical to avoid significant genetic

differences among subjects to decrease the introduction of possible confounding factors that could compromise the results. Figure 4.1b shows the result of the same PCA analysis after the inclusion of additional 487 individuals from a closed Angus herd selected for minimized influence of MHC [156]. The Wye Angus animals grouped totally separated from the external Angus, however they were clearly randomly assorted regarding diet style.

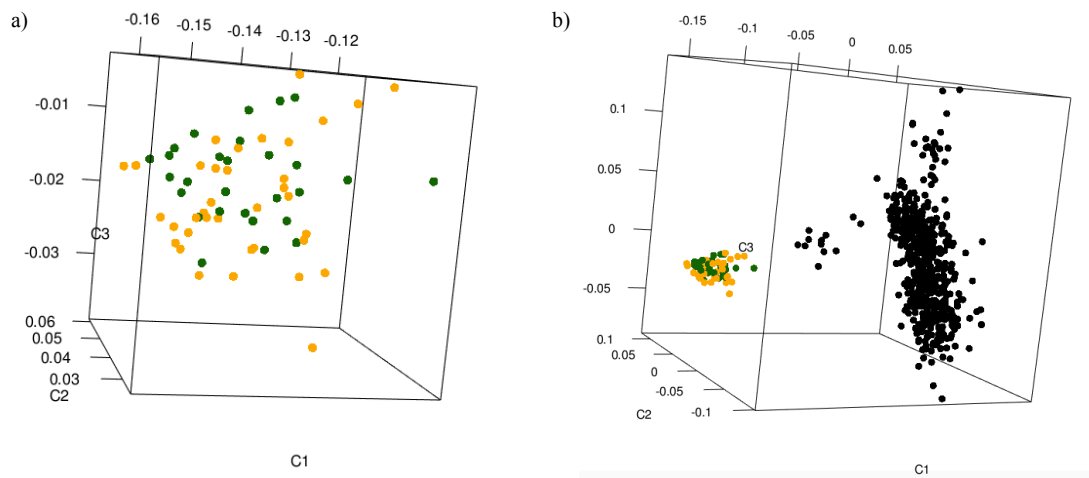


Figure 4.1 PCA Analysis from 80K SNPs
a) WYE Angus animals from both diet types b) WYE Angus steers plus external Angus individuals from a closed herd. Green and yellow dots represent the grass-fed and grain-fed diet respectively. Black dots denote the non-WYE Angus animals

MBD-Seq Analysis

Sequencing quality and alignment of reads

After filtration following a rigorous quality control process, the samples comprised around 9.8 million reads on average. The similar library size among individuals and the alignment level in all samples, which consistently surpassed 95%, demonstrate the quality and robustness of our method (Table 4.1).

Table 4.1 Sample descriptors for MBD-Seq analysis

Sample	Reads Processed	Aligned Reads	Aligned (%)
Grain-fed 1	9,750,290	9,408,286	96.49
Grain-fed 2	10,601,268	10,235,765	96.55
Grass-fed 1	9,244,190	8,866,996	95.92
Grass-fed 2	9,629,234	9,298,484	96.57

Peaks detection and overlapping peaks

The method Model-based Analysis for ChIP-Seq (MACS) detected comparable number of peaks in all individuals, except for the grass-fed 1 that has at least 4,000 less intervals than the rest of samples. Accordingly, the variation of number of peaks in the grass-fed group was larger than the observed within the grain-fed cluster. The average numbers of peaks were 22,893 and 25,236 for the grass-fed and grain-fed groups, respectively (Table 4.2). The resulting matrix contains 24,313 rows that represent the peaks that overlap at least in two-samples. After merging the overlapping peaks 46,258 unique peaks were identified.

Table 4.2 Number of peaks detected by MACS in each sample

Sample ID	Tissue	Condition	Replicate	Peak Caller	Intervals
Grass-fed 1	Muscle	Grass	1	MACS	20,203
Grass-fed 2	Muscle	Grass	2	MACS	25,582
Grain-fed 1	Muscle	Grain	1	MACS	26,140
Grain-fed 2	Muscle	Grain	2	MACS	24,386
24,313 sites in matrix (46,258 total unique peaks)					

Differentially Methylated Regions (DMRs)

Employing the previously generated matrix with the corresponding peak caller score and read count value for each row, heatmaps have been obtained using cross-correlation among samples. Figure 4.2a demonstrates that although the correlation

among replicates was low, a perfect clustering of samples was obtained based on the scores for peaks detected by MACS. Interestingly, grass-fed individuals were more consistent than its grain-fed counterpart regarding peak score values. Nevertheless, Figure 4.2b determines that the number of aligned reads in each peak was more consistent in the grain group. In the same chart, the dendrogram shows a perfect clustering for grain-fed samples, however grass-fed individuals differed from the grain-fed group but without grouping together, suggesting more variation in this group regarding reads number within consensus peaks. This observation raised a flag regarding the employed technic therefore we repeated it accordingly, achieving similar results that validated our approach.

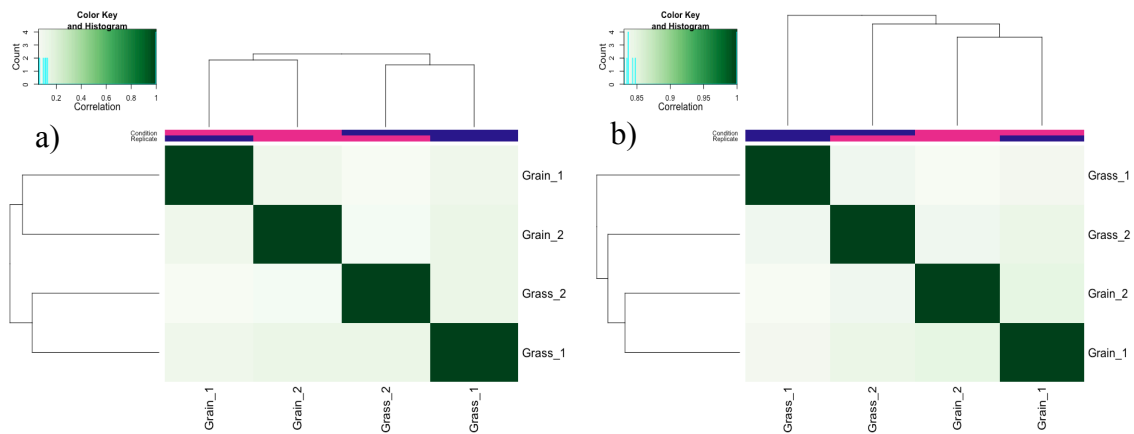


Figure 4.2 Cross-correlation of samples

a) Heatmap, using peak caller scores from MACS b) Heatmap from read count data within overlapping peaks

The DiffBind software implemented in R, identified 60 genomic regions with different methylation level between muscle samples from grain-fed and grass-fed individuals, for < 0.1 false discovery rate (FDR) (Figure 4.3a). As can be seen in

Figure 4.3b, the methylation profile of both grass-fed individuals was more stable than in grain-fed steers. Interestingly, the lateral dendrogram shows the groups of DMRs that present particular patterns regarding diet styles. Notice that this effect is particularly notorious in the last two clusters, having clearly opposite directions in both feeding styles.

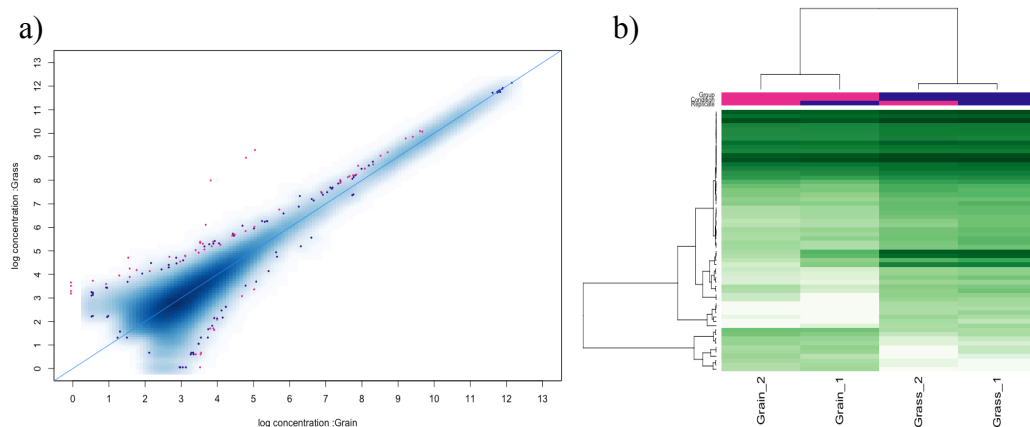


Figure 4.3 Differentially methylated regions in muscle from grass-fed Angus steers
a) Methylation level in beef from grass-fed versus grain-fed steers, red dots represent the genomic regions in which the methylation level differ between diet styles b) Heatmap obtained from methylation levels at the 60 differentially methylated regions (DMRs)

Generally, methylation increases at the DMRs in grass-fed individuals; 84% of DMRs show higher methylation levels than grain-fed animals (Figure 4.4a). Interestingly, the difference of methylation distribution observed in the DMRs that increase in grain-fed individuals was apparently larger than in DMRs that increase methylation in grass-fed animals, suggesting that these DMRs could be relevant for determining distinct phenotypes in animals that receive different types of diet. According to the Mann-Whitney-Wilcoxon test, the significance of the overall difference in distribution of methylation levels in the two groups was 0.00024, while those between

grain-fed and grass-fed in the individual cases (increased in grain-fed or increased in grass-fed) were 0.001 and 7.79^{-10} correspondingly. The plot of the first two principal components obtained from the PCA shows a clear separation of animals regarding diet styles, implying that the identified DMRs could be indicative of different diet regimens (Figure 4.4b). The PCA chart summarizes the information from the 60 DMRs and displays the clustering according to their similarities.

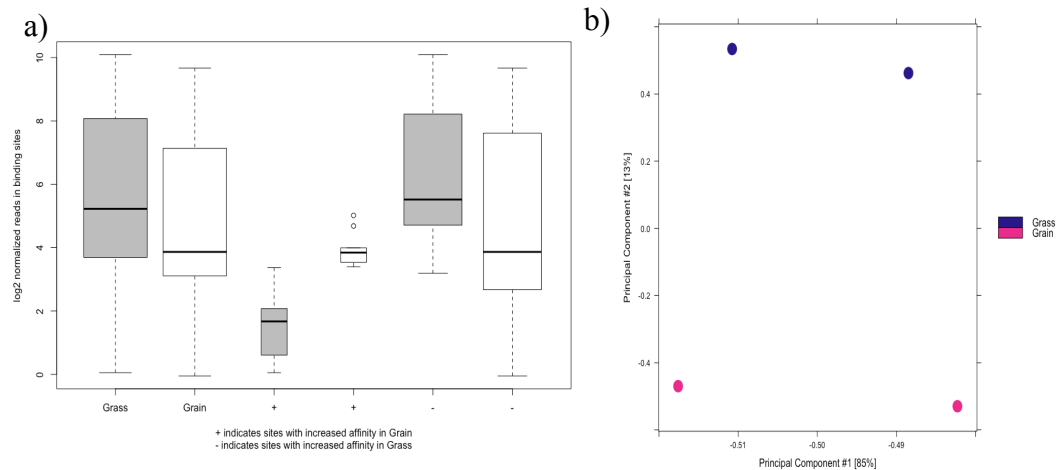


Figure 4.4 DMRs reads distribution and PCA analysis

a) The left two boxes represent the reads distribution over all DMRs; middle two boxes show reads distribution of DMRs that increase methylation level in the grain-fed group; the last two boxes illustrate the reads distribution of DMRs that increase methylation in the grass-fed group b) First two principal components from PCA using the 60 identified DMRs

A graphical representation of few DMRs detected in chromosome 14, with their correspondent peaks, can be seen in Figure 4.5. The four exemplified DMRs, represented by the black bars, clearly include totally two non-coding genes and overlap completely or partially with many CpG islands as well. Gene ENSBTAG00000046592.1 (U6) is a snRNA located around 5Kb downstream of the CpG island 88; which is also entirely contained by a DMR. This setting could be a

typical example of different methylation levels in gene promoters that may influence transcription of the corresponding gene. According to the read aggregation seen in each sample, most of prominent peaks coincided with CpG islands, however the magnitude and shape of these peaks clearly differ at DMRs.

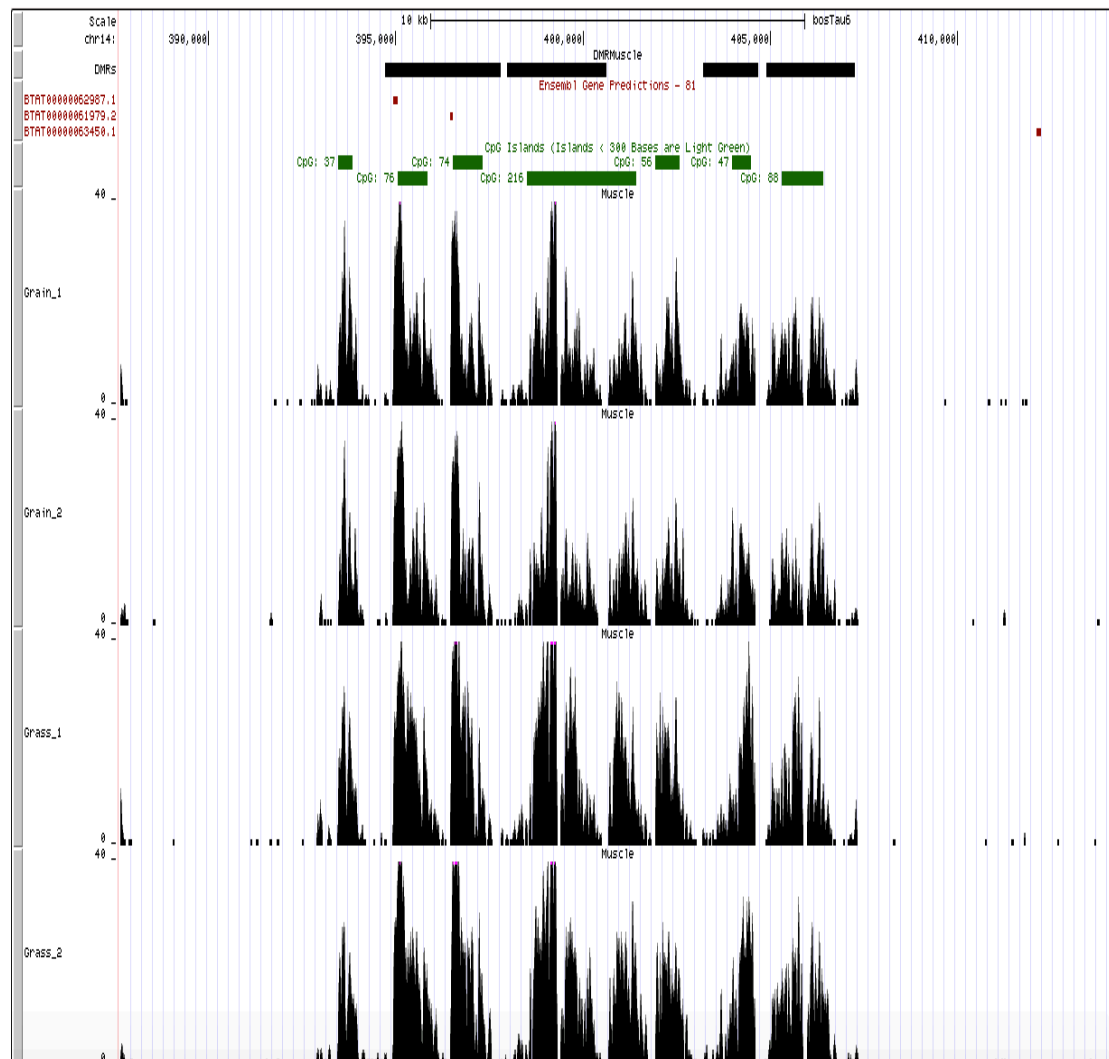


Figure 4.5 Example of DMRs identified in chr14
Black boxes represent DMRs, red and green boxes symbolize genes and CpG islands, respectively. The peaks denote the reads aggregation for the corresponding sample

Figure 4.6 shows the location of other two DMRs, in both cases CpG islands have been associated with them. In panel a, the DMR covers exonic and intronic regions of

gene ENSBTAG00000008421.5 and more than half of the CpG island 145. However, the DMR represented in panel b, overlaps almost entirely the CpG island 82, which lands completely in intron 11 of ENSBTAG00000009974.4 (TSNARE1) gene.

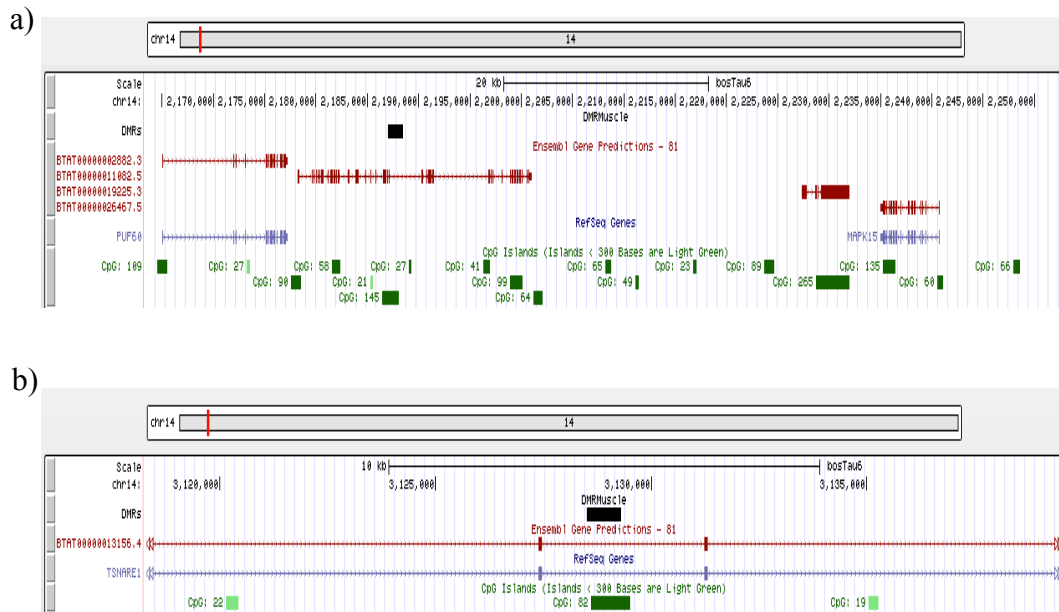


Figure 4.6 DMRs detected in chr14 represented as black boxes
a) DMR overlapping with exons and intron of gene ENSBTAG00000008421.5, and partially with CpG island 145
b) DMR overlapping intronic region of gene ENSBTAG00000009974.4 (TSNARE1) and almost entirely the CpG island 82

Consensus Peaks

From the total number of 24,313 peaks obtained during the consensus analysis, all samples share 8,787 peaks (Figure 4.7). Grain-fed animals showed a larger number of exclusive peaks than grass-fed individuals, accounting for 1,895 and 1,291 respectively. Although most of these sites were not recognized statistically as DMRs, they are unique for a specific type of diet thus they could also be potential markers for the feeding regimen employed during termination.

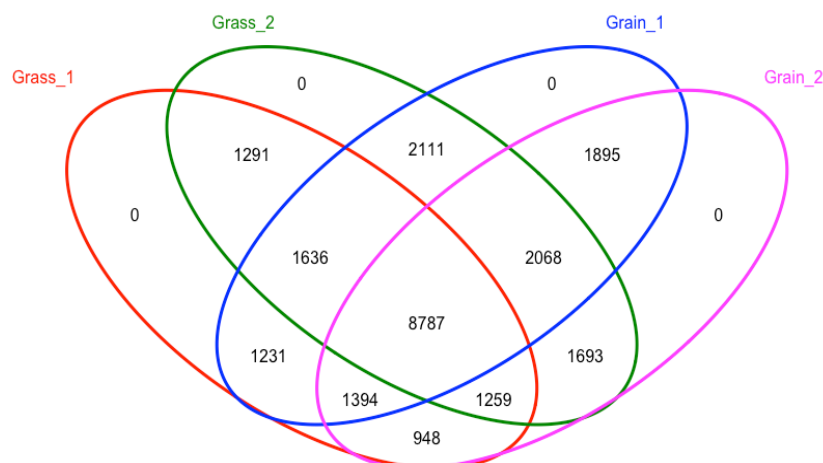


Figure 4.7 DNA Methylation Peaks Overlaps
Venn diagram shows the overlap of peaks considering a minimum overlap of two sites

Genome wide DNA methylation profiling

The whole genome DNA methylation profile regarding genes, TSS and CpG islands could be observed in Figure 4.8. It is clear that generally DNA methylation at genes remains constant disregarding the feed regimen (Figure 4.8a). The outline calculated using `ngs.plot.r` follows an expected pattern; methylation decreases immediately before TSS, increases rapidly at the TSS and then continuously through the gene body to diminish again at the TES. Figure 4.8b depicts DNA methylation in more detail for the TSS and its surroundings; although the magnification still reveal a similar shape in the promoter region, DNA methylation in grass-fed looks slightly higher right after the TSS and remains on top until the 5kb downstream from the TSS. Interestingly, the last panel (Figure 4.8c) reveals a different DNA methylation level at CpG islands; grass-fed shows superior levels within CpG islands, while on the outside DNA methylation remains stable.

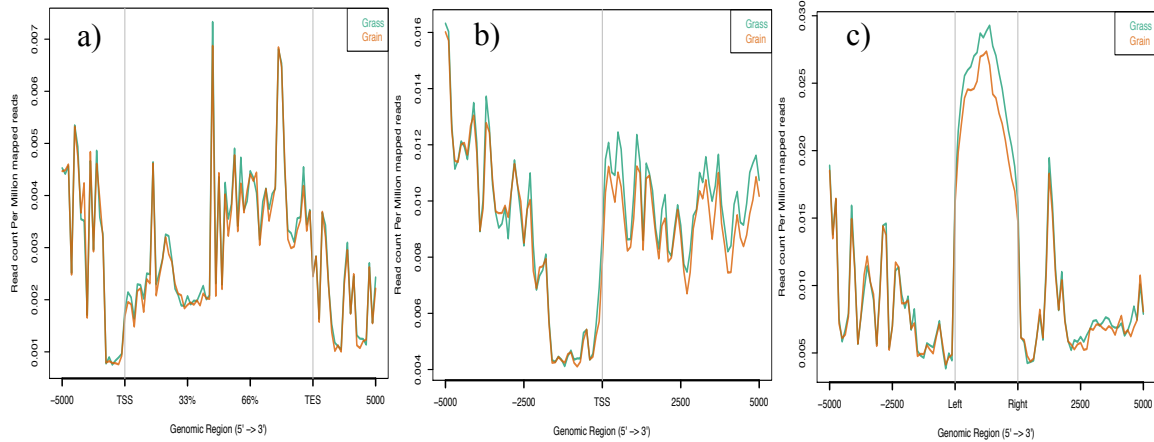


Figure 4.8 Genome-wide DNA methylation profile

a) Reads plotted against gene bodies b) Reads aggregation around transcription start sites (TSS) c) Accumulation of reads regarding CpG islands and CpG shores

Genomic Annotation

The identified DMRs ranged from 325 to a maximum of 3,429 base pairs (Figure 4.9a). Around 37% of DMRs comprises more than 1,000 bp and approximately 22% consists in less than 500 bp. The lengths mean and median for all identified DMRs were 1,043.6 and 802.5 bp, respectively. Only four DMRs extended beyond 3,000 bp, thus considering the size of other genomic features, DMRs could constitute relative small genomic portions important for relevant biological functions. Figure 4.9b shows the distribution of the DMRs regarding chromosome location. Chromosome 14 allocated the largest number of DMRs (12) followed by chromosomes 7, 10, 21 and 20 that included more than four DMRs individually. Around 22% of the DMRs were located in single DMR chromosomes.

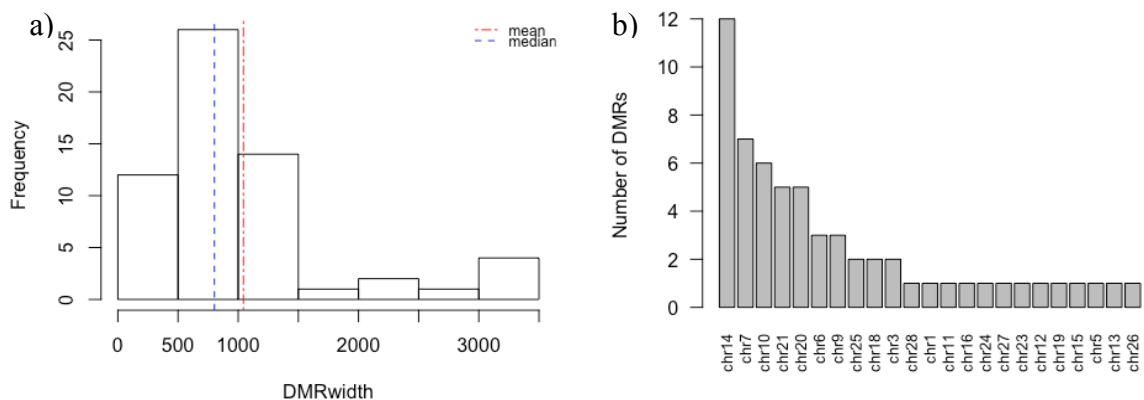


Figure 4.9 Histogram of DMRs regarding length and chromosomal location of DMRs
a) Histogram of DMR width in base pairs, red and blue lines indicate the mean and median, respectively b) Distribution of DMRs regarding chromosomes

The genomic annotation of DMRs has been performed employing ChIPpeakAnno. The results of this step regarding genes are summarized in Figure 4.10. Considering that DMRs could have an important role in regulation of gene expression we calculated the distance between DMRs and the closest transcription start site (TSS). As expected, 45% of the DMRs were situated within 5,000 bp to the closest TSS, contrarily to some of them that were located certainly far from any TSS; four and two DMRs exceeded 100,000 and 200,000 bp, correspondingly. The pie chart in Figure 4.10b summarizes the location of DMRs regarding genes following the next criteria; upstream: DMR resides upstream of the feature; downstream: DMR resides downstream of the feature; inside: DMR resides inside the feature; overlapStart: DMR overlaps with the start of the feature; overlapEnd: DMR overlaps with the end of the feature; includeFeature: DMR includes the feature completely. Around 45% of DMRs were located totally within genes boundaries and another 35% occupied the upstream area of genes; the smallest proportions of DMRs overlapped with the ends of genes.

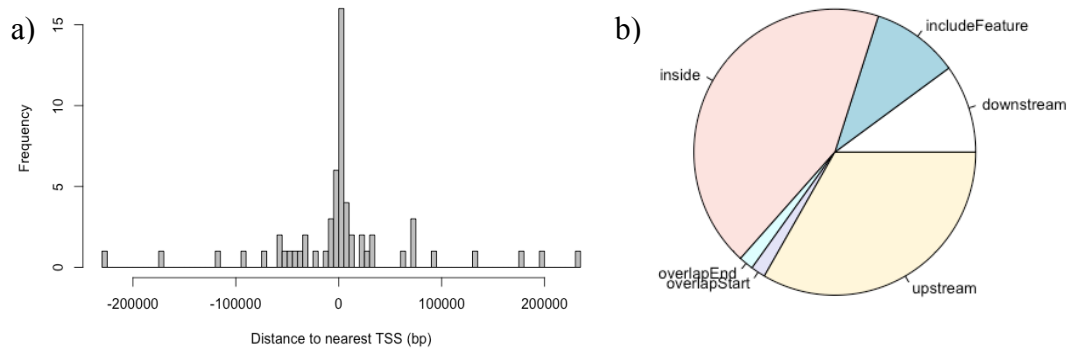


Figure 4.10 DMRs annotation regarding Genes
a) Histogram of the distance of the DMRs to the closest transcription start site (TSS) b) Proportion of the location of DMRs regarding genes

The Figure 4.11a shows the distribution of the distances of DMRs to the center of CpG islands. CpG islands lack specific start or end site thus we decide to use the center of the island as a reference point for measuring the distance. Accordingly, the histogram comprises exclusively positive numbers and shows that most of the DMRs resided close to the middle of CpG islands; only 11 DMRs located further than 20,000 bp from the center of the CpG islands. Figure 4.11b summarizes the position of DMRs respecting CpG islands; almost half of the identified DMRs contain a CpG island entirely and approximately a third resides upstream or overlap with the “start” of CpG islands. Interestingly, only a small proportion of DMRs are contained completely within CpG island boundaries, as is reflected by the pink portion in the pie chart. Considering the average size of CpG islands, those founding correspond and were expected.

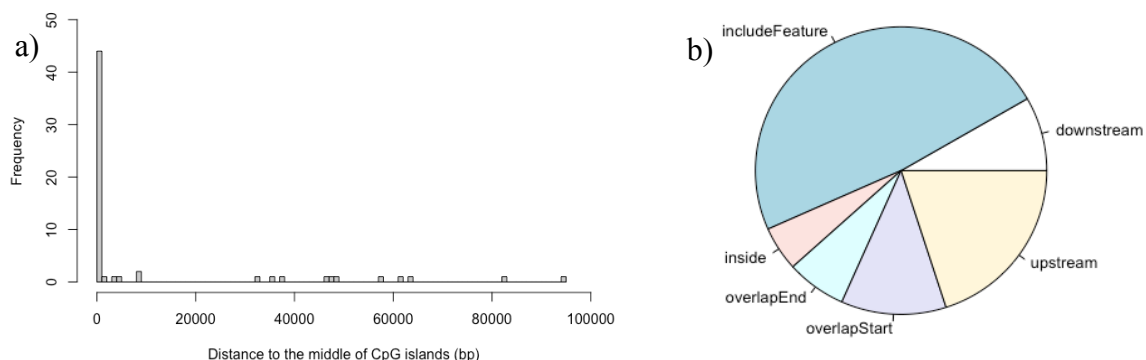


Figure 4.11 DMRs annotation regarding CpG Islands
a) Histogram of the distance of the DMRs to the center of the closest CpG island b) Pie chart considering location of DMRs regarding CpG islands

Most of DMRs were located in introns and intergenic regions. However, a significant proportion (>30%) was situated in exons. Promoters and downstream areas allocated similar numbers of DMRs. The genomic location defined as 5' UTR contained only a few DMRs, constituting the genomic feature with less number of DMRs in our data (Figure 4.12). The counting was performed allowing more than one feature per DMRs (not exclusive), thus each DMRs could allocate or overlap more than one genomic area.

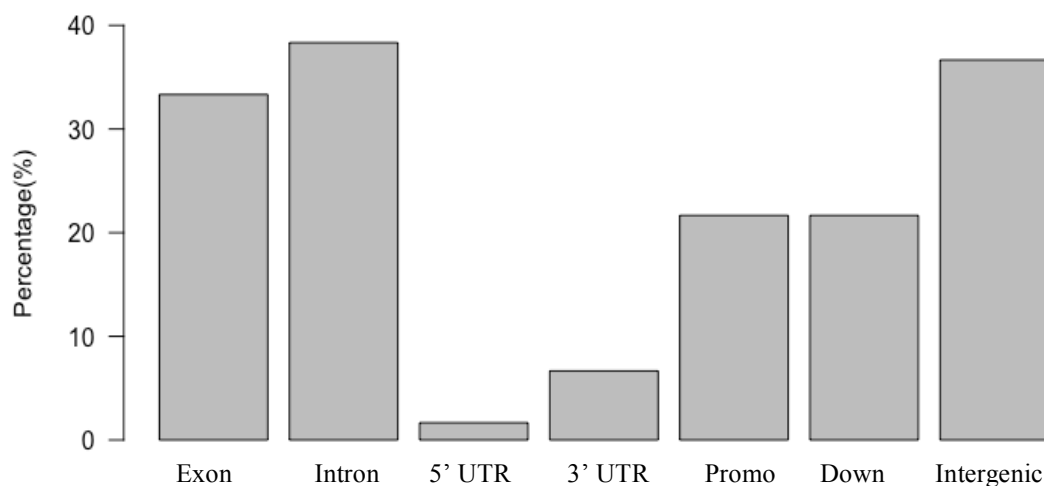


Figure 4.12 Distribution of the DMRs respecting different genomic features.

Motifs detected in DMRs

From the genomic regions that demonstrated different DNA methylation level, we extracted the genomic sequence of the DMRs ± 20 bp for performing a motif identification analysis. We employed the ChIPpeakAnno software in combination with the motifStack program for obtaining the two motifs identified in the DMRs (Figure 4.13). The two identified motifs were TACAAA and TATGTA as they are shown in panel a and b, respectively.

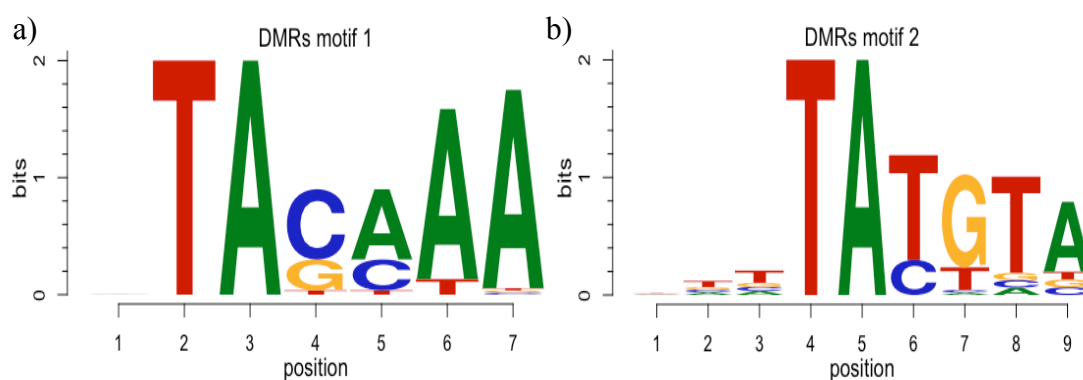


Figure 4.13 Motifs identified in DMRs

Functional Annotation

The DMRs were annotated using ChIPpeakAnno and the adjacent genes have been employed to identify associated Gene Ontology (GO) terms and perform an Ingenuity Pathway Analysis (IPA), which provided relevant biological information based on the comprehensive and manually curated content of the Ingenuity Data Base. Table 4.3 summarizes the more relevant results obtained from the IPA analysis. In the Canonical pathways, the significant pathways were cardiomyocyte differentiation via BMP receptors and coagulation system. For the molecular and cellular functions report, the five members of the group were significant for a p-value of <0.05 , calling our attention the amino acid metabolism and carbohydrate metabolism, which are critical functions related to the difference in nutrients on the provided diets. The physiological system and development and function list, includes skeletal and muscular system development and function as the top element. Tissue development and organismal function appear in the third and fifth position respectively, being statistically significant as well. Lastly, the members of the top networks that we considered relevant for this study are cell-to-cell signaling and interaction, organismal injury and abnormalities; and cell death and survival, nervous system development and function, tissue morphology, which have a score of 23 and 21 correspondingly.

Table 4.3 Ingenuity Pathway Analysis

Top Canonical Pathways		
Name	p-value	Overlap
Cardiomyocyte Differentiation via BMP Receptors	3.46E-02	5.0 % 1/20
Coagulation System	5.98E-02	2.9 % 1/35
GABA Receptor Signaling	1.21E-01	1.4 % 1/73
Ephrin B Signaling	1.24E-01	1.3 % 1/75
Chemokine Signaling	1.24E-01	1.3 % 1/75
Molecular and Cellular Functions		
Name	p-value	#Molecules
Cell-To-Cell Signaling and Interaction	2.95E-02 - 8.33E-04	14
Cellular Movement	3.12E-02 - 8.33E-04	9
Amino Acid Metabolism	2.26E-02 - 1.76E-03	2
Carbohydrate Metabolism	1.80E-02 - 1.76E-03	4
Cell Cycle	2.78E-02 - 1.76E-03	4
Physiological System Development and Function		
Name	p-value	#Molecules
Skeletal and Muscular System Development and Function	2.99E-02 - 1.77E-04	8
Nervous System Development and Function	3.12E-02 - 1.19E-03	14
Tissue Development	3.12E-02 - 1.47E-03	15
Hematological System Development and Function	3.22E-02 - 1.68E-03	6
Organismal Functions	1.57E-02 - 1.68E-03	4
Top Networks		
ID Associated Network Functions	Score	
1 Inflammatory Disease, Ophthalmic Disease, Organismal Injury and Abnormalities	32	
2 Cell-To-Cell Signaling and Interaction, Carbohydrate Metabolism, Lipid Metabolism	23	
3 Cell Death and Survival, Nervous System Development and Function, Tissue Morphology	21	
4 Hereditary Disorder, Neurological Disease, Organismal Injury and Abnormalities	3	
5 Hereditary Disorder, Neurological Disease, Organismal Injury and Abnormalities	2	

The resulting network 2 and 3 from the IPA analysis can be observed in Figure 4.14. We included this networks since their functions are more related to the differences observed in the phenotypic and metabolomics observations. Besides the identification of the molecules that are included or not in the dataset, the network visualization reveals the type of molecules and the nature of the relationship among their members, utilizing previously known information. It also allows the detection of critical molecules that play central roles in determined networks. The gray color represents the molecules that were included in the dataset while the white ones were absent. The colored lines symbolize the direct relationship of the enquired molecules with other network elements. Interestingly, G-protein-coupled receptors (GPCR) and Amyloid precursor protein (APP) were identified as “hub” molecules in network 2 and 3

changes in DNA methylation was 100% compatible with the results obtained by the MBD-Seq method, demonstrating the robustness of our results. Figure 4.15 illustrates one of the regions that have been validated. The top information corresponds to the MBD-Seq result in the enquired DMR. It is noticed from the concentration values (expressed in log2) that DNA methylation is higher in grass-fed than in the grain-fed group; correspondingly the DNA methylation level estimated from the proportion of black dots in the corresponding panel, was also higher in the grass-fed group achieving 86.5% compared to the 43.9% obtained in the grain-fed panel.

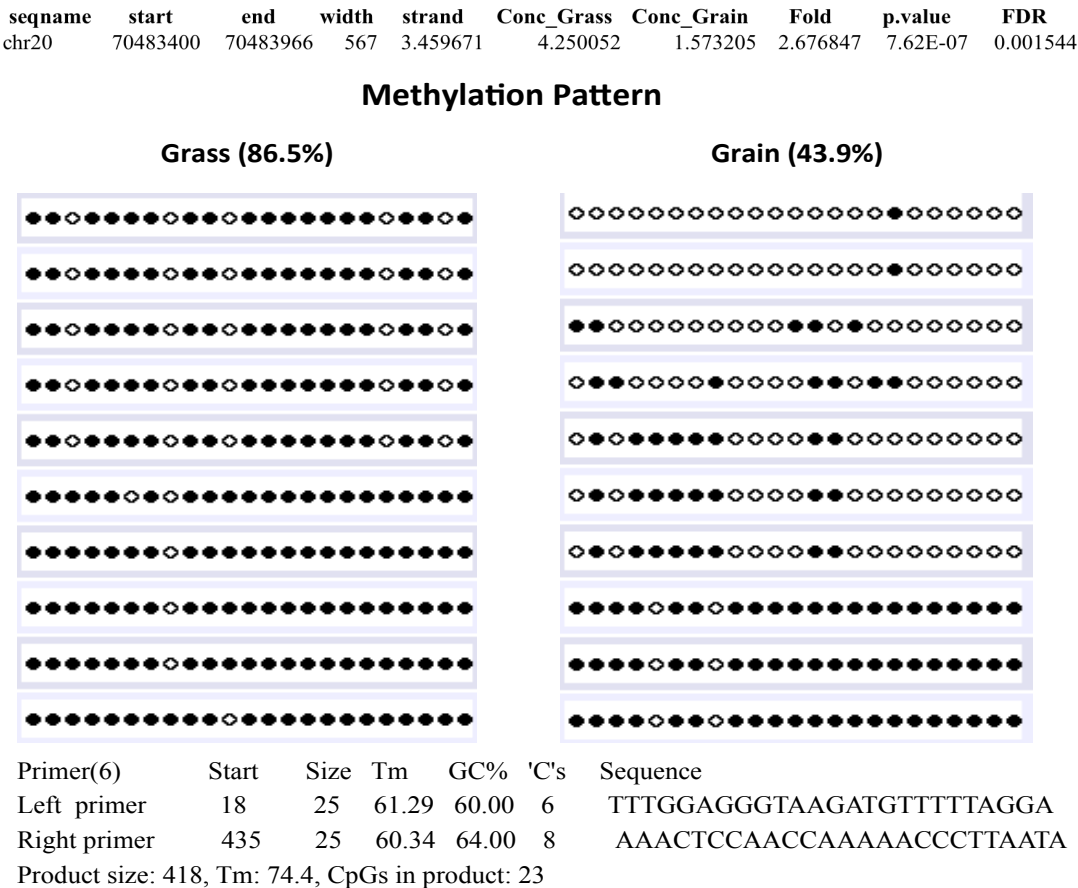


Figure 4.15 Bisulfite sequencing validation of MBD-Seq results
Each dot represents a CpG site; the black dots imply methylation at the corresponding CpG. The percentage value is estimated from the proportion of black dots to the total number of evaluated sites

Discussion

DNA methylation constitutes the first discovered, most studied and systematically understood epigenetic modification [251]. However the implication of DNA methylation in mature muscle formation remains unclear. During cellular differentiation, originally pluripotent cells become specific types of cells that usually are maintained through dynamic epigenetic mechanisms, including DNA methylation. A study analyzing 17,000 CpG islands in different tissues identified 178 of them significantly hypermethylated in muscle and found that CpG islands in skeletal muscle reach the highest percentage (8.3%) compared to other tissues [270]. Therefore we anticipated that phenotypically different muscles should embrace distinctive methylome profiles, considering the diet regimen that animals received from weaning until termination.

In our study, we compared adult muscle tissue obtained immediately after termination. However, during the previous periods of growing and finishing, in which muscle formation and fat deposition still occurs actively in the animal body, the feeding regimen was consistently dissimilar between the two groups. Consequently, we observed 60 differentially methylated regions with diverse localization across the whole genome. Recognizing the similar genetic background of individuals, we assumed that most of the alterations in the DNA methylation landscape was produced by the diet style. The number of DMRs seems small nonetheless it was estimated employing a well-established pipeline that has been used previously in other studies [271]. Among these 60 regions, there are two sets of elements that displayed particular patterns, suggesting synchrony or possible synergy among their members.

Nevertheless, we could not determine whether this DNA methylation variation is a mechanism to establish the phenotypic differentiation observed in muscle tissue from both diets or is a consequence of this differentiation. DNA methylation is a dynamic process that occurs continuously at different stages over the course of growing [272], however technical and logistical limitations prevented us to obtain samples at different time points for determining these fluctuations until termination of animals.

We observed that most of DMRs, 50 out of 60 displayed increased levels of methylation and showed more stability in grass-fed animals. Interestingly, the number of peaks detected individually for each sample comprised consistently large numbers of intervals in grain-fed samples, suggesting possible higher methylation levels in grain-fed animals. However, after considering peaks that overlaps in at least two samples, grass-fed individuals show more methylation at the identified DMRs. Transcriptome and methylome association studies in blood cells demonstrated that only around 2% of changes observed in gene expression could be explained by intra-individual DNA methylation variation, implying that DNA methylation markers are valuable for estimating the risk of disease [272]. Accordingly, our whole set of 60 DMRs or the previously two described clusters could be indicative for the type of diet employed during termination, having a potential practical application in the meat industry as a quality control method. A possible alternative to look for specific markers of diet during the finishing period could be the peaks detected uniquely in samples from those types of diets during the peak calling step; this approach could save time because it requires less processing but should be investigated in more details for possible practical implementation.

Chromosome 14 allocates the largest number of DRMs with 12 units. This chromosome has been extensively explored for quantitative trait loci and genes regarding economically relevant traits in beef and dairy cattle. A review based on 40 investigations reported 126 QTL anchored to the Btau 4_0 genome assembly [273]. The same study found the majority of QTL in beef cattle are located between 15-45 MB, and suggested genes *CYP11B1*, *CRH*, *DGATI*, *TG* and *FABP4* as potential candidates for those QTL. The targeted phenotypes in those reviewed studies comprised ADG, IMF, hot carcass weight, back fat and REA. All these economic important traits were also examined in this project. Interestingly, all DMRs that we found, except one, remain in the first 5MB genomic region of chromosome 14, coinciding with the location of most QTL found in dairy cattle (0MB-10MB). However, DNA methylation could influence transcription of distal regions through modification of the state of DNA, in conjunction with other epigenetic modifications [274]. It has been found that genomic organization of CG-rich areas varies substantially from that of GC-depleted regions. For instance, genomic regions with high GC content present higher gene densities and higher number of CpG islands as well [275, 276]. Furthermore, short interspersed repetitive DNA elements density is also greater in CG-enriched regions [277]. CpG islands have been considered gene markers and consist a relevant feature of mammalian genomes [278]. Chromosome 14 comprises 84,648,390 bp with an overall GC content of 41.4%; enclosing 427 coding genes, 121 non-coding genes and 3,018,079 short variants [279]. The largest number of protein coding genes are located in the first 5MB of the chromosome; region that also contains most of the DMRs detected in this study. It is well known

that chromosome 14 (BTA14) harbors several quantitative trait nucleotides (QTN) with large effect on milk fat percentage and composition, and intramuscular fat (marbling) [280]. A study using purebred Wagyu animals identified a critical 1.1 MB region on chromosome 14 for carcass weight [281]. Also, an identical-by-descent haplotype-sharing analysis performed in a commercial line of *Bos taurus* have fine mapped a QTL for back-fat in chromosome 14 [282]. Remarkably, all those mentioned traits were significantly different between grain- and grass-fed animals according to the phenotypic and metabolomics analyses performed previously in the same group of individuals [247]. *FAM84B*, *TRAPPC9*, *TSNARE1* and *SCRIB* were the relevant protein coding genes in chromosome 14 associated with the identified DMRs; the rest of the annotated genes in chromosome 14 comprised non-protein coding genes. Interestingly, the unique GO term regarding biological process shared among those genes was “protein binding”.

In bovines similarly as in humans, DNA methylation decreases severely before the transcription start site, increments abruptly towards the gene body regions and plateaus until the transcription termination site [283, 284]. We found the same pattern of DNA methylation in muscle, however we observed two strong peaks within gene bodies that should be investigated more for functional studies. These two peaks could be a singular print of muscle methylome since both were observed in the two groups with analogous intensities regardless the type of diet. A study using human embryos reported that the inverse correlation between DNA methylation level at promoters and gene expression progressively strengthens throughout early embryonic development, reaching its peak at the post-implantation stage [284]. Accordingly, we observed

slight higher intensities at three different peaks in promoters of the grass-fed group (around -4,200, -3,850 and -2,450 bp from the TSS), these peaks could be related to the larger number of up-regulated genes found in the same individuals (128 up-regulated vs 59 down-regulated) in a previous publication [247].

Transcription units enclose CpG islands, however expression of CpG islands associated genes in normal tissues was not assumed to be under control of DNA methylation. Interestingly, there are various CpG islands embracing tissue-dependent and differentially methylated regions (T-DMRs) which are prospective methylation sites in normal tissues [285]. Correspondingly, we found higher DNA methylation level inside CpG islands of grass-fed samples, contrarily to what occurs outside of the CpG island boundaries where methylation remains considerable stable. Most of CpG islands are sites for transcription initiation, and many of them are located remotely from the corresponding annotated promoter [286]. Common DNA sequence features adjust CpG islands for becoming promoters by disrupting nucleosomes and attracting proteins to create euchromatin [287]. Dense CpG island methylation silences the respective promoter employing particular DNA sequences. Therefore CpG islands influence chromatin structure and facilitate regulation of gene activity [287]. Considering all these, we speculated that the difference of DNA methylation in CpG islands could be related to the differentially expressed genes. Contrarily, none of the DMRs annotated genes were found in the list of differentially expressed genes reported by the RNA-Seq analysis [247]. The method that DMRs have been annotated and the small number of differentially expressed genes could explain this observation. However, the general profile of DNA methylation at CpG islands clearly

diverges between both conditions according to feeding style, making CpG islands potential biomarkers for type of diet during the finishing period as well.

The pathway analysis mention different canonical pathways that are not related directly to the muscular system, additionally all of them have just one member annotated to each pathway, constituting weak support for biological inferences. Contrarily, the list of physiological system development and function integrates “Skeletal and Muscular System Development and Function” as the top member and “Tissue Development” in third position with 8 and 15 molecules respectively and being statistically significant as well. Molecules BDKRB2, CXCL12, GPER1, GTF2IRD1, PLAGL1, PTPRJ, SOX9 and SMAD were affiliated to the first group, and CXCL12, PFKFB3, PTPRJ, RASA3, SCRIB, SOX9, SMAD6, F2RL3, BDKRB2, GPER1, PLAGL1, KIF26A, TRAF3IP1, CEP120 and TRAPPC9 for the tissue development cluster correspondingly.

The networks report includes “Carbohydrate Metabolism”, “Lipid Metabolism” and “Tissue Morphology”, functions that are directly related to the differences found in the metabolomics and phenotypic analyses as well [247]. Interestingly, network number 2 shows molecule GPCR (G-Protein-couple receptor) in the center of the system, having direct influence on many other molecules and acting as a hub for the correspondent network. This property makes GPCR a critical component for the biological functions related to this network. Network number 3 appears less radiated than network 2 but still shows APP (Amyloid beta precursor protein) molecule as the component with more interactions without appearing in the input data. (GPCR, also known as seven-transmembrane domain receptors, comprise a large protein family of

receptors that activate inside signal transduction pathways. There are two principal pathways involving GPCR; the cAMP and phosphatidylinositol pathways [288]. APP is also a membrane protein expressed in different tissues, however it is concentrated in synapses of neurons. APP is considered the precursor of beta amyloid ($A\beta$), which is a polypeptide that is the main component of amyloid plaques found in patients with Alzheimer's disease [288]. APP is cleaved by secretases to form various peptides, which are secreted and bind to acetyltransferases complex APBB1/TIP60, promoting transcriptional activation, while others form the plaques observed in Alzheimer's disease [289]. Interestingly, two of the secreted peptides have demonstrated bactericidal and antifungal properties, characteristic that could be relevant for the incidence of diseases in animals maintained under determined type of diet, however this effect has not been tested in the present study. Moreover, it has been mentioned that APP peptides may regulate the function of certain other proteins by inhibiting their activity [288], controlling other biochemical functions as well.

The motifs identified in the DMRs are TACAAA and TATGTA. The TACAAA sequence has been reported as a poor TATA box for the human IL-1 α gene at position -31 from the TSS [290]. The 5'upstream regions of IL-1 α and IL-1 β include sequence similarities to the immunoglobulin core enhancer octamer binding site and E motif, however functional analysis of these elements have not been yet performed [290]. The motif TATGTA, also identified in DMRs, has been linked to a transposon (Tc1) insertion site in *Caenorhabditis elegans*. This Tc1 insertion, followed by a typical Tc1 excision, generates a sequence that possibly acts as a splice donor site [291]. Additionally, the TATGTA sequence has been detected as a footprint of excision for

posterior transposition in Tc1/mariner family elements of *Cyanobacterium* insertion element in *Escherichia coli* [292]. More investigation regarding these possible regulatory motifs are still needed to understand their biological function in mammals. These results deliver general information about DNA methylation and the characteristics of beef under distinct feeding systems. Nonetheless, we are aware of the limitations of our study. Regardless MBD and bisulfite validation steps, all other analyses were performed using bioinformatic tools thus extensive experimental and functional validation is still required. Accordingly, screening of DNA methylation in local genomic regions or following more restricted criterion, such as marbling gene-set, should be recommended to validate and expand our results.

Conclusion

We found 60 genomic regions that display different level of methylation in muscle from grass-fed Angus steers. Remarkably, chromosome 14 allocated the largest number of DMRs (12 of 60); this chromosome has been linked to large effect on milk fat percentage and composition, marbling, back-fat and carcass weight, traits that also differed between both types of diet. Most of these DMRs (84%) increased their methylation level in grass-fed steers and were located inside (~45%) or upstream (~30%) of genes. Almost half of the DMRs and approximately a fifth of them were totally contained and remained in the upstream region of GpG islands, respectively. The pathway analysis revealed skeletal and muscular system as the top physiological system and function, and identified carbohydrates metabolism, lipid metabolism and tissue morphology among the top networks.

5. Conclusions and Future Directions

The ultimate goal of this project is to unveil the real differences between the traditional and grass-fed beef regarding meat quality and the biological processes that occur and affect the animals under these two types of production systems. Understanding the underlying mechanisms that cause this differentiation is critical for improving the quality of beef, management practices and economic revenue as well. We recognize the importance and applicability of these objectives for grass-fed operations, thus we included them in the first part of the project. However our interest extends beyond these applications, we want to identify the genomic, metabolomic and specific epigenomic profiles for each type of diet to understand the mechanisms that determine the corresponding phenotype. Additionally, we would like to combine the information generated at different –omics levels to reveal their complex interactions and joint functions in diverse biological processes. Accordingly, the focus of my graduate research comprises three components: a) Analysis of the growth curve, important economic characteristics and beef quality traits, b) Integrated transcriptomic and metabolomic examination and c) DNA methylation analysis regarding a particular type of feeding system. The present project presents novel contributions in many of these areas.

The selected model to achieve our objectives is a highly genetically related population (Wye Angus herd), which has been closed for almost 60 years and constitutes a valuable resource for performing epigenomic studies. The genetic similarity among individuals diminishes the genetic effect, allowing a better experimental detection of the treatment effect, in this case diet.

Firstly, we identified a distinct growth curve in each diet and observed that grass-fed animals require nearly 180 extra days to achieve the required market final weight; the ADG was superior by approximately 70 % in grain-fed animals. Regarding beef quality traits, grass-fed carcasses were lighter, with reduced subcutaneous fat and less dressing than traditional carcasses. All these findings were obtained from an experimental setting that emulates as much as possible the conditions that producers face in real-life situations, in order to serve as a management decision tool and become a reference source for future studies. Remarkably, although the tenderness of both types of beef were similar, the traditional USDA grading system categorized grain-fed carcasses as better than their grass-fed counterpart. This implies the existence of an economic risk in producing high-quality grass-fed beef that will not be recognized as it under the traditional grading system. Particularly, we believe it is critical to develop a distinct grass-fed grading system to guarantee grass-fed beef consumers would receive the correct information about their decision and to further foster the natural products industry as well.

In the second section, we integrated the transcriptomic and metabolomics results, which have been independently obtained, employing the web-based Integrated Molecular Pathway-Level Analysis (IMPaLA). It runs an over-representation analysis with the provided lists of genes and metabolites employing 11 databases, which included more than 3000 pre-annotated pathways [194]. The software identifies the pathways that may be altered at the transcriptional level, the metabolomic level, or both. Based on this approach, our results indicate that grass-fed animals yield tender beef with lower total fat, higher omega3/omega6 ratio and greater protein content

than conventional beef, characteristics that are beneficial for human health. Respecting animal welfare, cortisol levels detected in blood by the standard and metabolomics analyses suggests that animals maintained under grass-fed regimen could experience less stress, which might be promoted by the more natural conditions maintained in grass feeding systems, according to the animal innate behavior. Additionally, we detected the set of genes that were up- and down-regulated in the grass-fed beef and recognized 13 mitochondrial genes as potential markers for diet style in beef cattle. In the final part of this step, we identified oxidative phosphorylation as the main pathway that differs between both conditions. Interestingly, this pathway only includes up-regulated genes suggesting a speed up of oxidative phosphorylation in grass-fed animals.

In the last part of the project, we investigated the DNA methylation profile for each diet regimen. This step was based on the hypothesis that transcription of genetic information encoded in DNA is partially regulated by chemical modifications, such as DNA methylation and histone alterations. All these chemical alterations together represent the “epigenetic code”. Therefore DNA methylation constitutes just one layer of epigenetic regulation, however its characterization is critical to understand the relationship with the transcriptome and other epigenomic modifications under the same biological conditions. Hence, we identified 60 differentially methylated regions; most of them (50) displayed increased levels of methylation and showed more stability in grass-fed animals, suggesting a global increase of DNA methylation in grass-fed individuals. Remarkably, 12 DMRs were located within the first 16 MB region of chromosome 14. According to 40 studies, this chromosome has been linked

to 126 QTL in the Btau 4_0 genome [273]. Interestingly, the location of the DMRs corresponds with the area of QTL detected in dairy cattle (0-10 MB). However, DNA methylation could influence transcription of distal regions through modification of the state of DNA, in conjunction with other epigenetic modifications [274]. Thus, the identified DMRs also could influence the QTL found in beef cattle, which remain in the area located between 15-45 MB. Correspondingly, genes *CYP11B1*, *CRH*, *DGAT1*, *TG* and *FABP4* were suggested as potential candidates for those QTL [273]. According to our results *FAM84B*, *TRAPPC9*, *TSNARE1* and *SCRIB* were the relevant protein coding genes in chromosome 14 associated with the identified DMRs; the rest of the annotated genes in chromosome 14 comprised non-protein coding genes. Almost 50% of DMRs were contained completely within CpG islands, demonstrating its association with DNA methylation and its possible implication in gene expression regulation. The pathways analysis reported skeletal and muscular system as the top physiological system, serving as a validation for our findings in this part of the project.

We understand that gene regulation and phenotypic differentiation are co-regulated by different epigenetic mechanisms that interact dynamically among them. Therefore, instead of examining each of them individually, integrative approaches including other sources of information, such as gene expression, DNA methylation, histone modifications, miRNAs, lincRNAs and CNVs, are still necessary for a better understanding of this complex process. In the near future, we would like to combine the information provided in this project with miRNA and lincRNA datasets obtained

from the same individuals in order to expands our knowledge regarding epigenomics as a whole system.

Appendices

Appendix I. Ingenuity Pathway Analysis of all differentially expressed genes

Top Diseases and Bio Functions

Diseases and Disorders

Name	p-value	# Molecules
Neurological Disease	4.28E-06 - 1.66E-02	53
Psychological Disorders	4.28E-06 - 1.55E-02	40
Auditory Disease	4.97E-06 - 1.70E-02	8
Skeletal and Muscular Disorders	1.29E-05 - 1.21E-02	47
Metabolic Disease	2.74E-05 - 1.55E-02	46

Molecular and Cellular Functions

Name	p-value	# Molecules
Nucleic Acid Metabolism	1.15E-06 - 1.41E-02	20
Small Molecule Biochemistry	1.15E-06 - 1.60E-02	47
DNA Replication, Recombination, and Repair	2.30E-06 - 9.13E-03	12
Energy Production	2.30E-06 - 9.14E-03	14
Cell-To-Cell Signaling and Interaction	8.29E-05 - 1.70E-02	22

Physiological System Development and Function

Name	p-value	# Molecules
Organ Morphology	1.62E-05 - 1.63E-02	23
Skeletal and Muscular System Development and Function	1.62E-05 - 1.41E-02	13
Hematological System Development and Function	8.29E-05 - 1.70E-02	34
Immune Cell Trafficking	8.29E-05 - 1.70E-02	25
Humoral Immune Response	4.63E-04 - 2.86E-03	4

Top Canonical Pathways

Name	p-value	Ratio
Oxidative Phosphorylation	2.14E-11	13/109 (0.119)
Mitochondrial Dysfunction	6.03E-09	13/171 (0.076)
Acute Phase Response Signaling	1.65E-04	8/169 (0.047)
FXR/RXR Activation	1.67E-04	7/127 (0.055)
Protein Ubiquitination Pathway	2.43E-03	8/255 (0.031)

Top Networks

I Associated Network Functions	Score
D	
1 Nucleic Acid Metabolism, Small Molecule Biochemistry, DNA Replication, Recombination, and Repair	51
2 Cell-mediated Immune Response, Cellular Development, Cellular Function and Maintenance	48
3 Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry	35
4 Cellular Assembly and Organization, Cellular Function and Maintenance, Hereditary Disorder	26
5 Connective Tissue Disorders, Inflammatory Disease, Skeletal and Muscular Disorders	26

Top Tox Lists

Name	p-value	Ratio
Mitochondrial Dysfunction	7.44E-09	13/174 (0.075)
Renal Safety Biomarker Panel (PSTC)	1.47E-05	3/6 (0.5)
FXR/RXR Activation	1.67E-04	7/127 (0.055)
Positive Acute Phase Response Proteins	2.53E-03	3/30 (0.1)
Glutathione Depletion - CYP Induction and Reactive Metabolites	5.15E-03	2/12 (0.167)

Top Molecules

Log Ratio up-regulated

Molecules	Exp. Value	Exp. Chart
RBP2	↑10.995	
CCL5	↑10.706	
VIL1	↑10.500	
FCGBP	↑10.255	
ALPI	↑9.773	
FABP2	↑9.727	
CDHR2	↑9.473	
GIMAP7	↑9.413	
KRT20	↑9.156	
LCK	↑9.025	

Log Ratio down-regulated

Molecules	Exp. Value	Exp. Chart
SERPINA3*	↓-10.589	
KRT5	↓-10.266	
TTR	↓-10.201	
CAMK1D	↓-10.143	
A2ML1	↓-10.000	
PRSS35	↓-9.980	
HELQ	↓-9.710	
CA12	↓-9.616	
TLR9	↓-9.613	
PLA2G2A	↓-9.552	

Appendix II. Ingenuity Pathway Analysis according to direction of gene expression in grass-fed individuals

Down-regulated genes Top Canonical Pathways

Name	p-value	Ratio
FXR/RXR Activation	2.05E-07	6/127 (0.047)
Acute Phase Response Signaling	1.1E-06	6/169 (0.036)
LXR/RXR Activation	1.05E-04	4/121 (0.033)
Complement System	2.05E-03	2/33 (0.061)
Arginine Degradation I (Arginase Pathway)	8.11E-03	1/4 (0.25)

Up-regulated genes Top Canonical Pathways

Name	p-value	Ratio
Oxidative Phosphorylation	8.45E-13	13/109 (0.119)
Mitochondrial Dysfunction	2.68E-10	13/171 (0.076)
Protein Ubiquitination Pathway	4.79E-04	8/255 (0.031)
Telomere Extension by Telomerase	4.94E-03	2/15 (0.133)
Phospholipase C Signaling	7.23E-03	6/239 (0.025)

Appendix III. Primers for validation of RNA-Seq

Gene	Ensemble Gene ID	Purpose	Primers	Sequence
YBX1	ENSBTAG00000017368	Gene expression	F	5'-TCGACCACAGTACTCCAACC-3'
			R	5'-AGCACCCCTCCATCACTTCTC-3'
FGB	ENSBTAG00000022120	Gene expression	F	5'-CGTGGTCACTGAGTTAGGCTT-3'
			R	5'-GGTCCTCAAGTCTACCTCCCT-3'
HSDL2	ENSBTAG00000031295	Gene expression	F	5'-TGCATTGTGGCCTAAAAACAGC-3'
			R	5'-CCCAGCATATCCATAGCAGC-3'
Novel gene	ENSBTAG00000016688	Gene expression	F	5'-AGTTCACCCATTAAGCTGCCA-3'
			R	5'-GCACACTAGAGTCACCATGCT-3'
KRT5	ENSBTAG00000038384	Gene expression	F	5'-GGTATCGTTGGGGAGAGAGG-3'
			R	5'-CATCAGTGCATCGACCTTGG-3'
MDH1	ENSBTAG00000019295	Gene expression	F	5'-ATCCAGCCAATACCAACTGC-3'
			R	5'-TGAAGTTCTCCTTGGGGATG-3'
GJA1	ENSBTAG00000001835	Gene expression	F	5'-CGGAGAAAACCATCTTCATCA-3'
			R	5'-CCCTTCACACGATCCTTAACA-3'
GSTT3	ENSBTAG00000008587	Gene expression	F	5'-CCATGAAGGAGAGGCTGAAG-3'
			R	5'-GGTGAAGCTAGTGGGCAGAC-3'
CHST13	ENSBTAG00000008778	Gene expression	F	5'-CCTTCTTGGCCTCAGTCCAG-3'
			R	5'-ACGGTCCTGTCTACTCTGCT-3'
CDHR5	ENSBTAG00000047914	Gene expression	F	5'-AGCAGCTTCTTCTCCTTGGT-3'
			R	5'-CTCTGTGGTCTCCTCCTGTG-3'

Appendix IV. Down-regulated genes in grass-fed samples

Gene	logFC	logCPM	LR	PValue	FDR
ENSBTAG00000038384	-10.26634742	3.55996361	37.61019458	8.64E-10	1.06E-05
ENSBTAG00000001835	-5.252818634	4.830731217	34.09818675	5.24E-09	4.30E-05
ENSBTAG00000019390	-9.616257792	2.920874812	28.07546014	1.17E-07	0.000501563
ENSBTAG00000008778	-8.225612355	3.727174636	27.98510223	1.22E-07	0.000501563
ENSBTAG00000015676	-9.382261868	4.908643962	27.48104522	1.59E-07	0.000557875
ENSBTAG00000008587	-7.612045082	3.151526929	24.45098575	7.62E-07	0.002345428
ENSBTAG00000015988	-3.13954922	6.356304436	23.56578366	1.21E-06	0.002971523
ENSBTAG00000033365	-10.94030438	4.237089352	20.56210246	5.77E-06	0.00793753
ENSBTAG00000022120	-6.418660799	6.073271149	20.2668121	6.74E-06	0.008726881
ENSBTAG00000036116	-4.168586793	4.26475709	19.97399058	7.85E-06	0.008903216
ENSBTAG00000018151	-9.709798277	3.033675504	19.51406752	9.99E-06	0.010170584
ENSBTAG00000017305	-9.930703009	3.199778605	19.31115996	1.11E-05	0.010514379
ENSBTAG00000046540	-10.5888075	3.856151254	18.89004476	1.38E-05	0.010995683
ENSBTAG00000032236	-5.036162037	3.911310877	18.61618432	1.60E-05	0.011040095
ENSBTAG00000039425	-7.374547236	2.958933968	18.59720201	1.61E-05	0.011040095
ENSBTAG00000017280	-3.692685525	4.584900662	17.86954881	2.37E-05	0.013402826
ENSBTAG00000047986	-8.847460878	2.146671431	17.615507	2.70E-05	0.01381514
ENSBTAG00000010991	-10.20126148	3.468789554	17.44750728	2.95E-05	0.014255624
ENSBTAG00000008650	-10.14311385	3.410768306	17.09570588	3.55E-05	0.016202149
ENSBTAG00000045836	-10.11917975	3.386898824	17.00405455	3.73E-05	0.016451204
ENSBTAG00000026708	-9.979506296	3.29617921	16.88576753	3.97E-05	0.016848435
ENSBTAG00000004097	-9.999732734	3.267872142	16.57260103	4.68E-05	0.018635349
ENSBTAG00000022395	-5.680735406	3.50940194	15.92693025	6.58E-05	0.023486979
ENSBTAG00000045808	-9.663870645	2.990459552	15.71808183	7.35E-05	0.025032155
ENSBTAG00000002055	-4.857111434	2.955196884	15.56849161	7.96E-05	0.026115797
ENSBTAG00000018198	-9.612556116	2.940974969	15.42281613	8.59E-05	0.027210271
ENSBTAG00000047885	-9.605077064	2.933765159	15.41675563	8.62E-05	0.027210271
ENSBTAG00000002985	-6.88139235	3.268932359	15.3070196	9.14E-05	0.02748224
ENSBTAG00000048135	-6.000869916	2.444307394	15.28619641	9.24E-05	0.02748224
ENSBTAG00000013039	-9.551593072	2.882261989	15.17927681	9.78E-05	0.028314344
ENSBTAG00000012104	-9.49605744	2.828852528	15.02753581	0.000105954	0.029978861
ENSBTAG00000038627	-9.44668775	2.781445175	14.8433859	0.000116817	0.031773785
ENSBTAG00000010978	-5.043390095	4.333794568	14.72715544	0.000124244	0.031883249
ENSBTAG00000017121	-5.655238151	2.575035101	14.62288444	0.00013131	0.031883249
ENSBTAG00000019423	-9.388730209	2.662577623	14.26155444	0.000159081	0.034963771
ENSBTAG00000002430	-6.385144972	2.809137371	14.15366864	0.000168468	0.036060999
ENSBTAG00000004903	-4.601263965	4.549351535	14.06568962	0.000176535	0.036517509
ENSBTAG00000002292	-4.799301766	3.56865606	13.9315997	0.000189584	0.037941524
ENSBTAG00000010062	-9.1503636	2.498380566	13.69793725	0.00021469	0.042021322
ENSBTAG00000039422	-9.160601325	2.508120267	13.68726327	0.000215914	0.042021322
ENSBTAG00000037903	-9.173222538	2.451143582	13.40258528	0.000251278	0.046160094
ENSBTAG00000039237	-8.129324134	1.477421074	13.34647402	0.000258909	0.047209653
ENSBTAG00000030683	-9.125965454	2.404978671	13.26293714	0.000270705	0.047960767
ENSBTAG00000040133	-9.12596555	2.404978671	13.26213049	0.000270822	0.047960767
ENSBTAG00000017827	-5.292807533	2.900005963	12.88547044	0.000331143	0.054707473
ENSBTAG00000013532	-8.960749412	2.244189618	12.61289806	0.000383094	0.059284094
ENSBTAG00000012728	-8.867318375	2.230720546	12.59924063	0.000385904	0.059284094
ENSBTAG00000045632	-1.779581974	8.959279513	12.4343069	0.000421519	0.063578397
ENSBTAG00000045532	-1.780861089	8.944447467	12.25122391	0.000464953	0.066914134
ENSBTAG00000021844	-8.86328494	2.149830957	12.17155903	0.000485237	0.067874197
ENSBTAG00000001638	-3.010202349	4.181697141	12.12604748	0.000497224	0.068378014
ENSBTAG00000003774	-6.043204591	3.638358132	12.08086471	0.00050942	0.069120344
ENSBTAG00000048000	-1.742780665	8.952726271	11.90181318	0.000560793	0.071898373
ENSBTAG00000006350	-7.34233072	2.837288768	11.49592464	0.00069749	0.08375318
ENSBTAG00000045494	-1.717198088	8.940747299	11.37458662	0.000744558	0.08686274
ENSBTAG00000012403	-6.939420389	3.330164593	11.27787323	0.000784364	0.088568326
ENSBTAG00000002253	-8.64606298	1.941076772	11.24183394	0.000799741	0.088798868
ENSBTAG00000020957	-8.546378372	1.931151567	11.22170011	0.000808464	0.088798868
ENSBTAG00000026917	-7.19488639	2.738308303	11.02233494	0.000900206	0.095859809

Appendix V. Up-regulated genes in grass-fed samples

Gene	logFC	logCPM	LR	PValue	FDR
ENSBTAG000000006133	3.667723345	7.824277125	51.57131136	6.90E-13	1.70E-08
ENSBTAG00000019295	3.276500089	6.321670456	28.10358306	1.15E-07	0.000501563
ENSBTAG00000011145	2.335538102	7.574937268	24.21187103	8.63E-07	0.002360366
ENSBTAG00000017368	2.392394799	8.828298855	22.22247852	2.43E-06	0.005433743
ENSBTAG00000016688	2.40414632	7.200455763	21.96012183	2.78E-06	0.005710396
ENSBTAG00000001521	3.071342306	5.776322479	21.74345827	3.12E-06	0.005901227
ENSBTAG00000002590	5.351005951	3.460181871	21.38378291	3.76E-06	0.006610057
ENSBTAG00000018669	7.348059211	3.978613375	21.09008952	4.38E-06	0.006797753
ENSBTAG00000002507	2.276784881	7.233248914	21.07417534	4.42E-06	0.006797753
ENSBTAG000000022715	10.38599052	3.703552929	20.55171604	5.80E-06	0.00793753
ENSBTAG000000031295	4.45134361	3.90019603	19.98936555	7.79E-06	0.008903216
ENSBTAG00000018801	2.909704927	5.691028883	19.94815973	7.96E-06	0.008903216
ENSBTAG00000015056	10.99516546	4.298761064	19.52579822	9.93E-06	0.010170584
ENSBTAG000000047029	3.411318095	4.938077318	19.44954563	1.03E-05	0.010170584
ENSBTAG00000001144	3.253949491	5.795508054	19.0307218	1.29E-05	0.010995683
ENSBTAG00000013930	2.06341773	7.419703201	18.98689028	1.32E-05	0.010995683
ENSBTAG00000012589	2.813892624	6.161260204	18.92420846	1.36E-05	0.010995683
ENSBTAG00000007782	2.499861784	9.949095327	18.91704609	1.37E-05	0.010995683
ENSBTAG000000022203	2.052056686	8.059865734	18.81774022	1.44E-05	0.011040095
ENSBTAG00000001003	2.560727485	10.06428107	18.64441293	1.58E-05	0.011040095
ENSBTAG00000007191	10.70607268	4.015555284	18.63616018	1.58E-05	0.011040095
ENSBTAG00000016709	6.818374723	2.640670419	18.39508567	1.80E-05	0.011943443
ENSBTAG00000006359	8.702349746	2.123978833	18.28776831	1.90E-05	0.012302995
ENSBTAG000000030940	9.194475172	2.562280591	18.15010606	2.04E-05	0.012886038
ENSBTAG00000012335	5.730090121	3.244425449	17.95243158	2.26E-05	0.013402826
ENSBTAG00000018543	10.49997533	3.814351611	17.87563227	2.36E-05	0.013402826
ENSBTAG000000046786	2.290800289	6.874999386	17.8540748	2.39E-05	0.013402826
ENSBTAG00000008505	10.49608369	3.810558186	17.84562432	2.40E-05	0.013402826
ENSBTAG000000046158	8.875403477	2.267056308	17.75515561	2.51E-05	0.013637305
ENSBTAG00000008550	9.412838787	2.767399658	17.72805163	2.55E-05	0.013637305
ENSBTAG00000005760	4.012727118	3.970380585	17.60032824	2.73E-05	0.01381514
ENSBTAG000000046385	8.643625928	2.072451434	17.58324427	2.75E-05	0.01381514
ENSBTAG00000003330	6.70693049	2.502874424	17.54252317	2.81E-05	0.013831896
ENSBTAG000000020641	3.089325404	4.987867961	17.25900009	3.26E-05	0.01543939
ENSBTAG00000018011	6.578241213	3.73485617	17.2172457	3.33E-05	0.015484675
ENSBTAG00000005259	2.168018333	8.50986103	16.99767835	3.74E-05	0.016451204
ENSBTAG00000005261	10.25485257	3.575921568	16.93798831	3.86E-05	0.016678814
ENSBTAG00000011465	2.217133591	6.545069001	16.80803184	4.14E-05	0.017255351
ENSBTAG00000013905	8.547824549	1.97965093	16.73379728	4.30E-05	0.017644739
ENSBTAG00000013162	2.23762747	8.633581117	16.55433695	4.73E-05	0.018635349
ENSBTAG00000006816	8.504757591	1.940166155	16.53764761	4.77E-05	0.018635349
ENSBTAG00000016363	4.119984959	3.722679671	16.47174664	4.94E-05	0.018721235
ENSBTAG00000012695	9.025093137	2.402156251	16.469673	4.94E-05	0.018721235
ENSBTAG00000006441	2.738759254	6.191078507	16.11748186	5.95E-05	0.022203617
ENSBTAG00000014675	6.005220056	2.648316177	16.04827288	6.17E-05	0.022401395
ENSBTAG00000008224	2.57977354	5.800682507	16.04415657	6.19E-05	0.022401395
ENSBTAG000000046335	2.108754199	6.962380944	15.80029441	7.04E-05	0.024753693
ENSBTAG000000015204	2.560739144	5.70401295	15.77239999	7.14E-05	0.024767533
ENSBTAG00000011713	8.533206526	4.288338844	15.69974621	7.42E-05	0.025032155
ENSBTAG000000022058	1.96650426	7.378998846	15.57275446	7.94E-05	0.026115797
ENSBTAG00000017077	4.296737359	3.582842057	15.46543396	8.40E-05	0.027210271
ENSBTAG00000013015	8.434024065	1.891437473	15.37360897	8.82E-05	0.027433601
ENSBTAG00000006607	2.489630945	5.720790635	15.35347116	8.92E-05	0.027433601
ENSBTAG00000011045	4.724911774	3.105174333	15.26226568	9.36E-05	0.02748224
ENSBTAG00000012363	9.772530655	3.110093184	15.25795772	9.38E-05	0.02748224
ENSBTAG00000017045	9.726767871	3.066158388	15.06364334	0.000103946	0.02975278
ENSBTAG00000007836	5.534899387	2.792107484	14.99040684	0.000108059	0.030227081
ENSBTAG00000015053	2.097406854	6.387220249	14.86522658	0.000115472	0.031773785
ENSBTAG000000046468	1.913402008	6.858388445	14.82995255	0.000117652	0.031773785
ENSBTAG000000021020	8.311860651	1.779970507	14.81240868	0.000118752	0.031773785
ENSBTAG000000031383	9.061972957	2.435151957	14.76224238	0.000121953	0.031883249
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ENSBTAG00000002408	8.322388116	1.765488408	14.72131469	0.000124629	0.031883249
ENSBTAG00000019552	5.732382383	2.431269686	14.67858655	0.000127486	0.031883249
ENSBTAG000000012490	3.123449697	4.481906231	14.67421472	0.000127782	0.031883249
ENSBTAG00000002973	4.86292717	2.989692504	14.630299	0.000130795	0.031883249
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ENSBTAG00000013358	2.943602593	4.90562688	13.93376308	0.000189366	0.037941524
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ENSBTAG000000019770	6.593549486	4.179311759	10.87076157	0.000976943	0.099786062

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